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Development of Nanospheres Formation with Sulfasalazine Drug and its degradation studies

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ABSTRACT: This research focuses on the development of nanospheres containing sulfasalazine encapsulated with polyhydroxybutyrate (PHB) and their degradation behavior. Nanospheres were fabricated with biodegradable polymers Poly Hydroxy Butyrate(PHB) by using a sol gel method yielding uniform spherical particles and efficient drug loading. In vitro release studies demonstrated sustained drug release, promising extended therapeutic benefits. Accelerated degradation tests under varying conditions revealed the nanospheres stability, exhibiting minimal drug degradation. The findings suggest that nanospheres containing sulfasalazine encapsulated with polyhydroxybutyrate (PHB) hold potential for enhanced drug delivery with reduced degradation concerns, offering a viable strategy for improving therapeutic outcomes in inflammatory disease treatments such as Inflammatory bowel disease (IBD),Ulcerative colitis,Crohn's disease and Colon cancer.

Keywords: Sulfasalazine, Nanospheres, polymer, PHB, Invitro release, Drug Degradation, Inflammatory Disease.

I. INTRODUCTION

1.1 GENERAL

Nanospheres are colloidal particles with diameters typically in the nanometer range (1-1000 nm)[1]. The goal of drug nanospheres is to improve slow drug delivery by encapsulating the drug in nano-sized microspheres. These nanospheres can improve drug solubility, stability and bioavailability, leading to better therapeutic outcomes and reduced side effects. They can target specific cells or tissues, improve controlled release and allow for more precise dosing, making them a promising method in the field of pharmaceutical research [2].The development of nanospheres loaded with drugs and subsequent degradation studies are essential steps in ensuring the effectiveness and safety of drug delivery systems.The first step in developing nanospheres involves selecting suitable biocompatible polymers. The selected drug is then incorporated into the polymer matrix through various techniques, such as solvent evaporation, emulsion-solvent diffusion, or nanoprecipitation.The nanospheres are characterized for size, shape, drug loading, and encapsulation efficiency using techniques like dynamic light scattering, scanning electron microscopy, and high-performance liquid chromatography [3].

Degradation Studies to evaluate drug release from the nanospheres, in vitro release studies are conducted. Nanospheres are suspended in a release medium, typically simulated body fluids or buffers, and sampled at regular intervals. The concentration of released drugs is quantified using analytical methods. This

study helps assess the drug release kinetics and the duration of sustained release.Nanospheres stability is evaluated under various storage conditions (e.g., temperature, humidity, light).Biodegradable nanospheres are designed to break down gradually in the body. In vivo studies or accelerated degradation tests simulate the conditions under which these nanospheres degrade.The trials involve different phases and aim to establish the optimal dosage, administration route, and therapeutic benefits [4].

1.2 NANOSPHERES

Nanospheres are the division of polymeric nanoparticles. Nanospheres are matrix type structures, which the spherical particulate systems are characterized by a size range between 10-200 nm-are widely used as carriers in drug delivery systems in clinical application [1]. Basically the drug dissolved, encapsulated, entrapped and attached to the matrix of polymer. The drug is uniformly dispersed to form a homogeneous structure. Nanospheres can be amorphous or crystalline in nature, and also they have protected the drug from enzymatic and chemical degradation 5]. Nanospheres can be biodegradable or non- degradable. Some biodegradable nanospheres include modified starch nanospheres, albumin nanospheres, gelatin nanospheres, polypropylene dextran nanospheres and polylactic acid nanospheres. Administration of medication via systems offers high advantages, they can be ingested or injected. Nanospheres can be used for the organ targeted release of drugs. Nanospheres offer high potential to formulate diverse range systems that can be given by new patent life to known effective pharmacophores [3]. Nanospheres can be used to target the organs like liver, spleen, lungs, spinal cord [6].

1.3 CHARACTERISTICS OF SULFASALAZINE - DRUG

Sulfasalazine is a disease-modifying antirheumatic drug (DMARD) used to treat and manage autoimmune diseases, including rheumatoid arthritis and an inflammatory bowel disease called ulcerative colitis. It's a slow acting DMARD that takes time to build up and start working. Sulfasalazine helps reduce pain, swelling, and inflammation in your body [2]. It limits the damage that rheumatoid arthritis causes to your joints, helping to stop the progression of the disease.Sulfasalazine consists of the anti-inflammatory agent 5-aminosalicylic acid (5-ASA, mesalamine or mesalazine) and the antibiotic sulfapyridine, linked by a bond. The way sulfasalazine works is still not fully understood. Sulfasalazine is too bulky to be absorbed by your small intestine, but bacteria in your colon can break the bond between 5-ASA and sulfapyridine, releasing 5-ASA to work locally in your colon to help fight ulcerative colitis [7].

1.4 POLYHYDROXYBUTYRATE(PHB)

Poly(3-hydroxybutyrate) (PHB) is a biopolymer of 3-hydroxybutyrate and the most significant member of the biodegradable thermoplastic polyhydroxyalkanoate family, with characteristics of high melting temperature, a high degree of crystallinity, and low permeability to O2,H2O, and CO2. Polyhydroxybutyrate can be classified into different monomers based on the carbon chain attached: short length (contains 5 carbon PHB units), medium length (contains 6–14 carbon PHB units), and long length (contains more than 14 carbon PHB) [8]. PHB is a good replacement for synthetic polymer, and its mechanical properties are comparable to that of polypro-pylene [9]. PHB possesses the characteristics of thermoplasticity and biodegradability in compost which has attracted commercial attention. PHB has great potential in food packaging applications with better water vapor barrier properties than polypropylene and better oxygen barrier properties than both polyethene terephthalate and polypropylene [10]. PHB *in vitro* biocompatibility has been established on different cell lines such as fibroblasts, osteoblasts, bone marrow cells, endothelial cells, smooth muscle cells, etc. The PHB homopolymer is rigid and brittle in nature [11]. More than 20 bacterial species such as

Bacillus megaterium, Methylobacterium rhodesianum, Alcaligenes eutrophus, M. extorquens, P. Putida, Sphaerotilus natans, Escherichia coli, etc. are known as effective producers of PHB [12]. Among gram-positive bacteria, *Bacillus spp., B. subtilis, Bacillus thuringiensis*, and *B. cereus* and among gram-negative bacteria, *Pseudomonas spp.* are notable for the production of PHB [13].

1.5 ANTIMICROBIAL ACTIVITY

Antimicrobial activity has been demonstrated *in vitro* for several herb and spice extracts and essential oils from thyme, oregano, parsley, cilantro, and cinnamon. Growth of several bacteria strains has been shown to be inhibited by various concentrations of these culinary herb and spice extracts in the culture medium. The well diffusion test (also known as the agar diffusion test, Kirby–Bauer test, disc-diffusion antibiotic susceptibility test, disc-diffusion antibiotic sensitivity test and KB test) is a culture-based microbiology assay used in diagnostic and drug discovery laboratories. In diagnostic labs, the assay is used to determine the susceptibility of bacteria isolated from a patient's infection to clinically approved antibiotics. This allows physicians to prescribe the most appropriate antibiotic treatment[14]. In drug discovery labs, especially bioprospecting labs, the assay is used to screen biological material (e.g. plant extracts, bacterial fermentation broths) and drug candidates for antibacterial activity. When bioprospecting, the assay can be performed with paired strains of bacteria to achieve dereplication and provisionally identify antibacterial mechanisms of action [15]. Caffeic acid phenethyl ester on is suggested to disrupt the bacterial outer membrane and to provoke superoxide radical stress in bacteria, leading to protein and DNA damage [16]. An in vitro study of K21 coated sutures at concentrations ranging from 5 to 25% showed a dose and suture-dependent antimicrobial activity [17].

1.8 DRUG DEGRADATION

Forced degradation involves degradation of drug and drug product in circumstances that are more rigorous in comparison to accelerated conditions. It offers an insight into the degradation pathways and the degradation products of the drug, as well as assisting in the elucidation of the degradation products structure. In the regulatory compliant stability program design of drug and drug products forced degradation study is an essential step. In 1993 such studies were formalized as a regulatory requirement in ICH Guideline Q1A [4]. The principal cause of impurities in drugs and drug products is degradation. The chemical, as well as physical degradation of the drug and drug product in conditions such as heat, humidity, solvent, pH, and light, occur at the time of manufacturing, isolation, purification, drying, storage, transportation, and formulation are the main reasons for its instability [18]. FDA guidance states that stress testing should be performed in phase III of the regulatory submission process. Stress studies should be done in different pH solutions, in the presence of oxygen and light, and at elevated temperatures and humidity levels to determine the stability of the drug substance. These stress studies are conducted on a single batch. The results should be summarized and submitted in an annual report [19]. Degradation of drug substances between 5% and 20% has been accepted as reasonable for validation of chromatographic assays [20].

II. MATERIALS AND METHODS

2.1 ISOLATION OF *Bacillus sp.* FROM SOIL

The soil sample with a depth of 1-3 cm was obtained from Sowripalayam (latitude: 11.0115913, longitude: 77.0089332) in Coimbatore, Tamil Nadu, India, with a sterile spatula. About 20 g of soil sample was obtained. From the sample 1g of soil is taken and dissolved in 100ml of distilled water. The serial dilution was performed (10^{-1} to 10^{-7}) and plated on nutrient agar using spread plate technique. The

plates were incubated at 37°C for 24hrs. Microscopic views of selected strains were performed. For further research, the isolated *bacillus* colonies were chosen and subcultured on nutrient agar and stored at 4°C [21].

2.2 SCREENING OF PHB PRODUCING *Bacillus sp.* FROM SUDAN BLACK TECHNIQUE:

0.1g of sudan black powder was prepared by dissolving 75ml of 95% ethanol & adding 25 ml of distilled water and it was made up to 100 ml. The mixture was filtered twice and it was stored. The selected strains were taken and flooded with the sudan black for 20 mins and viewed under the microscope [21].

2.3 EXTRACTION OF PHB FROM SCREENED *Bacillus sp* BY USING CHLOROFORM EXTRACTION METHOD

10 ml of screened *Bacillus* sp broth was taken in a centrifuge tube and centrifuged for 10 minutes at 5000 rpm. Discard the supernatant and to the pellet 2 ml of acetone and ethanol was added to remove the unwanted dirt. 2 ml of 4% sodium hypochloride was added and incubated for 30 minutes at 38°C in a waterbath. After the incubation period, the whole mixture was added with equal volume of ethanol and acetone. i.e. 1 ml each and 2 ml of chloroform were added to the tubes. The upper phase which consisted of sodium hypochlorite solution was removed and the middle phase (chloroform Containing undisturbed) was separated by filtration from the bottom phase (chloroform with PHB) [21].

2.4 FORMATION OF NANOSPHERES WITH PHB BY USING SOL GEL METHOD:

20 ml of distilled water was taken and added 2g PVA (poly vinyl alcohol) as a first binder. Polyvinyl alcohol (PVA) was stirred in a magnetic stirrer until PVA dissolved. 0.1g poly Hydroxy Butyrate (PHB), 0.2 g of sodium alginate as a second binder and 0.5 g of drug sulfasalazine was added. Made the solution to thick paste (solid consistency). prepared 5% CaCl2 in 50 ml of distilled water and poured it on a petri dish. The prepared drug solution was poured drop by drop by syringe. After 30 mins, nanospheres were formed and microscopic views were observed [22].

2.5 FORMATION OF NANOSPHERES WITHOUT PHB BY USING SOL GEL METHOD:

20 ml of distilled water was taken and added PVA (poly vinyl alcohol) as a first binder.Then stirring of polyvinyl alcohol (PVA) with a magnetic stirrer until PVA dissolved. Added 0.2 g of sodium alginate as a second binder and then added 0.5 g of drug sulfasalazine. Make the solution to thick paste (solid consistency). prepared 5% CaCl2 in 50 ml of distilled water and poured it on a petri dish. The prepared drug solution was poured drop by drop by syringe. After 30 mins, nanospheres were formed and microscopic views were observed [22].

2.6 FTIR (FOURIER TRANSFORM INFRARED SPECTROSCOPY)

The extracted PHB was characterized.The sample was exposed to infrared radiation from an infrared source.The functional groups of PHB, drug (sulfasalazine) and PHB with drug(sulfasalazine) were made on comparision.

2.7 ANTIMICROBIAL ACTIVITY

To perform antimicrobial activity using different bacterial species Escherichia coli, Staphylococcus aureus. All bacterial strains were obtained from Microbial Type Collection and Culture (MTCC) in Chandigarh, India. Peptone broth, beef extract, yeast extract, sodium chloride, and nutritional agar were used throughout the study to determine antimicrobial

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dosage. The medium was adjusted to pH and autoclaved at 121°C for 15 minutes [23].

2.7.1 WELL-DIFFUSION METHOD

Test drug solution (1000 µg) PHB coated with sulfasalazine was prepared by dissolving 10 µg, 20 µg, 30 µg, 40 µg. 50 µg of sample was collected separately in Eppendorf tubes and returned to 1000 µg with distilled water. The tested strains of E. coli and staphylococci were inoculated onto the agar plates with cotton swabs. Then, sterile wells containing each PHB test solution with the drug (200 µl) were placed on beaded agar plates such that there were no overlapping zones of inhibition. The plates were kept at room temperature for two hours to allow the drug to diffuse into the agar; they were incubated for 24 hours at 37°C for zone growth.

2.8 DEGRADATION STUDIES

In-vitro drug release is a unique technique in which the prepared encapsulated nanospheres are placed in equal volumes of distilled water in the dissolution medium used. Different pH and temperature conditions were maintained. The pH was adjusted to the required values (2,5,7) and each adjusted pH value was maintained at +27°C, +37°C, +40°C.Absorbance was taken on every hour continuously from initial to 8th hour on each PHB at all temperatures in the calorimeter [24].

III. RESULT AND DISCUSSION

3.1 ISOLATION OF *Bacillus sp.* FROM SOIL

Microorganisms were isolated from the soil sample, and isolated colonies were created through serial dilution. Bacterial colonies with diverse morphological characteristics were chosen, and a number was assigned to each colony. By streaking these colonies on nutrient agar plates, the colonies were preserved for further study. Five different bacterial colonies from the crowded plate technique were selected as strains B1, B2, B3, B4 and B5.

***Bacillus* sample**	**Colony Morphology**
B1	Large dried edged colonies
B2	Large colonies
B3	Large irregular colonies
B4	Small dried colonies
B5	Small irregular colonies

Table 3.1: Colony Morphology

Colony Bl is large and dried-edged, which is a typical morphology for *Bacillus sp.* Colonies B2 and B3 are also large, but they have irregular edges. This could be due to a number of factors, such as the culture conditions or the presence of other organisms in the sample. Colony B4 is small and dried, which is another common morphology for *Bacillus sp*. Colony B5 is small and irregular. This could be due to the same factors as for colonies B2 and B3.

3.2 PRELIMINARY SCREENING OF PHB PRODUCING *Bacillus sp.* FROM SUDAN BLACK TECHNIQUE

Five colonies were displayed in Sudan black staining. They are B1, B2, B3, B4 and B5. These isolates tested positive for the presence of Sudan-black absorption patterns and lipophilic PHB granules.

Table 3.2: PHB Production efficiency

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Bacillus Sample	Colony Morphology
B1	Minimum absorption of Sudan Black
B2	Minimum absorption of Sudan Black
B3	Moderate absorption of Sudan Black
B4	Minimum absorption of Sudan Black
B5	Maximum absorption of Sudan Black

The table shows that all five *Bacillus* samples absorbed Sudan Black B1 to B5 indicating that all of them are capable of producing PHB. However, there is a significant variation in the amount of PHB produced by the different strains. Colony B5 showed the maximum absorption of Sudan Black, indicating that it is the most efficient PHB producer of the five strains tested.

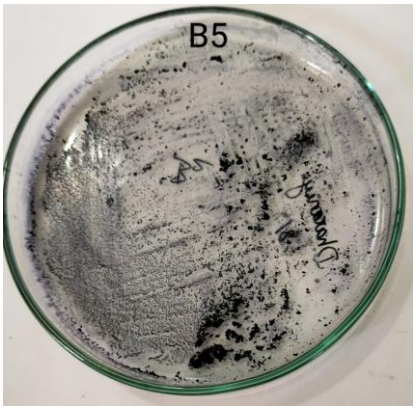

Figure 3.1: Strains Of Sudan Black Stain B5

3.3 EXTRACTION OF PHB FROM SCREENED *Bacillus sp* BY USING CHLOROFORM EXTRACTION METHOD

The centrifuge tube produces three different phases. Sodium hypochlorite solution is present in the top phase, undisturbed cells are present in the middle phase of chloroform, and PHB is present in the bottom phase of chloroform. A micropipette was used to transfer the bottom phase, which contained chloroform and PHB, into a petri dish, where it was left to evaporate for 20 minutes at 70 °C in a water bath. By scratching, PHB powder is removed from the petri plate and placed in an eppendorf tube.

Figure 3.2: PHB Extraction Using Chloroform Extraction Method

3.4 FORMATION OF NANOSPHERES WITH PHB BY USING SOL GEL METHOD:

Nanospheres formed on the CaCl2 solution's surface. For further studies, the nanospheres were stored in eppendorf tubes.

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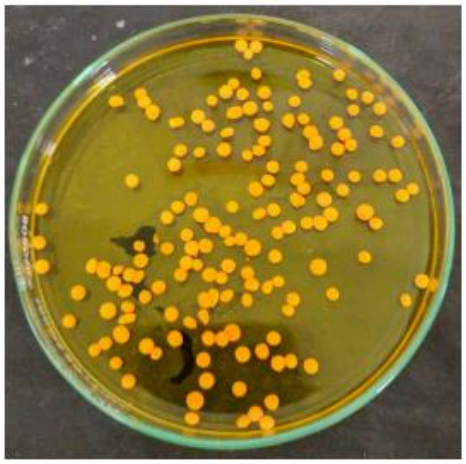


Figure 3.3: Formation of nanospheres with PHB by using sol gel method

Nanospheres formed on the CaCl2 solution's surface. For further studies, the nanospheres were stored in eppendorf tubes.

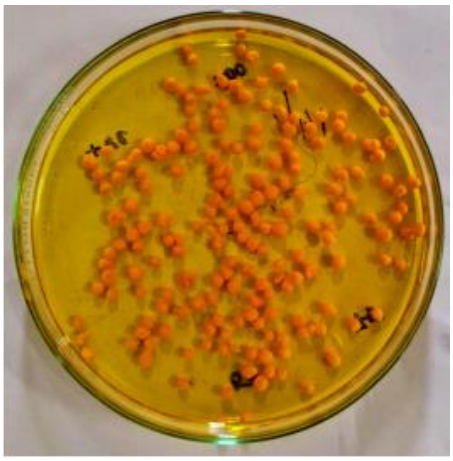


Figure 3.4: Formation of nanospheres without PHB by using sol gel method

3.5 FORMATION OF NANOSPHERES WITHOUT PHB BY USING SOL GEL METHOD:

3.6 COMPARATIVE STUDIES OF FTIR ANALYSIS OF PHB , SULFASALAZINE, SULFASALAZINE WITH PHB

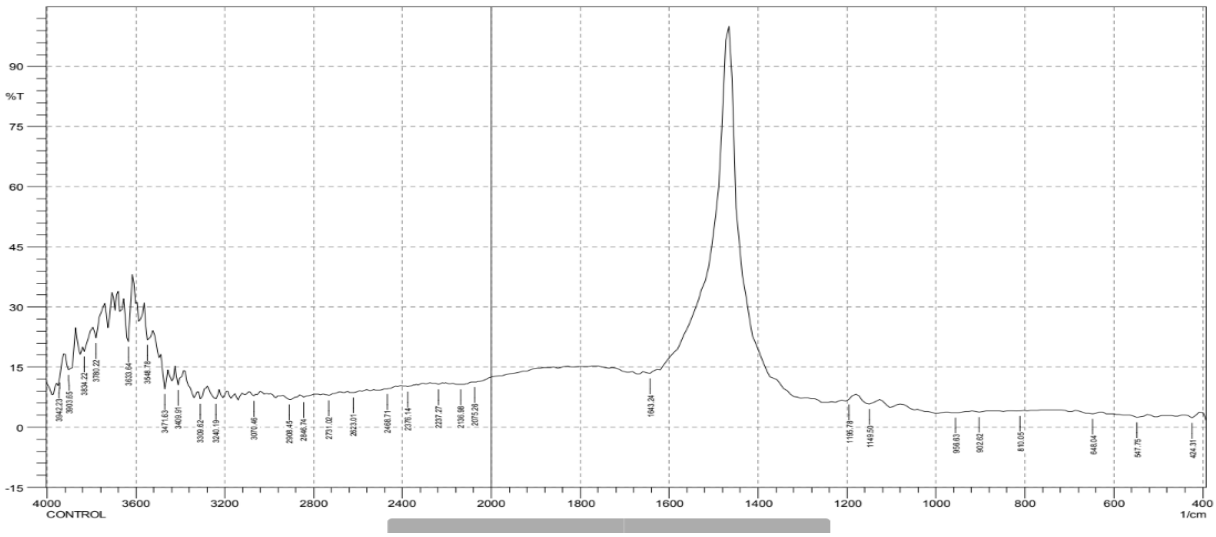


Figure 3.5: FTIR Analysis of PHB

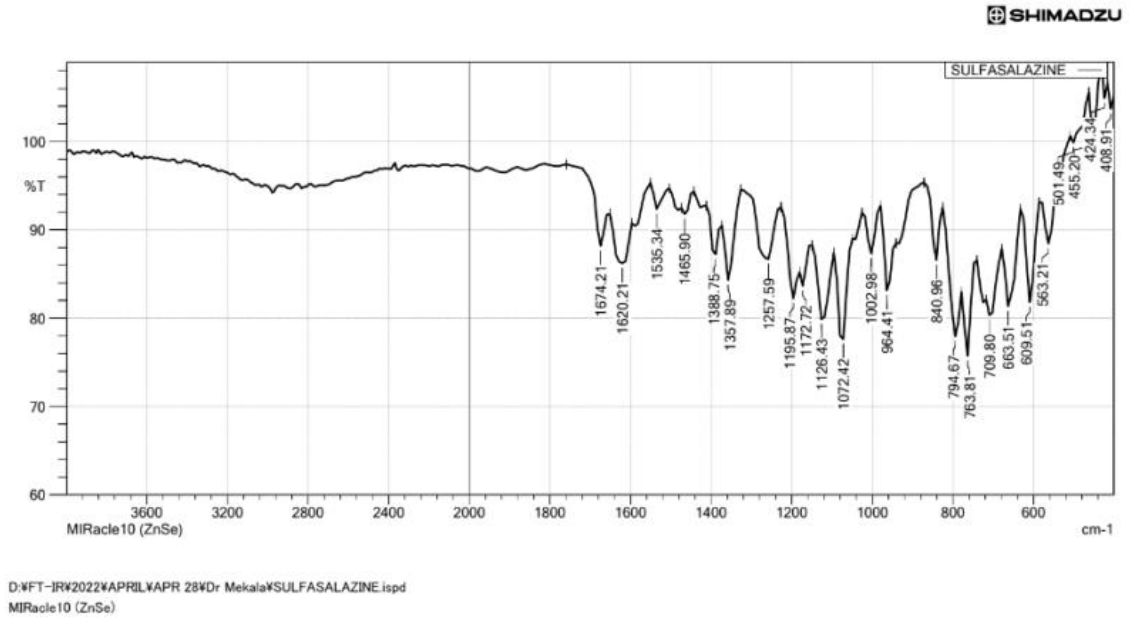
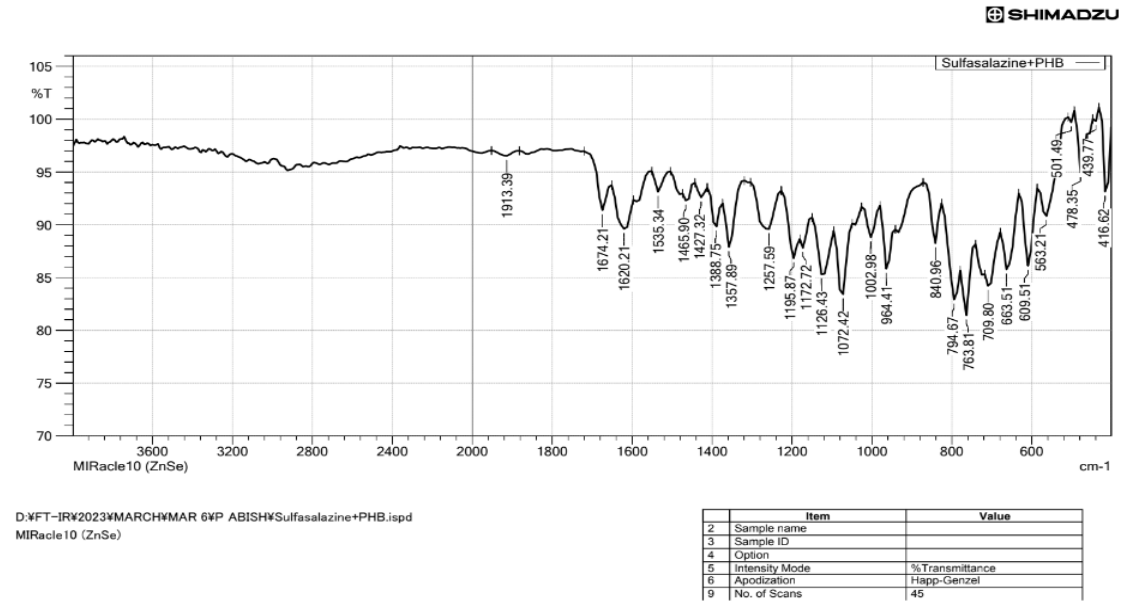


Figure 3.6: FTIR Analysis of sulfasalazine



	Item	Value
2	Sample name	
3	Sample ID	
4	Option	
5	Intensity Mode	%Transmittance
6	Apodization	Happ-Genzel
9	No. of Scans	45

Figure 3.7: FTIR Analysis of Sulfasalazine and PHB

SNO	PHB cm	SULFASALAZINE	SULFASALAZINE +PHB	FUNCTIONAL GROUP & COMPOUND	APPEARANCE

1	1643.24	1674.21	1674.21	C=N (inime, oxime)	medium
2	1195.78	1195.87	1195.87	C-H(aromatic compound)	weak
3	1149.50	1126.43	1126.43	C-O (tertiary alcohol)	strong
4	810.05	840.96	840.96	C-Cl (halo compound)	strong
5	640.04	663.51	663.51	C-Br (halo compound)	strong
6	547.75	563.21	563.21	C-l (halo compound)	strong

Table 3.3: Comparison of FTIR results

The FTIR spectrum obtained at peak 1 revealed that presence of C=N(1643.24cm-1) stretching seems to be similar in PHB and the PHB with Sulfasalazine.The FTIR spectra obtained at peak 2 show the presence of prolonged C-H(1195.78cm-1) stretching which seems to be similar in PHB and PHB with Sulfasalazine. Band observations at peak 3 revealed the presence of C-O(1149.50cm-1) seems to be similar in PHB and PHB with sulfasalazine. Band observed at peak 4 revealed the presence of C-Cl(810.05cm-1) seems to be similar in PHB and PHB with sulfasalazine.The FTIR spectrum obtained at peak 5 revealed that presence of C-Br(648.04cm-1) stretching seems to be similar in PHB and the PHB with Sulfasalazine.The FTIR spectrum obtained at peak 6 revealed that presence of C-I (547.75cm-1) stretching seems to be similar in PHB and the PHB with Sulfasalazine. It concludes the presence of Nanospheres in PHB with sulfasalazine.

3.7 ANTIMICROBIAL ACTIVITY

Figure 3.8: Sulfasalazine against *S.aureus*

Figure 3.9: sulfasalazine against *E.coli*

No zone was formed. The test organism *E.coli* and *S.aureus* are resistant to sulfasalazine

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3.8 DEGRADATION STUDIES

SNO	pH	Temperature	1st hr (absorbance)	2nd hr (absorbance)	3rd hr (absorbance)	4th hr (absorbance)	5th hr (absorbance)	6th hr (absorbance)	7th hr (absorbance)	8th hr (absorbance)
1	pH 2	27ºC	0.02	0.033	0.041	0.052	0.055	0.052	0.048	0.045
		37ºC	0.08	0.16	0.21	0.25	0.27	0.26	0.23	0.21
		40ºC	0.04	0.07	0.09	0.11	0.12	0.1	0.08	0.07
2	pH 5	27ºC	0.03	0.05	0.07	0.09	0.1	0.09	0.08	0.07
		37ºC	0.021	0.033	0.045	0.058	0.06	0.08	0.039	0.033
		40ºC	0.07	0.15	0.2	0.26	0.28	0.26	0.24	0.19
3	pH 7	27ºC	0.09	0.16	0.2	0.24	0.26	0.23	0.2	0.18
		37ºC	0.03	0.05	0.06	0.09	0.1	0.09	0.07	0.05
		40ºC	0.02	0.03	0.04	0.049	0.05	0.048	0.039	0.035

Table 3.4: Degradation studies on sulfasalazine

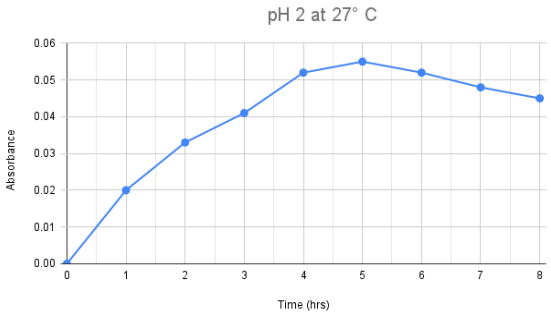

Figure 3.10: Plot of absorbance versus time (pH 2 and 27°C temperature)

Nanospheres are kept at 27°C,the maximum observation peak on 5th hr at pH 2 (0.055) and degradation continuous till 8th hr here the value is 0.045.

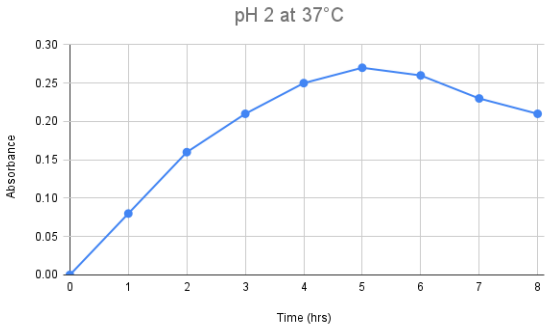

Figure 3.11: Plot of absorbance versus time (pH 2 and 37°C temperature)

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Nanospheres are kept at 37°C,the maximum observation peak on 5th hr at. pH 2 (0.27) and degradation continuous till 8 hr here the value is 0.21.

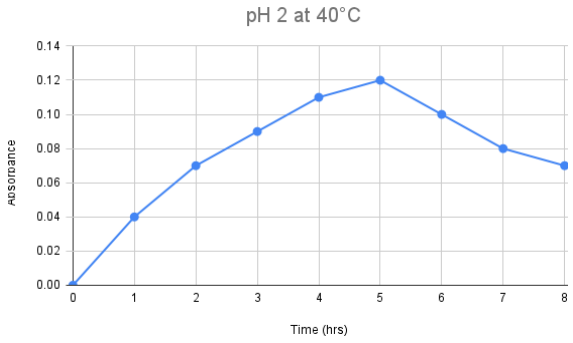

Figure 3.12: Plot of absorbance versus time (pH 2 and 40°C temperature)

Nanospheres are kept at 40°C,the maximum observation peak on 5th hr at pH 2 (0.12) and degradation continuous till 8 hr here the value is 0.07.

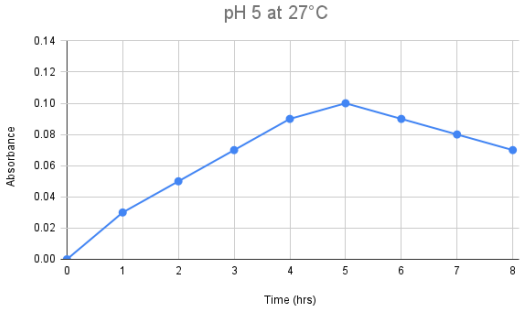

Figure 3.13: Plot of absorbance versus time (pH 5 and 27°C temperature)

At pH 5, for 27°C temperature the maximum observation peak is 5th hr (0.1) and degradation continues to 8th hr value (0.07).

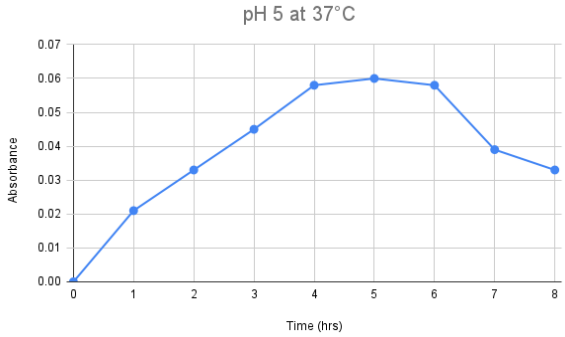

Figure 3.14: Plot of absorbance versus time (pH 5 and 37°C temperature)

At pH 5, for 37°C temperature the maximum observation peak is 5th hr (0.06)and degradation continues to 8th hr value (0.033).

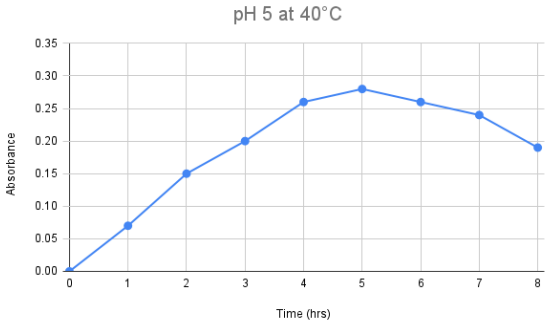

Figure 3.15: Plot of absorbance versus time (pH 5 and 40°C temperature)

At pH 5, for 40°C temperature the maximum observation peak on 5th hr (0.28)and degradation continues to 8th hr value (0.19).

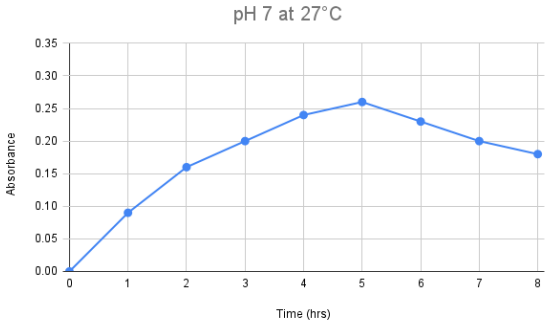

Figure 3.16: Plot of absorbance versus time (pH 7 and 27°C temperature)

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At pH 7, for 27°C temperature the maximum observation peak is on the 5th hr (0.26) and the degradation continues till 8 hr to 0.18 .

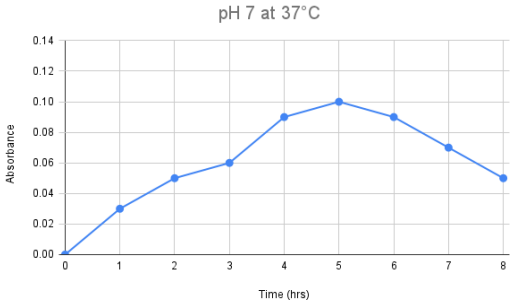

Figure 3.17: Plot of absorbance versus time (pH 7 and 37°C temperature)

At pH 7, for 37°C temperature the maximum observation peak is on the 5th hr (0.1) and the degradation continues till 8 hr to 0.05.

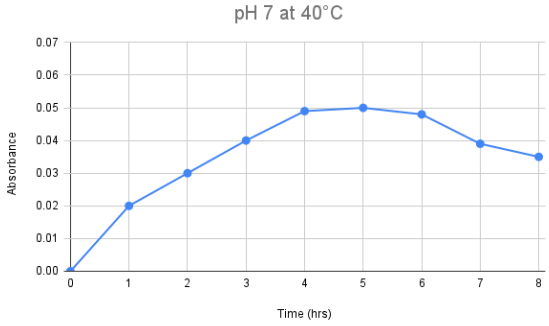

Figure 3.18: Plot of absorbance versus time (pH 7 and 40°C temperature)

At pH 7, for 40°C temperature the maximum observation peak is on the 5th hr (0.05) and the degradation continues till 8 hr to 0.035 .

The result shows the outcomes of a study on the in-vitro drug release of sulfasalazine nanospheres in distilled water under various pH and temperature conditions. The table suggests that sulfasalazine degrades more quickly at higher temperatures and lower pH values.

The enhanced swelling of the nanospheres and the higher solubility of sulfasalazine in acidic solutions are likely to blame for the quicker release at pH 2 and 40°C. Sulfasalazine is less soluble and the nanospheres are less likely to swell at pH 7, which results in a slower release.

The effect of temperature on drug release is also complex. In general, greater temperatures speed up diffusion, which can result in quicker drug release. High temperatures, however, can potentially cause the nanospheres to disintegrate, slowing the release of the medicine. The higher diffusion at 40°C in the case of the encapsulated sulfasalazine nanospheres surpassed the degradation of the nanospheres, leading to a quicker drug release.

Overall, the in-vitro drug release study's findings indicate that by adjusting the pH and temperature levels, encapsulated sulfasalazine nanospheres can be created to release the drug at various rates and intervals. This may be helpful for targeting particular body tissues or cells or for administering sulfasalazine to various regions of the gastrointestinal tract.

IV. CONCLUSION

The development of Nanosphere formation of sulfasalazine with the biodegradable polymer PHB(Polyhydroxybutyrate) which extracted from *bacillus sp* is achieved.The development of nanospheres for sulfasalazine delivery is a promising approach for improving the efficacy and safety of this important drug. Nanospheres can protect sulfasalazine from degradation, target it to the site of inflammation, and release it over a sustained period of time. These properties could lead to improved outcomes for patients with inflammatory bowel disease and other conditions treated with sulfasalazine.Therefore the developed sulfasalazine nanospheres improve the slow drug delivery and can improve drug solubility, stability and bioavailability, leading to better therapeutic outcomes. They can target specific cells or tissues, improve controlled release of drugs.

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