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In vitro STUDIES ON CHARACTERIZATION OF POLYHYDROXYBUTYRATE

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AUTHOR'S CONTRIBUTION

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

The bacterial polyesters may be considered as "Green Plastics", because of their biodegradable nature. These polyesters can be employed for packaging and coating materials / as biodegradable carriers and applied in the biomedical field. Twelve bacterial isolates were isolated from the dairy industry effluent sample. Screening for polyhydroxybutyrate (PHB) was done by Sudan black staining. PHB extraction was carried out by the chloroform digestion method. Biochemical and 16s rRNA analysis showed that PHB producing bacteria belong to *Bacillus* genera with Maximum production of PHB was analyzed by U.V spectrophotometer and finally it was characterized by FTIR, NMR, and GC MS.

Keywords: Biopolymer; polyhydroxybutyrate (PHB); dairy industry effluent.

1. INTRODUCTION

Industrialization is not only responsible for economic development, but the release of hazardous substances heavily affects the environment. The health of an ecosystem, flora, fauna is largely disturbed by industrial pollutants. In recent days, people are more interested in the treatment of industrial waste, if left untreated harmfully affects the soil and water ecosystem [1]. Nowadays the number of physicochemical techniques is extensively studied by a number of researchers [2]. Treatment of effluent through the use of microorganisms largely removes the sludge particles, the removal of sludge is more through the aerobic system than anaerobic system [3] it also reduces the COD value. The ecosystem is greatly affected by the discharge of dairy wastewater with a high pollution load [4]. The presence of large amounts of nutrients in dairy wastewater, cause eutrophication in receiving water bodies [5].

The bioremediation process converts the complex toxic substances into simple organic substances, with the commonly available indigenous microbiota [6]. The treatment of effluent using biological organisms is the commonly accepted method [7]. The efficiency or rate of biodegradation can be increased by the use of a consortium of microorganisms [8,9]. The need for plastic is increasing day by day due to population explosion, and petrochemical plastic is used in many applications, but at the same time, it releases more toxic gases during the degradation. Plastic posses more advantages due to its easy availability and durability. One of the major drawbacks of synthetic plastic was its nonbiodegradability [10,11,12]. Polyhydorxybutyarte is a homopolymer of polyhydroxyalkanoate, which is synthesized by bacteria under nutrient-limiting conditions. Biopolymers are biodegradable in nature which are completely oxidized into CO_2 and H_2O by PHA hydrolases and PHA depolymerase [13,14].

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Renewable sources are the precursor for biopolymer production [15,16,17]. This research work mainly studied the PHB producing strains, extraction, and characterization of PHB.

2. MATERIALS AND METHODS

The chemicals used for the preparation of reagent, solutions, and microbiological growth media were purchased from Hi-media Laboratories Pvt Ltd, Mumbai, India. Solvents used in the studies were of AR grade and were purchased from Merck Pvt Ltd. *Bacillus cereus* showing appreciable PHB production was isolated from dairy industry effluent sample. The bacterium was identified based on their biochemical and molecular characterization. The PCR amplification and DNA sequencing of the 16S rRNA gene fragment of the bacterial strains was carried out by isolating genomic DNA from the pure culture pellet using 27F forward primer and 1492 reverse primer, the gene fragment was amplified using MJ Research Peltier thermal cycler. The PCR product was sequenced bidirectionally using the 785 forward and 907 reverse primers. The sequence data were aligned and analyzed to identify the bacterium and its closest neighbors [18,19].

Bacillus cereus colonies were initially maintained in nutrient agar medium and were selected and sub-cultured on a minimal agar medium for PHB production [20]. Sudan black B staining was used to screen the bacterial isolates for PHB production [21]. For quantitative screening, PHB was extracted from bacterial isolate by solvent extraction method [22] and the study was proceeded up to 4 days to quantify the production of PHB. The produced PHB was weighed and the percentage was determined by Hungund [23].

The PHB was extracted from the bacterial isolate *Bacillus cereus* by sodium hypochlorite method and was analyzed by FT IR spectroscopy. The FT IR spectrum of the PHB was obtained under the spectral range 400 – 4000cm^{-1}. ^{1}H NMR spectra were acquired by dissolving the primer in deuteron chloroform ($CDCl_3$) as a concentration of 32mg/ml and analyzed on AV300 spectrometer at 300K with 9.65 ms pulse width, 2-sec pulse repetition. H^1 NMR spectrum was recorded at 6172.8 Hz spectral width at 24^0c using Brucker spectrometer.

GC MS analysis of the sample was carried out after methanolysis of PHB [24]. For methanolysis of PHB, the polymer sample was suspended in 1 ml chloroform and 1 ml methanol containing 2.8 M H_2SO_4 in a screw capped tube and then incubated at 100^0c for 2 hours. After cooling, 0.5 ml demineralized water was added, and then the organic phase containing the resulting methyl esters of 4-hydroxy alkanoic acids was analyzed by using GC- MS QP 2010 MS spectrometer. The column used was VF-5 ms, 30 m x 0.250 mm dia with the film thickness of 0.25 µm, and the column oven was programmed between 70 and 300^0C at the rate of 10^0c/min with the injection temperature of 240^0C. Mass spectra were recorded in underscan mode in the range of 40 – 1000m/z. Compounds were identified using NIST 11 library.

3. RESULTS

The bacterium showing an appreciable amount of PHB production was isolated from the dairy industry effluent sample. The bacterial isolate was initially characterized using various microbiological and biochemical tests and was identified to be gram-positive *Bacillus* sp. Analysis of the 16S rRNA gene sequence of *Bacillus cereus* was performed using NCBI BLAST. The complete sequence was aligned to the homologous sequence available for *Bacillus cereus* was performed using NCBI – BLAST. The complete sequence was aligned to the homologous sequence available for *Bacillus* strains. The test organism sequence showed 100% similarity in the BLAST sequence of *Bacillus cereus*. The nucleotide sequence of bacterial isolate determined in this study has been deposited in the Gen Bank database under accession number MK920175.

3.1 Optimization of Parameters for PHB Production

The bacterial growth and PHB production by *Bacillus cereus* was followed simultaneously and the results are given in Fig. 2. The highest amount of PHB was observed at P^H 7 by the isolate after 48 hours of incubation. The amount of PHB was observed is found to be 14.63 ± 1.20 mg/L with a % of 64.03 ± 0.003.

The optimum temperature for PHB production was found to be 35^0c, the amount of PHB produced was 15.5 ± 1.95 mg/L with a percentage of 62.46 ± 4.52 (Fig. 3).

The production of PHB from different carbon sources (glucose, fructose, maltose, mannitol, sucrose, and succinate at 0.2 % to 2% were studied. *Bacillus cereus* exhibited a maximum PHB production in 2% sucrose supplementation medium and the optimization of nitrogen source for the isolate *Bacillus cereus* and the results were given in Figs. 4 and 5.

select all *100 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus cereus strain 01 16S ribosomal RNA gene, partial sequence	1770	1770	100%	0.0	100.00%	MK920175.1
Bacillus anthracis strain ABU-3 16S ribosomal RNA gene, partial sequence	1620	1620	96%	0.0	98.28%	KX951940.1
Bacillus aryabhattai strain ABU-2 16S ribosomal RNA gene, partial sequence	1620	1620	96%	0.0	98.28%	KX951938.1
Uncultured bacterium clone nck210d08c1 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	KF095543.1
Enterobacter cloacae strain TR20T2 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	KJ206932.1
Bacillus sp. D22 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	KF788129.1
Bacillaceae bacterium 11_St_14 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	JX064814.1
Uncultured bacterium clone b11_62 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	JN236281.1
Bacterium 2 amp 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	JN392009.1
Bacillus sp. USTB-O 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	HQ916661.1
Bacillus sp. cp-h21 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	EU558971.1
Bacillus cereus strain L8 16S ribosomal RNA gene, partial sequence	1615	1615	96%	0.0	98.17%	DQ486872.1
Bacillus cereus strain SIJB13 16S ribosomal RNA gene, partial sequence	1613	1613	96%	0.0	98.17%	MK775246.1
Bacillus sp. (in: Bacteria) strain PUP 27 16S ribosomal RNA gene, partial sequence	1613	1613	98%	0.0	97.77%	MH223457.1
Bacillus thuringiensis strain CH 05 80 16S ribosomal RNA gene, partial sequence	1613	1613	96%	0.0	98.07%	KY510936.1
Bacillus cereus strain LPB4 3 16S ribosomal RNA gene, partial sequence	1613	1613	98%	0.0	97.77%	JQ308569.1

Fig. 1. Sequences producing significant alignments with *Bacillus cereus*

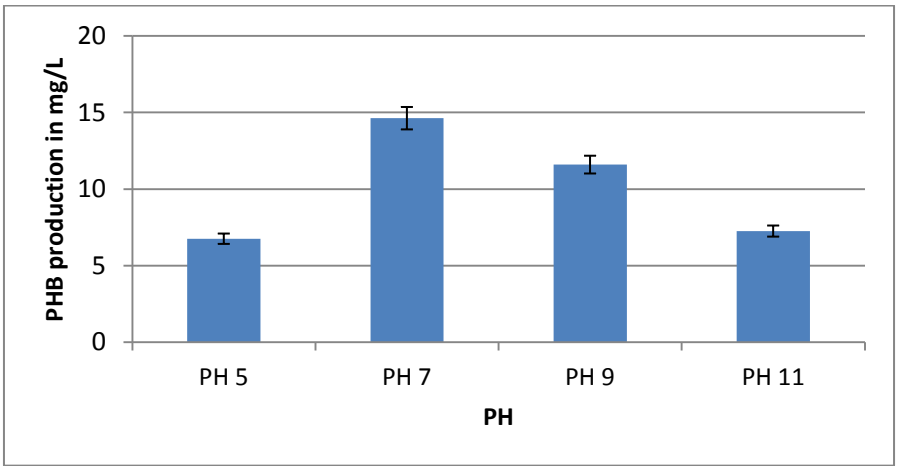

Fig. 2. Optimization of PH for PHB production

3.2 PHB Characterization

3.2.1 FTIR analysis of polymer

The FTIR spectra of PHB from isolate DIB1 were given in Fig. 6. The IR spectra of PHB from *Bacillus cereus* show peaks at 1725.5 cm^{-1} represent the ester functional of PHB and fatty acids. The absorption band at 2957.61 cm^{-1} represents CH_2 Stretch. The peak at 1636.81cm^{-1} resembles amide carbonyl stretch. The peak at 1565.96 cm^{-1} represents the NH bending.

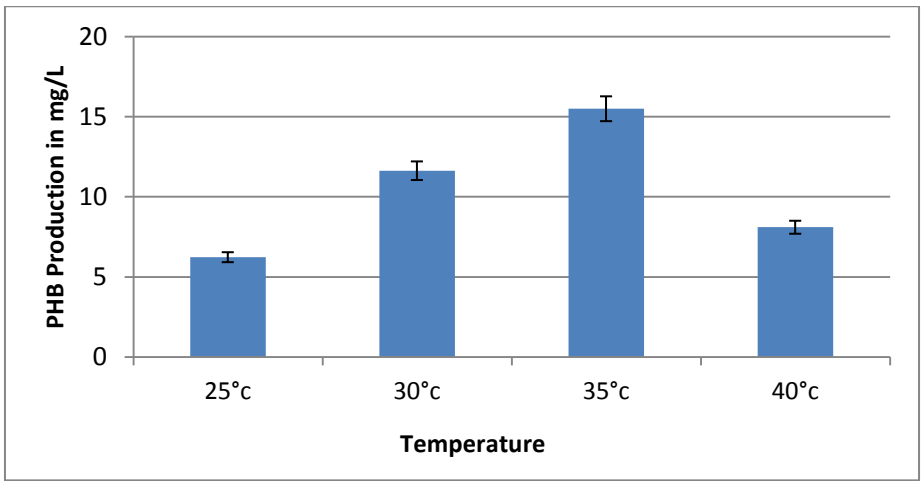

Fig. 3. Optimization of temperature for PHB production

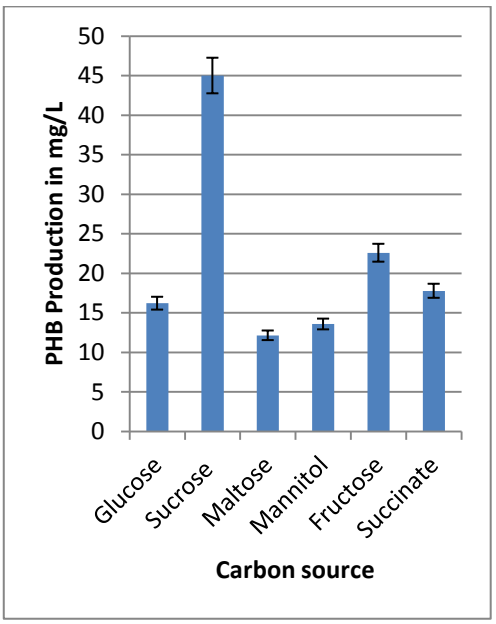

Fig. 4. Optimization of carbon source for PHB production

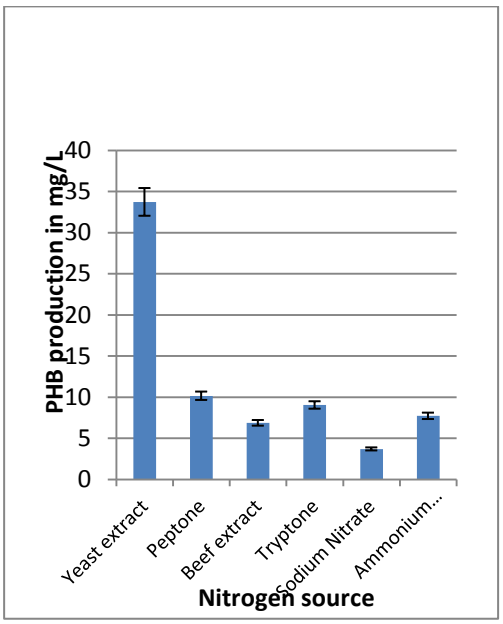

Fig. 5. Optimization of nitrogen source for PHB Production

3.2.2 NMR analysis of biopolymer

The NMR characterization of PHB from *Bacillus cereus* was presented in Fig. 7. The structures of polyesters were analyzed by ^{1}H-NMR. The structures of polyesters were analyzed by ^{1}H-NMR. The ^{1}H-NMR spectra of the PHB extracted from *Bacillus cereus* show the following resonance signals (Fig. 6) CH_2O COOH bond at 1.880ppm, a high signal at 0.8ppm belong to the hydrogen of methylene in the saturated lateral chain of the terminal CH_3 group.

3.2.3 GC MS analysis of polymer

The methanolic extracts of PHB from *Bacillus cereus* polymer were dried and analyzed by GC MS Perkin Elmer Clarus 500 make. Table 1 shows the result of GC MS analysis, where twelve different biodegradable compounds were found from chloroform extract.

The major among the analyzed compounds were n-hexadecanoic acid, octadecanoic acid, and benzene propionic acid. The n-hexadecanoic acid is an aliphatic polymer ester. This aliphatic biodegradable polyester family due to hydrolyzable ester bonds was reported by Dawes [25] and others [26].

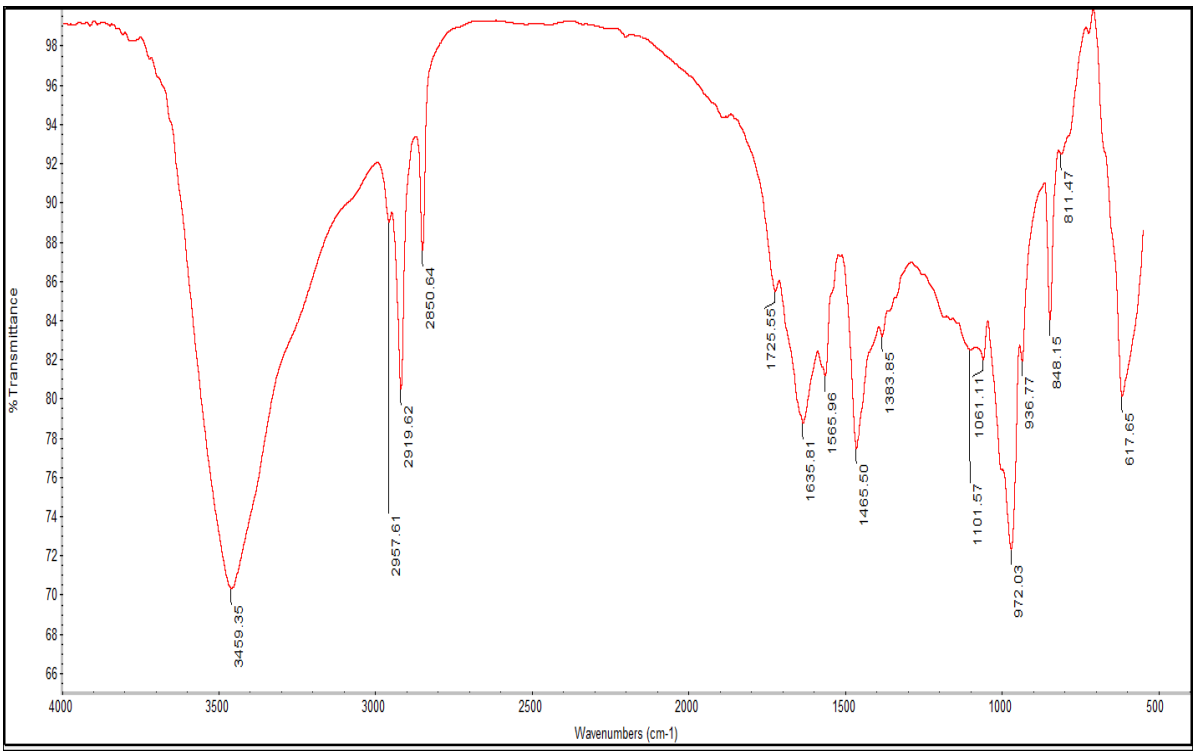

Fig. 6. FT – IR Analysis of polymer from *Bacillus cereus*

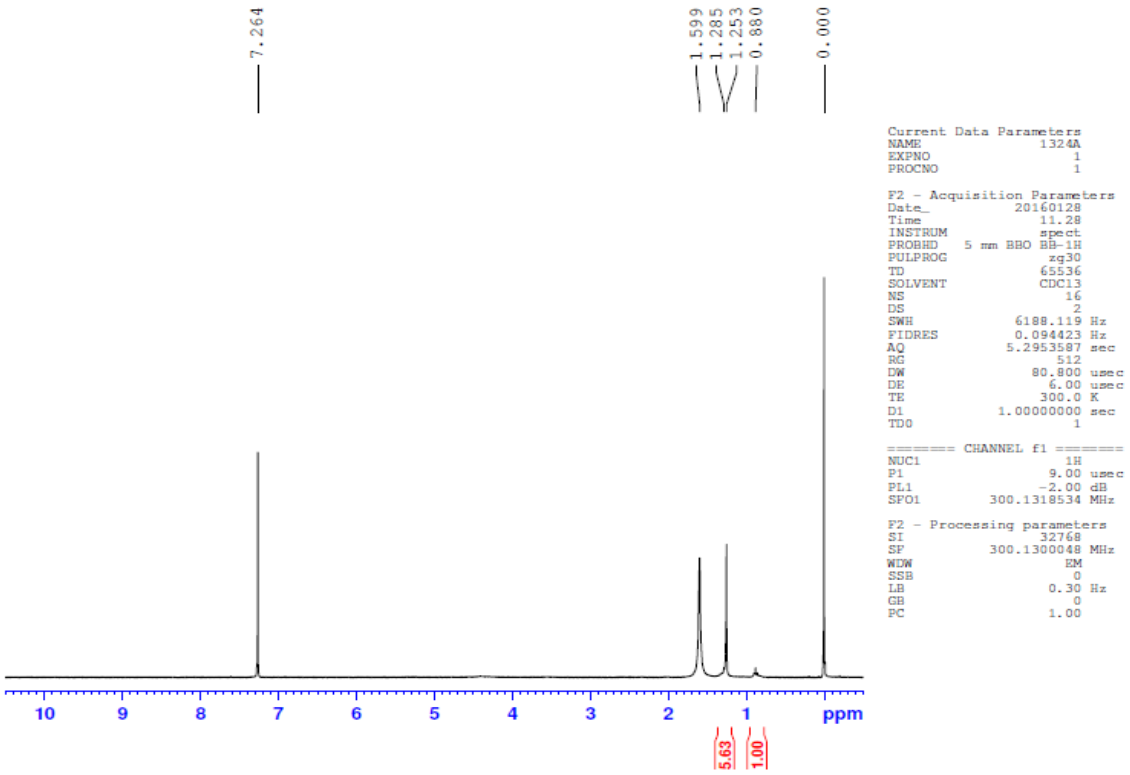

Fig. 7. NMR Analysis of polymer from *Bacillus cereus*

Table 1. Chemical Composition of the Biodegradable Polymer of *B.cereus* as revealed by GCMS analysis

S. No	Compound Name	Retention time	Molecular weight	Peak area	% of peak area
1	Octanol	6.33	128	243994	0.8822
2	2-Nananone	8.28	142	514842	1.8615
3	1-methyldodecylamine	10.93	199	180857	0.6539
4	Phenol-2,4-bis 1,1-dimethylethyl	19.00	206	1116896	4.0384
5	Tridecanoic acid methyl ester	24.24	228	1146749	4.1463
6	E9- Tetradecanoic acid	28.96	226	428696	1.5500
7	Hexadecanic acid methyl ester	29.50	270	8188739	29.60181
8	Benzenepropanoic acid 3,5- bis(1,1 – dimethyl – 4-hydroxy methyl ester	30.11	292	3051825	11.0345
9	n-Hexadecanoic acid	31.14	256	7514607	27.1706
10	15- octadecanoic acid methyl ester	33.74	296	661562	2.3920
11	Octadecanoic methyl ester	34.35	298	4469045	16.1588
12	E-2 Octadecadecen-1-ol	35.83	268	139284	0.5036

4. DISCUSSION

The dairy industry is one of the important agricultural industry, found all over the country but the product production varies from place to place [3]. Dairy industry effluent contains a large number of pollutants, which may be in organic or inorganic form [5,27]. In India, the dairy industry is one of the prime industries [28,29]. The dairy industry consumes a huge volume of water for their processing, hence it releases more amount of effluent, so more treatment methods are necessary [30]. A number of researchers have been extensively studied the biological treatment of dairy industry effluent [31]. Treatment of waster waters through microorganisms is one of the commonly used mechanisms, because of its low cost and easy maintenance [32]. So the bioremediation is one of the widely accepted methods for the treatment of effluent, because of its low cost, less manpower, and easy techniques [33].

People are taking an interest in searching for biodegradable plastic, an alternative to synthetic plastic. Due to the elastic and biodegradable nature bioplastic can be used for a variety of applications such as packaging material. The extensively studied biopolymer is the polyhydroxybutyrate (PHB), which acts as a green alternative to synthetic plastics. A lot of interest has been created for production and large scale cultivation of biopolymer producing microorganisms, but the major limitation was the production cost and time for PHB production by bacteria [34,35,36], Haas *et al.,* 2015. Low-cost and effective PHB production methods are studied by many researchers. The utilization of effluents and low-cost substrates such as corn starch, whey for biopolymer production are taking an interest.

5. CONCLUSION

This study concluded that PHB has been considered to be a good candidate for biodegradable material. 12 bacterial isolates were isolated from dairy industry effluent samples, best PHB producing strains, and identified by morphological and biochemical techniques using a taxonomic scheme of Bergey's Manual of Bacteriology. Optimization of physical and chemical parameters was the P^H 7, 35^{o}C, and 48 hrs incubation and the optimum inoculum size for PHB production was 30% DAIE, optimum carbon and nitrogen source was the 2.0% sucrose and 1% yeast extract. The *Bacillus cereus* regarding PHB production was confirmed by gene sequencing studies. FT-IR, NMR, and GC MS analysis of PHB from *Bacillus cereus* show peaks similar to standard PHB.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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