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Bioprospecting antimicrobial biopolymer PHB from *Bacillus chungangensis* as an efficient antimicrobial agent to control human pathogens in the healthcare sector

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Abstract

The increasing environmental concerns of synthetic polymers, particularly their non-biodegradability and pollution, have led to a search for sustainable alternatives. Multidrug antibiotic-resistant microorganisms are increased in number due to the continuous use of synthetic antibiotics. Biopolymers, such as polyhydroxybutyrate (PHB), offer a promising solution to petroleum-based plastics and synthetic antibiotics. *Bacillus chungangensis* bacteria was isolated from the soil samples of the Western Ghats of Tamil Nadu, India, and studied for the synthesis of PHB using various carbon sources including the waste sugarcane bagasse to enhance both sustainability and cost-efficiency. The antimicrobial efficiency of biopolymer from *Bacillus chungangensis* was evaluated against multiple human pathogens. It exhibited excellent antibacterial activity compared to the standard antibiotics against a range of pathogens such as *Escherichia coli* (25 mm), *Pseudomonas aeruginosa* (24 mm), *Proteus vulgaris* (20 mm), *Shigella flexneri* (20 mm), *Pseudomonas fluorescens* (23 mm), *Klebsiella pneumonia* (15 mm) and *Staphylococcus aureus* (15 mm). The standard antibiotics gentamycin, tetracycline, methicillin, and kanamycin did not produce the zone against *Klebsiella pneumonia*. Similarly ampicillin and methicillin not produced the zone of inhibition against *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. These results suggest that biopolymers produced by *Bacillus chungangensis* are highly effective antibacterial agents against human pathogenic bacteria.

Keywords: Polyhydroxybutyrate (PHB); *Bacillus chungangensis*; antimicrobial biopolymer, antimicrobial agent, human pathogens, health care sector

Introduction

The widespread use of synthetic polymers, particularly plastics, has transformed various sectors due to their versatility, durability, and cost-effectiveness. However, the environmental challenges posed by these materials, including their non-biodegradability and significant contribution to pollution, have raised serious concerns (Haward, 2018). The accumulation of plastics resistant to microbial degradation continues to exacerbate environmental and health issues (Adam et al., 2020). Additionally, the use of synthetic antibiotic agents paved the way for the development of multiresistant antibiotic microorganisms which cause severe health issues and is very difficult to control. This highlights a concerning trend of antibiotic resistance among these pathogens, which is increasingly prevalent in clinical settings (Hasan et al., 2020). The resistance observed in these pathogens may be attributed to intrinsic mechanisms, such as the production of beta-lactamases or efflux pumps, which are known to confer resistance to multiple antibiotic classes (Askar et al., 2018).

Human pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Shigella flexneri*, *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, and *Staphylococcus aureu*s, are well-known for causing serious infections in humans. *Escherichia coli* is one of the main causes of urinary tract infections (UTIs) and stomach illnesses, especially due to harmful strains like enterohemorrhagic and uropathogenic *E. coli* (Peng et al., 2024). *Pseudomonas aeruginosa* is a dangerous pathogen that causes lung infections, especially in cystic fibrosis patients, as well as wound infections and blood infections in people with weakened immune systems (Moradali and Rehm, 2020). *Proteus vulgaris* is linked to UTIs, often in patients with catheters and hospital-acquired infections (Armbruster and Mobley, 2012). *Shigella flexneri* is a major cause of dysentery leading to severe stomach problems (Anderson et al., 2016). *Pseudomonas fluorescens* is not usually harmful but can cause infections in hospitals, mainly in people with weak immune systems (Hernandez et al., 2020). *Klebsiella pneumoniae* is becoming more dangerous due to drug-resistant strains, causing pneumonia, UTIs, and blood infections, especially in hospitals (Paczosa and Mecsas, 2016). Lastly, *Staphylococcus aureus*, particularly the methicillin-resistant strain (MRSA), is a major cause of skin infections, blood infections, and pneumonia, with drug-resistant strains making treatment difficult (Tong et al., 2015). This situation has created an urgent need for eco-friendly alternatives to harmful synthetic polymers, leading to a growing interest in naturally synthesized biopolymers and antibiotic agents.

Biopolymers, such as polyhydroxybutyrate (PHB), are gaining traction as sustainable replacements for conventional plastics due to their non-toxic, renewable, biocompatible, and biodegradable properties (Endres, 2017). PHB, a type of polyhydroxyalkanoate (PHA), is produced by various microorganisms and has emerged as a promising substitute because of its biodegradability and material properties comparable to those of traditional plastics (Ciesielska & Kiewisz, 2016). Among the different forms of PHAs, poly-3-hydroxybutyrate (PHB) is the most prevalent, synthesized by bacteria through the polymerization of hydroxybutyrate monomers (Adam et al., 2020). The economic and environmental significance of developing such materials is underscored by Lu et al. (2004), who highlight the potential of PHB to address the issues associated with synthetic plastics while promoting waste valorization.

The production of PHB from renewable resources, particularly agro-industrial waste, not only mitigates the environmental impacts of synthetic plastics but also enhances sustainability (Liau et al., 2014). Among the microbial producers of PHB, Bacillus species have gained attention for their ability to utilize a wide range of substrates, including agricultural residues (Nair et al., 2014). Researchers have explored the synthesis of biopolymers using various carbon sources, including sugarcane bagasse, to optimize PHB production (Hong et al., 2019; Koch et al., 2019; Lamberti et al., 2020). Utilizing agro-waste as a feedstock for PHB synthesis can significantly reduce production costs (Dietrich et al., 2020). Previous studies have demonstrated that optimizing fermentation conditions and selecting appropriate carbon sources can enhance PHB yields significantly. For example, Sheekh et al. (2015) reported the production of PHB by *Bacillus flexus* ME-77 using molasses, while Sakthiselvan and Madumathi (2018) observed PHB production in *Bacillus safensis* EBT1 with sugarcane bagasse as a feedstock. In another study, Danial et al., (2021) found that the *B. wiedmannii* AS-02 OK576278 strain synthesized PHB using diverse agricultural waste materials, including onion, banana, mango, orange, and rice straw peels. This study aims to evaluate the efficiency of *Bacillus chungangensis* in synthesizing biopolymer PHB using various carbon sources and also bioprospecting its antimicrobial efficacy against several human pathogens in the healthcare sector.

Methods and Methodology

Sample collection

Soil samples were collected from various forest locations within the Western Ghats region of Tamil Nadu, India. This area is known for its rich biodiversity and distinct

ecological characteristics. In addition to soil samples, agro-waste materials such as sugarcane bagasse were sourced from local markets in Coimbatore to serve as potential substrates for microbial growth.

Isolation and screening of bacteria

The isolation of bacterial species was conducted using a serial dilution and spread plate technique on a nutrient agar medium (NAM). This method facilitated the effective separation and enumeration of bacterial colonies from the collected soil samples (Williams et al., 1989). For the screening of isolates, the bacteria were stained with Sudan Black B to enhance the identification of positive isolates. The staining procedure involved immersing the isolates in Sudan Black B for 30 minutes, followed by washing with 90% ethanol to remove any excess stains. The presence of bluish colonies indicated positive results, confirming the successful identification of the isolates.

Molecular Identification of Isolate by 16S rRNA Gene Sequencing

Genomic DNA was extracted from the bacterial samples using a lysis method and amplified through polymerase chain reaction (PCR) with universal primers targeting the 16S rRNA gene. The resulting amplified DNA was sequenced using single-pass sequencing with fluorescently labelled terminators. Following purification of the labeled fragments via ethanol precipitation, the 16S rRNA sequence was subjected to a similarity search using the NCBI BLAST tool to identify closely related sequences. Multiple sequence alignment was performed to confirm the taxonomic classification of the isolate, and the generated sequence was subsequently deposited in the GenBank database to contribute to microbial diversity research.

Biopolymer production using various carbon sources

The optimization of biopolymer production by the isolates was performed using a variety of traditional carbon sources, including starch, glucose, lactose, and fructose. Nutrient broths containing 1% of each carbon source were prepared in 250 mL volumes, supplemented with essential salts to support microbial growth. A 10% inoculum was introduced into each flask. Agro-waste substrate such as sugarcane bagasse, was initially shade-dried, pulverized, and sieved through a 2.0 mm pore-sized sieve for uniformity. Subsequently, the substrate underwent hot water hydrolysis, where 5 g of substrate was treated to improve easy nutrient availability. The pre-treated agro-waste substrate was used as growth media for the isolate, with a 10% inoculum introduced into each flask. All the flasks were incubated at 37°C and

160 rpm for 12 days, alongside a control setup with only nutrient broth. The experiments were conducted in triplicate.

Extraction and purification of biopolymer from biomass

Following a 12-day incubation period, the culture broth was centrifuged at 8000 rpm for 15 minutes to separate the biomass from the liquid medium. The resulting supernatant was discarded, and the pellet was extracted with ethanol. This mixture was then centrifuged at 3000 rpm for 10 minutes to further purify the biopolymer. The dry weight of the final pellet was recorded for subsequent analysis. For purification, 50 mg of the dried pellet was treated with 3 mL of 4% sodium hypochlorite at 50°C for 1 hour. After treatment, the mixture was centrifuged again at 3000 rpm for 15 minutes. The polymer was then dissolved in chloroform, and the solution was evaporated to remove the solvent. The remaining material was scraped for characterization, following the methodology outlined by Law and Slepecky (1961).

Antimicrobial activity testing for PHB biopolymer

The antimicrobial activity of the extracted PHB biopolymer was evaluated using the agar well diffusion method. Sterile agar plates were inoculated with various bacterial pathogens, including *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Shigella flexneri*, *Proteus vulgaris, Pseudomonas aeruginosa, and Pseudomonas fluorescens.* Wells was created in the agar, which was subsequently filled with 12mg concentrations of the PHB biopolymer solution. Antibiotic susceptibility tests were conducted, using standard antibiotics such as gentamicin, tetracycline, ampicillin, methicillin, and kanamycin. The plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition surrounding the wells were measured to assess the antimicrobial efficacy of the PHB biopolymer against the tested human pathogens.

Results and Discussion

Soil samples were collected from various locations in the Western Ghats of Tamil Nadu, India, to isolate and screen bacterial strains for polyhydroxybutyrate (PHB) production. A total of eleven bacterial strains were successfully isolated and screened using the Sudan Black B test, which is a reliable method for detecting PHB granules (Devadass et al., 2016). This approach is consistent with previous studies, such as that by Vasava et al. (2022), which also focused on microbial diversity in the Western Ghats. The screening indicated a significant presence of PHB-producing isolates, characterized by their rod-shaped

morphology and Gram-positive properties. Subsequent molecular identification through 16S rRNA gene sequencing confirmed the isolate as *Bacillus chungangensis*. The genetic sequence was submitted to GenBank, where it received the accession number MZ474520.1, contributing to the understanding of microbial diversity in this ecologically rich region (Saravanan et al., 2013; Mistry et al., 2022).

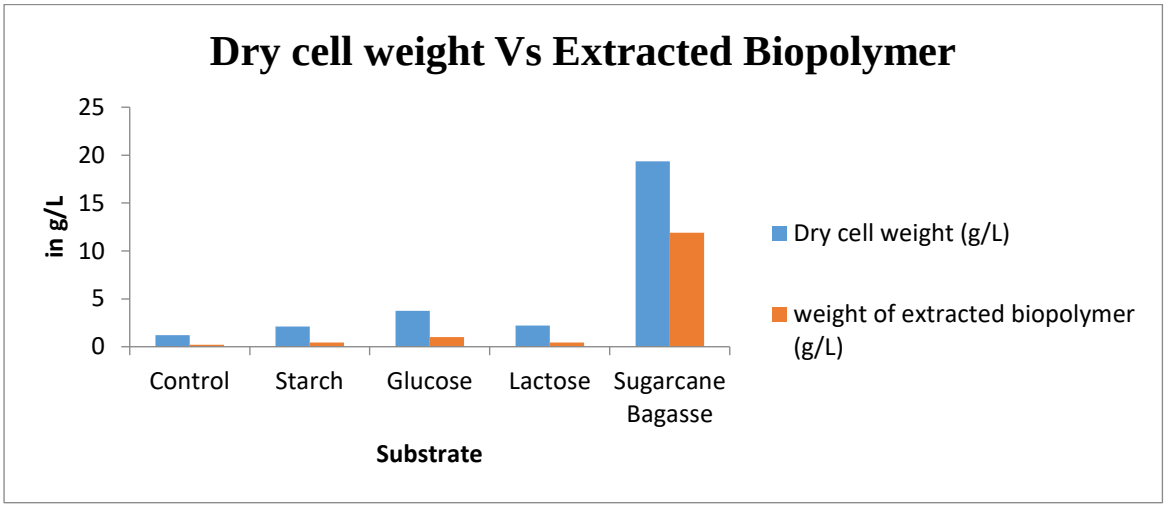

Figure 1: Dry cell weight Vs. Extracted biopolymer PHB from Bacillus chungangensis

In this study, *Bacillus chungangensis* was used to produce polyhydroxybutyrate (PHB) utilizing various carbon sources, including starch, glucose, lactose, and sugarcane bagasse. The results for dry cell weight and extracted biopolymer are depicted in Fig. 1, where sugarcane bagasse showed the most significant enhancement in both bacterial growth and PHB production. Specifically, sugarcane bagasse resulted in a dry cell weight of 19.336 g/L and a biopolymer yield of 11.8860 g/L, which was considerably higher than the other substrates. In comparison, glucose produced 3.74 g/L dry cell weight and 1.0098 g/L extracted biopolymer, while the control yielded only 1.204 g/L dry cell weight and 0.2047 g/L biopolymer. The increased growth and biopolymer production using sugarcane bagasse could be attributed to its nutrient-rich composition, making it an ideal feedstock for PHB biosynthesis.

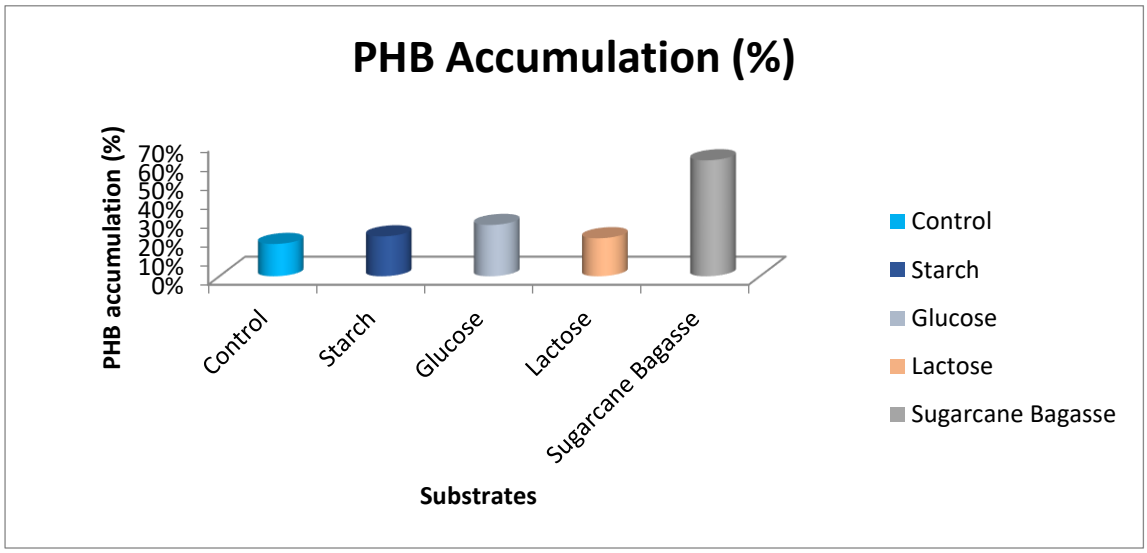

Figure 2: Percentage of PHB accumulation in biomass of *Bacillus chungangensis* using different carbon sources.

The PHB accumulation data is presented in Fig. 2, highlighting that *Bacillus chungangensis* grown on sugarcane bagasse exhibited the highest PHB accumulation, reaching 61%. Glucose and starch substrates supported moderate PHB production, with accumulations of 27% and 21%, respectively. Meanwhile, lactose and the control showed lower PHB yields of 20% and 17%, respectively. These findings suggest that sugarcane bagasse, as an agro-waste material, is highly effective for maximizing PHB accumulation, indicating its potential for bio-plastic production. The high PHB yield observed with sugarcane bagasse reinforces its suitability as a sustainable carbon source. These results align with other studies, further validating sugarcane bagasse's utility in PHB production. Sakthiselvan and Madhumathi (2018) reported a PHB yield of 32% and a dry cell weight of 5.90 g/L using sugarcane bagasse with *Bacillus safensis* EBT1. Suwannasing et al. (2015) demonstrated a PHB yield of 53% when *Bacillus* strains were grown on pineapple and sugarcane agro-waste, while Ashby et al. (2011) found a PHB yield of 38% with *Pseudomonas oleovorans* using crude glycerol. Similarly, Krueger et al. (2012) observed a PHB accumulation of 29.6% with *Bacillus megaterium* when using cassava starch as the substrate. In other studies, Sukan et al. (2014) reported a PHB yield of 41% (1.24 g/L) from *Bacillus subtilis* OK2 grown on orange peel, and Dalsasso et al. (2019) observed a PHB yield of 56% using *Cupriavidus necator* grown on sugarcane molasses. The PHB accumulation in *Bacillus chungangensis* observed in our study (61%) surpasses several of these previously reported yields, indicating that it is highly competitive in utilizing sugarcane bagasse as a substrate.

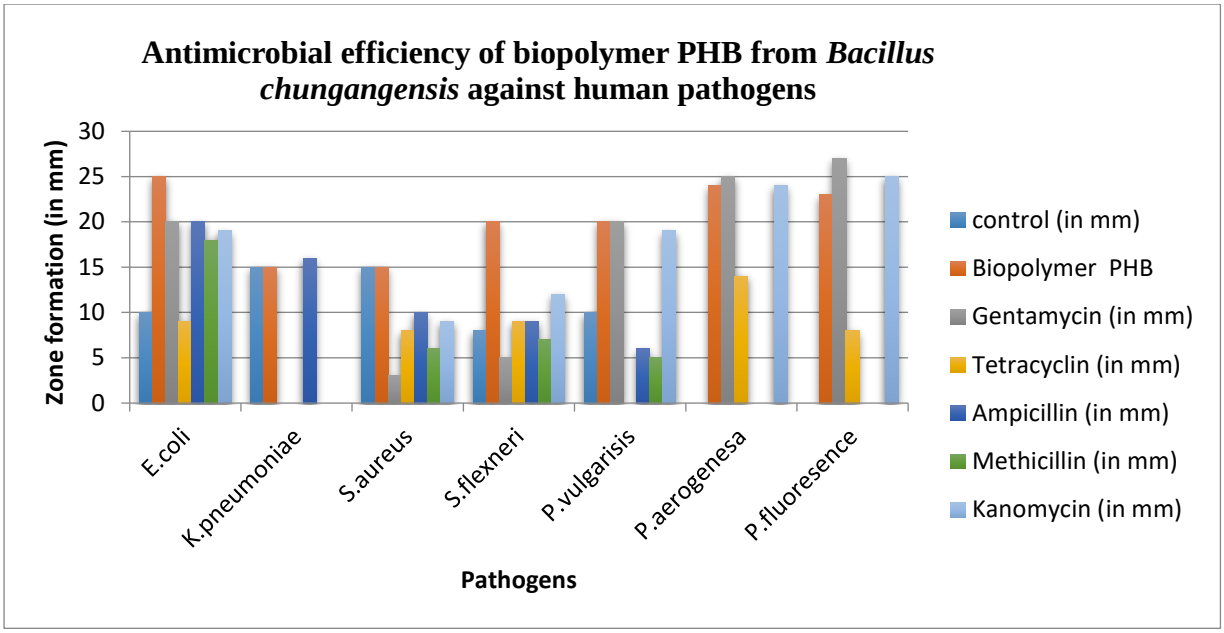

Fig 3: Antimicrobial efficiency of biopolymer PHB extracted from *Bacillus chungangensis* and standard antibiotics against human pathogens

Thapa et al. (2018) reported a PHB yield of 36.41% from *Bacillus pasteurii*, while Gowda and Shivakumar (2014) demonstrated promising results using *Bacillus thuringiensis* IAM 12077 on agro-waste. Getachew and Fantahun (2016) achieved a PHB yield of 55.6% using glucose and peptone, along with pre-treated sugarcane bagasse.

The antibacterial activity of biopolymer PHB from *Bacillus chungangensis* was evaluated against a range of pathogenic bacteria, and the findings were presented in Fig. 3. The biopolymer exhibited significant antibacterial effects, particularly against *Escherichia coli*, where sugarcane bagasse demonstrated the highest inhibition zone of 25 mm, outperforming than standard antibiotics such as Gentamycin (20 mm), Tetracycline (9 mm), and Ampicillin (20 mm). Similarly, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* were highly sensitive to the biopolymers, with inhibition zones of 24 mm and 23 mm, respectively. However, some standard antibiotics such as Tetracycline produced very little zone of inhibition whereas Ampicillin and Methicillin did not produce the zone of inhibition. The biopolymer also exhibited good antimicrobial activity against *Klebsiella pneumoniae* (15 mm) whereas other standards such as Gentamycin, Tetracycline, Methicillin, and Kanamycin did not produce the zone of inhibition against the pathogens. The control exhibited a 15 mm zone of inhibition against *Staphylococcus aureus*. The zone of inhibition was higher than other antibiotics such as Tetracycline (8 mm), Gentamycin (3mm), Methicillin (6mm), Kanamycin (9mm) and Ampicillin (10 mm). Similarly, previous studies have shown that biopolymers can be effective against a range of pathogens, including *Klebsiella pneumoniae*

and *Staphylococcus aureus* (Glick et al., 2007; Singh et al., 2011). In the case of *Shigella flexneri*, the biopolymer PHB exhibited a significant inhibition zone of 20 mm, markedly outperforming all other antibiotics including Gentamycin (5 mm). Similarly, *Proteus vulgaris* also showed substantial sensitivity to the biopolymers, with an inhibition zone of 20 mm, similar to Gentamycin whereas other antibiotics produced very less zone of inhibition and tetracycline produced no zone. The metabolites produced by *Bacillus* species can disrupt bacterial cell membranes or interfere with cellular processes such as protein synthesis and biofilm formation (Cheng et al., 2018; Sumi et al., 2015). According to Beric et al. (2012), natural isolates of *Bacillus* species exhibit antimicrobial activity, highlighting their potential for use in the biocontrol of pathogenic bacteria. Jeon et al., 2022 studied the antibacterial activity of *Bacillus* strains against *Vibrio* sp. Devadass et al., 2016 noted the antibacterial activity of actinomycetes against *E.coli* sp. Abdelwahab et al., 2019 studied the antibacterial activity of biopolymer PHB against *E.coli, S. aureus, P. aeruginosa* and *K. pneumoniae*.

These findings indicate that the antimicrobial biopolymer PHB derived from *Bacillus chungangensis* could be a powerful alternative to conventional antibiotics in the healthcare sector. This study underscores the potential of PHB synthesized from agro waste as a biodegradable alternative to plastics and highlights its dual functionality as an antimicrobial agent, contributing to environmental sustainability and healthcare innovation.

Conclusion

This study demonstrates the significant potential of *Bacillus chungangensis* as a producer of polyhydroxybutyrate (PHB) using agro waste sugarcane bagasse and also an efficient antimicrobial agent against various human pathogens. The biopolymer exhibited remarkable antibacterial activity against a range of pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Shigella flexneri*, *Pseudomonas fluorescens*, *Klebsiella pneumonia*, and *Staphylococcus aeruginosa*. The results showed that biopolymer PHB has more efficient antimicrobial activity on tested human pathogens than the known standard antibiotics. These findings confirmed that biopolymer PHB derived from *Bacillus chungangensis* not only serves as an effective biodegradable alternative to synthetic plastics but also possesses significant antimicrobial properties, making it a valuable candidate for both environmental and healthcare applications. The use of agro waste for PHB production not only enhances sustainability but also reduces production costs, contributing to a more eco-friendly approach to managing plastic waste. Overall, this research supports the development of biopolymers as a dual-purpose solution, addressing both environmental and

antimicrobial challenges, and paves the way for further exploration of microbial biopolymers in sustainable development and public health. This study also increases focus on environmental sustainability and the need for innovative approaches in healthcare.

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