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RESEARCH ARTICLE

PRODUCTION AND OPTIMIZATION OF BIOPLASTIC FROM BACILLUS ISOLATED FROM ENVIRONMENTAL SAMPLE

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Abstract

Biopolymers represent a major alternative to petroleum-derived plastics, with poly-3-hydroxybutyrate (PHB) being the most common. To reduce the cost of production and expand the use of such biopolymers, our research focused on identifying microorganisms with effective PHB-producing capabilities. Through standard microbiological procedures, we isolated six different samples and used Sudan Black B colony staining to determine their PHB-producing capabilities. The Crotonic acid method revealed a 90.1% yield of PHB in the sample. Biochemical, morphological, and molecular analyses - including 16S rRNA gene sequencing - confirmed the bacterium as Bacillus cereus. FTIR analysis was also used to confirm the polymer as PHB. Furthermore, the ability of Bacillus cereus to use various carbon sources, coupled with gentamycin encapsulated in PHB polymer sheets to effectively demonstrate its antimicrobial properties, and a cell viability analysis to show its biocompatibility, highlight this microorganism as an effective candidate for large-scale industrial production of bacterial polyhydroxybutyrate.

Introduction:-

Biodegradable materials with plastic-like properties have been gaining traction from researchers around the world due to a range of factors, such as the growing awareness of environmental issues caused by plastic waste, the microplastic crisis, strict regulations regarding plastic use and disposal, and the constant increasing amount of greenhouse gas emissions leading to global warming and climate change. Furthermore, as fossil fuels are being depleted, research in biodegradable materials has become increasingly important. One such polyester made up of microbial storage compounds with plastic-like properties is Polyhydroxyalkanoate (PHA), which is considered a major solution to these pressing issues. PHA production is achieved through renewable resources and biological catalysis under in vivo conditions by the action of living prokaryotic cells (Narodoslawsky et al. 2015).

Polyhydroxyalkanoates (PHAs) are a type of biodegradable, biocompatible thermoplastic stored in bacteria as intracellular inclusions. They have many desirable properties, such as being hydrophobic, impermeable to gases, non-toxic, and piezoelectric. PHAs are also enantiomerically pure, meaning that they have a high degree of polymerization with molecular weights ranging from 20,000 to 30 million Daltons.Enhanced synthesis of PHAs occurs when bacteria are grown in rich carbon mediums with inadequate concentrations of other nutrients. This

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production of PHAs makes them attractive for a broad range of uses, from packaging to medical devices. For example, PHAs can be used to create drug-releasing medical implants or as a biodegradable packaging material. They can also be used to create bulk items such as plastic plates and cutlery, as well as in 3D printing and fabric coatings. Additionally, PHAs can be used to produce green solvents, surfactants, and lubricants, or as a raw material for the synthesis of biodiesel (Muller and Seebach 1993; Andreeben et al. 2010; Chen et al. 2010). The core focus of biopolymer research is to develop economically viable approaches to the large-scale production of biopolymers with superior quality. To achieve this, researchers are currently working on enhancing the properties of biopolymers to make them more suitable for their intended applications. This includes improving their mechanical properties and thermal stability, while at the same time maintaining biocompatibility. Furthermore, researchers are also working on methods to reduce the cost of production and make biopolymers more accessible to a wider range of end users. Overall, biopolymers offer many advantages over traditional polymers and have an array of potential applications. With ongoing research and development, biopolymers are set to become an even more important part of our future. (Muller and Seebach 1993; Steinbuchel and Fuchtenbush 1998; Poli et al. 2011).

Polyhydroxyalkanoates (PHAs) are a diverse group of biopolymers found within a variety of microbial species, including bacteria and archaea. PHAs are energy-efficient, sustainable, and biodegradable materials that have high commercial potential. They can be used for a variety of applications, including food packaging, construction materials, wound dressing, and drug delivery. Prokaryotic microorganisms are the major sources of PHAs, with the highest accumulation capability being observed in the Gram-positive bacteria Bacillus, Clostridium, Corynebacterium, Nocardia, Rhodococcus, Streptomyces, and Staphylococcus. Gram-negative bacteria such as Cupriavidus and Pseudomonas also demonstrate PHA accumulation capabilities, as do certain archaeal strains such as Halobacterium, Haloarcula, Haloquadratum, and Haloferax. Of all the microbial species that produce PHAs, Bacillus spp. isthe most notable. Bacillus spp. are capable of accumulating poly-3-hydroxybutyrate (PHB), which is the most prevalent and simplest form of PHA found in bacteria. As such, PHB has become the most researched biopolymer and has a wide range of commercial applications, from food packaging to drug delivery and construction materials.

The present study aimed to identify a bacterial strain with the highest potential for polyhydroxybutyrate (PHB) production from a specific region, as well as to determine the effect of changing carbon sources on polymer accumulation. Furthermore, a time course analysis of polymer accumulation was conducted, and the biocompatibility and other characteristics of the produced polymer were evaluated.

Materials And Methods:-

Sampling, isolation, and maintenance of bacterial strains

Soil samples were collected from the banks of an irrigation canal from Kalliyoor (8.431777° N 77.017019°E), Thiruvananthapuram district, Kerala, India. Bacterial strains were isolated on nutrient agar medium (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract, 1.5 g of yeast extract, and 15 g of agar per liter at pH 7.4) by serial dilution method and incubated at room temperature (30°C) for 48 h. Based on colony morphology and pigmentation, bacterial isolates were selected, and pure cultures were prepared and maintained on nutrient agar slants and stored at 4^0 C (Shrivastav et al. 2010; Singh et al. 2015).

Screening of isolates for PHB production

Initially, the PHB-producing strains were screened by direct colony staining method on bacterial isolates grown on half-strength nutrient agar (2.5 g of peptone, 2.5 g of sodium chloride, 0.75 g of beef extract, 0.75 g of yeast extract, and 15 g agar per liter at pH 7.4) supplemented with 20 g/L glucose, after 48 h of incubation at room temperature (Liu et al. 1998). The bacterial colonies on Petri plates were flooded with Sudan Black B solution (0.03% in ethanol) and kept undisturbed for 30 min. The excess stain was washed out by sterile saline and the dark blue coloured colonies were identified as PHB positive. The PHB-producing strains screened were further confirmed by microscopy.

Staining for PHB accumulation and microscopy

48 h old bacterial cultures grown in basal medium (1.5 of peptone, 1.5 g of yeast extract, 1 g of Na2HPO4and 0.2 g of MgSO4·7H2O per liter, pH 7.2) supplemented with 20 g/L glucose at room temperature were taken for staining and microscopic analysis. Sudan Black B stained smear was observed under 100X oil immersion objective lens of a light microscope (Burdon 1946).

Evaluation of bacterial strains for PHB production

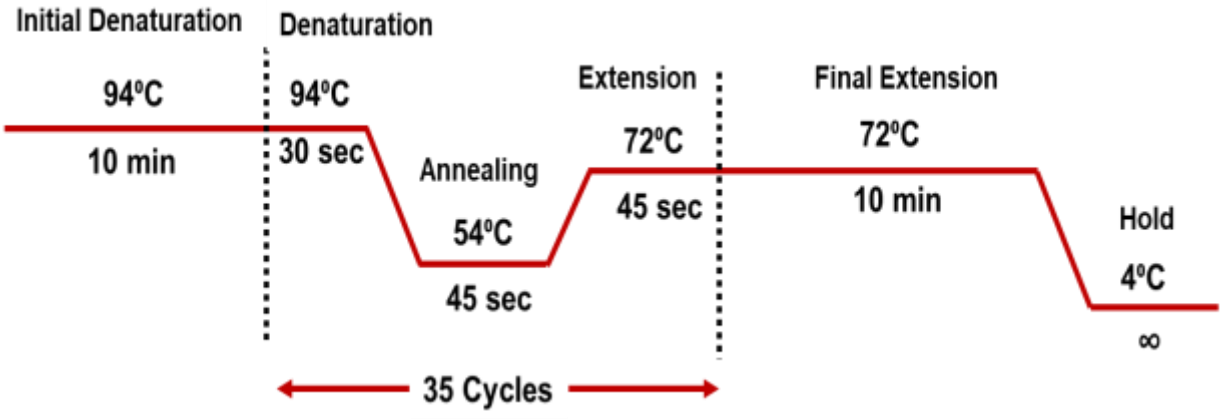
From the isolates, strains producing a considerable amount of PHB granules were inoculated in 1000 mL basal medium with 20 g/L of glucose and incubated at room temperature for 48 h at an agitation rate of 150 rpm. The cells were harvested and washed with sterile normal saline. The biomass obtained was dried overnight and the cell dry mass (CDM) was calculated. The polymer was extracted from the dried cells, weighed, and estimated the yield was in percentage (w/w) (Shi et al. 1997; Aneesh et al. 2016).

Molecular identification of the most potent PHB-producing bacterial strain

Genomic DNA isolation 1.5mL of one-night bacterial culture was taken and pelleted by centrifugation at 14000 rpm for 1 min. The supernatant was discarded without disturbing the pellet. Resuspended the pellet in 600µL of lysis buffer containing lysozyme, incubated at 37°C for 1 h, and added an equal volume of phenol: chloroform (1:1) or phenol: chloroform: iso amyl alcohol (25:24:1). The components were mixed well by inverting followed by centrifugation at 14000 rpm at room temperature for 5 min. A white layer was formed at the interface between phenol: chloroform and aqueous phases. The upper aqueous phase was carefully transferred to a new tube. The steps were repeated until protein precipitation stopped. To remove phenol equal volumes of chloroform were added to the aqueous layer. Mix well by inverting and centrifuging at 14000 rpm at room temperature for 5 min. The aqueous layer was transferred to a new tube and add chilled (-20°C) absolute ethanol (2.5-3 vol) was added to it. The tubes were kept at -20°C for 30 min or overnight for maximum yield. After the incubation period, centrifugation was repeated at 14000rpm for 15 min at 4°C. The supernatant was discarded and rinsed with 1 mL of 70% ethanol at room temperature and centrifuged at 14000 rpm for 2 min followed by discarding the supernatant. The pellets were air-dried and then resuspended in nuclease-free water.

Bacterial 16S rRNA gene- PCR

The PCR reaction mixture used for the experiment comprised 300 ng template DNA, 10 X PCR buffer, 25 mM MgCl2, 10 pmol of both forward (27f) and reverse primers (1492r), 1U Taq polymerase, 1mM dNTPs and deionized distilled water (Hall, 1999). The PCR was done with the following cycling conditions



3.6. PHB production process optimization (Belal and Farid, 2016)

Effect of different carbon sources

To detect the effect of carbon sources, a nutrient broth medium was prepared and supplemented with different carbon sources at the optimum culture conditions (pH 7 and 30ºC). The carbon sources included sucrose, glucose, maltose, and starch. After 48 h the PHB yields were extracted and quantified using the sodium hypochlorite-chloroform method.

pH and temperature optimization

To determine the optimum pH, experiments were carried out at pH 3, 4, 5, 6, 7, and 8. Cultures were incubated on a rotary shaker at 30°C and 150 rpm for 48 h. To determine the effect of temperature starch medium with pH 7 was incubated at 25, 37, and 45ºC under 150 rpm for 48 h. After 48 h, PHB yields were extracted and quantified as described before.

Optimization of incubation time

To determine the effect of the incubation period, the inoculated starch liquid medium were incubated at 30ºC and pH 7 at 150 rpm under different incubation period (12, 24, 36, 48, 72, 84, and 96 h). The optical density was read at 600 nm at these time intervals. PHB yields were extracted and quantified as described before.

Effect of different C: N ratios on PHB

Different C: N ratios such as 1:1, 1:2, 1:3, and 1:4 using the best C and N sources were done after inoculation and incubated on a rotary shaker (150 rpm) at 30°C. After 48 h, PHB yields were extracted and quantified as described before.

Biocompatibility studies of PHB

MTT assay is a simple colorimetric assay to measure cell cytotoxicity, proliferation, and viability (Mansur and Costa, 2008). Cytotoxicity effects of PHB polymer sheets were investigated on cultured Chang liver cells. 100 µl of appropriately diluted cells were seeded into each well of the 96 well plates and incubated the plates for 24 h. Extracted and purified PHB from the bacterial isolate was surface sterilized with 70% ethanol and inoculated into the wells (20 mg/well). The plates were incubated for 24 h at 37^0 C in a CO2 incubator. After incubation, the media was discarded from the well plate. 50 µL of serum-free media and 50 µL of MTT (1 mg/ml) solution were added to each well and incubated at 37^0 C for 3 hours. After incubation, 150 µL of MTT solvent (100% DMSO) was added to each well. Followed by incubation at 37°C for 10 minutes, the absorbance was read at 570 nm. The percentage of cell viability was calculated by comparing it with the control.

PHB production using food waste

Two hundred grams of banana and tapioca peels each were washed thoroughly, peeled, sliced, and chopped into small chunks. Distilled water was added and the extraction process was carried out through the use of a centrifuge at different speeds (1000, 2000, 4000) rpm for different periods (5, 10, and 15 min). Thereafter, the centrifuged samples were filtered using Whatman no. 1 filter paper and the supernatant was neglected to obtain wet starch (Altemimi, 2018). The wet starch was dried at room temperature for 5 h, then crushed into a fine powder and used as a substrate for PHB production.

PHB polymer characterization by FTIR

The PHB extracted from the strain PHB2 was analyzed by FT-IR spectroscopy. The presence of different functional groups in PHB was checked by FTIR. Extracted PHB (2 mg) was dissolved in 500 µl of chloroform. After evaporation of chloroform, PHB polymer film was subjected to FTIR under the spectral range of 4000-400 cm^{-1} to confirm the functional groups of the extracted polymer.

Results:-

Biodegradable plastics are becoming increasingly important as a sustainable alternative to regular plastic, which is highly resistant to natural breakdown and is causing a major environmental crisis. However, their production costs remain high, posing a major problem for their widespread adoption. To combat this, scientists have been searching for bacterial strains that can produce these plastics in higher quantities and more efficient ways. Additionally, they have been attempting to optimize the culture conditions to further reduce the cost of production. These approaches can provide a more economical solution to environmental protection by allowing more widespread use of biodegradable plastics.

Isolation and screening of PHB-producing isolates

From the soil sample collected, a large number of white and ivory-coloured colonies were isolated on the nutrient agar plates by serial dilution spread plate method (**Figure 1A & B**). Based on colony morphology and characteristics, 15 colonies were selected for downstream study (**Figure 1C**). Based on colony staining results, six isolates were found to be PHB accumulating (**Figure 1D**) and were designated as PHB 1-6. These isolates were further confirmed by microscopy for their PHB accumulation by Sudan black B staining (**Figure 1E**).

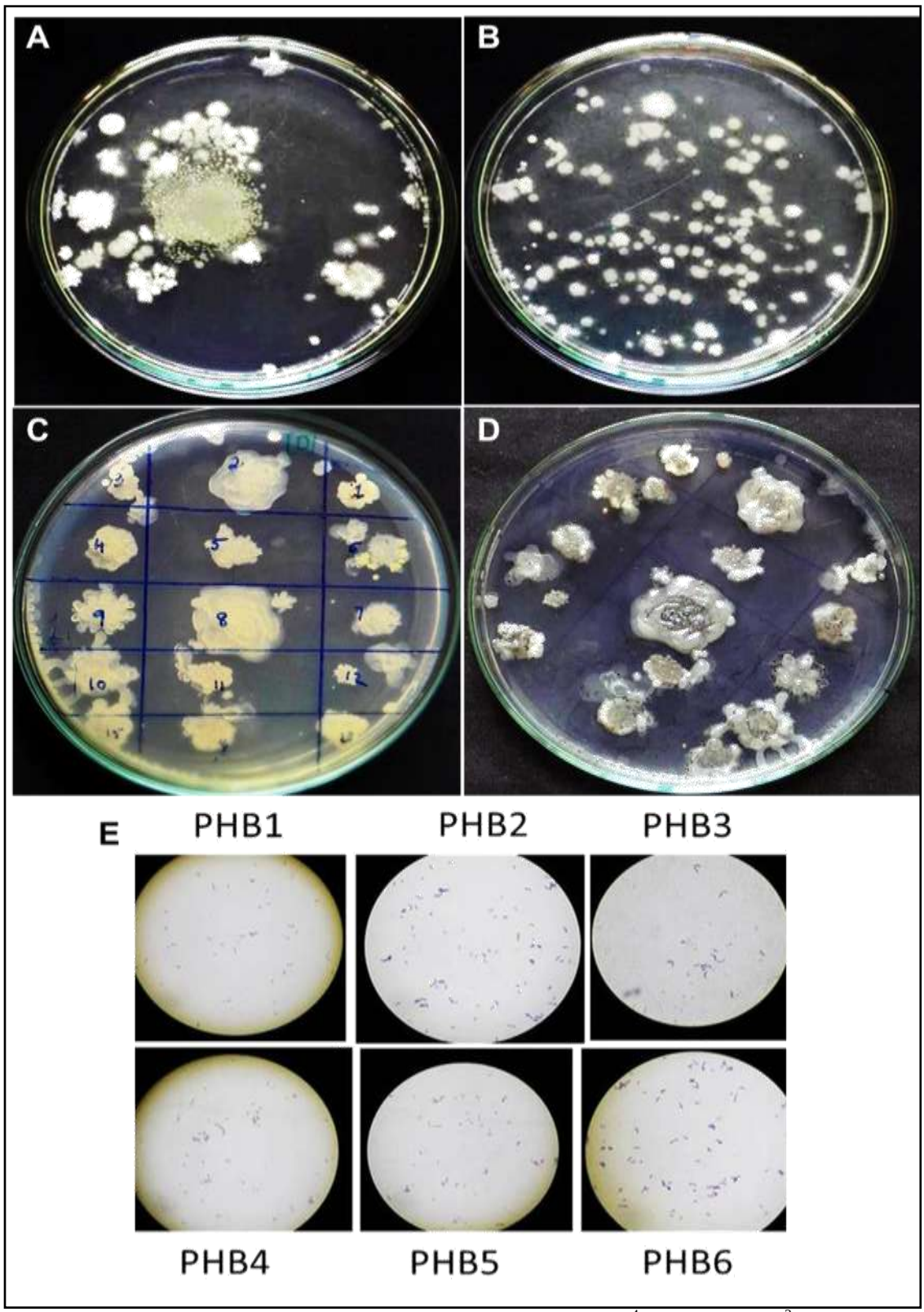


Figure 1:- Bacterial colonies from environmental samples (A) 10^{-4} dilution; (B) 10^{-3} dilution; Morphologically different colonies isolated (C); Six isolates (D); Sudan Black B Staining (E).

For quantification of PHB produced by different bacterial strains isolated crotonic acid method was used. A standard crotonic acid curve was generated (**Supplementary Figure 1**). The PHB-producing capacity of different bacterial strains isolated was depicted in **Supplementary Table 1**. The strain PHB 2 was found to be the most potent one and the downstream workwas done in it. Morphological and biochemical characterization of the isolate PHB 2 is given in **Supplementary Table 2**. The strain PHB 2 was found to be starch-utilizing which depicts itspotential to use agricultural waste as a substrate for PHB production.

The present investigation was focused on isolating a large number of bacterial colonies, to find those that accumulate polyhydroxyalkanoates (PHA). The colony staining method was used as a simple and speedy way to detect and isolate PHA-accumulating bacteria from environmental sources. After the screening, 6 colonies were identified as having PHA accumulation potential. Secondary screening of these isolates revealed that the bacterial isolate PHB 2 had much higher polyhydroxybutyrate (PHB) accumulation potential, with results from the crotonic acid method showing 90.10% PHB production. This isolate was found to be more efficient at PHB accumulation than the other strains, and thus could be used to produce polyester polymers with great efficiency.

Molecular identification of the strain PHB2

The genomic DNA from strain PHB 2 was isolated using the phenol: chloroform: isoamyl alcohol extraction method. The genomic DNA integrity was checked by agarose gel electrophoresis (**Figure 2A**). The purity of the DNA was analyzed using spectrophotometrically.

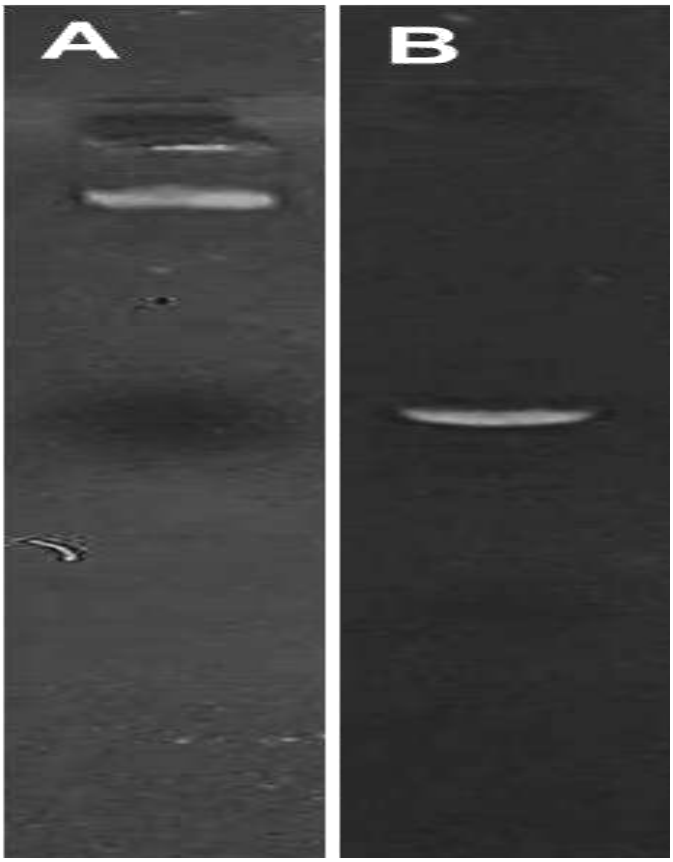


Figure 2:- Genomic DNA isolated from strain PHB2 (A); 16S rRNA gene amplified from the strain PHB 2 (B).

A260/A280 was found to be 1.91 (1.8 for pure DNA) and was found to be compatible with downstream molecular biology works. The 16S rRNA gene was amplified successfully from the DNA (**Figure 2B**) and was given for sequencing for strain identification.

PHB extraction and sheet production

The selected isolate was screened for PHB production. The production of PHB granule was confirmed by Sudan Black staining. After 48 h of incubation, the PHB granule was extracted using chloroform. A thin PHB sheet was formed after the chloroform was completely vaporized. Different stages of PHB polymer extraction and sheet production was depicted in **Figure 3**.

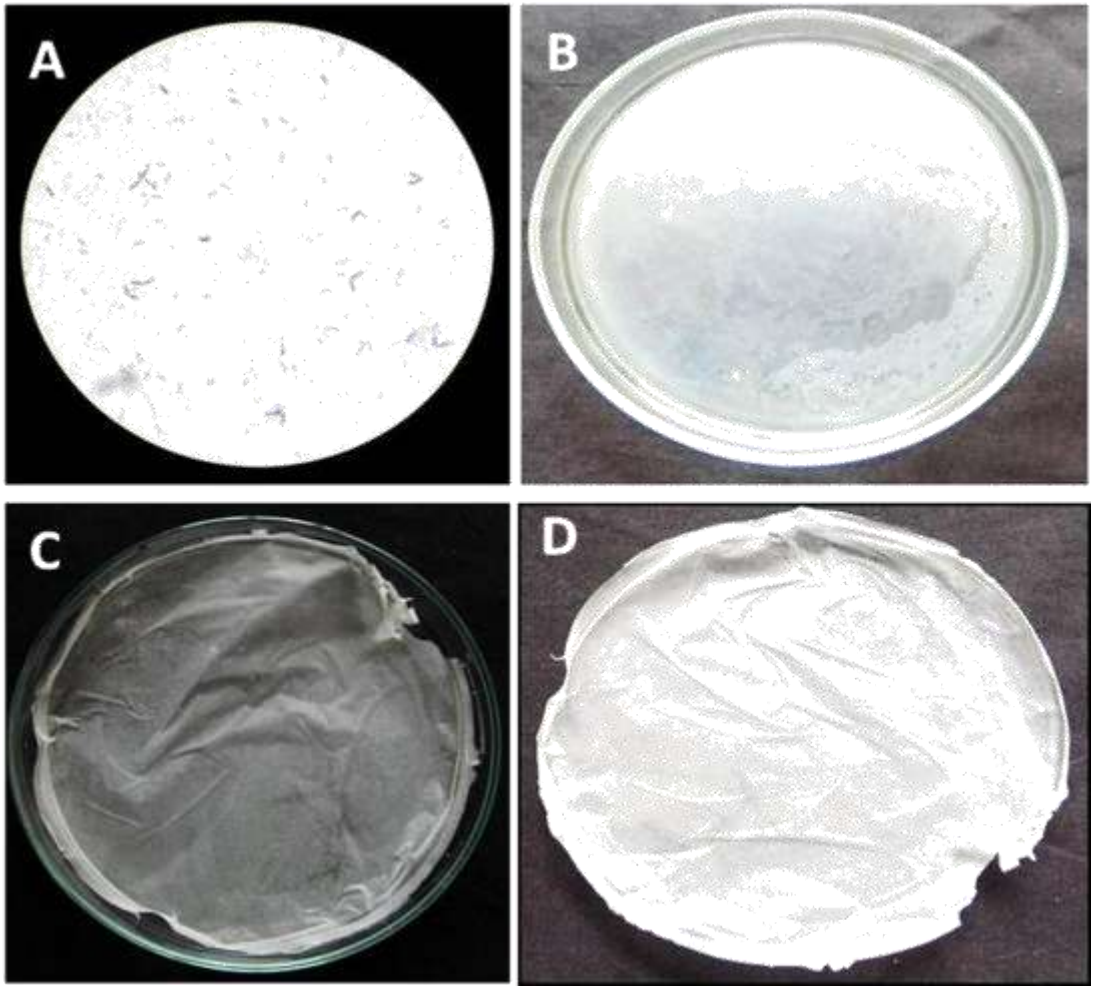


Figure 3:- PHB extraction different stages; A- PHB granules inside the cell- Sudan black B staining; B- PHB extracted with chloroform; C- Solvent evaporation to form PHB sheet; D- Dry PHB sheet.

The PHB sheet produced was used in downstream works such as drug encapsulation and biocompatibility studies.

Process optimization for PHB production using the strain PHB 2

The PHB production process with PHB 2 was optimized with different carbon sources, pH, temperature, incubation time, and different starch concentrations. The results were summarized in **Figure 4**.

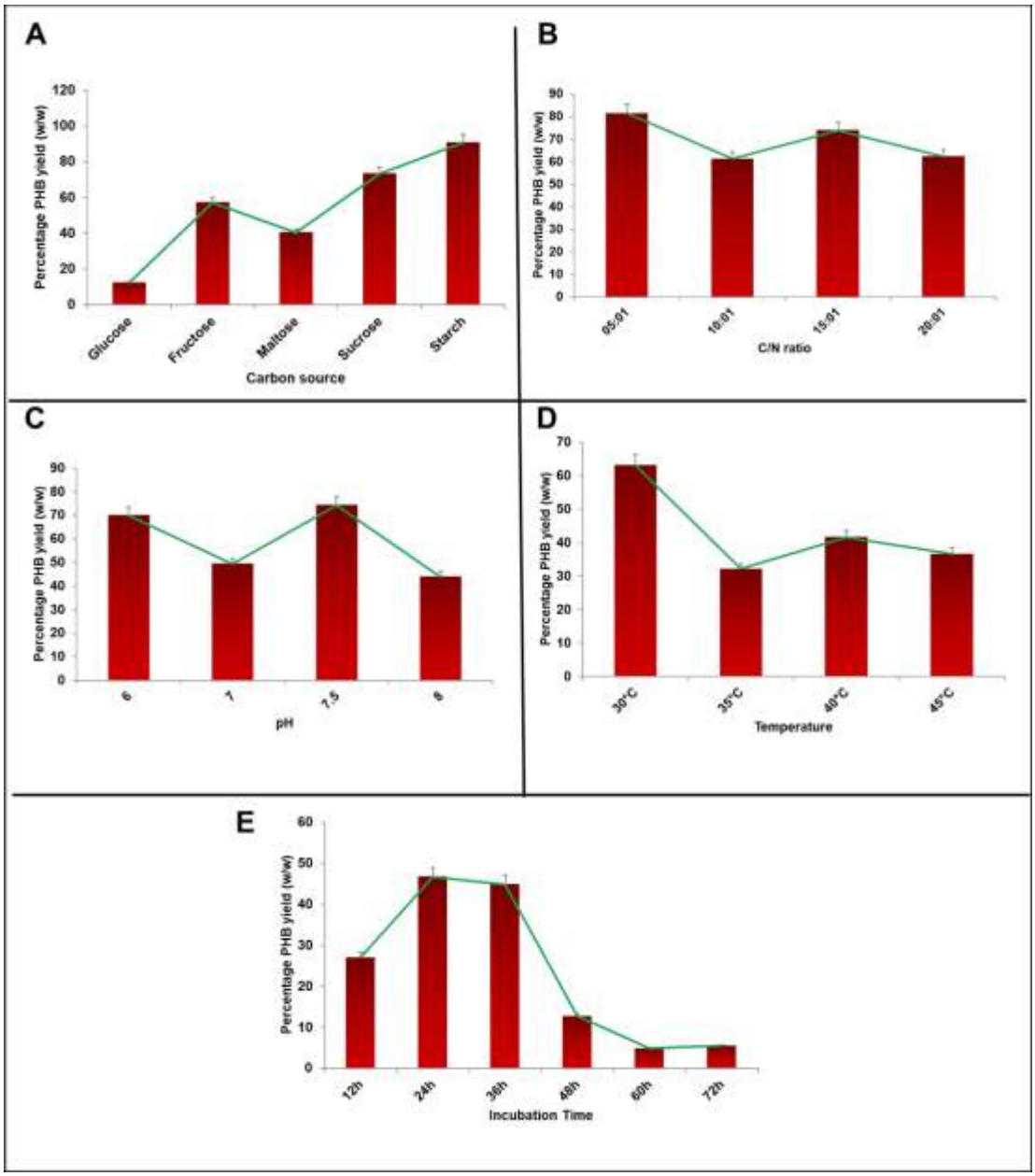


Figure 4:- Effect of carbon source (A); C/N ratio (B); pH (C); temperature (D) and incubation time (E).

Antibacterial study

The inhibitory effect of PHB films incorporated with gentamycin was tested against bacterial strains using an agar diffusion assay (**Figure 5**). The antimicrobial activity of gentamycin against bacteria is dependent on the time of exposure, concentration, and target organism.

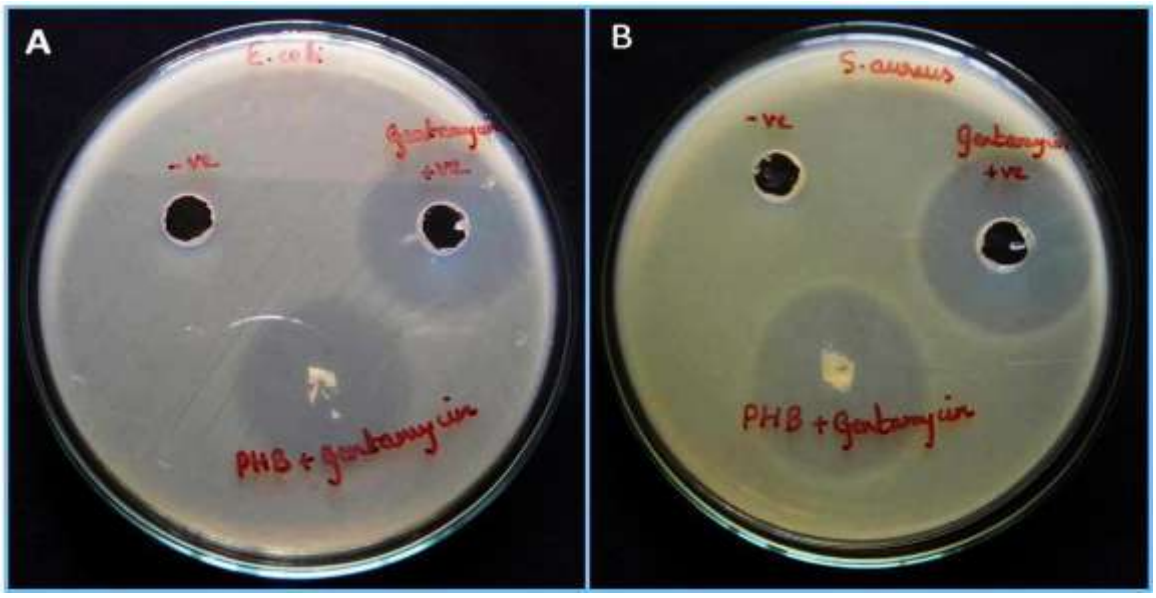


Figure 5:- Antibacterial activity of drug-conjugated PHB sheet against (A) E. coli (B) S. aureus.

The drug encapsulation results are promising which reveals the potential of PHB 2 to be developed as a drug delivery system.

PHB biocompatibility study in Chang liver cell line

To check the biocompatibility of PHB sheets produced by the strain PHB-2, an MTT assay was performed in a normal liver cell line (Chang's liver). The PHB sheets did not show significant cytotoxicity towards thenormal liver cell lines. This indicates the potential of biopolymers in biomedical applications. The biopolymer developed in this study is highly biocompatible, as evidenced by the results of the MTT assay. This viability assay revealed no significant reduction in cell viability when PHB sheets derived from the strain PHB 2 were administered to the normal human liver cell line, Chang liver cell line. This indicates that the developed compound is not cytotoxic to normal human cells and is thus fit for use in humans. The findings of this study also highlight the importance of using high-quality biopolymer materials for drug delivery systems, as biocompatibility is an important factor for ensuring in the safety of any medical application. Additionally, further studies should be conducted to evaluate the biocompatibility of other biopolymers and delivery systems to ensure their safety and effectiveness when used in clinical applications.

PHB production with natural starch

The PHB produced with natural starch as the substrate is visualized by Sudan black B staining (Figure 6). The PHB production yield is summarized in **table 2**.

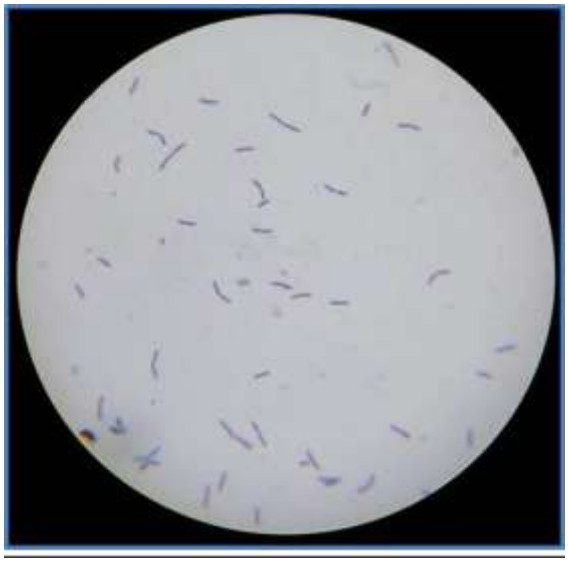


Figure 6:- PHB production with natural starch extracted from food waste (24 hours).

Table 2:- PHB production with starch extracted from food waste.

Tapioca/Banana peel	Cell Dry weight (g/L)	Absorbance at 235 nm	PHB yield (µg/ml)	PHB yield (g/L)	Percentage PHB yield
1:1	0.048	0.77	38.5	0.039	81.25

The results are promising and depicted the potential of the strain PHB 2 to utilize low-cost agricultural and food waste as a substrate for PHB production. The strain PHB 2 has been identified as a potential source for producingpolyhydroxybutyrate (PHB). It can utilize starch from agricultural waste as a major substrate for PHB production. This can help to reduce the cost of polymer production. The optimal conditions for maximum PHB production have been identified as a C/N ratio of 5:1, a pH of 7.5, and a temperature of 30°C within 24 to 36 hours. The PHB sheets produced by this strain have been studied for biomedical applications such as drug encapsulation and biocompatibility tests. The results of these tests have shown the promising potential of PHB sheets as a drug delivery system. Overall, the strain PHB 2 has been identified as a reliable source of PHB with various potential applications in the healthcare industry.

Molecular Identification of the bacterial strain PHB 2

Based on the 16S rRNA gene sequence, the strain PHB2 was identified as Bacillus cereus. The sequence is given in Figure 7. The quality of the sequence is depicted in Fig 10. Only the high-quality bases were selected for further analysis after quality trimming.

GAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGG AAGA

ACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGG CTAA

CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTG GGCG

TAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGT GGAG

GGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGT AGCG

Figure 7:- 16S rRNA gene sequence from PHB2.

Polymer Characterization The summary of the FTIR analyses of PHB extracted from the strain Bacillus cereus PHB 2 was depicted in Figure 8.

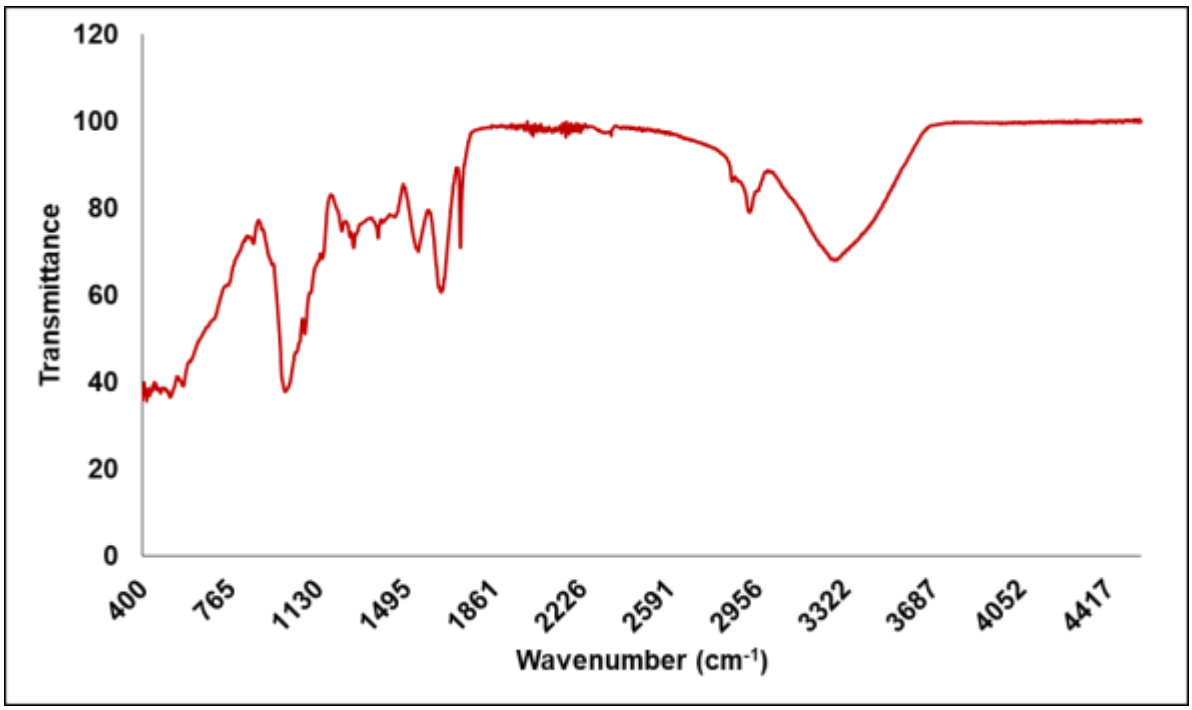


Figure 8:- FTIR analysis of PHB polymer extracted from strain PHB 2.

The characteristic peaks of PHB at 1723 cm^{-1} corresponding to the bond C=O and 1271 cm^{-1} corresponding to the functional group –CH were identified which beyond doubt confirms that the extracted polymer is PHB. The other peaks identified and relevant bonds in the PHB monomer. All these results depict the potential of the strain PHB 2 as a good source for the production of PHB.

Conclusion:-

Bacillus cereus is a Gram-positive bacterium known for its ability to produce polyhydroxyalkanoates (PHAs) such as polyhydroxybutyrate (PHB). PHB is a biopolymer that has been studied extensively for its potential to be used as a replacement for petroleum-derived plastics. Studies have shown that PHB produced by Bacillus cereus has promising antibacterial properties and higher levels of biocompatibility. These properties indicate that it could be used in medical and other allied areas. Furthermore, the cost of producing PHB from Bacillus cereus is relatively low, which is an important factor when considering its use as a replacement for petroleum-based plastics. Therefore, further exploration into PHB produced by Bacillus cereus could result in it becoming a major substitute for plastics derived from petroleum.

Declaration of Competing Interest

The authors declare no competing interest.

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