ISOLATION, PRODUCTION, EXTRACTION, OPTIMIZATION AND FORTIFICATION OF PHB USING SILVER NANOPARTICLES FROM *LACTOBACILLUS CASEI*

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Abstract

In this study, bacteria were first isolated to produce the biopolymer PHB (poly - 3 hydroxybutyrate) as a substitute for plastic, which is a major contributor to soil pollution and excess plastic wastage worldwide. Plastic lacks the property to degrade in soil but is used in large forms. To overcome this problem, bioplastics are synthesized which are biodegradable compounds that are completely sustainable and can substitute fossil fuels and non-degradable plastic. In this study, the ideal organism for the production of PHB was first isolated from soil samples and then grown on suitable media. After identifying the isolate as Lactobacillus by performing various biochemical tests. The organism was then mass-produced to extract PHB by differential digestion treatment to extract the bacterial pellet containing PHB. The estimation of PHB was done by using a double-beam UV-VIS spectrophotometer, the readings were taken and maximum absorbance was noted. Optimization is performed to increase the polymer production in PHB-producing organisms, in optimization we check for the optimal conditions of different concentrations of carbon and nitrogen sources where we estimate the dry weight and check for the source showing the maximum dry weight. The post-synthesis of PHB nanoparticles was done to enhance its properties and increase the strength of the polymer. It is done by introducing silver nitrate to the bacterial pellet containing PHB. The presence of silver PHB nanoparticles was estimated using a double-beam UV-VIS spectrophotometer, the readings were taken and maximum absorbance was noted.

Keywords. PHB, Bioplastics, Differential digestion treatment Optimization, Silver nanoparticles.

1. Introduction

Because naturally occurring soils cannot break down plastics in their native form, the overproduction of plastics has become a major pollution-related problem. Plastics, including microplastics, are now ubiquitous in our natural environment. Systemic changes are needed to stem the flow of plastic waste into the environment. To date, less than 10% of the billions

of tons of plastic waste generated worldwide has been recycled. Millions of plastic wastes are lost from the environment, sometimes transported thousands of kilometres to destinations where most are burned or dumped. Rivers and lakes carry plastic waste deep inland to the ocean and are a major source of marine pollution. Excessive Plastic production and its improper disposal have proven to be a significant source of pollution, especially since plastic wasteland is easily biodegradable. As a result, plastic waste accumulates in our environment, including the oceans, posing a significant threat to marine life and ecosystems. Recycling is one way to manage plastic waste, but it is clear that we need to address this problem with a more comprehensive approach that goes beyond recycling. This includes reducing plastic production, improving waste management methods, and developing alternative materials such as bioplastics **[Rosenboom, J., Langer, R., & Traverso, G. (2022)]**. Plastics are Considered durable and resistant to degradation, but this same property is a major drawback because it makes it impossible to degrade plastic. Bioplastics are fully sustainable, biodegradable compounds that replace fossil fuels and non-degradable plastics. Analysts have been searching for modern, selectable materials that can be used as excellent substitutes for conventional plastics. One of them is the generation of bio-based biodegradable plastics [BP]. These days, bioplastics account for almost 1% of the nearly 300 million tons of plastics shipped each year. Bioplastics are promising alternatives to conventional plastics. They are made from natural, renewable resources such as corn starch, sugarcane, and potato starch, and they biodegrade naturally in the environment. It Should Be Noted, however, that not all bioplastics are created equal, and some can be harmful to the environment if not disposed of properly. Ultimately, addressing the plastic pollution crisis will require systemic change, which will require the cooperation of individuals, governments, and businesses around the world. We must reduce our dependence on single-use plastics, improve waste management practices, and invest in the development of sustainable materials and technologies **[Atiwesh, G., Mikhael, A., Parrish, C. C., Banoub, J., & Le, A. T. (2021)]**. Bioplastics are plastics made from renewable resources such as vegetable oil and cornstarch. They can be biodegradable or non-biodegradable. Biodegradable plastics break down naturally, but this is dependent on specific conditions, such as high temperatures. Compared To Conventional plastics, bioplastics emit fewer greenhouse gases during production and reduce dependence on fossil fuels. However, bioplastics aren't always recyclable and may require specific disposal methods. Bioplastics do not harm the natural environment. Biodegradation is the partial or complete hydrolysis of polymers by microbial activity, with the positive effect of photodegradation. However, this process is time-consuming and often requires high temperatures.

- **PHA and Synthesis of 'PHB'**

Bioplastics are defined as a class of plastics synthesized from renewable sources such as plant starch and microbial species. Some of the bioplastics are natural biodegradable polymers made from polyhydroxyalkanoates (PHAs). PHA is a carbon-neutral and valuable polymer that is produced by microorganisms from many renewable carbon sources, making it a sustainable and environmentally friendly material **[Vigneswari, S., Noor, M. S. M.,**

Amelia, T. S. M., Balakrishnan, K., Adnan, A., Bhubalan, K., Amirul, A. A., & Ramakrishna, S. (2021)]. Currently, PHAs are not competitive compared to products derived from fossil sources. Promoting and strengthening research work on PHA is expected to improve future economic viability. The development of various biomolecular and chemical techniques for PHA analysis has led to the identification of many PHA-producing microbial strains, some of which have been deposited in culture collections. These ready-to-use techniques and microbial strains enable immediate PHA research **[Isabel, A. (2024)].** From an industrial biotechnology perspective, the biodegradability of bioplastics makes them a desirable alternative to petrochemical-based plastics, which are environmental pollutants. Increased production of bioplastics will significantly reduce carbon emissions, limit plastic waste generation and reduce fossil fuel consumption **[McAdam, B., Brennan Fournet, M., McDonald, P., & Mojicevic, M. (2020)].** In addition to PHA's biocompatibility, it has polypropylene-like properties, biodegradability, high hydrophobicity and thermoplasticity, high crystallinity, high melting temperature, and excellent resistance to organic solvents. In some microbial species, PHA accumulation occurs between the presence of excess carbon and limitation of nitrogen sources **[Samadhiya, K., Sangtani, R., Nogueira, R., & Bala, K. (2022)]**. PHA is produced in response to stressful situations and functions as an energy storage molecule that is used in the absence of a common energy source. The plastic polymer accumulates as refractive amorphous storage particles within the cells of these organisms. The chemical and physical properties of PHAs vary greatly with monomer composition. PHA is insoluble in water. Polymers are commonly characterized using parameters such as hydrophobicity, melting point, glass transition temperature, and crystallinity. The PHA family exhibits a range of mechanical properties, from hard crystalline to elastic. Many microorganisms have been discovered that can produce PHA as insoluble granules in cells under stress conditions, intracellularly. PHAs are produced in large amounts by microorganisms, whether they are Gram-positive or Gram-negative. Various microorganisms synthesize polyhydroxyalkanoates (PHAs) as physiological strategies related to food resource utilization in ecosystems. PHA accumulates to approximately 9 % (w/w) of the dry cell mass within bacterial cells **[Verlinden, R. A., Hill, D. J., Kenward, M. A., Williams, C. D., & Radecka, I. (2007)].** Based on organisms, PHAs vary greatly in structure, physicochemical properties, monomer composition, mass and size. The granules of the PHAs are coated with phospholipids and proteins called phasins, and these phasins play a vital role in size of the PHA granule. Although the high cost of industrial production and recovery of bioplastics currently cannot compete with traditional routes of synthetic plastics production, Fermentation processes have been greatly improved by using recombinant organisms that can produce these polymers at high rates using cheap carbon sources. PHA patents cover a wide range of PHAs products such as coating and packaging, bottles, cosmetic containers, golf tees, and pens. PHAs have also been processed into fibers, for a non-woven fabric's material **[Trakunjae, C., Boondaeng, A., Apiwatanapiwat, W., Kosugi, A., Arai, T., Sudesh, K., & Vaithanomsat, P. (2021)].** PHAs can be used for all sorts of biodegradable packaging materials, including composting bags, food packaging, sanitary articles like diapers and fishing nets, biodegradable rubbers. Despite extensive research into

the physiology of PHB formation, little is known about the enzymes that catalyze its synthesis and few genetic studies have been done. Although many bacterial species that produce PHB have been identified, the potential to discover and identify new species with exceptional production capacity remains unchanged. The isolation, identification, and genetic engineering of other microbes that produce many bioplastics with different structures, properties, and their uses show a promising future for bioplastics industrialization since the full potential of bioplastics remains unexploited. A key factor in the commercialization of bioplastic production is optimizing conditions for maximal synthesis of PHB by varying culture parameters such as temperature, pH and type of carbon source and nitrogen source. PHB has been shown to be produced naturally by different bacteria including Alcaligenes, Pseudomonas, Bacillus, Rhodococcus, Staphylococcus and Micrococcus. The favorable mechanical properties and amenability to biodegradation when exposed to certain active biological environments, earmark PHB as a high potential replacement for petrochemical based polymers such as ubiquitous high-density polyethylene (HDPE).

- **Lactobacillus**

PHB can be synthesized by species from over 50 different genera. Although PHB has been found in actinomycetes and yeasts, it is most commonly found in bacteria of various morphological and physiological groups **[Chee, Jiun Yee & Yoga, S. & Lau, Nyok-Sean & Ling, S. & Abed, Raeid & Sudesh, Kumar. (2010)].** In the mycelium of micromycetes, only the monomer was found. The amount of PHB produced by the Lactobacillus, Streptococcus, and Pediococcus genera has not been well studied or documented. The genus Lactobacillus is a taxonomically complex and is composed of over 170 species that cannot be easily differentiated phenotypically and often require molecular identification. Lactobacillus spp. are facultative anaerobic, catalase-negative, Gram-positive, non-spore-forming rods that often grow better under microaerophilic conditions. Some strains exhibit bipolar bodies, internal granulations, or a barred appearance with the Gram-reaction or methylene blue stain. Their Gram stain morphology can vary, including as short, plump rods, long, slender rods, in chains or palisades **[Nicolescu, C. M., Bumbac, M., Buruleanu, C. L., Popescu, E. C., Stanescu, S. G., Georgescu, A. A., & Toma, S. M. (2023)].**

- **Nanoparticles Obtained from PHB Granules**

The unique physicochemical properties of PHAs have made them applicable to nanotechnology, tissue engineering, and other biomedical applications **[Babos, G., Rydz, J., Kawalec, M., Klim, M., Fodor-Kardos, A., Trif, L., & Feczkó, T. (2020)]**. Nanotechnology deals with particles up to billionths of a meter in size and is a pervasive and critically important research for present and future discoveries to improve our world. The confiscated size of the nanoparticles enables many applications similar to their originally dominant counterpart in the construction of structural and architectural metal frameworks. The PHB which is isolated from the samples is further utilized to produce PHB nanoparticles which can be used to produce hydrophilic/hydrophobic drugs and also in various aspects such as

biosensors, fertilizers, pesticides and can be used as antimicrobial food packaging which show greater shelf life and stability **[Ibrahim, M. I., Alsafadi, D., Alamry, K. A., Oves, M., Alosaimi, A. M., & Hussein, M. A. (2022)].** Degradation of PHAs embedded in specialized cells of the desired host organism raises the possibility of accompanying the release of bioactive compounds such as antibacterial, antifungal and anticancer agents. For example, if a cell is saturated with a substance, degradation over a period of time will release the substance and replace it as a self-activating substance. PHB is widely used as an implant material. The importance of using an implant made of PHB is that the body does not see it as a foreign substance, so the immune system does not react to or reject the implant. makes it an ideal material for Nano encapsulation and delivery of antimicrobial compounds and strengthening the already present polymer compound. Food packaging is the primary motive for the synthesis of PHB nanoparticles as using these instead of regular plastic makes them more sustainable and eco friendly. But the actual purpose of incorporation of these nanoparticles is to increase the strength of the existing PHB particles and reduce their brittle nature **[Matos, R. S., S Monteiro, M. D., Santos, S. B., F Filho, H. D., S Andrade, G. R., Salerno, M., & Almeida, L. E. (2022)**

2. MATERIALS AND METHODS.

The present study was performed in St. Francis Degree College, Department of Microbiology, Louis Pasteur Research Laboratory, Hyderabad. The study was carried out in the duration of 2 months' time.

- **Materials required [Apparatus and Media, and Reagents].**
  - ***Apparatus***

Petri dishes, Test tubes & boiling tubes, Beakers, Glass slides & coverslips, Glass rods, Compound microscope, Rotary shaker, Cold centrifuge, UV-VIS spectrophotometer.

  - ***Culture Media***

Nutrient Agar (NA), DeManRogosa and Sharpe (MRS) Agar & Broth, Simmons Citrate Agar, Glucose Phosphate Peptone Medium, Tryptone Broth, Triple Sugar Iron (TSI) Agar, Nitrate Reduction Broth.

  - ***Reagents & Stains***

Silver nitrate solution. Crystal Violet. Gram's Iodine, Safranin, Alcohol (for Gram staining), Kovac's reagent, Methyl Red indicator, Barritt's A (α-naphthol in ethanol), Barritt's B (40% KOH), Phenol Red 1.0%, Mannitol 2%, Urea, Sudan Black B.

  - ***Chemicals & Solvents***

Sodium hypochlorite, chloroform, Distilled water, Concentrated sulfuric acid, Ethanol.

- **Isolation of PHB-Producing Bacteria from the Soil Samples**

1gm of the sample was obtained and inoculated into 100 ml of distilled water. 1 ml of the sample is then serially diluted to get the titer values of – 10^{-1} to 10^{-10} out of which five samples were chosen and inoculated on petri plates containing nutrient agar via spread plate technique. These plates were incubated at 37°c for 24 hours. Identification of the colonies via the colony characteristics was done. After which, identical isolated colonies were subcultured. To confirm the presence of Lactobacillus, the colonies from nutrient agar were

subcultured onto MRS media which is specific for Lactobacillus spp There was abundant growth seen in MRS media plates, hence the organism maybe Lactobacillus.

- **Identification of Bacteria and Screening of PHB in Bacteria**

For the identification of colonies, gram staining and various biochemical tests are employed based on Bergey's manual. The bacterial isolate was tested for Gram Staining, Endospore Staining, Sudan Black B, Indole Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, Catalase Test, Urease Test, Triple Sugar Iron Test, Oxidase Test, Mannitol Test and Glucose Test for further classification of Lactobacillus sub spp.

- **Production and Extraction of PHB.**

For the production of PHB, a loop full of the bacterial isolates are first inoculated into tubes containing 10ml of MRS broth. They are incubated for 48 hours at 37°c, to obtain a fresh culture. After 48 hours 2ml from the broth containing the bacterial culture, is inoculated into a flask containing 250ml of MRS broth and then incubated for 48 hrs at 37°c for mass production of PHB. The bacterial culture obtained is then subjected to extraction of PHB via differential digestion method. The extraction is done by following the differential digestion treatment by using sodium hypochlorite and chloroform. After 48hrs of incubation at 37°c the culture is collected and centrifuged at 10,000 rpm for 10 minutes at 10°c in the cold centrifuge. Discard the supernatant and collect the pellet, to the pellet add acetone and centrifuge it at 10,000 rpm for 5 minutes. Discard the supernatant and add ethanol to it and centrifuge again for 5 minutes at 10000 rpm. Discard the supernatant and collect the pellet. Add sodium hypochlorite solution and incubate at 37°c for 3 hours. After incubation, centrifuge the tubes for 10,000 rpm for 10 minutes. Discard the supernatant and collect the pellet, to the pellet add acetone, centrifuge for 10,000 rpm for 10 minutes. Repeat the previous step with ethanol. Discard the supernatant and add chloroform in hot conditions (60°c) for 10 minutes. Shake well and filter the solution by using filter paper, collect the pellet and proceed with estimation of PHB.

- **Estimation of PHB by UV-VIS Spectrophotometer**

Take 15mg of sample in a clean test-tube and add 10 ml of concentrated sulphuric acid, incubate in a hot water bath for 10 minutes at 60°c after 10 minutes read the absorbance at a range of 200 nm to 800 nm through double beam UV-VIS spectrophotometer. Concentrated sulphuric acid is used as blank to measure the absorbance of the sample.

- **Optimization of PHB by Studying the Effect of Carbon and Nitrogen Source**

Optimization is performed to increase the polymer production of each PHB positive organism. PHB extraction was performed considering the nitrogen sources [tryptone, peptone and ammonium sulphate] and carbon sources [dextrose, sucrose, lactose] for each organism. Extracted PHB was calculated as the recovery of the obtained cell dry weight.

- **Production of PHB Nanoparticles Using Agno3**

The bacterial isolate was cultured in MRS broth at 37° C for 48 hours, the culture was then subjected to differential digestion treatment for the production and extraction of PHB. The resulting biomass is then washed thoroughly with sterile water and subjected to intracellular biosynthesis of AgNo3 nanoparticles by adding 10 ml of 1mm AgNo3 to 15mg of the

bacterial pellet. The flask containing the mixture is then incubated in the rotary shaker for 24 hours. The colour of the solution changes to pale brown. The UV–Vis spectrum was determined using a SPECORD M-400 UV-Vis spectrophotometer. The absorbance was measured in the range of 400-800 nm. Sterile double distilled water was used as the blank.

3. Results and Discussions

a. Isolation, Identification and Screening of PHB Producing Bacteria

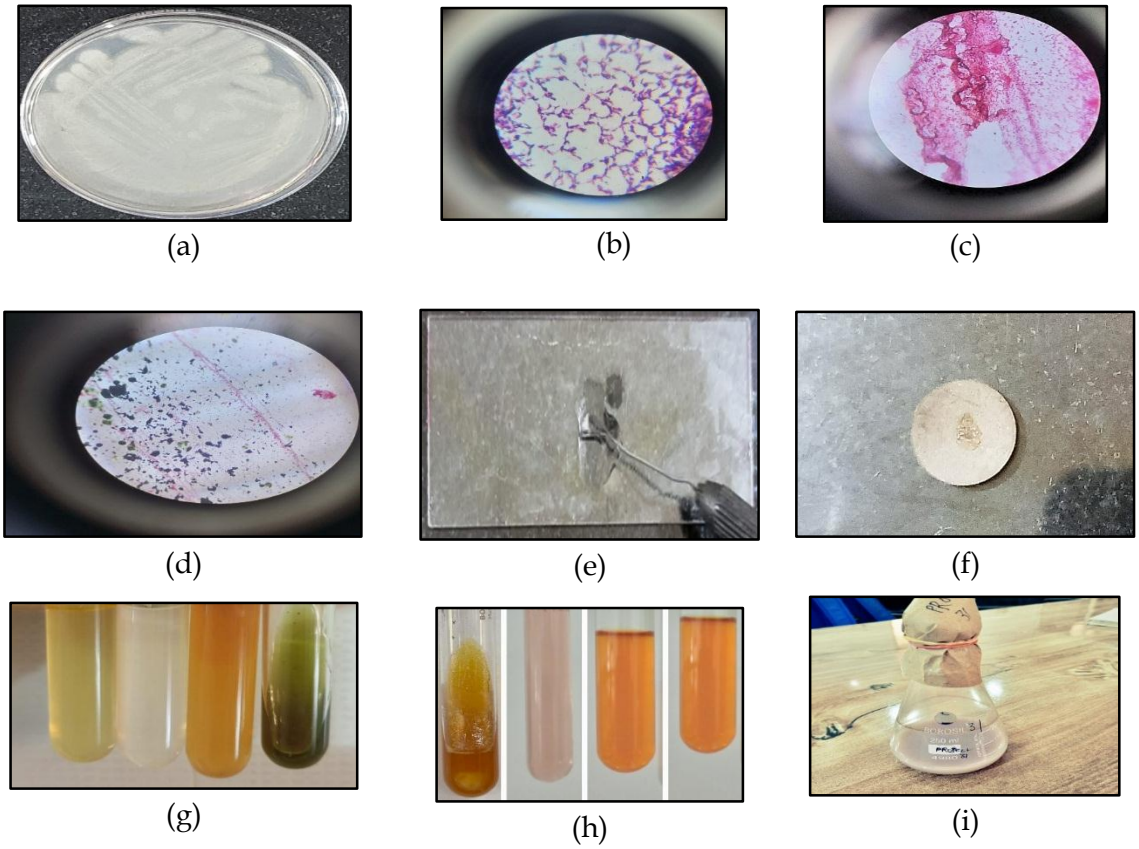
Tables – I) cultural characterization, II) Identification tests.

I.

S.No	Source	Cultural Characteristics				Morphological Characteristics	Organism Identified
		Colour	Texture	Elevation	Margin	Gram Nature	
1]	Soil sample	Opaque with creamy pigment	Smooth	Convex	Regular/round	Gram +ve rods	*Lactobacillus casei*

II.

S.No	Biochemical tests	Observation	Results
1.	Indole	No cherry red ring observed	Negative
2.	Methyl red	Yellow colour observed	Negative
3.	Voges-Proskauer	Colour change to red	Positive
4.	Citrate utilization	No change of colour in slant	Negative
5.	Catalase test	No effervescence was seen	Negative
6.	Oxidase test	No change in colour	Negative
7.	Triple sugar iron test	(Yellow slant/yellow butt)	Acid/acid
8.	Urease test	No change in colour	Negative
9.	Glucose test	Colour change from red to yellow	Positive
10.	Mannitol test	Colour change from red to yellow	Positive



Figures – a) white colonies with round edges were isolated from the soil sample, b) Microscopic view of the isolates showing gram positive rods in chains, c) Microscopic view of Sudan staining, d) Microscopic view of endospore staining, e) Oxidase test, f) Extended biochemical tests, g) IMVIC tests.

b. Estimation of PHB By UV-VIS Spectrophotometer

UV-VIS estimation of the PHB granules showed the followed results, the maximum absorbance was seen at 236nm where the absorbance was noted as 2.26335.

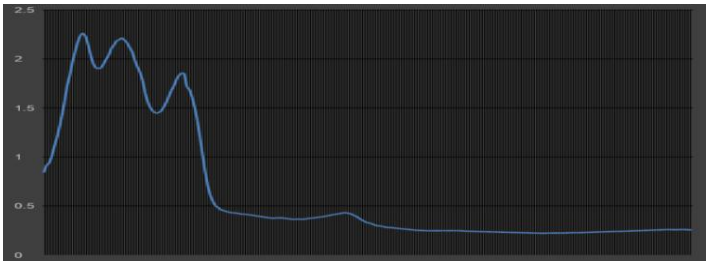


Figure h) - Absorbance Maxima of the PHB Granules

c. Optimization Of PHB By Studying the Effect of Carbon and Nitrogen Source

The two different optimization parameters; carbon source and nitrogen source have shown varied results. They are as follows;

S.No	Nitrogen Source	Dry Weight
1	Peptone	715Mg
2	Tryptone	597Mg
3	Ammonium sulphate	531Mg

Table III - Optimization of Nitrogen Source

S.No	Carbon Source	Dry Weight
1	Sucrose	584mg
2	Dextrose	257mg
3	Lactose	231mg

Table IV - Optimization of Carbon Source

d. **Post Synthesis of PHB Nanoparticles**

PHB nanoparticles were synthesis using intracellular biosynthesis by incubating the bacterial biomass in AgNo3 solution to obtain silver PHB Nano particles. The production of the nanoparticles was estimated using UV-VIS double beam spectrophotometer at 400nm – 800nm. The maximum absorbance was recorded at 450nm where the absorbance was noted 4.974567.

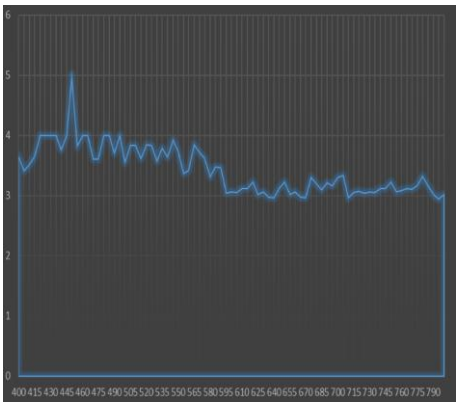


Figure i) Absorbance Maxima for PHB Nanoparticles

4. Conclusion

The study was conducted with the main objective to produce and extract PHB, which is a biopolymer, which can be used as an alternative for plastic. Bacteria were isolated from soil samples and were first grown on nutrient agar and then sub cultured on MRS agar since the organism was suspected to be Lactobacillus, the colonies were identified by various staining techniques and biochemical tests. Through staining and biochemical tests, the bacterial isolate was found to be Lactobacillus casei, and the isolated organism was subjected for production and extraction of PHB by differential digestion treatment.

The estimation of PHB was done by using UV-VIS double-beam spectrophotometer. Optimization techniques were used to find out the best carbon and nitrogen source for the maximum production of the cell biomass, and the dry weight of PHB was calculated. After

observing the dry weight of the bacterial PHB pellet through various optimization concentrations of carbon and nitrogen sources in the media prepared, sucrose (584mg) and peptone (715mg) turned to be the best carbon and nitrogen sources.

Post-synthesis strategies are usually employed to enhance the properties of the already existing PHB granules; hence we incorporated silver nitrate into the PHB pellet to decrease the brittle nature and increase the strength of the PHB granules.

This method of incorporating nanoparticles can be repeated with various other metal salt solutions to further observe the post-synthesis enhancement of PHB.

- **Future Prospects.**

PHB or bioplastics will significantly reduce carbon emissions, limit plastic waste generation and reduce fossil fuel consumption. Food packaging is the primary motive for the synthesis of PHB nanoparticles as using these instead of regular plastic makes them more sustainable, ecofriendly and can be used as antimicrobial food packaging which show greater shelf life and stability.

PHB which is isolated from the samples is further utilized to produce PHB nanoparticles which can be used to produce hydrophilic/hydrophobic drugs and also in various aspects such as biosensors, fertilizers, pesticides.

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