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Enhanced co-production of biopolymer polyhydroxybutyrate (PHB) and biopigment (carotenoid) through wild *Bacillus chungangensis* by utilizing agro-waste substrates

Sathya Priya B [1*], Soundrya S. L [1], Stalin T [2], Charles J. Banks [3], Huang-Mu Lo [4*]

[1] *Department of Environmental Sciences, Bharathiar University, Coimbatore, Tamil Nadu, India*
[2] *Clonal Propagation and Research Centre, Forestry Research & Development, Karur, Tamil Nadu, India*
[3] *University of Southampton, University Road, Southampton, United Kingdom*
[4] *Department of Environmental Engineering and Management, Chaoyang University of Technology, Taiwan*

ABSTRACT

The biopolymer polyhydroxybutyrate (PHB) and biopigment carotenoid produced by microbes holds significant industrial and environmental potential. This research aimed at isolating pigmented bacteria from the Western Ghats of Tamil Nadu, India, which effectively co-produced PHB, adding novelty to the study. The orange pigmented bacterium was further screened for PHB synthesis by Sudan Black B staining. Subsequent 16S rRNA gene sequencing identified the positive isolate as *Bacillus chungangensis*. The isolate exhibited optimal performance at a pH of 10, a temperature of 45°C, and a salinity of 6%, confirming its extremophilic nature. The isolate achieved cost-effective biopolymer and biopigment production by leveraging agro-waste substrates like sugarcane bagasse, groundnut shell, and coconut oil cake. Notably, sugarcane bagasse yielded a remarkable 63% PHB yield and groundnut shell yielded more biopigment, 4.89 g/L surpassing other waste substrates and control. The biopigment exhibited λ_{max} at 450 nm and the FTIR studies confirmed the carotenoid nature of biopigment. Fourier transform infrared (FTIR) analysis of extracted biopolymer from fermented samples of these substrates exhibited a C=O stretch at 1635.64/cm and 1627.92/cm, confirming the presence of polyhydroxybutyrates. This study unveils the potential of *Bacillus chungangensis* for sustainable PHB synthesis with biopigment carotenoid as a co-product by utilizing agro-waste substrates and contributing to ecofriendly biopigment and biopolymer.

Keywords: Polyhydroxybutyrate (PHB), Agro-waste, Biopigments, Biopolymer, *Bacillus*, Ecofriendly, Co-production, Carotenoid

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Corresponding Author:
Sathya Priya. B
sathyapriya@buc.edu.in
Huang-Mu Lo
hmlo@cyut.edu.tw

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1. INTRODUCTION

Synthetic polymers play a crucial role in contemporary society. However, the escalating volume of plastic waste they produce has raised worldwide alarms. The extensive utilization of non-biodegradable synthetic polymers has triggered a significant environmental predicament by Endres (2017). Throughout their life cycle, these synthetic polymers entail diverse hazards to human well-being and the environment.

Despite their manifold societal applications, the accumulation of polymers resistant to microbial degradation persists and grows, exacerbating the environmental and human issues caused by McAdam et al. (2020).

There is a current imperative to discover eco-friendly alternatives to harmful synthetic polymers. As a result, naturally synthesized biopolymers are experiencing a surge in demand as replacements for synthetic polymers due to their non-toxic, renewable, biocompatible, and biodegradable nature. Their popularity has been growing steadily in recent years by Endres (2017). Microbial bioplastics, such as polylactic acid and polyhydroxyalkanoates (PHAs), exemplify renewable raw materials that can be used to create bio-based plastics, according to Juengert et al. (2018). Among the various forms of PHAs, poly-3-hydroxybutyrate (PHB) is the most prevalent. This organic molecule is synthesized by bacteria through the attachment of hydroxybutyrate monomers to ester linkages by McAdam et al. (2020). Lu et al. (2004) highlight the economic and environmental significance of developing these materials. The toxic nature of synthetic dyes forced us to select an ecofriendly alternative. The microbial pigments are more favorable than plant-based derivatives due to their availability throughout the year and increased growth within a shorter period. There is a great demand for microbial-based natural dyes due to their ecofriendly nature and safety for human beings. Exploring a new microbial strain that synthesize pigments is the need of the hour to meet the rapidly growing global demand for cost-effective natural pigments. The bacterial pigments are more favorable due to their simple cultivation and scale-up process than other microorganisms by Numan et al. (2018). The carotenoid pigments obtained from microbes are widely studied due to their health benefits and also enormous applications in the food, feed, pharmaceutical, cosmetic, textile and dyeing industries. Cost-effective production is a major constraint even though the biopolymer PHB and biopigment carotenoid are industrially important bio metabolites obtained from microorganisms.

To address this challenge, researchers have explored the utilization of renewable substrates as substitute for traditional feed stocks. Agricultural waste (agro-waste), which is readily available and very inexpensive, has emerged as a promising solution for cost-effective PHB production and biopigment carotenoid. Agro-waste is more suitable for both microbial growth and effective PHB and carotenoid synthesis. Currently, there is a huge demand for natural based carotenoid due to their production rate being small, i.e. 24 percent compared to synthetic carotenoids by Ram et al. (2020). Kumar et al. (2021), who used *Iodobacter* sp. for the synthesis of PHB with violacein as a co-product by using different carbon and nitrogen sources. Pallath et al. (2023) observed orange carotenoid pigment production using *Planococcus maritimus*. Dawoud et al. (2020) studied the yellow pigment of *Bacillus* species. Vila et al. (2019) noted carotenoid pigment from *Flavobacterium*, Korumilli and Mishra (2014) showed the carotenoid production by *Bacillus claussi* using rice powder as substrate. Ratnakaran et al. (2020) synthesized carotenoid pigments using different bacterial isolates.

Numerous studies by Sawant et al. (2014), Kourmentza et al. (2017), Sathiyanarayanan et al. (2017), Bhatia et al. (2018), Pradhan et al. (2018), Hong et al. (2019), Koch et al. (2019), Choi et al. (2020), Lamberti et al. (2020) investigated the synthesis of biopolymer with the utilization of different carbon sources. The higher the utilization of carbon substrates elevates the expenses of PHB production. Hence, agro-waste could be used as a cost-effective feedstock for the synthesis of biopolymer PHB. El-Sheekh et al. (2015) reported the production of PHB by *Bacillus flexus* ME-77 using molasses. Getachew and Woldesenbet (2016) identified a *Bacillus* sp. that produced PHB by effectively utilized resources such as banana peels, maize cobs, sugarcane bagasse, corn cobs, and Teff straws. Sakthiselvan and Madumathi (2018), observed PHB production in *Bacillus safensis* EBT1 with sugarcane bagasse as a feedstock. Saratale et al. (2019) produced PHB from *Ralstonia eutropha* from Paddy straw and Kenaf biomass, respectively. Poomipuka et al. (2014) identified PHB production from the *Cupriavidus* sp., using Cassava starch. In a separate study, Danial et al. (2021) observed that *B. wiedmannii* AS-02 OK576278 strain synthesized PHB using diverse agricultural waste materials, including onion, banana, mango, orange, and rice straw peels. Kalaivani and Sukumaran (2015) and Desouky et al. (2017) studied maximum PHB production from molasses using *Bacillus thuringiensis* and *Bacillus* sp. KSN5.

The co-production of biopigment carotenoid and biopolymer PHB by single fermentation using agro-waste as a feedstock has not been reported so far. Therefore, the primary objective of this study was to isolate carotenoid-based pigmented bacteria from the Western Ghats region and employ them for the synthesis of biopolymer PHB with co-production of biopigment carotenoid by utilizing economical agricultural waste materials to mitigate production costs (Fig. 1). The resulting biopolymer and biopigment were subsequently subjected to FTIR analysis for characterization.

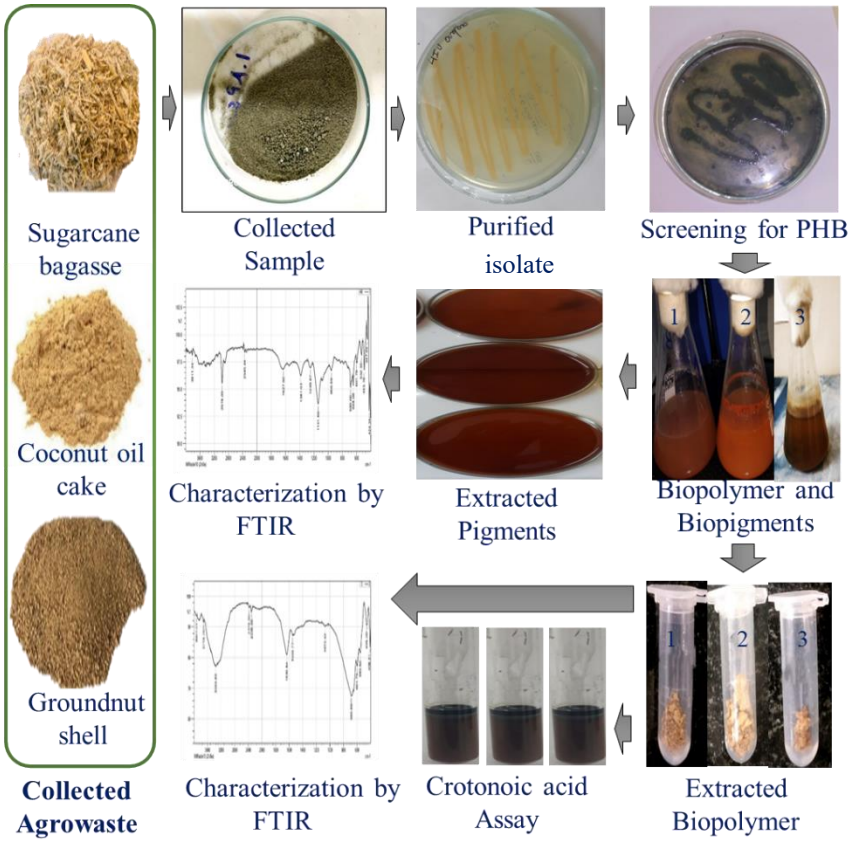

Fig. 1. Graphical scheme of the research work

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil and sediment samples were collected from various locations within the Western Ghats of Tamil Nadu, India. Clean, dry, and sterile containers were employed for collection and the samples were promptly transported to the laboratory. The agro-waste materials such as sugarcane bagasse, groundnut shell and coconut oil cake were collected from the local market in Coimbatore.

2.2 Bacterial Isolation

The bacterial species were isolated using a serial dilution and spread plate technique on nutrient agar medium (NAM). The medium's (Himedia) composition comprised: peptone (5 g), yeast extract (1.5 g), sodium chloride (5 g), and agar (15 g), which is dissolved in 1000 mL of distilled water and sterilized in an autoclave at 121°C at 15 lbs for 20 min. For sample preparation, 1 g of the sample was mixed with 100 mL of distilled water and vigorously vortexed for 15 min to get a sample suspension. Serial dilutions were then performed, ranging from 10^{-1} to 10^{-6} (which corresponds to the dilution factor of 1/10 to 1/1000000). A volume of 0.1 mL from each suspension was spread on NAM plates using the spread plate method. The plates were incubated at 37°C in an incubator to facilitate bacterial growth. Isolated colonies were selected, pure-cultured, and preserved on nutrient agar slants at 4°C for future use. The isolate's morphological characteristics were studied, and a Gram staining procedure was conducted following the guidelines outlined in Bergey's Manual of Systematic Bacteriology by Williams et al. (1989).

2.3 Screening for PHB-producing bacteria

The pure culture of the carotenoid based pigmented bacteria, red, orange and yellow were selected for biopigment synthesis and further screened for PHB production. The isolates were stained in Sudan Black B and kept for 30 min, followed by washing with 90% ethanol to remove the excess stain. The positive isolates were confirmed by the presence of bluish colonies.

2.4 Molecular Identification of Isolate via 16S rRNA Gene Sequencing

Genomic DNA was extracted from the bacterial samples using the lysis method and subsequently amplified via polymerase chain reaction (PCR). The resulting amplified DNA served as the basis for identifying the isolate through the 16S rRNA gene sequencing technique. For this purpose, single-pass sequencing was executed for each template, utilizing 16S rRNA universal primers. Fluorescent-labeled fragments were then purified from unincorporated

terminators through ethanol precipitation. The obtained 16S rRNA sequence was subjected to a similarity search using the NCBI BLAST tool. The analysis was performed using closely related sequences from the BLAST results, followed by multiple sequence alignment. The generated sequences were subsequently deposited in GenBank.

2.5 pH, Salinity, and Temperature Optimization

The adaptability of the isolates to different pH levels, temperatures, and salinities was evaluated. Sterile nutrient broth (approximately 15 mL) was dispensed into distinct test tubes, and the pH was modulated across a range of 6 to 12 using 1 N solutions of hydrochloric acid and sodium hydroxide. Salinity variations from 1% to 10% were achieved through the addition of sodium chloride solution, while temperatures of 37, 40, 45, and 50°C were employed. A loopful of the isolated culture was inoculated into each test tube, followed by incubation at 37°C in an incubator. The growth of the isolate was tracked using a UV-visible spectrophotometer set at 600 nm.

2.6 Hydrolysis Test

2.6.1 Starch hydrolysis

The isolate under examination was introduced into starch agar media and subsequently incubated at 37ºC for 24 h. Following the incubation period, an iodine solution was introduced. The presence of a clear zone surrounding the tested isolate signified the successful hydrolysis of starch.

2.6.2 Cellulose hydrolysis

The isolate's cellulose hydrolysis capabilities were assessed. Carboxymethyl cellulose agar plates were inoculated with the test culture and subsequently incubated at 37°C for 24 h. Following the incubation period, the plates were scrutinized for the development of any observable zones.

2.6.3 Lipid hydrolysis

The test isolate was introduced onto a Tributyrin agar plate, followed by incubation at a temperature of 37ºC for 24 h. Organisms capable of lipase production and subsequent tributyrin breakdown would manifest as a discernible clear halo around the application areas.

2.7 Co-Production of Biopigment and Biopolymer using Different Waste Substrates

Agro-waste substrates including coconut oil cake, groundnut shell, and sugarcane bagasse were procured and subjected to shade drying. Post-drying, the waste materials were finely pulverized and sieved through a 2.0 mm pore-sized sieve. For pretreatment, 5 g of each agro-waste substrate underwent hot water hydrolysis. To prepare the inoculum, a loopful of the isolate was introduced into 100 mL of sterile nutrient broth. This mixture was incubated in a shaker at 37°C and 160 rpm for 24 h. This cultivated inoculum was used subsequently. Each pretreated agro-waste substrate serves as a growth medium for PHB and biopigment synthesis by the isolate. A control setup was established using nutrient broth. In all flasks, a 10 percent inoculum was introduced alongside the three distinct waste substrates. The conical flasks were placed in an incubator shaker at 37°C and 160 rpm for a 12-day incubation period. All experiments were conducted in triplicate.

2.8 Extraction of Biopigment and Biopolymer from Biomass

Following a 12-day incubation period, the broth was subjected to centrifugation at 8000 rpm for 15 min. The resultant supernatant and the pellet were extracted by using ethanol with centrifugation at 3000 rpm for 10 min. The dry weight of the final pellets was then measured.

2.9 Characterization of Biopigment and Biopolymer

The extracted biopigment was further subjected to UV-visible spectroscopy analysis to find out the maximum absorbance λ_{max} by using the scanning range of 300–900 nm.

2.10 Biopolymer Purification

The pellets were subjected to purification using the sodium hypochlorite-chloroform method. Approximately 50 mg of the dried pellet was employed for this process. To this, 3 mL of 4% sodium hypochlorite was introduced, and the mixture was heated to 50°C for 1 h. Subsequently, the solution was subjected to centrifugation at around 3000 rpm for 15 min. The resultant polymer was then dissolved in chloroform and left for evaporation. After evaporation, the samples were carefully scraped and designated for subsequent characterization, following the methodology outlined by Law and Slepecky (1961).

2.11 Crotonic Acid Assay

The confirmation of the resulting biopolymer was extended through the crotonic acid assay method. In this process, 0.5 g of biopolymer was combined with 10 mL of concentrated sulfuric acid, and the mixture was subjected to a 100°C water bath for 10 min. During this period, an observable brown color in the solution was noted. The release of crotonic acid was quantified using spectrophotometric analysis, employing a UV-visible spectrophotometer set at 235 nm, following the approach described by Law and Slepecky (1961).

2.12 FTIR Analysis

The purified biopigment and biopolymer underwent FTIR spectroscopic analysis over a frequency range of 4000–400/cm. This analysis aimed to discern the diverse functional groups within the biopigment and biopolymer. The KBr Pellet method was employed for this analysis.

3. RESULTS AND DISCUSSION

3.1 Bacterial Isolation and Identification

The bacterial species with red, orange and yellow pigment were isolated from the Western Ghats of Tamil Nadu by serial dilution and spread plate technique. Based on the efficiency, the orange pigmented bacterial species was selected from 10 bacterial isolates and screened for PHB synthesis by Sudan Black B staining. It showed a greater prevalence of positive results indicating the presence of PHB granules in the screening test. Similarly, Mayeli et al. (2015), Juengert et al. (2018) and Mostafa et al. (2020) investigated the presence of PHB granules using Sudan Black B staining. In the primary screening, the test isolate showed a positive sign for PHB accumulation, evidenced by a change in colony color to dark greenish-blue. To further confirm the PHB-producing competence of the test isolate, microscopic observations were conducted. The Sudan Black B-stained isolate exhibited a slight reddish-dark color with vesicle-like granules under standard microscopic observation, aligning with observations by Kalaivani and Sukumaran (2015) and Mahitha and Madhuri (2015). The strong attraction of Sudan Black-B to lipid-interacted biopolymer further validates its significance in screening PHB producers, as discussed by Moorkoth and Nampoothiri, (2016). The isolate was rod-shaped and exhibited gram-positive characteristics. Subsequently, 16S rRNA gene sequencing affirmed the identity of the isolate as *Bacillus chungangensis*. This genetic sequence was duly submitted to GenBank and assigned the accession number MZ474520.1.

3.2. Optimization of Isolate and Enzymatic Hydrolysis

Optimization experiments revealed that the isolate's optimal conditions included a pH of 10, a temperature of 45°C, and a salinity level of 6%. It confirmed the extremophilic nature of the isolate adapted to unique environmental conditions within the Western Ghats. Hence it favors the dual production of biopigment and biopolymer. Notably, *Bacillus chungangensis* was previously identified as a halophilic species isolated from sea sand (Cho et al., 2010). The isolate showed positive results for starch, cellulose and lipid hydrolysis. It confirmed the efficiency of the isolate to produce multi enzymes such as amylase, cellulase and lipase.

3.3 Co-production of Biopigment and Biopolymer using Different Agro-waste

The isolate's proficiency in hydrolyzing starch, cellulose, and lipids was evaluated, revealing positive outcomes for each respective hydrolysis process. Abundant agro-waste materials, namely sugarcane bagasse, groundnut shell, and coconut oil cake, underwent hot water pretreatment. This pretreatment and the efficiency of isolate for multi enzyme production facilitated the hydrolysis of the waste, generating easily accessible fermentable substrates. Consequently, the isolate's fermentation process was augmented, benefitting from both the readily available nutrient substrates and its inherent hydrolytic enzymes. It enhanced the co- production of biopigment and biopolymer. The control was maintained without any agro-waste. The effective utilization of complex waste materials by the isolate for biopolymer PHB synthesis and reddish orange biopigments were underscored, emphasizing an eco-friendly approach that concurrently curtailed production costs. This eco-friendly approach simultaneously minimized the production costs. These studies altogether demonstrate the feasibility of using agro-waste materials as substrates for the co-production of biopigment and biopolymer. *Bacillus thuringiensis* IAM 12077, a PHB-producing strain, has shown positive results in agro-waste based PHA production using the innate enzymatic potential (amylase) by Gowda and Shivakumar (2014).

3.4. Mechanism of Biomass Digestion by *Bacillus* to Produce Biopolymer PHB

The consumption of amino acids, sugar, and fatty acids produces PHB through the acetyl-CoA, and various routes are represented in Fig. 2. Microorganisms synthesize PHB by concentrating two acetyl-CoA molecules, resulting in the formation of acetoacetyl-CoA, which is then condensed to hydroxybutyryl-CoA. This complex is a crucial component in the synthesis of PHB. PHA particles are extracted by disrupting the structure of the cell. PHB, an environmentally friendly plastic with biopigmentation was first discovered in *Bacillus chungangensis*. Various bacterial species accumulate biodegradable PHAs offering thermomechanical properties similar to synthetic polymers like polypropylene (Kumalaningsih et al., 2011). The molecular structures of PHBs serve as an energy reserve and develop in environments rich in carbon. PHB production in acetyl-CoA producing bacteria involves a series of enzymatic reactions. Firstly, 3-ketothiolase (encoded by the phbA gene) catalyzes the formation of a carbon-carbon bond through the condensation of two acetyl-CoA molecules. Secondly, acetoacetyl-CoA is reduced to R-3-hydroxybutyryl CoA by NADPH-dependent acetoacetyl-CoA reductase. Lastly, PHB is synthesized when PHB synthase catalyzes the polymerization of R-3-hydroxybutyryl-CoA. This biosynthetic process generates a PHB polymer with partial crystalline properties, resembling polypropylene and polyethylene. PHB is considered a promising biodegradable plastic and an alternative to synthetic and petrochemical plastics due to its biocompatibility, biodegradability, and versatile applications.

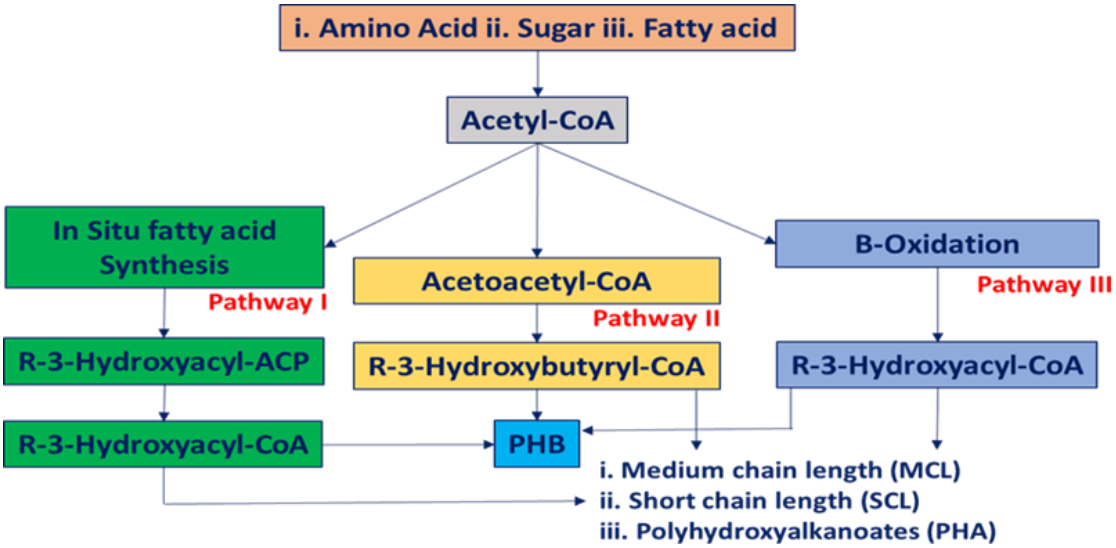

Fig. 2. Mechanism of biomass digestion with isolated *Bacillus* to produce PHB

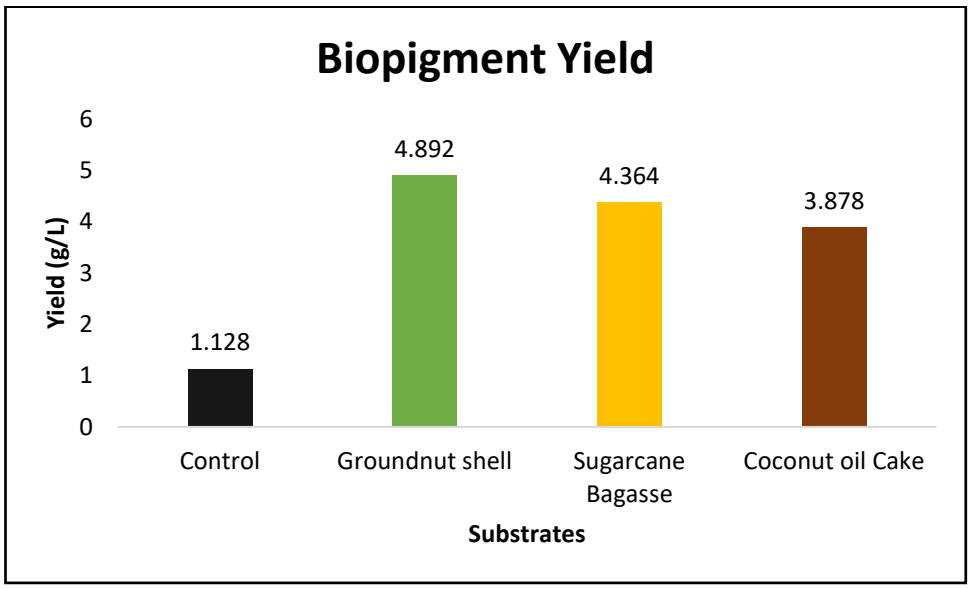

Fig. 3. Yield of biopigment by *Bacillus chungangensis* using different agro-waste

3.5. Yield of Biopigment and Biopolymer

Fig. 3 shows the results of the biopigment yield using different agro-wastes as feedstock. The biopigment yield was very high in groundnut shell, with 4.89 g/L, compared to sugarcane bagasse and coconut oil cake. The yield was very low in control compared with the agro-waste. Kumar et al. (2021) observed that the pigment yield of violacein was 1.50 g/L for *Iodobacter sp*. Kazi et al. (2022) observed a pigmentation of 3.00 g/L in *Streptomyces sp*. This shows the yield of carotenoid pigment by *Bacillus chungangensis* was very high using agro-waste as feedstock.

Analysis of the results from Figs. 4 and 5 depicted the weight of dry cell, which is the quantitative assessment of the cellular biomass vs the weight of accumulated biopolymer and its percentage. The results showed the highest PHB accumulation of 63% with 12.29 g/L as biopolymer yield was observed in the fermentation medium with sugarcane bagasse. The biopolymer yield of 9.43 g/L and 5.88 g/L with 56% and 48% was noticed in groundnut shell and coconut oil cake fermentation media. Whereas, in control 14% of PHB accumulation was found. Among the three agro-wastes, sugarcane bagasse established the highest PHB production efficiency and the control showed the lowest efficiency at 14%. Sakthiselvan and Madhumathi (2018) reported 5.90 g/L with 32% yield from sugarcane bagasse using *Bacillus safensis* EBT1. Similarly, *Bacillus* strain produced PHB using pineapple and sugarcane agro-waste with a yield of 53% reported by Suwannasing et al. (2015). Ashby et al. (2011) observed 38% of PHB yield *by Pseudomonas oleovorans* from crude glycerol. The isolate *Bacillus megaterium* yielded 29.6% PHB from the cassava starch was reported by Krueger et al. (2012). Sukan et al.

(2014) observed a PHB production of 1.24 g/L with a 41% yield from *Bacillus subtilis* OK2 using orange peel. Furthermore, an observation of *Cupriavidus necator* using sugarcane molasses yielded 56% was noted by Dalsasso et al. (2019). Our findings suggest that *Bacillus chungangensis* exhibits a competitive or even superior performance compared to other reported strains in utilizing certain agro-waste materials for PHB production. The FTIR results confirmed the biopigment as carotenoid.

3.6 Characterization of Biopigment

Fig. 6 demonstrated the UV-visible spectrophotometric analysis results of the extracted pigment from *Bacillus chungangensis*. The λ_{max} at 450 nm confirmed the carotenoid nature of the pigment. Mostly the absorption of carotenoids occurs in the visible range of 400–500 nm (Ram et al., 2020). Similarly, Pallath et al. (2023) confirmed the presence of carotenoid pigment at 447 nm. Priya et al. (2017) observed the peak for carotenoid pigments at 450 nm. Ratnakaran et al. (2020) studied the λ_{max} of carotenoid pigment from bacterial isolate at 420 nm. Korumilli and Mishra et al. (2014) obtained the peak at 447 nm for carotenoid pigment from *Bacillus clausii*. The results confirmed that the pigment extracted from *Bacillus chungangensis* was carotenoid due to the characteristic reddish orange colour and exhibited λ_{max} peak at 450 nm, which has a similarity with other reports.

Fig. 7 demonstrated the FTIR spectrum of biopigment extracted from *Bacillus chungangensis* using agro-waste groundnut shell as feed stock. It showed a peak at 3423.03 /cm which corresponds to the O-H stretching of alcohol. Another peak at 2927.41/cm depicted the C-H bonds indicating the presence of amines. Additionally, a peak at 2364.30/cm showed the presence of nitrile compounds. The peaks at 1644.98, 1405.85/cm indicated the presence of alkenes and C-C aromatics. The peak at 1105.01/cm was attributed to alkyl amines and 615.18/cm indicated the halogen compounds. The results have the similarity with standard beta carotene, which showed the peaks at 2972.4, 2874.9, 1717.4, 1447.6, 1174.6, 1070.6, 967.4/cm (Sharma and Ghosal, 2021). Similarly, Pallath et al. (2023) obtained the peaks for carotenoid pigment at 3345.36, 1635.25, 1403.47, 1085.62 and 1045.60/cm. Korumilli and Mishra (2014) studied the FTIR spectrum peaks at 3320, 2943, 2832, 1652, 1448, 1405, 1100 and 1020/cm for carotenoid pigment extracted from *Bacillus species*. The FTIR spectrum showed the similarity with peaks obtained from standard carotene and other studies related with carotenoid pigment. It confirmed the presence of carotenoids in biopigment synthesized by *Bacillus chungangensis* using agro-waste.

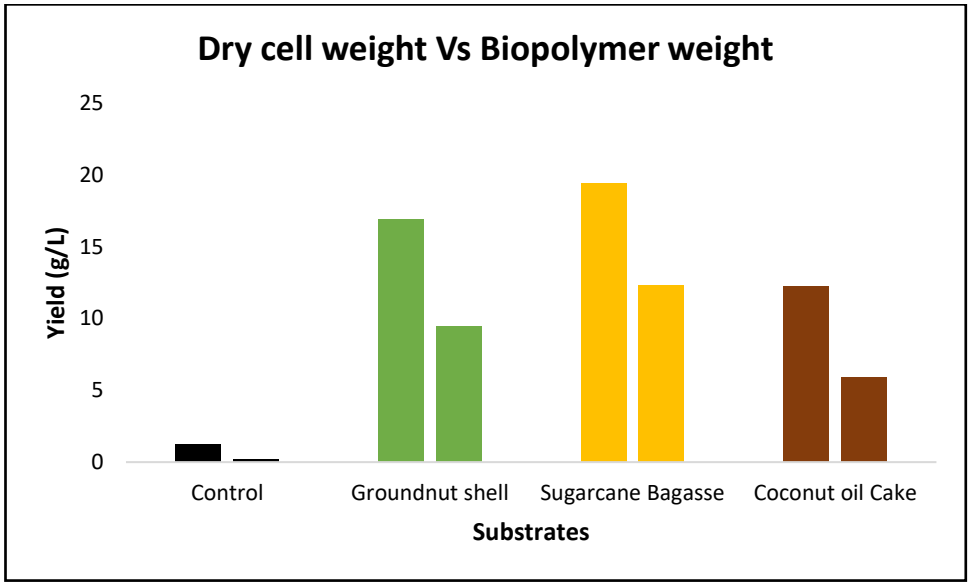

Fig. 4. Weight of dry cell (biomass) and biopolymer obtained from *Bacillus chungangensis* using different agro-waste

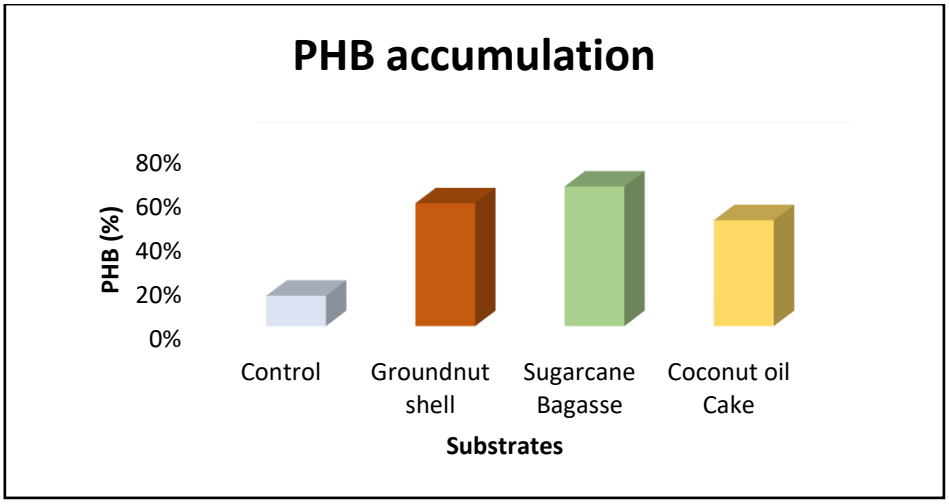

Fig. 5. Percentage of accumulated PHB from *Bacillus chungangensis* using different agro-waste

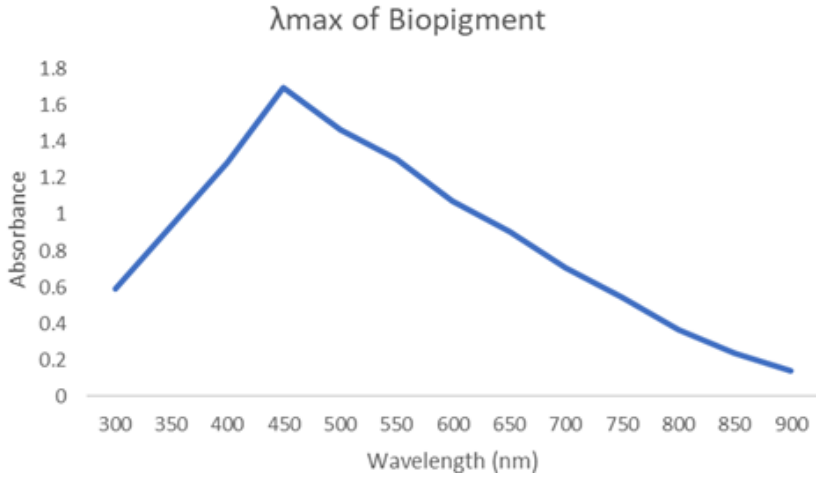

Fig. 6. UV-visible spectrophotometric analysis of reddish orange pigment synthesized by *Bacillus chungangensis*

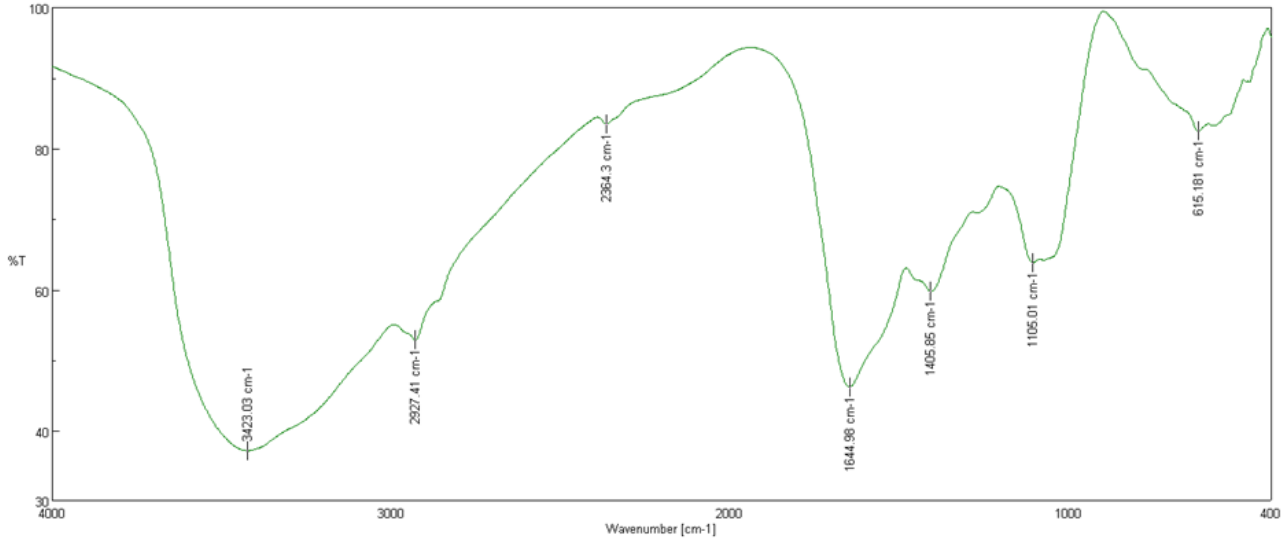

Fig. 7. FTIR spectrum of biopigment produced by *Bacillus chungangensis* using agro-waste groundnut shell

3.7. Characterization of Biopolymer PHB

In the FTIR spectrum shown in Fig. 8, acquired for the PHB extracted from *Bacillus chungangensis* strain using sugarcane bagasse as the agro-waste substrate, distinct bands were observed that corresponded to different functional groups. A peak at 3363.86/cm was associated with the hydroxyl (O-H) group of alcohols. Smaller peaks at 2376.30/cm and 2306.86/cm indicated the presence of nitrile compounds. A prominent peak at 1635.64/cm was attributed to the C=O stretch of a ketone compound. Another observed band at 1550.77/cm was linked to NH, indicating asymmetrical stretching in the amide group, while 1072.42/cm was suggestive of PO_3 stretching, signifying phosphate ion compounds. The remaining peaks were closely aligned within the range of 3430 to 400/cm. The presence of the C=O bond at 1635.64/cm in the FTIR spectrum of the extracted PHB confirms the successful production of the biopolymer by *Bacillus chungangensis* using sugarcane bagasse as the substrate. This finding is crucial for validating the results of the study and demonstrating the potential of this approach for sustainable and eco-friendly biopolymer production. Similar studies conducted by Getachew and Woldesenbet (2016) also observed peaks indicating C=O bond extension in various waste substrates. Gowda and Shivakumar (2014) observed a peak at 1629/cm attributed to the C=O stretching vibration in PHB produced by *Bacillus thuringiensis* using mango peel extract. A peak at 1714/cm was reported by Shah (2014) for PHB synthesized by G1S1 *Bacillus subtilis*. Aryaraj and Pramitha (2021) observed a peak at 1692/cm with the C=O stretching vibration for PHB produced by *Bacillus flexus*.

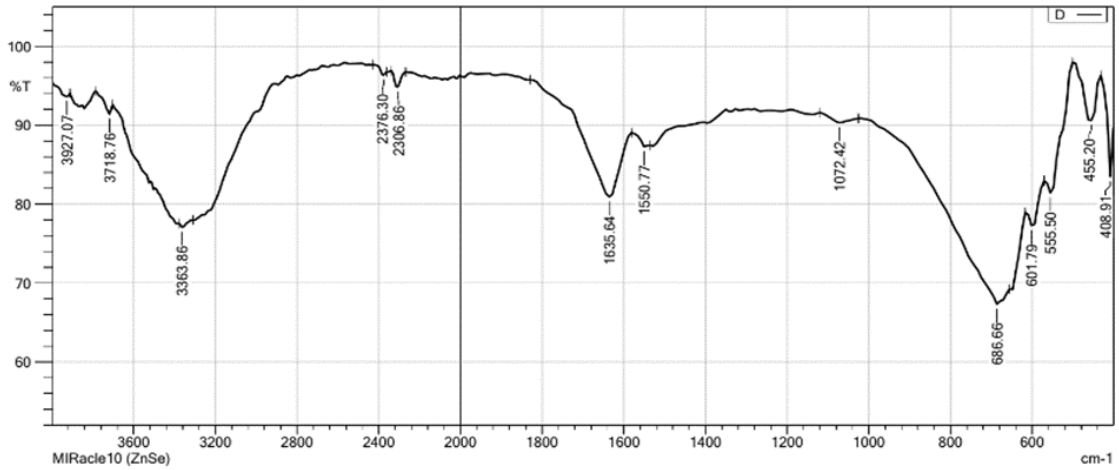

Fig. 8. FTIR analysis of PHB synthesized by *Bacillus chungangensis* using sugarcane bagasse

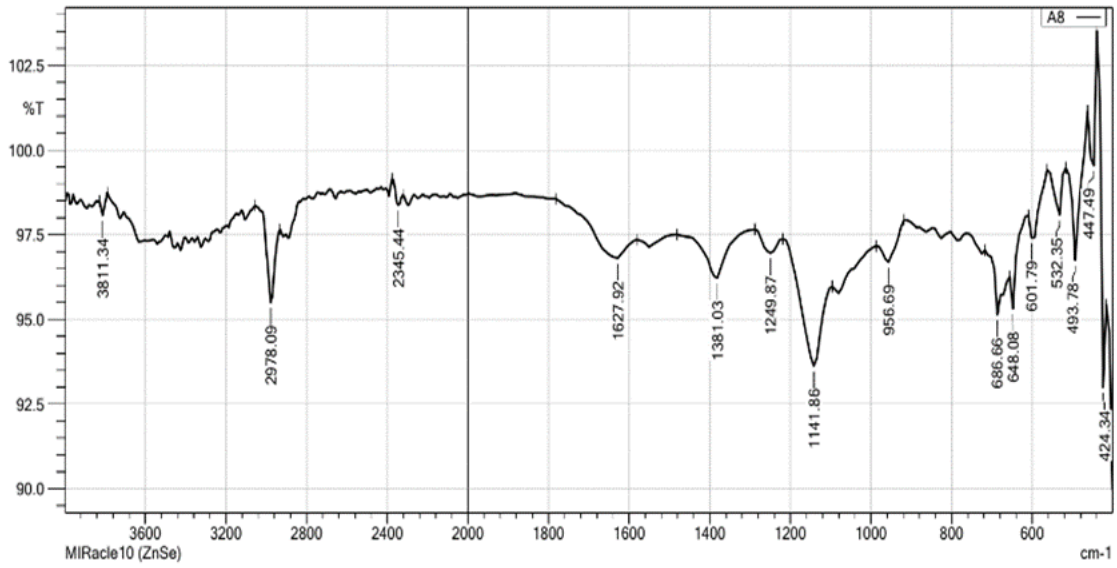

Fig. 9. FTIR analysis of PHB synthesized by *Bacillus chungangensis* using groundnut shell

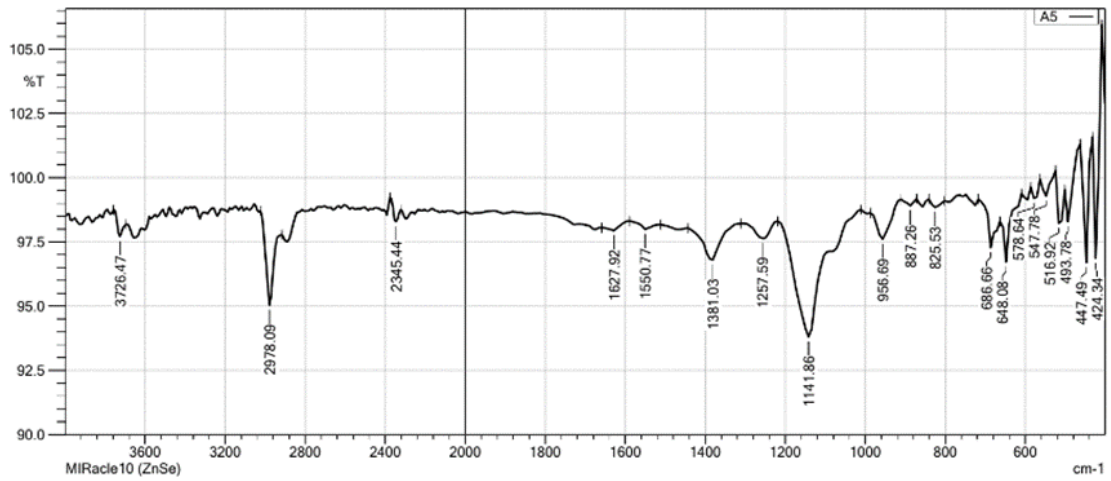

Fig. 10. FTIR analysis of PHB synthesized by *Bacillus chungangensis* using coconut oil cake

For PHB extracted from *Bacillus chungangensis* using groundnut shell as a feedstock, the IR spectra (Fig. 9) displayed distinct bands. The peak at 3811.34/cm indicated a non-bonded OH stretch, representing a hydroxyl group. The presence of (C-H) stretching vibrations of methyl and methylene groups was evident at 2978.09/cm. The appearance of 2345.44/cm was attributed to the C=N stretch, indicative of nitrile compounds. A significant peak at 1627.92/cm pointed towards C=O stretching vibrations, affirming the presence of the ketone compound and confirming the polyhydroxybutyrate structure. This aligns with observations by Bhuwal et al. (2013) and Anjali et al. (2014) regarding C=O stretching and C-H stretching vibrations. Ratnawati et al. (2024) also observed C=O stretching vibrations with peak of about 1642/cm. Shah (2012) detected a thioester bond in 1639/cm. Aluru (2020), had found 1609/cm a peak, from *Bacillus* sp., RR02 which represents C=O ester group, which also supports our results.

In the FTIR spectrum derived from PHB extracted from *Bacillus chungangensis* using coconut oil cake as the agro-waste substrate (Fig. 10), the band at 3726.47/cm was attributed to the hydroxyl (O-H) group of alcohols. A distinctive stretch at 1627.92/cm indicated the C=O stretch of ketone compounds, reinforcing the presence of PHB. The appearance of 2978.09/cm correlated with CH (CH_2) stretching bonds. These observations parallel those made by Kumalaningsih et al. (2011). The peak at 1550.77/cm suggested C=C-C, an aromatic compound. Joseph and Chithira (2021), observed a peak in 1636.61/cm. The functional groups of the polymer reported were at 1720 (ester C=O), 1639/cm (thiosester C=O). The FTIR spectrum is found very close to FTIR spectra of PHB extracted from *Bacillus shackletonii* K5 by Liu et al. (2014), *Bacillus magaterium* by Dhangdhariya et al. (2015) and commercial PHB by Han et al. (2015) and the results of standard PHB by Ramezani et al. (2015). This finding is in line with results of Nair et al. (2014) and Sabapathy et al. (2019), which confirmed the presence of PHB through similar stretching patterns.

The findings highlight the potential of *Bacillus chungangensis* for efficient production of cost-effective PHB and biopigment carotenoid from readily available agro-waste resources. The screening of the isolate from the extreme niches such as the Western Ghats of Tamil Nadu adds another advantage to its distinctive extremophile nature of isolate. The expense for individual production of microbial metabolites is very high. Hence, co-production of two metabolites by single fermentation using agro-waste reduced the production cost. This method is economically viable and industrially most significant. The multienzymatic hydrolysis and extremophile nature of the isolate enhanced the breakdown and easy availability of waste substrate to the isolate. The distinctive nature of *Bacillus chungangensis* enhanced the production and yield of the two industrially important metabolites biopolymer PHB and biopigment carotenoid using inexpensive waste substrates.

CONCLUSION

In this study, *Bacillus chungangensis* was effectively harnessed for biopigment carotenoid and biopolymer polyhydroxybutyrate (PHB) production as a coproduct using diverse agro-waste substrates, including sugarcane bagasse, groundnut shell, and coconut oil cake. Optimization of key parameters pH (10), temperature (45°C), and salinity (6%) underscored the isolate's extremophilic nature and industrial viability. Leveraging its hydrolytic enzymes, the isolate efficiently converted agro-waste into valuable PHB with co-product biopigment carotenoid, showcasing a promising avenue for cost-effective nutrient sourcing in PHB and biopigment manufacturing. These findings hold the potential to mitigate the high production costs associated with biodegradable

plastics and biopigments offer a sustainable alternative to petroleum-based plastics and synthetic dyes in industrial contexts. As a substantial contribution, this research underscores *Bacillus chungangensis*-mediated PHB synthesis with biopigmentation from agro-waste as an environmentally-friendly solution, bridging the gap towards more sustainable plastic and natural dye production.

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