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*Original Article*

# Novel Application of a Greenly Synthesized Biopolymer as an Azithromycin Drug Delivery System Against Antibiotic-Resistant *Salmonella*

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## ABSTRACT

Bacterium multidrug resistance are significant health concerns. Antimicrobial resistance, also referred to as AMR, is a significant worldwide health concern exacerbated by the misuse and overuse of antibiotics across multiple industries, resulting in the proliferation of resistant microbes. Antimicrobial resistance (AMR) confirms the imperative necessity of health-related solutions. The present investigation evaluated the generation of polyhydroxybutyrate (PHB) utilizing the bacteria Escherichia coli and Pseudomonas. The mixed fermentation demonstrating the best yield 42.2% compared to other cultures. The production identified by IR and HPLC indicated a singular signal with an average retention time of 1.57 minutes, indicating the analysis results for standard PHB and the sample at the same retention time. The co-polymer was effectively synthesized from PHB and PEG, and the drug encapsulation efficiency was around 45.3%. Additionally, concerning the totality of the carrier exhibiting 25.4% mg. The drug loading efficiency of AZI-PHB-PEG was superior to that of blank particles. Exhibiting the maximum release from microspheres, the starting point release of 18% occurring within the first two hours During the subsequent 4 to 8 hours, the dialysis bag method was employed to illustrate the release of Azithromycin. This release was achieved using a solution with a pH of 7.4, identified as the optimal swelling medium for the polymer. The synthesized co-polymer was characterized using X-ray diffraction and the Fourier transformation in infrared spectroscopy. The XRD sample, when thoroughly packed, exhibited distinguishing characteristics as the peak intensity increased with a higher percentage of crystalline Azithromycin dihydrate within the polymer network. The antibacterial effectiveness of the bio-polymer drug delivery system was assessed against bacterial strains about 71% of those strains were responsive to all dosages, as groups with higher of MIC. Generally. Fisher's exact test indicated that DDS was substantially more efficacious than Azithromycin ($p = 0.021$). This work is the first to report the preparation of an azithromycin-loaded PHB–PEG delivery system for use against Salmonella, which provides a new drug-polymer formulation to be tested for its antimicrobial potential.



*Keywords: PHB, Drug Delivery Systems, Salmonella, Azithromycin.*

## 1 Introduction

The incidence of multidrug resistance (MDR) is rising across numerous harmful bacteria, including *E. coli, Salmonella*, and *Staphylococcus*, globally, resulting in the ineffectiveness of antibiotics in managing infectious disorders. Biodegradable polymeric nanoparticles (BPNPs) are well-established drug delivery vehicles that enhance drug solubility, stability, bioavailability and allow sustained release. Poly (lactic-co-glycolic acid) (PLGA) is the most widely used platform, accounting for more than 60-70% of the polymeric nanocarriers being explored clinically, and is widely used in cancer, inflammatory, eye and systemic drug delivery. In recent years (2022-2025), polyhydroxybutyrate (PHB) and other polyhydroxyalkanoates (PHAs) have shown great potential as next-generation candidates with high drug encapsulation (>70-85%), controlled release and biocompatibility. Polyhydroxybutyrate- co-valerate (PHBV), a copolymer of PHB, offers enhanced drug stability and efficacy, especially in cancer and microbial therapies. But despite promising preclinical outcomes, PHA-based systems are yet to be approved and are in early development, with low clinical translation so far (<1%) [1-3]MDR bacteria provide a worldwide challenge in ruminant,

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owing to the extensive application of antibiotics as growth enhancers and for preventing and treating illnesses. Resistant infectious strains in animals, as well as emerging species of multidrug-resistant bacteria, can be transmitted to people via the intake of food (meat and milk) as well as either direct or indirect interaction with animals or their excrement in the surroundings [4, 5].

The emergence of antimicrobial resistance (AMR) in numerous strains of *Salmonella* to various antibiotics, including trimethoprim-sulfamethoxazole, tetracyclines, and chloramphenicol, complicates therapy and poses a significant health risk [6, 7]. In a European Union investigation, Salmonella spp. isolates exhibited antimicrobial resistance, with a notable increase in susceptibility found from 2009 to 2012 against sulphonamides, ampicillin, and tetracycline, with rates varying from forty percent to eighty percent [8]. External variables influencing antibiotic selection, such as patient age particularly in young people and geriatric populations must be taken into account, as these demographics bear the greatest healthcare burden and are particularly susceptible to drug side effects, alongside the sensitivity of *Salmonella* strains [9]. Since the intracellular infection, *Salmonella* is able to penetrate and persist inside many host types of cells, evading cellular defenses. Antibiotics are used for severe cases of salmonellosis, particularly serious infections or in patients with weakened immune systems; Azithromycin is progressively employed for the oral management of enteric fever. We analyzed the susceptibility to antibiotic profile of azithromycin in *Salmonella spp*. due to its extensive use, isolating samples from a tertiary care hospital to detect emerging resistance. Resistant to azithromycin is emerging among both typhoidal and nontyphoidal Salmonella [10, 11]. Poly-3-hydroxybutyrate (PHB) is a natural polymer classified within the polyhydroxyalkanoates (PHA) due to its characteristics akin to synthetic polymers such as polypropylene (PP). The composition of carbon sources utilized by microbial producers influences the properties of PHB, which consists of three to five chain carbons and can be synthesized by various bacteria under limited nutrient circumstances[12-14]. PHA-based films that decompose have garnered considerable attention recently owing to their rapid biodegradability in composting environments, resulting in no toxic residues, along with favorable biodegradability and biocompatibility [15-17]. Excellent biodegradability and biocompatibility, along with non-toxicity and property modification, have garnered interest as a replacement material for microbeads in personal care and cosmetic goods [2]. Polymers are prevalent in our everyday environments, including businesses and residences. Biopolymers are increasingly favored as substitutes for petroleum-based polymers due to their reduced environmental effect, characterized by a minimal carbon footprint and ease of breakdown. The principal objective of technological development is to enhance the quality of living. Enhanced therapies and customized treatments are presently the emphasis of researchers. Nonetheless, the administration of pharmaceuticals has long posed a challenge in the medical industry. Consequently, researchers have developed numerous drug delivery systems (DDSs) to achieve this objective. Among these are drug delivery systems based on nanotechnology [18]. The present study aimed to sustainably synthesize biopolymers to enhance drug delivery systems (DDSs) that utilize these biopolymers, due to their various characteristics and advantages. While PHB carriers have been extensively studied for drug delivery, PHB-based carriers for the delivery of azithromycin with antibacterial activity against Salmonella have not previously been reported. Thus, the present research is the first to develop a PHB-PEG-azithromycin drug delivery system and explore its use as a new delivery method for the treatment of Salmonella.

In the present work we will develop and investigate a poly(3-hydroxybutyrate)–poly (ethylene glycol) (PHB-PEG) drug delivery system for enhanced antimicrobial effectiveness. We expect that the addition of PEG chains into the PHB matrix will increase hydrophilicity, promote Azethromycin encapsulation and allow for sustained release of the loaded antimicrobial agent, thus improving its effectiveness against multidrug-resistant Salmonella enterica in vitro.

## 2 Materials and methods

### 2. 1 Chemicals

All reagents and chemicals utilized in this work were of analytical grade: P3HB (Sigma Aldrich), nutritional broth (Himedia), Mueller-Hinton agar (LAB), and Xylose Lysine Deoxycholate (Scharlau). The formulation of Azithromycin employed in the specificity study was obtained from Awamedica Company in Erbil, Iraq.

### 2. 2 Bacterial Strains

Various strains of bacteria were tested, specifically *Salmonella enterica* (CP029958.1, CP029952.1, CP029885.1, CP029896.1, CP029881.1, CP029870.1, CP120398.1, LT905064.1). Additionally, cultures for PHB production are including ATCC reference strains of *E. coli* and *Pseudomonas*.

### 2. 3 Conformation of PHB-producing bacteria using

#### *2. 3. 1 Sudan Black B (SBB):*

The bacteria (*E. coli* and *Pseudomonas*) were cultivated on nutritional agar media enriched with one percent glucose and incubated at 37ºC for 24 hours.[19, 20].

#### *2. 3. 2 Nile blue A (NBA) staining:*

Sudan Black B-positive isolates were evaluated for polyhydroxybutyrate (PHB) synthesis using Nile Blue A staining, a more specific and sensitive approach for live colonies. Coloring heat-fixed bacterial cell smear using Nile blue A solution at 55°C for 10 minutes in a staining jar, utilizing a Florence microscopy with a connected digital camera for picture acquisition[21].

### 2. 4 Production and extraction of PHB and Estimation of cell dry weight

The positive strains for PHB synthesis, both individually and in a combined culture system of *E. coli* and *Pseudomonas*, were introduced in Minimal Davis Media, supplemented with glucose as the carbon sources (at a final concentration of 1%), inoculate with 5 ml which contain 0.5 McFarland of each of the test isolates. Use optimum conditions were preformed incubated at 37 °C at 150 rpm for 72 hours. $P_3HB$ was recovered from the cultured growth using the sodium hypochlorite-chloroform technique Five millimeters of culture were centrifuged at 10,000 rpm for10 minutes; the supernatant was collected and subsequently utilized to determine dry cell weight. The pellet was suspended in 2.5 ml of 4 % sodium hypochlorite for digestion followed by incubation with 2.5 ml of hot chloroform was incubated at 37°C for 1 hour. The suspension underwent centrifugation at 1500 rpm for 10 minutes. The lower phase containing biopolymer with chloroform was collected after that extracted with hot chloroform then precipitated using a mixture of ethanol and acetone (1:1) while the non-soluble residue was eliminated to purified. The precipitate was permitted to evaporate for dryness at 30º C until dry, yielding white P3HB crystals. following the incubation procedure P($_3$HB) granules were used to ascertain the highest synthesis of PHB at identical carbon source conditions [22], Subsequently, the measurement of cell dry weight was conducted by re-suspending the washed pellet in 1 ml of distilled water, transferring it to plates, and drying to a consistent weight at 60˚C.

$$\text{PHB Content}(\%)=\frac{\text{Weight of extracted PHB}}{\text{Dry cell weight(DCW)}}\times 100.....(1)$$ [23].

## 2. 5 The measurement of swelling property of PHB

The swelling test was conducted. The weight of the dried beads was initially measured. The beads were subsequently submerged in a phosphate-buffered saline solution (NaH2PO4) under varying pH settings (pH 7.4, 1.3), while distilled water and ethanol served as swelling media at room temperature. Each experimental condition was evaluated in duplicate. Subsequent to quantifying the weight of the as-treated beads, the swelling ratio (S) was calculated utilizing equation (2). [24].

$$S(\%)=\frac{W_s-W_d}{W_d}\times 100 \quad \text{..... (2).}$$

* Where Ws and Wd represent the weights of swelling and as-dried beads, respectively.

## 2. 6 Preparation of biopolymer-based DDS and determinant of yield

The biopolymer-based drug delivery system approach was employed to prepare both blank and drug-loaded biopolymers with specific alterations [25]. In summary, to prepare AZI-loaded PHB-PEG, PHB (50 milligrams) and PEG (50 milligrams) were solubilized in 3 mL of acetone as the organic phase. The Azithromycin is was solubilized in an aqueous solution with a sonicator at 6 Watts for 2 minutes. The stage known as organic was incrementally introduced into the water phase while stirring at ambient temperature. The amalgamation was meticulously agitated overnight at 400 rpm. The drug-encapsulated particles were recovered via centrifugation at 10,000 rpm, after a third rinse with deionized water and subsequent drying in a vacuum heating oven for evaporation of solvent. The blank PHB particles were synthesized using the previously disclosed methodology reported, excluding the incorporation of the medication in the aqueous phase. The purified PHB was utilized for the production of blank PHB and AZI-PHB-PEG particles loaded with Dig.

The procedure yield was quantified as the ratio of the weight of microparticles (Wm) to the weight of polymer (Wp) in the starting solution, as illustrated in Eq. (3):

$$\text{yield}\ (\%)=\frac{\text{Wm}}{\text{Wp}}\times 100.....(3)$$

## 2. 7 Drug Loading and Encapsulation Efficiency

This section outlines the computation and assessment of Drug Loading Efficiency (DLE percentage) and Drug Loading Capacity (DLC percentage) Drug Loading Efficiency (DLE%) and Drug Loading Capacity (DLC%) for the formulated drug delivery system (DDS). These metrics evaluate the efficacy of drug incorporation into the polymer carrier matrix of drugs. The encapsulation efficiency (EE) is characterized as the proportion of the drug's weight in small particles (Wm) to the initial drug weight (Wi), as illustrated in Equation. (4) [26]

$$EE\%=\frac{\text{Amount of Azithromycin available in the formulation (mg)}}{\text{Amount of Azithromycin incorporated in the formulation (mg)}}\times 100 \quad (4)$$

The amount of drugs percentage was calculated by comparing the quantity of drug present in the mixture to the amount of recovered particles based on Equation (5) [26] :

$$\text{Drug content (w)}\%=\frac{\text{Amount of Azithromycin available in the formulation (mg)}}{\text{Amount of Azithromycin incorporated DDS recovery (mg)}}\times 100 \quad (5)$$

### 2. 8 Calibration Curves of Azithromycin

The calibration Azithromycin Curves: Instrumentation and Reagents:A Shimadzu Corporation UV/VIS dual-beam spectrometer (model number 1800) with 1 cm corresponding quartz cells was employed for spectral studies. All remaining compounds were of analytical quality. Creation of a standard stock solution along with working standard solutions. Ten milligrams of precisely. measured AZI was put into a 10 mL volumetric flask, and its volume was adjusted accordingly. From the standard stock solution, 1 mL was extracted and mixed with the phosphate buffer to achieve a final volume of 10 mL, resulting a working standard solutions concentration of 100 milligrams per milliliter, followed by additional dilutions. Assessment of the wavelength corresponding to maximum absorbance A UV spectroscopy scan (200 - 400 nm) was conducted with the working standard solutions to ascertain the λ max for the identification of AZI, utilizing phosphate buffer as a blank [24].

### 2. 9 Characterization of Product PHB (Bulk Polymer)

The microbial synthesis of biopolymer (PHB) was initially validated through visual inspection and subsequently characterized using a Shimadzu UV/VIS double beam spectrometer (model 1800), IR spectroscopy (Shimadzu IR Japan, 400–4000 cm-1), and HPLC (SYKAM Germany, C18 size of 25 cm x 4.6 millimeter).

### 2. 10 Characterization of bio-polymer microsphere delivery systems

The analysis of biopolymer-based drug delivery systems (DDS) confirms the successful validates the effective formulation and guarantees product performance by FTIR (Shimadzu FTIR Japan, 400–4000 cm-1), XRD (PAN Analytical: XPERT-PRO), and FESEM (TESCAN, MIRA3, Czechia).

### 2. 11 In vitro investigation of pharmaceutical release

To quantify the released drug from the polymer matrix, precise quantities of the polymer matrix were positioned in donor spaces with 1 mL of phosphate buffer solution at a pH of 7.4, detached by a dialysis membrane (Delchimica Scientific Glassware) with a threshold for molecular weight of 12 - 14 kDa from recipient spaces filled with 19 mL of the buffer. The glass vials were incubated at 37 °C for 24 hours with continuous agitation using multi-position magnetic stirrer. The system was mechanically stirred at a velocity of 100 rpm, with temperature regulation to maintain a constant water circulation system (like a water bath) zero-order, first-order and mixed order kinetics of drug release use Higuchi, Korsmeyer- Peppas. A portion was extracted and substituted with an equivalent volume of fresh buffer at specified periods to maintain sustained sink conditions, and medication levels in the specimens were quantified using microbiological test assays. Tests were conducted in triplicate (n = 3) [27, 28]. Drug quantification was achieved using UV-Vis Spectroscopy at 210 nm use Hixson-Crowell model.

### 2. 12 Preparation of inoculum

Prior to experimentation, the microbes were cultured on Mueller-Hinton agar (Neogen, UK) and incubated at 37°C for 24 hours. On the following day, various bacterial dosages were created using normal saline and subsequently adjusted according to distinct McFarland standards to achieve final densities of roughly $1.5 \times 10^{\wedge 6}$, $1.5 \times 10^{\wedge 7}$, and $1.5 \times 10^{\wedge 8}$ CFU/ml [29].

### 2. 13 Antimicrobial Assays

*Salmonella enterica* Prior to experimentation, the bacteria underwent an antibiotic sensitivity test (AST) utilizing the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) to confirm their resistance characteristics against antibiotics [30]. Subsequently, other inoculation dosages were formulated. Antibacterial Efficacy of Biopolymer-based Drug Delivery Systems to evaluate the in vitro antibacterial efficacy of biopolymer-based drug delivery systems against chosen multidrug-resistant Gram-negative bacteria, specifically *Salmonella enterica*, a well diffusion method was employed to measure the zone of inhibition, as outlined by [30]. Fifty (50) µL of the chosen bacterial strain inocula ($1 \times 10^{\wedge 8}$ CFU/mL) were uniformly distributed on the surface of solid Mueller-Hinton Agar (MHA) using a sterile cotton swab. Five agar wells, each with a diameter of 6 mm, were created using a sterile cork borer [31]. The concentrations of 0.25, 0.50, and 1 mg/mL of biopolymer-based drug delivery systems were prepared using deionized water, alongside a negative control of the biopolymer and a 15-µg azithromycin disc. These were dispensed into separate wells using a micropipette. The plates were maintained at ambient temperature for 1 hour to facilitate uniform dispersion of the azithromycin disc into the agar, followed by incubation at 37 °C for 24 hours, after which the zone of inhibition was determined.

### Statistical Examination

Statistical analysis with Python (version 3.x). Experiment conducted with n= 2-3 independent biological replicates. Data were analyzed using pandas and NumPy, while statistical tests were performed with SciPy and stats statsmodels. Data are presented as mean stander deviation (SD). Data visualization and graphical analysis were performed using Matplotlib and Seaborn to illustrate trends, distributions, and relationships. A significance level of $p < 0.05$ was utilized.

## 3 Results and Discussions

### 3. 1 Screening of PHB-producing

Several strains of Bactria with the ability of PHB granules were identified during the screening of PHB producers, as evidenced by different stages of the screening process. The initial evaluation of Sudan Black B (SBB) staining on the cultivated sample in a petri dish demonstrated that the addition of Sudan Black B dye altered the colony's color to a deep blue-green hue, as shown in "Figures 1-A".

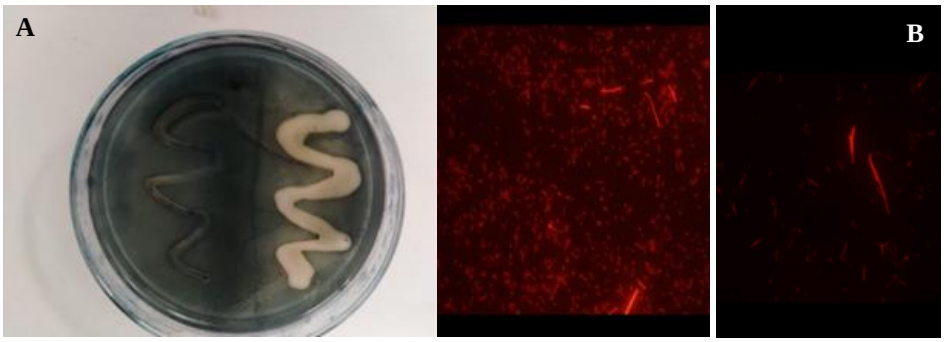


**Figure 1:** (A) Sudan Black B staining shows dark greenish-blue areas that show PHB production. (B) Nile Blue A fluorescence microscope shows that there's many bright fluorescent orange granules inside the cells.

This find concentrated on the green synthesis of a nontoxic, biodegradable and eco-friendly biopolymer to mitigate environmental pollution. These results were regarded as additional proof for the high sensitivity of the Sudan Black-B method in screening bacterial isolates that produce an internal lipid biopolymer. The deep blue colonies stained with Sudan Black-B exhibit high sensitivity for PHB screening due to the lipophilic nature of the dye. This staining distinctly reveals intracellular lipid matter as cytoplasmic inclusions or materials linked to the structural components of the cells, which weren't previously observed or suspected. Notably, there is a clear sign of SBB positive results when the isolate is obtained from sediments and subsequently stained with SBB colors [32].

The screening of PHB producers is significant, as all positive strains exhibited bright orange fluorescence within fluorescence microscopy at a wavelength of excitation of 460 nm. Hot-fixed smears of bacterial cells stained with Nile Blue A (NBA) showed multiple brightly fluorescent bright orange particles within the cells, as illustrated in Figures 1-B. Conversely, SBB positive was ultimately validated through the application of NBA, a particular dye that assesses PHA granule binding capability, employing fluorescent dye to differentiate among PHA-producing and non-PHA isolates. The staining dye employed for the screening of PHA-producing isolates from their natural habitats was NBA. According to prior publications, the dyes are the most widely used for the selectively labeling of PHA granules in bacterial cells. The Sudan Black-B exhibits a significant affinity for the lipid-interacting biopolymer, Nile Blue, along with corresponding data [33-35].

## 3. 2 Quantification of PHB

The isolate proved positive for PHA granules when stained with SBB and NBA dyes and methods of recovery for dry cell weight. Subsequently all the four isolates were subjected for PHA production under optimum condition, PHB5 (mixed culture) was found to produce maximum amount of PHB (82.52%) in modified growth medium whereas the PHB3 showing less PHB production (40.32%) compared with other strains [36].

Such PHA production rate appears to be the highest as recorded for all strains bacteria so far was estimated as reports suggested dry cell weight at this ratio for many bacterial species "Table 1," .According to [37], Polyhydroxyalkanoates (PHA) are increasingly recognized as valuable precursors for biopolymer manufacture because of their biocompatibility, biodegradability, and adjustable features. The combination can be accomplished via fermentation by microbes, which is a compelling option for the production of these biopolymers. Recent improvements in PHA production methodologies, particularly the utilization of mixed microbial cultures, have enhanced the features of PHA, including their molecular weight and physical attributes, rendering them appropriate for various uses [38]. The practical side loading of a medication in a drug delivery system (DDS) denotes the quantity of drug integrated into the carrier material, usually articulated as a percentage of 25.4% mg drug/mg DDS relative to the entirety of the carrier [39].

**Table 1:** Dry Cell Weight and Yield of PHB of Bacteria.

| Positive bacteria for PHB | Dry cell weight (g/L) | PHB (g/L) | Yield of PHB % |
|---|---|---|---|
| *PHB1* | 0.394 | 0.29 | 73.68 |
| *PHB2* | 0.92 | 0.371 | 40.32 |
| *PHB3* | 0.301 | 0.12 | 39.86 |
| *PHB4* | 0.93 | 0.246 | 26.451 |
| *PHB5(mixed culture)* | 0.412 | 0.34 | 82.52 |

## 3. 3 Swelling Behavior of PHB

Assessing the degree of swelling determines their optimum drug loading capacity and regulates their release behavior depended on water uptake. Computed using equation (2) Consequently, the swelling characteristics of polyhydroxybutyrate (PHB) in various duplicate in different media (pH 7.4 phosphate buffer, pH 1.3 HCl

solution, and distilled water) corresponding to acidic, neutral, and alcoholic environments incubated for 0, 1, 2, 4 and 6 h. The samples were incubated at 37 ± 0.5 °C were established and are illustrated in "Figure 2," encompassing each of these factors. The pH of the swelling medium significantly influences the degree of swelling during the initial six hours. At pH 7.4, the polyhydroxybutyrate (PHB) hydrogel exhibits the largest degree of swelling [28], as illustrated in Figure 2, followed by mild swelling at pH 1.3, while swelling in ethanol medium is the least. It is widely recognized as the dispersion efficiency of polymer compounds rapidly escalates due to the swelling of the substance [40]. In this work, we are designing and evaluating a poly(3-hydroxybutyrate)–poly (ethylene glycol) (PHB–PEG)-based drug delivery platform for enhanced antimicrobial treatment. We expect that the incorporation of PEG segments into the PHB matrix will increase the hydrophilicity of the polymer matrix and will increase the encapsulation efficiency of Azethromycin and allow the controlled release of the drug, thus enhancing the therapeutic effect of the antibiotic for resistant microorganisms.

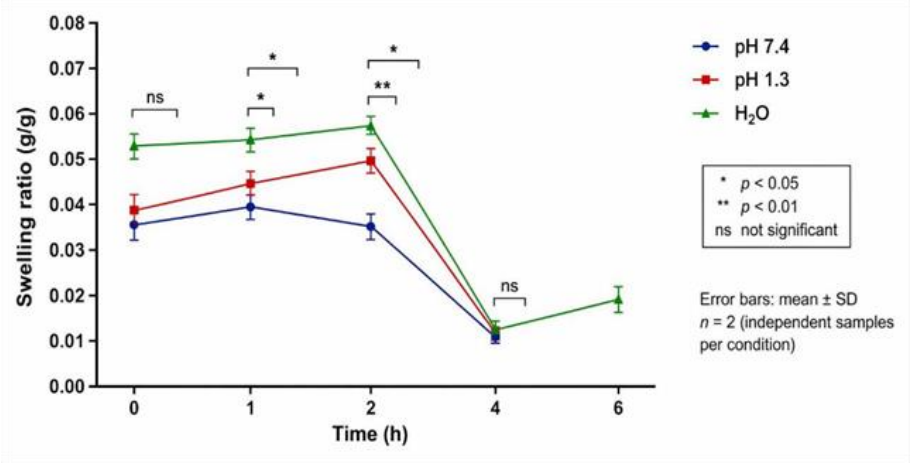


**Figure 2:** Time-dependent swelling of PHB in different media (pH 7.4, pH 1.3 and distilled water). Swelling ratio (Q) was defined as Q = (We-Wo)/Wo where Wo is the initial dry weight and We is the weight at time t after removing the surface water. Results are expressed as mean ± standard deviation (SD) (n = 2). Two-way ANOVA was used to determine statistical significance followed by Tukey's multiple comparisons test for each time point.*p < 0.05, **p < 0.01; ns, not significant.

## 3. 4 Calibration curve for azithromycin

A supersaturated solution (SSS) of AZI was formulated in a phosphate buffer, while the remaining work standard solution (WSS) was made by diluting the SSS with phosphate buffer. The UV spectra of AZI have a λ max at 210 nm; hence, the remainder of the experiments was conducted at this wavelength. The calibration curve for azithromycin in a buffer consisting of phosphate at pH 6.8 exhibited linearity within the dosage range of 0.25-2.50 mg/ml "Figure 3", with an $R^2$ value of 0.994. The linear regression equation was established as:

$A=0.021C+0.806A = 0.021C + 0.806A=0.021C+0.806$ [24].

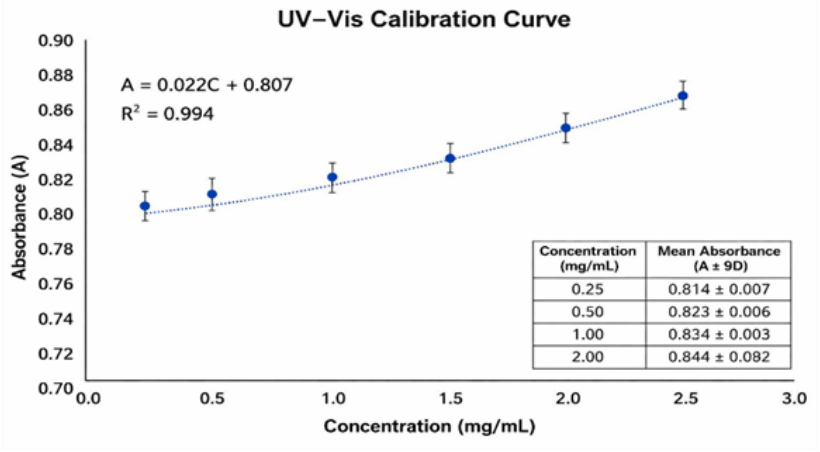


**Figure 3:** UV-Vis calibration curve of the analyte. The relationship between concentration (0.25–2.5 mg/mL) and absorbance was evaluated using UV-Vis spectrophotometry. The calibration curve shows a strong linear correlation.

## 3. 5 Drug loading efficiency, capacity and release

Subsequent to the preparation step, PEG was attached to the surface of PHB and CMC cross-linked structures. The loading of

Azithromycin, formulated for (PHB-co-PEG) with a 50/50 mole fraction, was conducted in a solution with a pH of 7.4 at room temperature. Evaluation data indicate that over 75 mg of the drug was introduced via the encapsulation process, with 34 mg effectively incorporated into the matrix of polymers, as detailed in "Table 2". The final extracted drug delivery system (DDS) weighed 134 mg, resulting in a drug delivery efficiency (DLE) of 45.3% and a medication load capacity (DLC) of 25.4% [41]. The dialysis bag method was employed to illustrate the release of Azithromycin, with an initial release of 18% occurring within the first two hours, representing the highest release from construction encompassing including zero- and first-order, Hixson-Crowell. In the subsequent 4 to 8 hour period, a consistent emission was observed, while 3/4 of the drug remained entrapped. during the 23 to 24 hour duration, a slower drug release. An increased loading capacity typically results in a greater therapy dose per delivery mechanism unit, enhancing efficacy, comparison to other known formations of biopolymer drug systems 30-40% DLE [42].

**Table 2:** Calculated of drug loading for the PHB-based drug delivery system, including total drug added, actual drug loaded, drug-loading efficiency (DLE %), and drug-loading capacity (DLC %).

| Parameter | Symbol | Formula | Result |
|---|---|---|---|
| ***Total Drug Added*** | DLE % | 15 × 5 | **75 mg** |
| ***Drug Loaded*** | DLC % | 134 – 100 | **34 mg** |
| ***Drug Loading Efficiency (DLE %)*** | DL | (34 / 75) × 100 | **45.3 %** |
| ***Drug Loading Capacity (DLC %)*** | - | (34 / 134) × 100 | **25.4 %** |

## 3. 6 Characterizations of bulk and Bio-polymer based drug delivery

### *3. 6. 1 Chromatographic performance*

The synthesis of PHB through mixed culture was validated using the HPLC method, which relies on the transformation of the extracted PHB with concentrated sulphuric acid into crotonic acid. Each sample was diluted five to one hundred times with 0.014 N H2SO4, which contained 0.8 mg of adipic acid (Sigma) per milliliter for the internal standard, before being filtered through a 0.45 µm-pore filtration membrane. Crotonic acid can be quantified in samples and standards analyzed on a Waters XSelect C18 (4.6 × 150 mm) utilizing a Waters 2998 PDA detector. Samples were evaluated using a high- performance liquid chromatography HPLC model from (SYKAM), Germany. The mobile phase consisted of (Methanol: D.W: formic acid) (70: 25: 5), the column utilized water C18-ODS, measuring 25 cm by 4.6 mm with a UV detector set at 280 nm at flow rate 1.0 ml /min.

The analysis of recrystallized crotonic acid exhibited a singular peak with a retention time of 1.57 minutes "Figure 4", indicating the results for both standard PHB and the specimen were obtained at the same retention time [24]. In spectroscopy, the base peak represents the peak with the highest intensity in a mass spectrum, with it typically correlating with the most stable ion generated during ionisation. This peak is essential for elucidating the stability and dissolution patterns of ions, as it frequently signifies the molecular structure. of the substance. The absence of second-order or break peaks in your analysis illustrates the high selectivity and particularity of the method toward crotonic acid, indicating that the peak is unaffected by other pieces or primary ions [43].

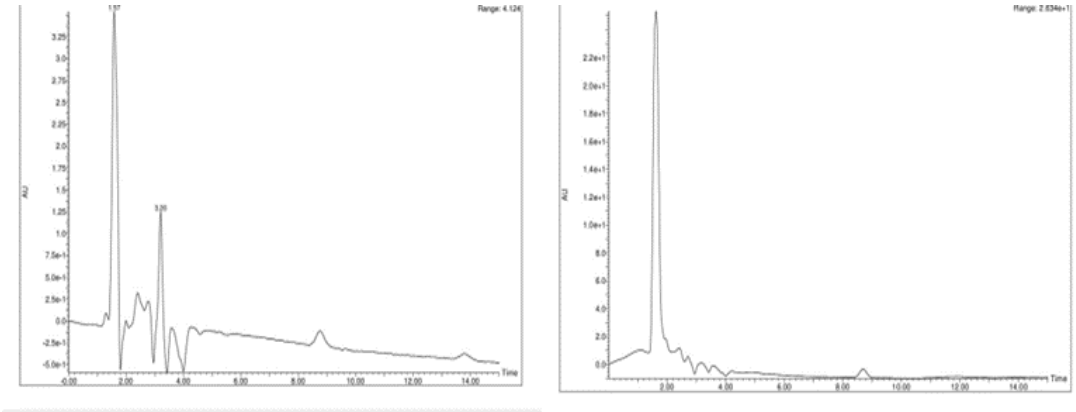

**Figure 4:** shows UV–visible chromatograms of PHB standard and PHB taken from a bacterial culture after sulfuric-acid hydrolysis.

The symmetrical peak in HPLC is a crucial indicator of technique specificity and sample purity. The separation by chromatography procedure is confirmed to be effective, enabling precise quantification of the analyse. Hydrogen bonding, electrostatic, and other kinds of non-covalent relationships are believed to facilitate the loading of AZI onto PHB and PEG. A comparable process for AZI load in poly(butyl cyanoacrylate) particles has been previously documented [44].

### *3. 6. 2 FTIR analysis of PHB and PHB-co-PEG loading Azithromycin*

Exploratory FTIR analysis was performed on both pure PHB and PHB isolated from cultures of bacteria, characterized using IR spectroscopy, as illustrated in "Figure 5 a". The spectrum exhibited absorption bands at 2926 and 2854 $cm^{-1}$ (CH stretching vibration) and 1465 and 1377 $cm^{-1}$, and the ester carbonyl band at approximately 1742 $cm^{-1}$ (C=O stretching). The absorption bands at 1465 and 1377 $cm^{-1}$ indicated the bending of $CH_3$ bonds, respectively[45]. linkage between monomers and the vibrations of the -C-O-C- bond in PHB, respectively. The infrared (of the pure substance) exhibited. Was similarly defined by the sample. The infrared spectrum of the co-polymer. In the PHB–PEG blend "Figure 5 b", the emergence of a broad –OH stretching band around ~3400 $cm^{-1}$ and increased intensity at ~1100 $cm^{-1}$ (ether linkage) confirm PEG incorporation. The slight shift and broadening of the carbonyl peak suggest hydrogen bonding between PHB and PEG, indicating improved compatibility and partial miscibility and drug-laden samples exhibited the characteristic absorbing bands of the raw components [28]. Significant variation in the strength of the absorption bands of both blank and drug-loaded Azithromycin (AZI) at the wide range of 3400 $cm^{-1}$ indicates the presence of O-H/NH bonds [46].

A prominent band with significant intensity in the range of 3410-3200 $cm^{-1}$ in the IR spectra of PEG vanished following the creation of microspheres and the interactions involving PEG and PHB, increased intensity in the ~1600–1500 $cm^{-1}$ region corresponding to amine-related vibrations. An absorption band at 1147-1107 cm-1 was detected in the FTIR spectrum of drug loading efficiency due to the encapsulation of AZI-PHB-PEG, as illustrated in "Figure 5 b". The distinctions encompass heightened crystallinity alongside diminished Young's modulus and extension at break. A stepwise shift in the carbonyl (C=O) peak was observed from 1724 $cm^{-1}$ (PHB) to 1719 $cm^{-1}$ (PHB–PEG), and further to 1715 $cm^{-1}$ after drug loading, indicating progressively increasing intermolecular interactions, this stepwise peak shifts and band broadening confirm increasing intermolecular interactions from PHB to PHB–PEG and further upon drug loading. The absence of new peaks indicates that azithromycin is physically encapsulated without chemical bond formation.

The copolymer also modifies the exterior shape of ultrathin papers and proteins, including collagen, fibrin, silk collagen, fibroin, and gelatin. These characteristics render PHB-PEG appropriate for applications in healthcare implants, biological manufacturing, and medicinal product formulations [47].

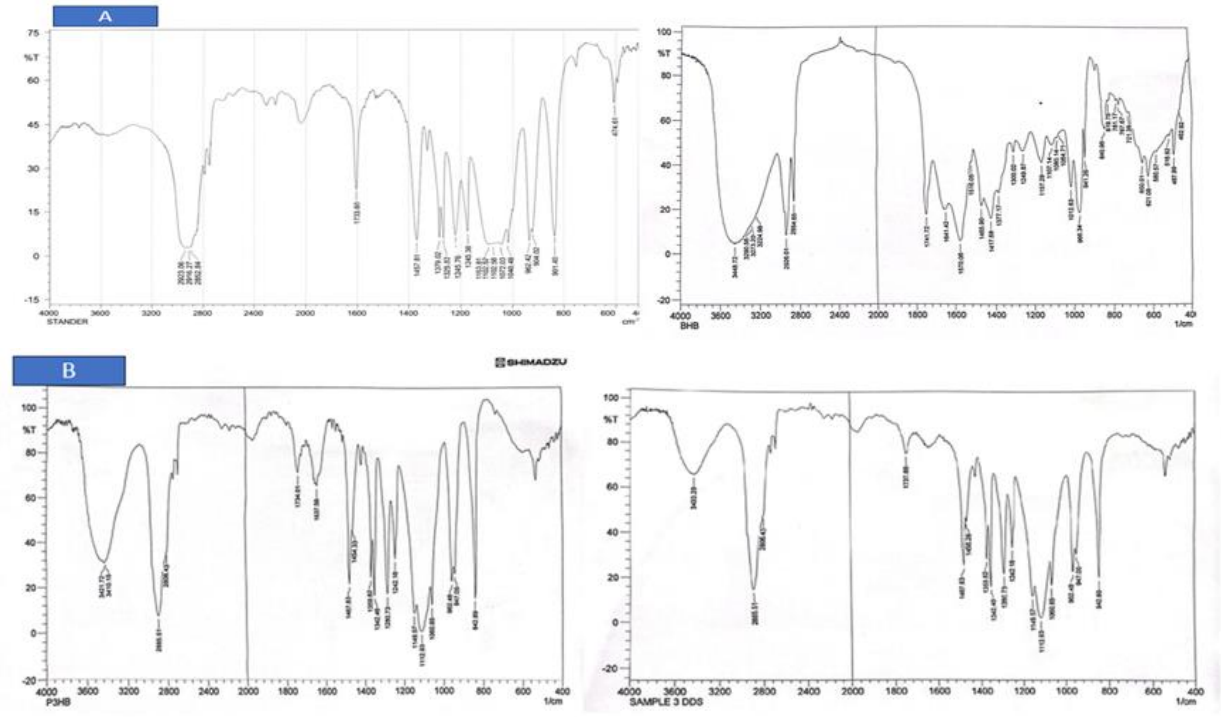


**Figure 5:** FTIR spectra, (a) of pure PHB and PHB isolated from cultures of bacteria, (b) of PHB-co-PEG and PHB-co-PEG with azithromycin loaded.

### *3. 6. 3 Surface morphology and particle structure (SEM analysis)*

The scanning electron microscopy (SEM) demonstrated significant differences between the pure PHB-PEG co-polymer and the drug loading PHB-based drug delivery system (DDS). At low magnifications, the co-polymer specimens showed flaky, fractured and porous surface suggesting heterogeneous and weak interaction between the polymer chains. The data suggest that the PHB-co-PEG particles have porous surface and thus may be heterogeneous and may represent the interparticle interaction distance and aggregation among the polymer chains as Shown

(Figure 6 A, C, & E) show. These rough structures offer a large surface area and considered as big particles in composites, allowing the drug absorption but may also induce burst drug release due to the weakness of the exposed active sites and making difficult to produce nanoscale particles with conventional production process.

The SEM images of drug loaded Azithromycin PHB-co-PEG hydrogels "Figure 6 B, D, & F ", show that the hydrogels are heavily loaded with drug as semi-microspheres are also present in between the folds of the hydrogels also showed smooth ~10-100µm with homogeneous surface topology and slight aggregation indicating the formation of stable self-organized structures [48]. The dense nature suggests that the drug molecules actively participated in the ordering of the polymer chains as in " Figure 6 B, D & F ", as stabilizers in the solvent evaporation and promoting cohesion among particles figure (6) The pure polymer has smooth, even surfaces, while the drug-loaded DDS has rough, granular surfaces that show that the drug has been added. The absence of cracks indicates strong polymer-drug interactions, highly drug loaded hydrogels as semi microspheres are interspersed between the hydrogel folds but even the hydrogel particles with drug molecules are formed with high aggregation in microspheres as showed with [49].

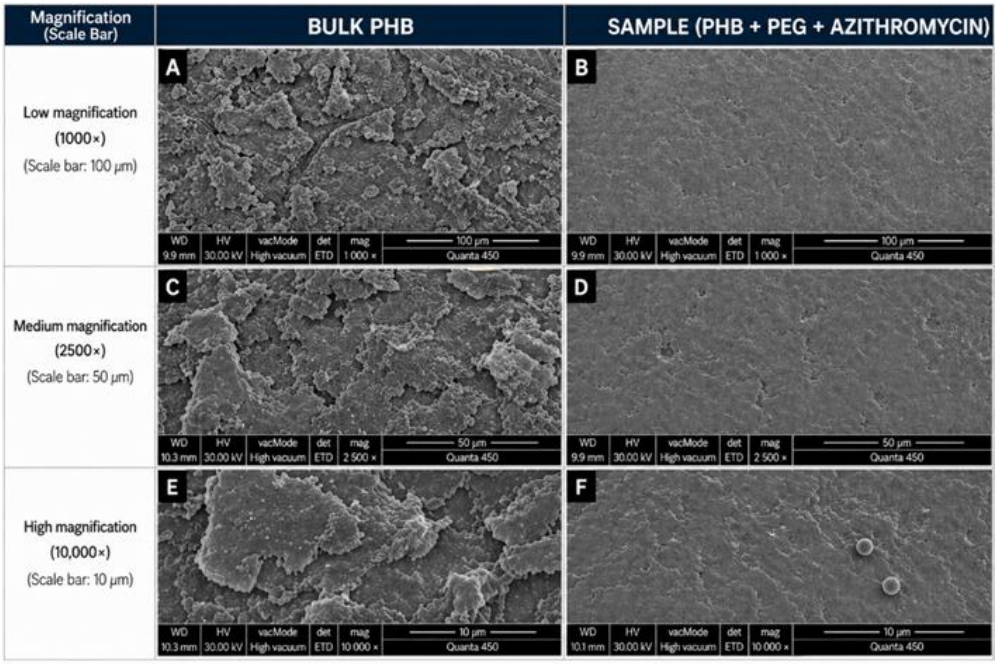


**Figure 6:** SEM pictures of PHB–PEG (A, C, & E) copolymer and a PHB-based DDS B, D, & F) that contains azithromycin at 1000×, 2500×, and 10,000× magnifications. Bulk PHB shows a rough, fractured, and porous morphology, while the DDS sample exhibits a smoother, more compact, and homogeneous surface. Scale bars: 100 µm, 50 µm, and 10 µm, respectively.

### *3. 6. 4 XRD Characterization of co-polymer loading azithromycin*

The results of XRD correlated well with the heat analysis data. The X-ray diffraction patterns in "Figure 7", indicated that pure azithromycin dihydrate was unequivocally in a crystalline condition, exhibiting crisp, identifiable peaks at 2θ diffraction angles of 18.05 °, 20.17 °, 21.00 °, 23.19 °, 23.78 °, 24.99 ° and 29.65, distinctly crystalline, exhibiting sharp peaks at 2θ reflection angles of 19° and 21°[46]. In contrast, X-ray analysis using two different stabilizers, PHB-co-PEG and AZI-PHB-PEG, displayed a weak reflection signal in contrast to the crystalline form. The emergence of several additional peaks and the displacement of specific peaks may indicate that alterations in the peaks of the pure medication contribute to enhanced solubility. In X-ray diffraction (XRD), its carrier polymer PHB-CO-PEG displays a broad halo distribution with minimal peak strength, signifying a feeble diffraction signal. This trend indicates that the polymer sample may exhibit low crystallinity or that the reflection method may be ineffective for this particular polymer. The intensity of peak in XRD patterns correlates with the quantity of crystalline material present; thus, a weak peak indicates less crystallinity or a more amorphous morphology[50].

The lack of distinctive peaks in the D curve for the drug-loaded biopolymer, coupled with the presence of a halo pattern spectrum, suggests that Azithromycin within the biopolymer exists in an amorphous state A representative high-angle (2θ ≈ 44.1–44.7°) shows only a broad, intensity feature with a maximum smoothed intensity of ~6.8 counts, compared to a baseline of ~4–5 counts. It is believed that, due to its significantly higher saturation solubility, the amorphous form of drug efficacy is a desirable attribute in a delivery mechanism. the combined qualitative (peak disappearance and halo formation) and semi-quantitative (reduced peak intensity and low signal-to-background ratio) analyses provide strong evidence of successful amorphization of the drug in the polymeric system [51, 52].

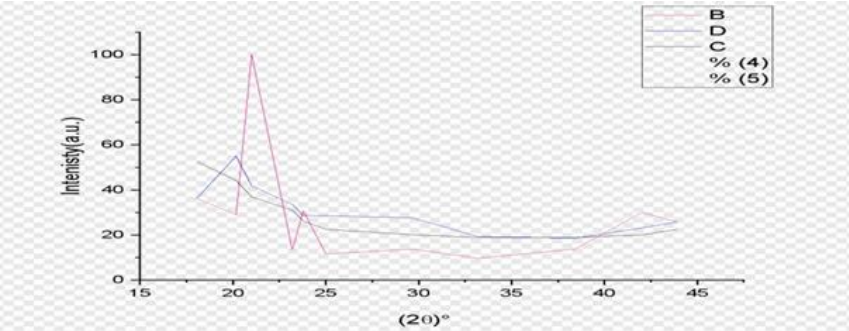


**Figure 7:** shows the XRD patterns of the PHB–PEG copolymer before and after azithromycin was added. Pure azithromycin (curve B) has sharp crystalline peaks at 2θ = 19–21°, but the drug-loaded copolymer (curve A) has lower intensity and broader peaks.

## 3. 7 Anti-bacterial activity

The efficacy of drug delivery systems (DDS) based on polymers has been evaluated at varying concentrations (MIC) of 250-1000 micrograms per millilitre against Azithromycin-resistant *Salmonella*, showing significant antibacterial activity. The loading data derived from "Figure 8, 9 ", are documented in "Table 3".

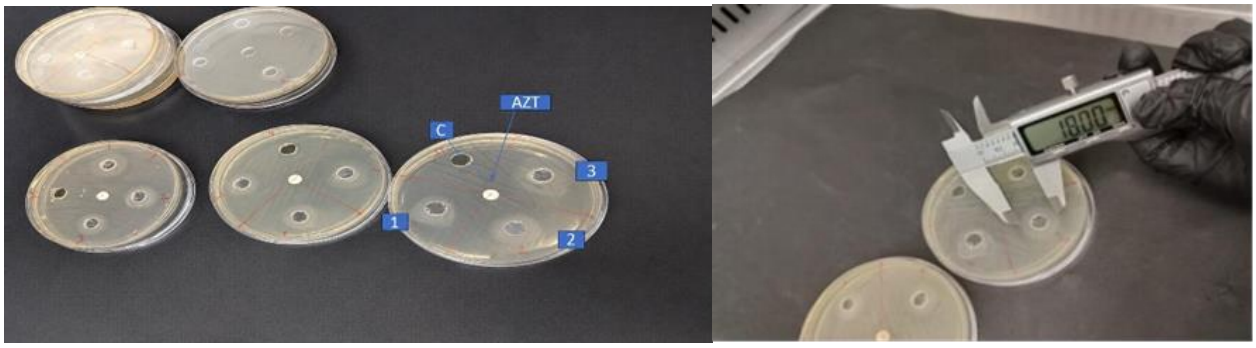


**Figure 8:** shows how well the PHB-based DDS works against *Salmonella enterica* resistance using agar well diffusion. As the concentration of DDS (1,2,3), AZT (Azithromycin disc) and C as control, the zones of inhibition grow, showing that the antimicrobial activity is dose-dependent.

Of the eight *Salmonella* strains tested, five (71%) were responsive to all dosages, while two strains (29%) remained resistant to DDS, lower MIC groups are S1-S6 at 250µg $mL^{-1}$, whereas the higher MIC strain S7 at 500 and S8 at 1000 µg $mL^{-1}$. However, when blank PHB-PEG were present, bacterial growth was visible, suggesting that they were not harmful to bacterial cells [53].

The fisher's exact test indicated that DDS was substantially more efficacious than Azithromycin (p = 0.021). The antibacterial activity assay of the DDS demonstrated higher efficacy against more resistant strains of *Salmonella*. Generally, AZI encapsulation inside a polymeric matrix maintains the therapeutic efficacy of AZI compared to pure AZI at a concentration of 1/8 of the total medicine [25].

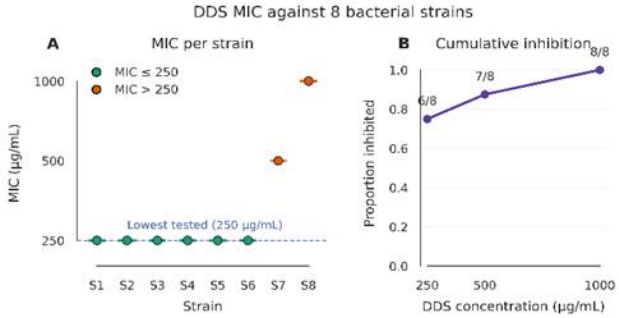


**Figure 9:** Antibacterial activity of the DDS across eight bacterial strains. (A) Minimum inhibitory concentration (MIC) for each strain (S1–S8) measured using a three-point concentration series (250, 500, 1000 µg/mL). Points show strain-level MICs plotted on a log2 scale; the dashed line marks the lowest tested concentration (250 µg/mL). Strains classified as *sensitive at all tested concentrations* were assigned MIC ≤ 250 µg/mL (plotted at 250 µg/mL). (B) Cumulative inhibition as a function of DDS concentration, expressed as the proportion of strains inhibited at each tested concentration; annotations indicate the number of inhibited strains out of the total (n/N).

**Table 3:** Accomplished this by quantifying their zones of inhibition of DDS in mm and contrasting them with the standard Azithromycin antibiotic disc (0.015 µg), against eight completely resistant strains of *Salmonella enterica*.

| *Sample ID* | *Zon of inhibition Azthromycin antibiotic disc* | *Zone of inhibition DDS mm* |
|---|---|---|
| *S1* | *0.0*± 0.0* | *25.4± 0.5* |
| *S2* | *0.0*± 0.0* | *25.8± 0.5* |
| *S3* | *0.0*± 0.0* | *35.0± 0.6* |
| *S4* | *0.0*± 0.0* | *32.9± 0.4* |
| *S5* | *0.0*± 0.0* | *36.0± 0.3* |
| *S6* | *0.0*± 0.0* | *38.0± 0.7* |
| *S7* | *0.0*± 0.0* | *0.0*± 0.0* |
| *S8* | *0.0*± 0.0* | *0.0*± 0.0* |

0* complete resistance (no zone of inhibition)

## 4 Conclusion

High-molecular-weight polymers stabilize and release drugs. However, SEM images show big and irregular particles. This is in agreement with the literature as such polymers are hard to prepare on the nanoscale owing to their high viscosity and entanglement. Therefore, their use in nano–drug delivery systems is limited. No molecular conformation of bacterial strains used for biopolymer production also; toxicity or in vitro biocompatibility tests were performed Future research should include investigations to reduce the molecular weight of these polymers or improve the fabrication process to make nanoscale systems.

This study is designed to select effective poly-hydroxybutyrate strains to maxim PHB yield under optimal conditions, subsequently exploring a formulation for drug delivery development using a PHB copolymer as a novel carrier for Azithromycin against antibiotic-resistant *Salmonella*. Our research indicates that efficient PHB-producing strains, together with an optimal fermentation approach, yield high production levels and encapsulate the medication azithromycin. In the final analysis, the utilization of a produced encapsulated medication, capable of encapsulating Azithromycin, may serve as a microsphere and environmentally friendly biopolymer that reduces pollution in the environment, while also addressing the resistance issue in *Salmonella*, demonstrating considerable therapeutic efficacy.

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## Conflict of interest

A conflict of interest does not exist.

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