

Original Research Article

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Synthesis of Biodegradable Packaging Material using Polyhydroxyalkanoate Produced by *Bacillus subtilis*

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ABSTRACT

Petroleum-based plastics persist in the environment, creating a need for biodegradable alternatives. This study investigated PHA production by a rhizosphere isolate identified as *Bacillus subtilis* and its incorporation into an agar–chitosan film. PHA production was screened using Mineral Davis Medium and Sudan Black B staining. The polymer was extracted using sodium hypochlorite–chloroform treatment and incorporated into the film with glycerol as a plasticizer. The film was evaluated by FTIR, solubility, melting-point, and soil-burial tests. PHA was insoluble in water but soluble in chloroform. The film showed a melting point of approximately 70°C and 26.9% weight loss after 20 days of soil burial. The findings support the potential of PHA-based films for biodegradable packaging applications.

Introduction

Petroleum-based plastics are widely used in packaging due to their strength, flexibility, and durability. However, their resistance to natural degradation contributes to the accumulation of plastic waste, posing significant environmental challenges. The environmental consequences associated with the global production, use and disposal of plastics have been widely recognized (Geyer *et al.*, 2017). Microbially produced biodegradable polymers are being explored as sustainable alternatives to conventional plastics. Polyhydroxyalkanoates are intracellular polyesters synthesized by microorganisms as carbon- storage compounds. Their biodegradability and versatile properties make them promising materials for

packaging and other industrial applications (Koller, 2018). *Bacillus subtilis* is a Gram-positive, endospore-forming, soil-associated bacterium with considerable biotechnological potential, particularly in the production of biodegradable microbial polymers. In this context, Joseph and Chithira (2021) characterized polyhydroxybutyrate produced by a *Bacillus subtilis* strain isolated from rhizosphere soil, highlighting its potential as a source of biopolymeric materials. In addition to microbial polymers, naturally materials such as agar and chitosan have also gained attention for their suitability in biodegradable film development. However, because films made from these polymers may be relatively rigid or brittle, glycerol is commonly added as a plasticizer to improve flexibility, extensibility, and

handling characteristics. The influence of agar–glycerol formulations and their compositional ratios on the resulting film properties has been reported by [Fransiska et al., \(2024\)](#). Furthermore, the potential of PHA-based films and PHA blends as biodegradable materials has been demonstrated by [Rebocho et al., \(2020\)](#).

The present study aimed to isolate and identify *Bacillus subtilis* from rhizosphere soil, screen for and extract PHA, develop an agar–chitosan–PHA film, and evaluate selected physicochemical properties and soil-burial mass loss.

Materials and Methods

Isolation and identification

Rhizosphere soil was suspended in sterile distilled water and serially diluted. Aliquots from selected dilutions were spread onto nutrient agar and incubated at 37°C for 24–48 h. A distinct colony was purified by repeated streak plating. Colony morphology, Gram staining, and endospore staining were examined for preliminary identification of the bacterial isolate. Biochemical tests included indole, methyl red, Voges–Proskauer, citrate utilization, triple sugar iron, urease, catalase, oxidase, gelatin hydrolysis, and starch hydrolysis. The isolate was identified as *Bacillus subtilis* based on its morphological, staining, and biochemical characteristics

PHA production and extraction

PHA production by the isolated *Bacillus subtilis* was carried out in nitrogen-limited Mineral Davis Medium (MDM), following the method described by [Mohammed et al., \(2022\)](#) with modifications. The medium was sterilized at 121°C for 15 min, adjusted to pH 7.0, inoculated with the bacterial culture, and incubated at 30–37°C for five days. Following incubation PHA accumulation was screened using Sudan Black B staining based on the method of [Joseph and Chithira \(2021\)](#), where the formation of dark intracellular granules was considered indicative of PHA accumulation.

PHA extraction was carried out using the modified sodium hypochlorite – chloroform method. For extraction, the bacterial culture was centrifuged at 10,000 rpm for 10 min to obtain the cell pellet. The pellet was washed thoroughly with distilled water, treated with 4% sodium hypochlorite at 37°C for 1 h, and centrifuged to

remove non-PHA cellular components. The pellet was washed with distilled water and 70% ethanol, followed by treatment with hot chloroform at 60°C for 30–60 min. The chloroform layer containing dissolved PHA was collected, and cold ethanol was added to precipitate the polymer. The precipitated PHA was subsequently dried for 5–6 days to obtain a dry polymer product. The resulting PHA powder was collected and collected and stored for further analysis.

Film preparation

The biodegradable film preparation was carried out following the method described by [Diop et al., \(2023\)](#), with modifications. The Agar-agar was dissolved in distilled water by heating. Chitosan was dissolved separately in 1% acetic acid. The two solutions were then combined, followed by the addition of extracted PHA and glycerol as a plasticizer. The mixture was stirred until a homogenous formulation was obtained. It was then cast into a Petri dish and dried to produce a continuous biodegradable film.

Characterization and degradation

The extracted PHA was examined by Fourier-transform infrared spectroscopy to identify characteristic functional groups associated with the polymer. Solubility was tested using water and chloroform. The melting behavior of the prepared film was determined by gradual heating.

For the degradation study, the method described by [Mohammed et al., \(2022\)](#) was followed with modifications. A pre-weighed film sample was buried in moist garden soil for 20 days. After incubation, the film was carefully recovered, washed carefully to remove adhering soil particles, dried, and weighed. The degradation-related weight reduction was calculated using the following equation:

$$\text{Weight reduction (\%)} = \frac{[(\text{Initial weight} - \text{Final weight}) / \text{Initial weight}] \times 100}$$

Results and Discussion

Isolation and identification

Three colony types were observed on nutrient agar, and one distinct colony was purified for further study. The isolate was Gram-positive, rod-shaped, and endospore-

forming. It was negative for indole, methyl red, and urease, but positive for Voges–Proskauer, citrate utilization, catalase, oxidase, gelatin, and starch hydrolysis. Based on these characteristics, the isolate was identified as *Bacillus subtilis* (Table 1). The observed characteristics were consistent with those reported for *Bacillus* species isolated from the rhizosphere soil by Joseph and Chithira (2021).

PHA production and recovery

After incubation of *B. subtilis* in Mineral Davis Media at 37°C for 5 days growth was observed in Medium and the medium turned into yellow color due to the presence of glucose. The composition of the medium (Table 2) was nitrogen- limited medium and carbon-rich medium. Nitrogen limitation triggers the metabolic stress in bacteria leading to excess carbon utilization and production of intracellular PHA accumulation. Mohammed *et al.*, 2022 demonstrated that nitrogen restricted media enhance the growth of PHA production in *B. subtilis*.

In accordance with earlier reports in this study, the accumulation of intracellular PHA Granules was confirmed using Sudan Black B Staining. It is a lipid soluble dye that specifically stains intracellular PHA granule, appearing as dark black colour. Sudan Black B staining observed under a microscope (Figure 4) showed the presence of distinct dark intracellular granules within the bacterial cells. This appearance confirms the synthesis and storage of PHA within the bacterial cell. Similar staining patterns were described by Joseph *et al.*, 2021, who used Sudan Black B as a rapid screening method for detecting PHA-producing microorganisms. When compared to Iftikhar *et al.*, 2024 study the results are same in the present study.

Extraction using sodium hypochlorite followed by chloroform treatment yielded a white to off - white polymer powder. Sodium hypochlorite removed cellular components, while chloroform facilitated polymer recovery. A similar extraction procedure was reported by Mohammed *et al.*, 2022 and Naseem *et al.*, 2024, who demonstrated that sodium hypochlorite effectively digests non-PHA cellular components while preserving the integrity of PHA granules, followed by dissolution in chloroform for polymer recovery. Since quantitative PHA yield was not determined, the result represents preliminary evidence of PHA production and recovery (Figure 2).

Film formation

The recovered PHA was incorporated into an agar–chitosan matrix using glycerol as a plasticizer. The resulting film (Figure 3) was smooth, uniform, and flexible. Agar and Chitosan provided the film-forming matrix, while glycerol improved flexibility and minimized brittleness. This interaction resulted in a homogeneous polymer matrix, which is crucial for producing films with consistent texture and strength. Similar findings were reported by Diop *et al.*, 2022, where agar-based composite films showed enhanced mechanical stability. The incorporation of PHA into the agar-chitosan matrix enhanced the mechanical strength and biodegradability of the film. Although PHA is water-insoluble, its fine dispersion within the polymer blend contributed to improved structural reinforcement. The casting and controlled drying process ensured uniform thickness and smooth surface characteristics of the final bioplastic film.

Similar to the Fransiska *et al.*, 2024 study, both works aim to improve biopolymer films for sustainable packaging. However, our study is slightly different because we added chitosan for antimicrobial activity and used PHA produced from bacteria, which makes the film more biodegradable and eco-friendlier. So, our work not only improves mechanical properties but also adds extra functional benefits. The composition of the prepared film is presented in (Table 2).

FTIR Analysis of PHA produced using *B. subtilis*

FTIR analysis showed hydroxyl, alkyl, carbonyl, and ester-related functional groups associated with the PHA-containing material (Figure 1). The FTIR spectral analysis of the sample indicated the presence of several functional groups as shown in Graph 1. A broad strong peak observed around 3350 cm⁻¹ 3000–3200 cm⁻¹ corresponds to O–H stretching vibrations of alcohol and hydroxyl groups. Peaks at 2960 cm⁻¹, 2920 cm⁻¹ and 2870 cm⁻¹ represent C–H stretching vibrations of alkyl and methyl groups present in fatty acids. The absence of peaks in the 2500–1650 cm⁻¹ region indicates that acidic O–H groups of carboxylic acids and ketone groups are not present. A peak observed at 1720–1740 cm⁻¹ corresponds to C=O ester stretching which resembles PHA. Peaks in the region 1470–1370 cm⁻¹ indicate C–H bending vibrations of alkyl substitution groups and polymer side chains. The range 1280–1050 cm⁻¹ denotes C–O–C ester stretching of ester linkage in PHA.

Figure.1 FTIR Analysis of PHA produced by *Bacillus subtilis*

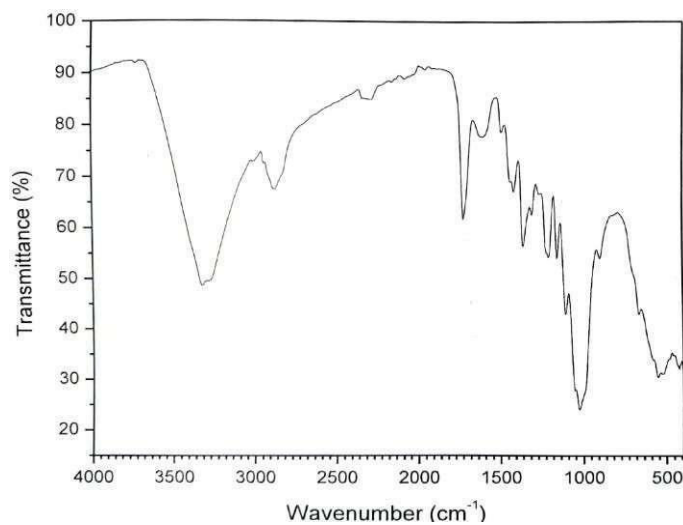


Figure.2 PHA Powder



Figure.3 Bioplastic Material



Figure.4 Presence of PHA Granules

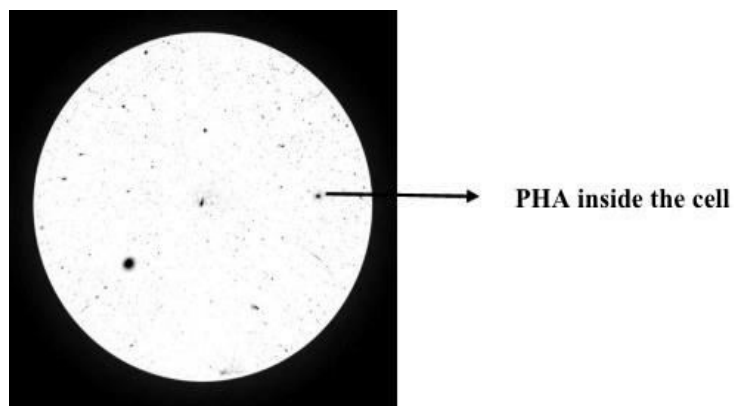


Figure.5 Before Soil Degradation



Figure.6 After Soil Degradation



Table.1 Morphological and Biochemical characteristics of *Bacillus subtilis* a

S. No	Test	Result
1	Colony morphology	White, circular, lobate, irregular margin, flat elevation
2	Gram staining	Positive
3	Endospore staining	Positive
4	Indole test	Negative
5	Methyl red test	Negative
6	Voges Proskauer test	Positive
7	Citrate utilization test	Positive
8	Triple sugar iron test	A/A
9	Urease	Negative
10	Catalase test	Positive
11	Oxidase test	Positive
12	Gelatin Hydrolysis test	Positive
13	Starch Hydrolysis test	Positive

Table.2 Composition of the prepared film

S. No	Composition	g/ml
1	Agar - agar	0.15
2	Chitosan [Dissolve 1% of acetic acid]	0.10
3	Polyhydroxyalkanoate	0.05
4	Glycerol	0.25
5	Distilled Water	10

Table.3 Soil-degradation data of the film after 20 days.

Parameter	Value
Initial weight (g)	0.227
Final weight after 20 days (g)	0.166
Weight reduction (%)	26.9

A peak at 1250 cm^{-1} indicates C–H stretching of alcohol groups, while a strong peak at 1050 cm^{-1} confirms the presence of R–OH groups of primary and secondary alcohols. Multiple peaks in the $1000\text{--}650\text{ cm}^{-1}$ region indicate the alkyl backbone, and peaks in the $980\text{--}800\text{ cm}^{-1}$ region represent the crystalline bands of PHA.

Solubility Testing

The extracted PHA was soluble in chloroform but insoluble in water. This solubility pattern confirms the polymeric nature of PHA. This result was similar to the study of [Mohammed *et al.*, 2022](#).

Melting Point Determination

The melting point of the prepared bioplastic film was determined. On gradual heating, the bioplastic film started softening and losing its original shape at around $60\text{--}65\text{ }^{\circ}\text{C}$. Thus, the melting point of the bioplastic film was found to be around 70°C , indicating its thermoplastic nature

Soil degradation

The film weight decreased from 0.227 g initially to 0.166 g after 20 days of soil burial, corresponding to a 26.9% weight reduction. The calculation is summarized below:

$$\text{Weight reduction (\%)} = [(0.227 - 0.166) / 0.227] \times 100 = 26.9\%$$

The result is presented in (Table 3 ; Figure 5, 6) Visible changes following soil exposure also indicated material deterioration under the tested conditions and indicated a degradation of the film to about 27% of its weight and thickness. The reduction in mass indicates microbial activity and environmental factors actively break down the polymer structure. However, because only short-term mass loss was measured, further studies are required to determine degradation of kinetics and the effects of individual film components and environmental conditions. The environmental relevance of biodegradable microbial polymers and their potential applications in reducing plastic waste have been discussed by [Koller \(2018\)](#), [Rebocho *et al.*, \(2020\)](#), and [Sharma *et al.*, \(2024\)](#). Hence the study strongly suggests the use of PHA as an environmentally friendly polymer and supports its potential use as sustainable alternative for packaging material. The usage of this can hence be

adopted. Overall, this study highlights the feasibility of producing biodegradable bioplastic material from bacterial sources using cost-effective methods. The developed packaging material with antimicrobial property showed promising potential as an eco-friendly alternative to conventional synthetic plastics, contributing toward sustainable material development and environmental protection.

This PHA-based antimicrobial packaging material can be widely applied in food industries, especially for dairy products, meat, poultry, seafood, bakery items, and ready-to-eat foods. Overall, the scope of biodegradable packaging material produced using *B. subtilis* is highly promising. With further research, industrial scaling, and regulatory approvals, this innovation has the potential to transform the packaging sector by offering a sustainable, safe, and environmentally responsible alternative to synthetic plastics.

Author Contributions

S. Amrithalakshmi: Conceived the original idea, designed the study protocol, and wrote the manuscript. S. Anusuya: Designed the model and the computational framework and analysed the data. N. Aarthi: Assisted in the research work and contributed to manuscript preparation. J. Afrose Fathima: Participated in the research work and contributed to revision of the Manuscript. V. Arthika: Supported the research work and contributed to data analysis and manuscript preparation. C. Soundhari: Developed and refined the experimental protocols, and reviewed the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

References

- Amaral-Zettler, L.A., Zettler, E.R., and Mincer, T.J. Ecology of the plastisphere. *Nature Reviews Microbiology*, 2020, 18(3):139–151. <https://doi.org/10.1038/s41579-019-0308-0>
- Akintunde, M.O., Adebayo-Tayo, B.C., Ishola, M.M., Zamani, A., and Sárvári Horváth, I. Bacterial cellulose production from agricultural residues by two *Komagataeibacter* sp. strains. *Bioengineered*, 2022, 13(4):10010–10025. <https://doi.org/10.1080/21655979.2022.2062970>
- Bouallegue, A., Sabbah, M., Di Pierro, P., Salamatullah, A.M., Bourhia, M., and Ellouz-Chaabouni, S. Properties of active levan-bitter vetch protein films for potential use in food packaging applications. *ACS Omega*, 2023, 8(45):42787–42796. <https://doi.org/10.1021/acsomega.3c05627>
- Diop, C.I.K., Beltrán, S., Sanz, M.T., García-Tojal, J., and Trigo-López, M. Designing bilayered composite films by direct agar/chitosan and citric acid-crosslinked PVA/agar layer-by-layer casting for packaging applications. *Food Hydrocolloids*, 2023, 144:108987. <https://doi.org/10.1016/j.foodhyd.2023.108987>
- Fransiska, D., Abdullah, A.H.D., Nurhayati, Irianto, H.E., Nissa, R.C., Sedayu, B.B., Syamani, F.A., Raharjo, S., Suwarti, and Agusman. Impact of agar-glycerol ratios on the physicochemical properties of biodegradable seaweed films: A compositional study. *International Journal of Biological Macromolecules*, 2024, 280(3):135855. <https://doi.org/10.1016/j.ijbiomac.2024.135855>
- Geyer, R., Jambeck, J.R., and Law, K.L. Production, use, and fate of all plastics ever made. *Science Advances*, 2017, 3(7):e1700782. <https://doi.org/10.1126/sciadv.1700782>
- Hahn, S.K., Chang, Y.K., and Lee, S.Y. Recovery and characterization of poly(3-hydroxybutyric acid) in *Alcaligenes eutrophus* H16. *Applied and Environmental Microbiology*, 1994, 60(9):3379–3384. <https://doi.org/10.1128/aem.60.9.3379-3384.1994>
- Iftikhar, N., Quddus, F., Nadeem, N., Ali, I., Raza, M.U., and Zaid, M. Production of polyhydroxyalkanoates (PHA) by *Bacillus* and *Pseudomonas* on cheap carbon substrates. *Brazilian Archives of Biology and Technology*, 2024, 67:e24230082. <https://doi.org/10.1590/1678-4324-2024230082>
- Joseph, J. and Chithira, O.S. Characterisation of polyhydroxybutyrate (PHB) from *Bacillus subtilis* strain P isolated from rhizosphere soil. *International Journal of Current Microbiology and Applied Sciences*, 2021, 10(1):3004–3014. <https://doi.org/10.20546/ijcmas.2021.1001.348>
- Khiabani, A., Sarabi-Jamab, M., Shakeri, M.S., Pahlevanlo, A., and Emadzadeh, B. Exploring the Acetobacteraceae family isolated from kombucha SCOBYs worldwide and comparing yield and characteristics of biocellulose under various fermentation conditions. *Scientific Reports*, 2024, 14:26616. <https://doi.org/10.1038/s41598-024-77305-w>
- Koller, M. Biodegradable and biocompatible polyhydroxyalkanoates as auspicious microbial macromolecules for pharmaceutical and therapeutic applications. *Materials*, 2018, 11(6):1006. <https://doi.org/10.3390/ma11061006>
- Mohammed, S. and Ray, L. Polyhydroxyalkanoate recovery from newly screened *Bacillus* sp. LPPI-18 using various methods of extraction from Loktak Lake sediment sample. *Journal of Genetic Engineering and Biotechnology*, 2022, 20:115. <https://doi.org/10.1186/s43141-022-00392-7>
- Naseem, A., Rasul, I., Raza, Z.A., Muneer, F., Rehman, A.U., and Nadeem, H. Bacterial production of polyhydroxyalkanoates (PHAs) using various waste carbon sources. *PeerJ*, 2024, 12:e17936. <https://doi.org/10.7717/peerj.17936>
- Norozi, M., Peighambaroust, S.H., Khorram, S., and Hamishehkar, H. Cold plasma-enhanced polylactic acid/chitosan films incorporating zein nanoparticles: a promising strategy for developing food packaging materials. *Carbohydrate Polymer Technologies and Applications*, 2025, 10:100852. <https://doi.org/10.1016/j.carpta.2025.100852>
- Rajasekharan, S.K., Paz-Aviram, T., Galili, S., Berkovich, Z., Reifen, R., and Shemesh, M. Biofilm formation onto starch fibres by *Bacillus subtilis* governs its successful adaptation to chickpea milk. *Microbial Biotechnology*, 2021, 14(4):1839–1846. <https://doi.org/10.1111/1751-7915.13665>
- Rebocho, A.T., Pereira, J.R., Neves, L.A., Alves, V.D., Sevrin, C., Grandfils, C., Freitas, F., and Reis, M.A.M. Preparation and characterization of films based on a natural P(3HB)/mcl-PHA blend obtained through the co-culture of *Cupriavidus necator* and *Pseudomonas citronellolis* in apple pulp waste. *Bioengineering*, 2020, 7(2):34. <https://doi.org/10.3390/bioengineering7020034>
- Rybalchenko, N.P., Demchenko, V.L., Hnatiuk, T.T., Vasyliuk, O.M., Iurzenko, M.V., Rybalchenko,

- T.V., Sytnyk, I.O., Shtepa, D.V., and Marynin, A.I. Antimicrobial effect of biopolymer packaging materials with silver nanoparticles for food storage. *Microbiological Journal*, 2024, 86(6):30–41. <https://doi.org/10.15407/microbiolj86.06.030>
- Sharma, D., Singh, S.S., Baindara, P., Sharma, S., Khatri, N., Grover, V., Patil, P.B., and Korpole, S. Surfactin-like broad spectrum antimicrobial lipopeptide co-produced with sublancin from *Bacillus subtilis* strain A52: dual reservoir of bioactives. *Frontiers in Microbiology*, 2020, 11:1167. <https://doi.org/10.3389/fmicb.2020.01167>
- Sharma, M., Tellili, N., Kacem, I., and Rouissi, T. Microbial biopolymers: from production to environmental applications—a review. *Applied Sciences*, 2024, 14(12):5081. <https://doi.org/10.3390/app14125081>
- To, H.T.A. Development of protective culture production from *Bacillus subtilis* P5-6 as food bio-preservative. Chulalongkorn University, 2020. <https://doi.org/10.58837/CHULA.THE.2020.1408>
- Wijayanti, S.D., Oliviani, K., Kusnadi, J., and Putri, R.A.C. Characterization of crude cellulase enzyme produced by *Bacillus licheniformis* P12 isolate. *IOP Conference Series: Earth and Environmental Science*, 2020, 475(1):012085. <https://doi.org/10.1088/1755-1315/475/1/012085>

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