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# Biosynthesis of biodegradable polymer by a potent soil bacterium from a stress-prone environment

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**ABSTRACT**

Polyhydroxybutyrate (PHB), produced by many microorganisms during stress condition, is an eco-friendly biodegradable polymer which can completely replace the petroleum-based commercial plastics. The study was aimed to screen a potent PHB-producing bacterium from municipal dumpsite areas and to analyze the effects of nutritional and environmental factors to enhance production of PHB. *Acinetobacter* sp. K3 was a potent PHB-producing soil bacterium identified by morphological, biochemical, and molecular techniques. The 16S rDNA nucleotide sequence of the bacterium was assigned the accession number: KT907046 in NCBI database. Effects of different carbon, nitrogen, pH, and temperatures on PHB production were analyzed to enhance its product yield. It grows at the rate of 0.105 g/h/L and PHB production achieved up to 4.8 g/L from 6.04 g/L dry cell weight (DCW) and PHB contents amount to 79.4 % w/w of total DCW. The above accumulated PHB was obtained at pH 8, temperature 40°C, mannitol, and urea used as carbon and nitrogen sources, respectively. Further, *phbA*, *phbB,* and *phbC* genes responsible for PHB production were amplified which confirms the presence of PHB using the gene-specific primers by polymerase chain reaction technique. This new strain could be used for further industrial production of biopolymer.

## 1. INTRODUCTION

Polyhydroxyalkanoates (PHAs) and their derivatives are microbial biopolymer which serves as an alternative means to the existing petroleum-based plastics as they can be diminished from the environment in an eco-friendly manner. Almost 150 constituents of PHAs have been identified, and among them, poly-3-hydroxybutyric acid (polyhydroxybutyrate [PHB]) is the principal constituent widely produced intracellular by many bacteria [1]. Nowadays, PHB is gaining much concern as it has similar properties to that of petroleum-based polypropylene [2]. Due to their biodegradability property, researchers have eyed on this biopolymer from decades. However, due to its high cost of production that is mainly attributed to the cost of the carbon source and polymer recovery, its widespread use as commodity plastic has been restricted [3]. In PHB production, 40–48% of the total production costs go to the raw materials where carbon source accounts for 70–80% of the total production cost. Therefore, using suitable cultural parameters to optimize its production is the need of the hour to reduce its overall production cost. Investigating a proper and suitable medium for maximum production of PHB is of critically important from an industrial point of view as the medium components significantly affect the product yield.

Moreover, the burning issues of global environment and solid waste management have also created lots of concern toward the development of biodegradable polymers. Besides solving the environmental problems, PHB can be effectively used in the field of medical science owing to its biocompatibility and biodegradability characteristics. It has been reported that the main advantage of using PHB in medical field is their biocompatibility and the degradation product, 3-hydroxybutyric acid is normally present in the human blood at concentrations of 1.3 mmol/L [4]. Furthermore, because of biodegradability, PHB fibers got thinner as time proceeded when used to suture wounds in mouse [5]. PHB can also be converted into nanoparticles, which can help to deliver drugs to all the body parts including the smallest capillaries of diameter 5–6 mm [6].

A large number of bacteria have the capability to produce this polymer in response to unfavorable growth conditions when a carbon source is readily available [7,8]. *Alcaligenes, Azotobacter, Bacillus, Nocardia, Pseudomonas,* and *Rhizobium* produce PHB as energy reserves [9]. *Alcaligenes eutrophus* is the most widely explored organism for PHB production as it is easy to grow, it accumulates large amount of PHB (up to 80% of dry cell weight [DCW]) in a simple medium, and its physiology and biochemistry leading to PHB synthesis are best understood [10]. Over 250 different types of bacteria have been reported as natural PHA producers [11]. In addition to natural

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producers, genetically modified organisms are also used for industrial production of PHA [12,13]. Although a lot of bacteria have identified as PHB producers, still there is a gap in discovering a more potential bacterium that could produce PHB very efficiently in an optimized medium.

PHB biosynthesis involves three important enzymatic steps, and for this, the starting material is acetyl-coenzyme A (acetyl-CoA). Two acetyl-CoA molecules undergo reversible condensation reaction into acetoacetyl-CoA which is catalyzed by β-ketothiolase (encoded by *phbA*). Another enzyme, NADPH-dependent acetoacetyl-CoA reductase (encoded by *phbB*) reduces acetoacetyl-CoA into 3-hydroxybutyryl-CoA, and finally, polymerization of 3-hydroxybutyryl-CoA monomers by PHB synthase (encoded by *phbC*) produces the polymer PHB [14].

In this report, an attempt was made to screen a potent PHB-producing bacterium from the soil samples of a stress-prone environment (municipal waste areas) and to analyze the effects of nutritional and environmental cultural parameters to enhance the production of PHB. This may help to reduce its cost of production in industrial point of context.

## 2. MATERIALS AND METHODS

### 2.1. Isolation, Screening, and Characterization of PHB-Producing Bacteria

The bacterium was isolated from the soil samples collected from municipal waste areas of Karimganj region of Southern Assam, India. For screening the PHB producing capability of the isolates, the pure cultures were grown in basally defined M9 medium (DM9 medium) and stained with Sudan Black B stain. The bacterial growth was evaluated by measuring the $OD_{600}$ in UV-Vis spectrophotometer (Spectrascan UV 2600). Further, the morphological and biochemical properties of the bacterium were investigated according to Bergey's manual of determinative bacteriology [15]. For molecular characterization of the bacteria, the genomic DNA was extracted using HiPurA™ Bacterial Genomic DNA Extraction Kit and nearly full-length 16S rDNA sequences were amplified by polymerase chain reaction (PCR) using primers 27F and 1492R [16]. PCR amplifications were carried out with the following temperature profile: 5 min at 95°C, 30 cycles of denaturation (60 s at 94°C), annealing (60 s at 55°C), extension (2 min at 72°C), and a final extension for 7 min at 72°C. Amplified products were separated on 1% agarose gel and observed with a UV transilluminator and documented with Gel Doc XR software (Biorad). The amplification product was purified using Genejet Gel Extraction PCR Purification kit according to the manufacturer's instruction. The purified PCR product was sequenced by ABI 3500 Genetic Analyzer. The 16S rDNA gene sequence analysis was carried out using NCBI-BLAST (National Centre for Biotechnology Information http://www.ncbi.nml.nih.gov) program. The sequences were aligned and the phylogenetic tree was constructed using the neighbor-joining method using MEGA 4.1.

### 2.2. Seed Culture Preparation

To 5 mL of the sterile nutrient broth containing (%): Peptone, 0.5; beef extract, 0.3; and sodium chloride, 0.5, one loopful of the bacterial stock culture was inoculated from the slant culture and incubated for 24 h at 30°C and 160 rpm. After incubation, 1% (v/v) of the bacterial culture was transferred to 50 mL sterile nutrient broth and again incubated in rotary shaker under the condition of growth at 30°C for 18 h.

### 2.3. Microscopic Detection of PHB Granules

PHB production was carried out on a rotary shaker at 150 rpm using 50 mL of DM9 medium containing 4 g/L glucose, 1 g/L $NH_4Cl$, 7 g/L $Na_2HPO_4$, 3 g/L $NaH_2PO_4$, 10 mL 0.01 M $CaCl_2$, and 10 mL 0.1 M $MgS0_4.7H_20$. The medium containing flask was autoclaved at 15 psi for 30 min, cooled and then, 1 % (v/v) of 18 h old culture was inoculated and incubated at 37°C for 48 h. The contents of the flask were then centrifuged at 8000 rpm for 10 min at 4°C, and the cell pellets were stained with Sudan Black B stain and observed under oil immersion microscope (Nikon Eclipse E200).

### 2.4. Production and Extraction of PHB

Hypochlorite method with slight modification [17] was used for the extraction of PHB from *Acinetobacter* sp. K3. For this, the bacterium was grown in 50 mL of DM9 medium and incubated at 37°C for 48 h on scientific rotary shaker at 160 rpm. 10 mL of the cell suspension was centrifuged at 6000 rpm for 10 min at 4°C. The cell pellet was washed with 10 mL saline and was recentrifuged to get the pellet. Cell pellet was then suspended in 5 mL sodium hypochlorite (4% active chlorine) and incubated at 37°C for 10 min with stirring. This extract was centrifuged at 8000 rpm for 20 min at 4°C, and the pellet of PHA was washed with 10 mL cold diethyl ether. The pellet was again centrifuged at 8000 rpm at 4°C to get the purified PHA and was dried to constant weight at 60°C.

### 2.5. PHB Production on Different Carbon Sources, Nitrogen Sources, pH, and Temperatures

By inoculating a single colony from nutrient agar plate into 10 mL Nutrient broth, and growing at 37°C for 16–20 h at 200 rpm, inoculum for PHB production was prepared. PHB production was monitored in DM9 medium (400 mL in 1000 mL conical flasks) at 37°C for 48 h in shaker at 160 rpm. To optimize the carbon source, the medium was supplemented separately with five different carbon sources (glucose, mannitol, fructose, starch, and sucrose) at a fixed concentration of 4 g/L. Among the nitrogen sources, peptone, yeast extract, tryptone, urea, and ammonium chloride were tested at fixed concentration of 1 g/L, different pH analyzed were 5, 6, 7, 8, and 9 and temperatures 25°C, 30°C, 35°C, 40°C, and 45°C were experimented for PHB production.

### 2.6. Evaluation of Kinetic Parameters

Specific growth rate (G) was determined by the formula:

$$G=\ln W2-\ln W1/T2-T1\times 100$$

Where, W2 is the dry cell biomass at time T2 and W1 is the initial dry cell biomass at time T1. It is expressed in terms of g/h/L. Content of the polymer was expressed as the ratio of PHB (P) to the biomass (X) multiplied by 100. PHB content=P×100/X. Specific PHB storage rate (μ) per unit time (t) was given as μ=P/X.t (g PHB produced/g biomass/h). PHB yield at the end of the fermentation ($Y_{p/s}$) was calculated as the mass of PHB obtained per gram of the substrate.

### 2.7. Analytical Procedure

Cell concentration was determined by measuring the DCW. For this, 5 mL broth culture was centrifuged, washed with distilled water and dried at 60°C until the weight does not decrease further. The residual mass was defined as total DCW minus PHB weight and PHB (%) as the percentage of the ratio of PHB to DCW.

### 2.8. Amplification of *phbA*, *phbB,* and *phbC* Genes

For amplifying *phbA, phbB,* and *phbC* genes in *Acinetobacter* sp. K3, the genomic DNA was extracted using HiPurA™ Bacterial Genomic DNA Extraction Kit and the genes were amplified by PCR using the specific primers [Table 1]. PCR amplifications were carried out with the following temperature profiles separately: *phbA:* Initial denaturation (5 min at 94°C), 35 cycles of denaturation (30 s at 94°C), annealing (40 s at 55°C), extension (40 s at 72°C), and a final extension for 5 min at 72°C; *phbB*: Initial denaturation (3 min at 94°C), 35 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), extension (30 s at 72°C), and a final extension for 7 min at 72°C; and *phbC*: Initial denaturation (5 min at 94°C), 35 cycles of denaturation (30 s at 94°C), annealing (40 s at 55°C), extension (1 min at 72°C), and a final extension for 7 min at 72°C.

### 2.9. Statistical Analysis

Analysis of the results obtained was done using SPSS 16.0 software, where $P < 0.05$ was considered to be statistically significant.

## 3. RESULTS AND DISCUSSION

### 3.1. Screening for PHB Accumulating Bacteria

A total of 20 bacterial cultures were isolated on nutrient agar medium from the soil samples collected from municipal wastes of Karimganj region of Southern Assam. These were screened for PHB producing capability by staining with Sudan Black B stain. Stained PHB granules appeared as dark spot inside the pink colored cells after 48 h of growth in the production medium (DM9 medium) which was revealed by microscopic analysis [Figure 1]. Accordingly, eight among the 20 isolates were found to be PHB positive [Table 2]. These positive isolates were again inoculated in DM9 medium for estimating the amount of PHB produced. Among these isolates, K3 was found to be a promising producer of PHB giving a production of 2.15 g/L PHB from 4.02 g/L DCW (53.4% w/w of the DCW). Therefore, based on the encouraging results, isolate K3 was selected for further optimizing its culture conditions.

### 3.2. Physiological and Biochemical Characterization of Isolate K3

The physiological and biochemical properties of the isolate K3 were determined according to the methods described in "Bergey's Manual of Systematic Bacteriology" [19]. The isolate K3 is a Gram-negative, rod-shaped, non-motile, aerobic, and non-spore forming bacterium. Its colony shape is round, colony color off white, 1–2 mm colony size, have smooth surface, raised, opaque, entire, and sticky. Its optimum temperature is 30°C–40°C and pH is 6–8. This bacterium can utilize glucose, mannitol, and rhamnose but unable to utilize adonitol, arabinose, lactose, sorbitol, and sucrose. Table 3 summarizes the biochemical characteristics of the isolate K3.

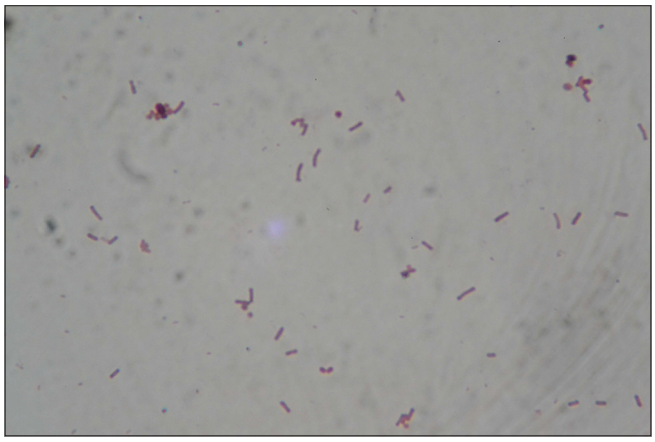

**Figure 1:** Cells of *Acinetobacter* sp. K3 stained with Sudan Black B.

**Table 1**: Primers used in this study.

| Name | Sequence (5'-3') | Tm (°C) | Product length | References |
|---|---|---|---|---|
| *PhbA*-F | CCATGACCATCAACAAGGTG | 58 | 262 bp | [18] |
| *PhbA*-R | TATTCCTTGGCCACGTTCTC | | | |
| *PhbB*-F | AATGTGGCTGACTGGGACTC | 60 | 174 bp | [18] |
| *PhbB*-R | GTTGAACAGCGAGGTCAGGT | | | |
| *PhbC*-F | CCTTCTCGCCTATGCTCTGG | 63 | 503 bp | For this study |
| *PhbC*-R | GCGTGACCTTGAATGGTTG | | | |

**Table 2**: Selected eight Sudan Black B positive isolates for PHB production.

| Sample number | Isolate code | Sudan Black B test | O.D | DCW (g/L) | PHB (g/L) | PHB content (%) |
|---|---|---|---|---|---|---|
| 1 | K1 | + | 0.165 | 2.51 | 0.94 | 37.4 |
| 2 | K3 | + | 0.965 | 4.02 | 2.15 | 53.4 |
| 3 | K2 | + | 0.153 | 2.45 | 0.90 | 36.7 |
| 4 | K4 | + | 0.150 | 2.30 | 0.87 | 37.8 |
| 5 | K7 | + | 0.147 | 2.29 | 0.83 | 36.2 |
| 6 | K5 | + | 0.145 | 2.08 | 0.70 | 33.6 |
| 7 | K6 | + | 0.132 | 1.68 | 0.67 | 39.8 |
| 8 | K15 | + | 0.097 | 1.05 | 0.23 | 21.9 |

DCW: Dry cell weight, PHB: Polyhydroxybutyrate.

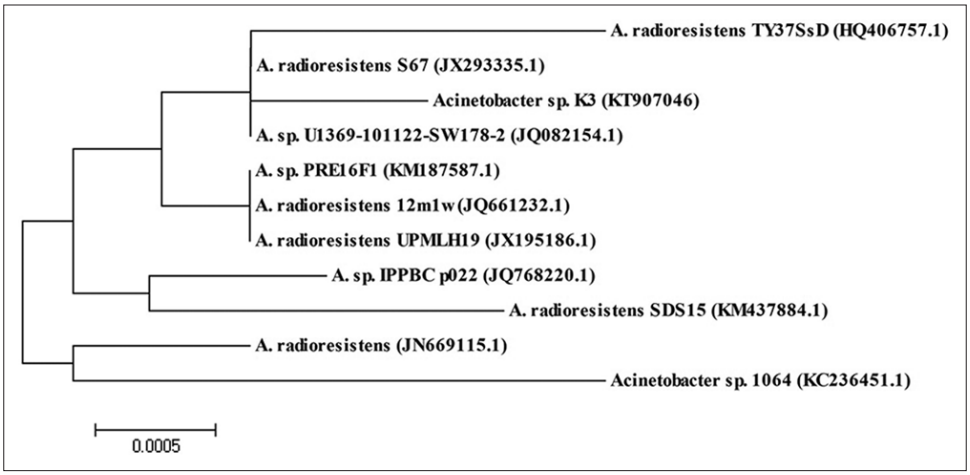


**Figure 2:** Phylogenetic analysis of 16S rDNA sequence of *Acinetobacter* sp. K3 and related strains. The tree was generated by neighbor-joining method using MEGA 4.1: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1. GenBank accession numbers are given in parentheses.

### 3.3. 16S rDNA Gene Sequence and Phylogenetic Analysis

The characterization of the isolate K3 was confirmed with 16S rDNA gene sequence analysis. The almost complete sequence of 16S rDNA gene (1388 bp) of the isolate K3 was amplified from the genomic DNA, purified and sequenced. The gene sequence of the isolate K3 following pair-wise alignment exhibited highest (99%) similarity with *Acinetobacter* sp. strain U1369-101122-SW178-2 (accession no. JQ082154.1). Therefore, the bacterium was identified as *Acinetobacter* sp. strain K3 and its nucleotide sequence has been deposited in NCBI database and the sequence was assigned the accession no: KT907046. The consensus sequence of the strain K3 and other related strains were aligned and the phylogenetic tree shown in Figure 2 was constructed. The evolutionary history was inferred using the neighbor-joining method [20]. The optimal tree with the sum of branch length = 0.00921500 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [21] and are in the units of the number of base substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+non-coding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1360 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [22].

### 3.4. Effects of carbon sources on PHB Production and Cell Concentration

Growth of organism and PHB accumulation depends on specific carbon source taken up by the microorganism. Hence, carbon source is the key material in producing PHB by the microorganisms and it serves three different functions: Synthesis of biomass, energy source for biosynthesis and cell maintenance, and carbon source for PHB polymerization. Figure 3 showed that mannitol was found to be the most suitable carbon source among different carbon sources tested producing 5.81 ± 0.01 g/L PHB, 71.46% DCW (significance at $P < 0.05$ level). Fructose and glucose are also suitable for growth and PHB accumulation after mannitol. Fructose and glucose also serves as suitable carbon sources by *Bacillus mycoides* [23]. It has been reported that 0.9% PHB on a dry weight basis were obtained when *Acinetobacter* strain RA3757 was grown on valerate as carbon source [24] which is very much less when compared with our *Acinetobacter sp.* K3 when

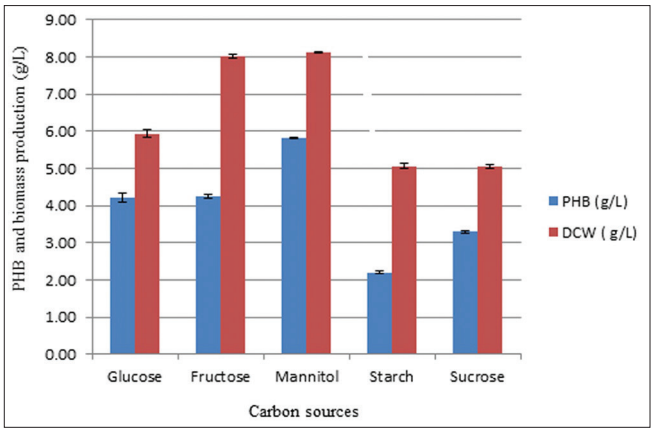


**Figure 3:** Effect of carbon sources on polyhydroxybutyrate (PHB) production by *Acinetobacter* sp. K3. PHB production was monitored in DM9 medium at 37°C for 48 h in shaker at 160 rpm.

mannitol was used as carbon source. *A. eutrophus* also shows high PHB yield on fructose utilization in mineral salt medium broth [8].

### 3.5. Effects of Nitrogen Sources on PHB Production and Cell Concentration

Different nitrogen sources were tried to select the best nitrogen source for PHB production by *Acinetobacter* sp. K3. In this study, urea was found to support maximum PHB production (significance at $P < 0.05$ level) followed by tryptone and yeast extract [Figure 4]. Ammonium chloride does not support growth and PHB accumulation in this study but supports maximum PHB production from *Bacillus subtilis* NG05 [25]. Literature also reveals that ammonium sulfate is the best nitrogen source for different microorganisms such as *A. eutrophus* [26], *Methylobacterium* sp. [27], and *Sinorhizobium fredii* [28]. Therefore, the type of nitrogen source used and the type of microorganisms utilizing it are also the main factor for efficient production of PHB. This may help in industrial application as waste liquids containing $NH_4^+$ can be utilized for polymer synthesis.

### 3.6. Effects of pH on PHB Production and Cell Concentration

Proper control of pH is very critical as slight change in pH affects the metabolic process of an organism. Therefore, growth of the cell and

PHB accumulation by *Acinetobacter* sp. K3 has also influenced by change in pH level. Here, Figure 5 indicates that PHB accumulated is maximum at pH 8 producing 2.77 ± 0.02 g/L PHB (significance at $P < 0.05$ level). This observation is in agreement with a previous report that pH 6.8 to 8.0 was optimum for PHB production by *A. eutrophus* [29]. Further, increase in pH decreases the yield of PHB, which may be due to the effect on the degradative enzymes of polymer breakdown, so that PHB is used up at a rate almost equal to the rate of its synthesis. In another report, maximum PHB was obtained at pH 7–7.5 by *Bacillus sphaericus* NII 0838 from crude glycerol [30].

### 3.7. Effects of Temperatures on PHB Production and Cell Concentration

Maximum PHB production was recorded at 40°C (significance at $P < 0.05$ level) after 48 h of growth and increase of temperature beyond 40°C have negative impact on PHB production [Figure 6]. This decrease in PHB at high temperatures may be due to low PHB polymerase enzyme activity [25]. This result slightly coincides with the earlier report that optimum incubation temperature for PHB production by *B. subtilis, Bacillus pumilus,* and *Bacillus thuringiensis* was 37°C [31,32].

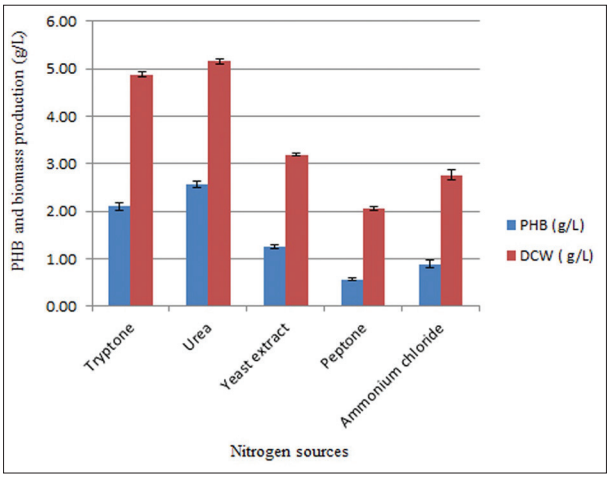


**Figure 4:** Effect of nitrogen sources on polyhydroxybutyrate (PHB) production by *Acinetobacter* sp. K3. PHB production was monitored in DM9 medium at 37°C for 48 h in shaker at 160 rpm.

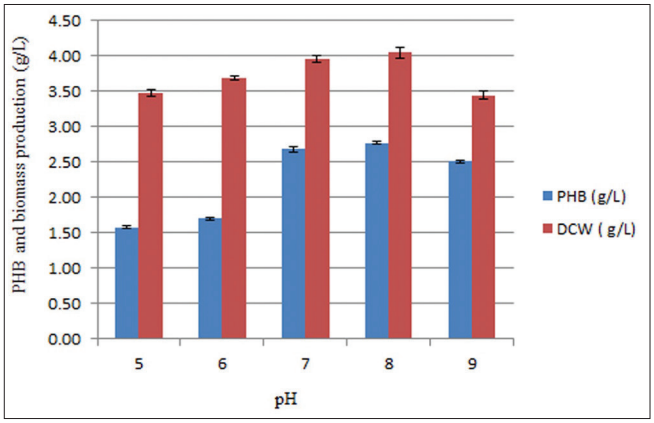


**Figure 5:** Effect of pH on polyhydroxybutyrate (PHB) production by *Acinetobacter* sp. K3. PHB production was monitored in DM9 medium at 37°C for 48 h in shaker at 160 rpm.

### 3.8. Evaluation of Kinetic Parameters

The kinetic parameters for the batch experiment were evaluated with respect to PHB and biomass production in the optimized culture medium. Table 4 summarizes that *Acinetobacter* sp. K3 grow at the rate of 0.105 g/h per liter of the production medium and produces 79.4% of PHB at the rate of 0.010 g/g of the biomass produced per hour.

### 3.9. Amplification of *phbA*, *phbB,* and *phbC* Genes

In this study, the PCR products of *phbA, phbB,* and *phbC* genes were amplified and separated on 1% agarose gel, observed with a UV transilluminator and documented with Gel Doc XR software (Biorad) [Figure 7]. Three fragments with the length 262 bp for *phbA* gene, 174 bp for *phbB* gene, and 503 bp for *phbC* gene have been obtained by PCR using forward and reverse primers as listed in Table 1. Amplification of these genes confirms the production of PHB by *Acinetobacter sp.* K3. This may help in studying the pathway and regulation of PHB biosynthesis in *Acinetobacter* sp. K3. PHB biosynthetic mechanism has been studied exhaustively in *A. eutrophus* [33] and *Azotobacter beijerinckii* [34]. However, in *Acinetobacter* sp., little knowledge is

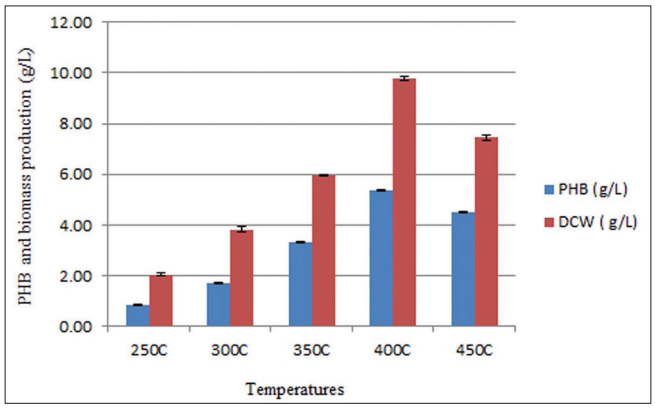


**Figure 6:** Effect of temperatures on polyhydroxybutyrate (PHB) production by *Acinetobacter* sp. K3. PHB production was monitored in DM9 medium at 37°C for 48 h in shaker at 160 rpm.

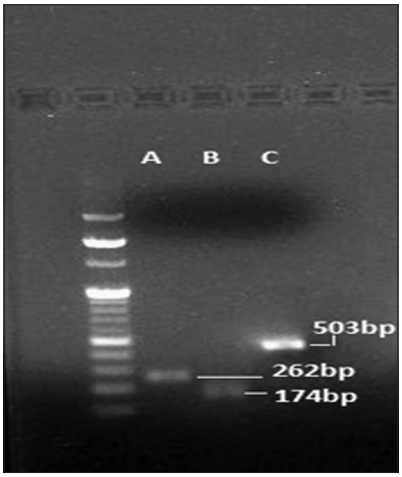


**Figure 7:** Amplified polymerase chain reaction products of polyhydroxybutyrate genes of *Acinetobacter* sp. K3. Lanes A, B, and C denotes *phbA, phbB, and phbC* genes, respectively, with fragment length of 262 bp, 174 bp, and 503 bp, respectively.

**Table 3**: Biochemical properties of *Acinetobacter* sp. K3.

| Characterization | Sample bacteria |
|---|---|
| Biochemical properties | |
| Indole | - |
| Methyl red | - |
| Voges-Proskauer | + |
| Citrate utilization | - |
| Nitrate reduction | - |
| Starch hydrolysis | - |
| Catalase | + |
| Oxidase | - |
| Urease | - |
| Gelatin liquefaction | - |
| Utilization of carbohydrates | |
| Glucose | + |
| Mannitol | + |
| Rhamnose | + |
| Adonitol | - |
| Arabinose | - |
| Lactose | - |
| Sorbitol | - |
| Sucrose | - |

**Table 4**: Evaluated kinetic parameters of *Acinetobacter* sp. K3 after optimization.

| Specific growth rate (G) | PHB contents (P) | PHB storage rate (μ) | PHB yield ($Y_{p/s}$) |
|---|---|---|---|
| 0.105 (g/h/L) | 79.4% | 0.010 ($g/g_x$/h) | 0.913 ($g/g_s$) |

PHB: Polyhydroxybutyrate.

known about the metabolism of PHB though activities of some of the enzymes involved in PHB synthesis were studied in two strains of *Acinetobacter* sp. [24]. Hence, in this study, amplification of PHB genes not only confirms the production of PHB but also provides a reference to study the effect of these enzymes on PHB production in *Acinetobacter* sp. K3.

## 4. CONCLUSION

The present investigation reported the importance of nutritional and environmental effects on the production of PHB by an efficient soil bacterium *Acinetobacter* sp. K3. It grows at the rate of 0.105 g/h/L of the optimized production medium. PHB production achieved under optimized conditions of growth was found to be 4.8 g/L from 6.04 g/L DCW and PHB contents amount to 79.4% w/w of total DCW. The above PHB production was obtained at pH 8, temperature 40°C, mannitol as carbon source, and urea as nitrogen source, respectively. Furthermore, *phbA, phbB,* and *phbC* genes were successfully amplified which confirms the production of PHB by *Acinetobacter* sp. K3. Production of PHB by *Acinetobacter* sp. K3 was optimized and this experimentation carried out at laboratory scale will provide very useful information for scale-up studies of PHB production from *Acinetobacter* sp. K3 which in turn may help to reduce its cost of production. *Acinetobacter* sp. K3 would be a good candidate for PHB production in further studies.

## 5. ACKNOWLEDGMENTS

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