Isolation, Screening and Identification of Biopolymer Producing ***Bacillus cereus*** From Vegetable Wastes

ABSTRACT

Plastic materials are causing tremendous damage to the environment. To mitigate synthetic plastics, an exploration for eco-friendly biopolymers emerged as an alternative to plastics synthesized from petrochemicals. Biodegradable plastics such as polyhydroxy butyrate (PHB) and polyhydroxyalkanoates (PHA) are currently used in several synthetic applications similar to biopolymers. The present research was conducted to screen bacteria from vegetable wastes for PHB production. PHB producing bacteria were screened using Sudan Black-B stain followed by microscopic examination for presence of PHB granules. The PHB was produced through shake flask fermentation method. Significant PHB-producing bacteria were identified using 16S rRNA sequencing (1200bp) (NCIMRef: 2023/Seq-160), outsourced from NCIM, Pune under CSIR, India. The sequencing report showed that our significant PHB-producing bacterial isolates have 99.93% closest homology to *Bacillus cereus*. Further, we have deposited this sequence in GenBank (**SUB14283541** 907R\_Seq160\_CB4 PP422177, **SUB14283541** 907R\_Seq160\_CB4\_RC PP422178, **SUB14283541** 704F\_Seq160\_CB4 PP422179, and **SUB14283541** 907RC\_704F\_Seq160\_CB4 PP422180) for further reference and studies. The ***Bacillus cereus* CB 4-derived** biopolymer precipitated with acetone has shown a maximum yield of 2.7 gL-1. Further research prospects would focus on the optimization of biopolymer production and its quality testing on various parameters.

**KEYWORDS:** *Bacillus cereus,* Vegetable waste,Biopolymer, PHB, 16S rRNA sequencing, GenBank,

## 1. INTRODUCTION

Plastic material has become an essential part of human life now this plastic material.  
They are used in human life, causing harmful environmental damage and severe issues (Porrier *et al.,* 1995). Since its first introduction in the 1950s, plastic has become an essential and absolute necessity. Research on biopolymers has emerged to clean the environment by mitigating the use of synthetic plastics to protect the environment. Researchers have been looking for alternatives like polymers made from organic materials in an environment-friendly manner, such as bioplastics or biopolymers(Zhang et al.,2018). Biopolymers are an alternative to petrochemical plastics (Anderson & Dawes, 1990; Sudesh *et al.,* 2000). Additionally, biopolymers, including polyhydroxyalkanoates (PHAs), polysaccharides, and extracellular polymeric substances (EPS), are biodegradable substitutes for synthetic plastics and have attracted much attention to the scientific approach to developing stable and rigid biopolymers. Numerous pieces of literature have recently reported Petroleum-based plastic contamination for a decade.

Biopolymers have unique properties and benefits, e.g., high bioavailability, less toxic, biodegradable, stable, and low cost. Over the past decades, there has been rapid growth and success in better understanding biopolymer science and technology, particularly its biological, physicochemical, morphological, and mechanical properties. Biopolymers are generally synthesized when the phosphorous and nitrogen content of the media becomes limited. This causes stress to the bacterial cell, which is then stimulated to produce polymer granules. This stress-induced response occurs during fermentation when nitrogen and phosphorus contents are unavailable, resulting in bioplastic production. Biopolymers are a broad term encompassing any polymer derived from biological sources (Upadhye *et al*., 2022). This includes natural polymers like cellulose and starch and those produced by living organisms, such as PHB. Biopolymers can have diverse structures and properties depending on their origin.

The bacteria produce PHB to store carbon and energy (Lee *et al*., 2021). It has gained significant interest due to its biodegradability and potential as a sustainable alternative to traditional plastics. Therefore, PHB falls under the umbrella of biopolymers, but not all biopolymers are PHB. Biopolymers are produced by living organisms under stress (Pittman, 2015), and sometimes as storage materials or structural components. Cabbage (*Brassica oleracea*) is a widely cultivated leafy vegetable which has been identified as a potential substrate for isolating biopolymer-producing bacteria due to its diverse microbiota, and rich nutrient profile (Shinali *et al*., 2024). In relation to the above statement, Wassermann *et al.* (2017) divulged that the *Brassica* vegetables harbor a diverse group of microbiota based on the results obtained from 16S rRNA gene amplicon sequencing. *Bacillus* species have been extensively studied in the PHA world since the exploration of poly-β-hydroxybutyrate (PHB) in the cytosol of *Bacillus megaterium* by the French Lemoigne in 1926 (Lemoigne, 1926). They have a quick production process in under-regulated settings. Further, Mahishi *et al.* (2003) revealed that the bacterial-based bioplasticsderived from *Azotobacter, Pseudomonas, Bacillus, Methylobacteria, Archaebacteria,* etc., that are easy to degrade under aerobic and anaerobic circumstances. Recently, Mahajan *et al*. (2024) have reported *Cellulosimicrobium cellulans* for effective polyhydroxyalkanoate (PHA)‐production.

Some *Bacillus* species have been reported to produce as much as 90% (w/w) PHAs of dry cells during nutrient imbalance (Madison & Huisman, 1999). *Bacillus* species are becoming model organisms in industry and academia due to their attribute primarily to their genetic stability (Biedendieck *et al.,* 2007). The major drawback of *Bacillus* species in PHA production is their sporulating nature. Practically, the sporulation and deposition of PHA granules are provoked by stress factors (Chen, 2010). To overcome the predicament, research on pilot-scale PHB productions by *Bacillus cereus* in the media that depresses sporulation under acidic pH (Valappil *et al.,* 2007) and potassium deficiency conditions (Wakisaka *et al.,* 1982). These pores-over strategies inhibit spore formation in *Bacillus* and can enhance PHA's productivity. With the concept mentioned earlier, the present research attempted to isolate, screen, and identify biopolymer-producing bacteria from vegetable wastes.

**2. MATERIALS AND METHODS**

The rotten leaves of cabbage were collected from a market area near D.L.S. P.G. College, Ashok Nagar, Sarkanda, in sterile bags and kept at four °C until used.

**2.1. Isolation and Screening of Bacteria for Biopolymer Production**

The samples were crushed and serially diluted up to 10-6. A 0.1 ml of each dilution was placed on Yeast Malt Agar (YMA) consisting of (g L-1) yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0 and agar 20.0 (Silva *et al*., 2009) and were incubated for 24 h at 30°C. After incubation, the total colony was counted and expressed as CFU g-1. The pure cultures of each bacterial isolate were streaked on the YMA plate for further work. Bacterial isolates were cultured in YMA at 30°C for two days. The bacterial isolates were screened using Sudan Black-B stain as mentioned by Mostafa *et al*. (2020) followed by microscopic examination based on the presence and absence of PHB granules.

2.4. Biopolymer production by fermentation

A shake flask fermentation method was used to produce PHB. A loopful of bacterial culture from the YMA plates was transferred to YM broth (25 ml) and was incubated at 30°C for 24 hours. This was eventually used as inoculums. The fermentation media comprised (g L-1) yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0, and agar 20.0 at a pH of 7 with 5% of inoculums. The fermentation was carried out in a shaker incubator for 72 hours at 30°C.

2.5. Separation of biopolymer by Alcoholic method and Acetonicmethod

The fermented broth was centrifuged at 10000 rpm for 20 min at 15oC. 10.0 ml of the cell-free supernatant was added to three volumes of ice-cold ethyl alcohol (95%), and then the mixture was kept for 12 h at four °C for PHB precipitation. The PHB was recovered by centrifugation at 10,000 rpm for 30 min at 4°C. The precipitated PHB was washed with ethyl alcohol and then dried in a hot oven at 40°C for 24 h (Salah *et al.,* 2010). Moreover, the same process mentioned above was carried out by replacing alcohol with acetone, and observations were recorded.

**2.6. Optimization of biopolymer production**

The biopolymer was extracted through the alcoholic and acetonic methods. The optimization parameters viz., pH (6.0, 7.0, and 8.0), temperature (20, 30, and 40), fermentation period (48, 72, and 96 h), and rotation rate (100, 150, and 200 rpm) were optimized at different ranges as per the experimental design to get the maximum possible weight of the biopolymer. Parameter A, Parameter B, and Parameter C were designed as per the literature outcomes to mitigate the repeatability of the research work. We have optimized two methods, viz., alcoholic and acetonic, to extract biopolymer from the fermented broth.

**2.7. Biochemical, Colony, and Molecular characterization of bacteria**

The bacterial isolates were characterized per the standard protocol outlined in Bergey’s Manual of Systematic Bacteriology (Bergey, 1923). After characterization, the most potential PHB producing bacteria were subjected to 16S RNA sequencing. The pure culture of a significant biopolymer-producing bacterial strain was sent to NCIM, Pune, for sequencing. The Genomic DNA was extracted using a HiPurA Bacterial DNA purification spin column kit (MB505-250PR, HiMedia, India). PCR amplification of bacterial-specific 16s rRNA gene (1500 bp) was carried out by using primers F27 (5 ’AGAGTTTGATCMTGGCTCAG 3’) and 1492R (5’ GGTTACCTTGTTACGACTT 3’) (Jill E. Clarridge, III, 2004). The sequencing reactions were run on a 3500xL Genetic Analyzer (Applied Biosystems, USA). Sequencing files (.ab1) edited using CHROMASLITE (version 1.5) and further analyzed by Basic Local Alignment Search Tool (BLAST) with the closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences (Altschul *et al*., 1990) to compare nucleotide to sequence databases and calculates the statistical significance of matches (Gertz, 2005). The statistical calculation, data processing, and graph generation were done using MS Office Excel for all data observed during the research.

**3. RESULTS AND DISCUSSIONS**

The bacteria were isolated from rotten cabbage leaves collected from a market near D.L.S. P.G. College, Ashok Nagar, Sarkanda.

**3.1. Isolation and Screening of Bacteria for Biopolymer Production**

The biopolymer-producing bacteria were isolated from rotten cabbage leaves (Fig 1). We have mentioned the colony and gram stain characteristics of bacterial isolates in Table 1. The bacterial isolates were screened using Sudan Black-B stain followed by microscopic examination based on the presence and absence of PHB granules. Out of 13 bacterial isolates, five bacterial colonies (CB1, CB2, CB3, CB4, and CB5) exhibited biopolymer-producing efficacy. Among them, the high density of PHB granules was noted with CB4, thereby further optimized for PHB production.

**Table 1. The morphological and cultural characteristics of bacterial isolates**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **S. No.** | **Colony** | **Shape appearance** | **Margin** | **Elevation** | **Front colour** | **Back colour** | **Gram staining** |
|  | CB1 | Circular | Undulate | Umbonate | Honeydew | Honeydew | + |
|  | CB2 | Irregular | Undulate | Umbonate | Oyster | Oyster | + |
|  | CB3 | Irregular | Undulate | Umbonate | Oyster | Oyster | + |
|  | CB4 | Circular | Undulate | Umbonate | Yellowish | Yellowish | + |
|  | CB5 | Irregular | Undulate | Convex | Yellowish | Yellowish | + |

|  |  |
| --- | --- |
|  |  |
| **Rotten cabbage (Sample)** | **Bacterial isolates (Master plates)** |

**Fig 1. Isolation of Biopolymer producing bacteria from cabbage**

|  |  |  |  |
| --- | --- | --- | --- |
| **S.No.** | **Solvent for extraction** | **Parameter** | **Product weight (gL-1)** |
|  | Ethanol (95%) | A\* | 0.59 |
|  | B# | 0.73 |
|  | **C^** | **1.03** |
|  | Absolute Acetone | A\* | 1.40 |
|  | B# | 1.89 |
|  | **C^** | **2.7** |
| **\*Parameter A:** pH- 6.0; Temperature- 20oC; Fermentation Time – 48; Rotation – 100 rpm  **#Parameter B:** pH- 7.0; Temperature- 30oC; Fermentation Time – 72; Rotation – 150 rpm  **^Parameter C:** pH- 8.0; Temperature- 40oC; Fermentation Time – 96; Rotation – 200 rpm | | | |

**Table 2. Optimization of Alcoholic and acetonic methods for the production of biopolymer using** *Bacillus cereus* CB-4

**3.2. Optimization of Biopolymer Production By Fermentation**

The biopolymer precipitation with ethyl alcohol exhibited the final weight of the biopolymer yield of 1.03 g L‑1, while the biopolymer precipitated with acetone showed a maximum yield of 2.7 gL-1 (Table 2, Fig. 3).

**3.5. Identification of bacterial using Phenotypic characterization and 16S RNA Sequencing**

The potent biopolymer-producing bacteria were primarily evaluated phenotypically using colony, microscopic, and biochemical examination (Table 1 and Table 4). Afterward, the sequencing report revealed that our significant PHB-producing bacterial isolate CB-4 has 99.93% closest homology to *Bacillus cereus*. NCIM report remarked that the strain showed the closest homology with *Bacillus* sp. (Closer to *B. cereus*) ++ Genus/genera was comprised of large number of species, hence 16S rRNA may not resolve species identity (Table 3). Electrophoresis Gel Images: Genomic DNA and PCR Product shown in Fig. 2.Biochemical/phenotypic tests/whole genome sequencing analysis might resolve species-level identity. We have deposited this sequence in GenBank (SUB14283541 907R\_Seq160\_CB4 PP422177, SUB14283541 907R\_Seq160\_CB4\_RC PP422178, SUB14283541 704F\_Seq160\_CB4 PP422179, and SUB14283541 907RC\_704F\_Seq160\_CB4 PP422180) for further reference and studies.

**Table 3. Result of 16 S RNA Sequecing of CB-4 (Seq160 CB4 NC281223 A)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S. No.** | **Strain** | **Aim** | **Primer** | | **NCBI BLAST (Type Strain)** |
| 1. | Seq160 CB4 NC281223 A | Identification of 16S rRNA gene ++ | 907RC\_7 04F (1503bp) | | Sequence ID: NR 115714  Taxon ID:  *Bacillus cereus* Identities: 1502/1503(99.93%)  Ref: FEMS Microbiol. Ecol. 60 (2), 341-350 (2007) ++  Sequence ID:  NR 175555  Taxon ID:  *Bacillus sanguinis* Identities: 1501/1503(99.87%)  Ref: Int J Syst Evol Microbiol 71 (9) (2021) |
|  | | | |  | |
| 1. **Genomic DNA**   L- Step-up 1 Kb DNA Ladder, Genei  1- Seq160\_CB4 | | | | 1. **16s rRNA PCR Product**   L- Step-up 1 Kb DNA Ladder, Genei  1- Seq160\_CB4 | |
| **Fig. 2. Electrophoresis Gel Images: Genomic DNA and PCR Product** | | | | | |

A recent article published by Hamdy *et al*. (2022) reported that *Bacillus cereus* SH-02 (OM992297) a potent PHB-producing bacterium was isolated from the Dirout channel at Assiut Governorate. Additionally, they reported maximum PHB content and concentration of 3100.799 mg/L and 28.799% respectively with *Bacillus cereus* SH-02 (OM992297) under optimized conditions. Notably, de Souza and Gupta (2024) stated that the microorganisms belongs to *Pseudomonas*, *Rhodobacter*, *Rhizobium*, *Halobacterium, Ralstonia*,*Spirulina Enterobacter* have been widely explored for PHA and PHB.  Valappil *et al*. (2007) reported that *Bacillus cereus* SPV-derived polymer was found suitable for their potential application in tissue engineering.

This work screened *Bacillus cereus* CB-4 from decaying cabbage leaves and processed them to assess their biopolymer-producing efficacy. A similar attempt was made by Sivkumar *et al*. (2013), they studied cauliflower rotten leaves and isolated biopolymer-producing bacteria such as *Panteo agglomerans, Bacillus subtilis, Enterobacter sp.* and *Pseudomonas sp.* Additionally, they reported maximum polymer production of 3.17g L-1 with *Pseudomonas sp.* Recently, Kumar *et al*. (2020) also isolated *Bacillus subtilis* and *Pseudomonas aeruginosa* strains from cabbage leaves, demonstrating their ability to produce PHAs under nitrogen-limited conditions.

**Table 4. Biochemical Feature of Bacterial Isolate** *Bacillus cereus* **CB-4**

|  |  |
| --- | --- |
| **Biochemical Examination** | **Observation** |
| Spore formation | + |
| Casein hydrolysis | + |
| Catalase test | + |
| Citrate utilization | + |
| Gelatin hydrolysis | + |
| Glucose fermentation | + |
| H2S production | – |
| Indole test | – |
| Mannitol fermentation | – |
| Oxidase test | – |
| Starch hydrolysis | + |
| Sucrose fermentation | + |
| Urease test | + |
| **+** represent positive result **-** represent negative result | |

The extraction of biopolymer-producing bacteria from decaying cabbage leaves underscores the potential of using waste materials for vital bioproducts. This emerged as an economical and readily available substrate for polymer synthesis utilizing bacterial strains. The Ellen MacArthur Foundation (2013) examines the principles of the circular economy, specifically focussing on utilizing cabbage waste as a resource for polymer production through microbial strains.

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
| **Biopolymer production by Shake flask fermentation** | | **Biopolymer film derived from Alcoholic Method** | **Biopolymer derived from Acetonic Method** |
| **Fig. 3 Production of Biopolymer using *Bacillus cereus* CB-4** | | | |

Further study is necessary to enhance polymer manufacturing with microbial strains at a commercial level. The capacity of these microbes to produce PHAs, EPS, and other biopolymers highlights their potential in tackling environmental and industrial issues. Additional investigation is required to enhance the production techniques that would facilitate sustainable solutions to worldwide plastic pollution. Biglari *et al*. (2018) mentioned that their Response Surface Methodology (RSM) based optimization of poly(3-hydroxybutyrate) (3HB) production showed that the glucose (37.70 g/L), urea (0.73 g/L), and 200 rpm of agitation speed were optimal. The maximum biomass of 12.84 g/L P(3HB) content of 92.16 wt%, and P(3HB) concentration of 11.83 g/L were recorded during the optimized validation experiments. Subsequently, Biglari (2020) reported that the gradual repeated fed-batch stage (RFB) mode significantly exceeded the decline mode and increased Cell Dry Weight (CDW) and P(3HB) productivities by 2.2 fold. In the incremental RFB-II mode, the P(3HB) concentration was stretched to 111 g/L after five cycles with CDW productivity of 1.3 g/L/h. Their work was effectively contributed to the upsurge in industrial biopolymer production. Present research work would align with the aforementioned findings to upscale the biopolymer production from our potent bacterial isolate *Bacillus cereus* CB-4.

Recent research suggested that the food waste can be utilized as an economical substrate for biopolymer production, with combined valorization strategies proving more efficient than individual approaches. Biopolymers derived from food waste have diverse applications, including healthcare and the food industry (Basumatary *et al*. 2022). Fresh produce, being highly perishable, requires protective packaging or coatings. Conventional plastics and waxes pose environmental and health risks, making biopolymer-based nanocomposites a sustainable alternative. These nanocomposite films and coatings effectively enhance shelf life (Gautam *et al*., 2022). But, the present research work was focused on the isolation, screening and identification of biopolymer producing *Bacillus cereus* from vegetable wastes because the screening, and identifying *Bacillus cereus* strains from vegetable waste is an important part of making biopolymers in a way that is both environmentally friendly and cost-effective. This approach supports waste valorization and reduces reliance on petroleum-based plastics by utilizing vegetable waste as a renewable substrate, . Biopolymers produced by *Bacillus cereus* are biodegradable, environmentally friendly, and have diverse applications in packaging, agriculture, biomedicine, and wastewater treatment. Screening is important for finding high-yield strains with the best metabolic efficiency and polymer composition, and genetic characterization helps strains get better by making fermentation more efficient. Different strains produce biopolymers with varying physicochemical properties, making strain selection vital for specific applications. Once an efficient strain is identified, it can be optimized for industrial-scale production, promoting a circular bioeconomy and reducing production costs. This process transforms organic waste into valuable materials, offering both environmental and economic benefits.

**CONCLUSION**

Cabbage is a nutritious and readily accessible agricultural resource, that offers a distinct environment for the isolation of biopolymer-producing bacteria. The current research demonstrated the biopolymer production efficiency of *Bacillus cereus* **CB-4** isolated from decayed cabbage leaves as a substitute for synthetic polymers. Biopolymers are frequently produced by particular microbial communities in reaction to surplus carbon sources or situations of vital nutrient scarcity. Biopolymers captivate the polymer industry owing to their entirely biodegradable characteristics in both aerobic and anaerobic environments, as well as their extensive uses in medicine, pharmaceuticals, agriculture, and food packaging, attributed to their superior biocompatibility and non-toxic properties.

**Disclaimer (Artificial intelligence)**

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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