Research Article

Isolation and Screening of Polyhydroxyalkanoates Producing Bacteria from Pulp, Paper, and Cardboard Industry Wastes

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Background. Polyhydroxyalkanoates (PHAs) are storage materials that accumulate by various bacteria as energy and carbon reserve materials. They are biodegradable, environmentally friendly, and also biocompatible bioplastics. Unlike petrochemical-based plastics that take several decades to fully degrade, PHAs can be completely degraded within a year by variety of microorganisms into CO2 and water. In the present study, we aim to utilize pulp, paper, and cardboard industry sludge and wastewater for the isolation and screening of polyhydroxyalkanoates (PHAs) accumulating bacteria and production of cost-effective PHB using cardboard industry waste water.

Results. A total of 42 isolates showed black-blue coloration when stained with Sudan black B, a preliminary screening agent for lipophilic compounds, and a total of 15 isolates showed positive result with Nile blue A staining, a more specific dye for PHA granules. The isolates NAP11 and NAC1 showed maximum PHA production 79.27% and 77.63% with polymer concentration of 5.236 g/L and 4.042 g/L with cardboard industry wastewater. Both of the selected isolates, NAPII and NAC1, were classified up to genus level by studying their morphological and biochemical characteristics and were found to be Enterococcus sp., Brevundimonas sp., and respectively.

Conclusion. The isolates Enterococcus sp. NAP11 and Brevundimonas sp. NAC1 can be considered as good candidates for industrial production of PHB from cardboard industry waste water. We are reporting for the first time the use of cardboard industry waste water as a cultivation medium for the PHB production.

1. Introduction

Plastic materials that have been universally used in our daily lives are now causing serious environmental problems. Millions of tons of these nondegradable plastics accumulate in the environment per year. For efficient management of used-plastic materials, recycling is one solution. Another solution to reduce plastic residue is the use of biodegradable plastics [1, 2] and among them polyhydroxyalkanoic acids (PHAs) are drawing much attention. Polyhydroxyalkanoic acids (PHAs) are common intracellular compounds found in bacteria, archaea, and in few eukaryotes such as yeasts and fungi. PHAs are carbon and energy reserve polymers produced in some microorganisms when carbon source is in plentiful and other nutrients such as nitrogen, phosphorus, oxygen or sulfur are limited. PHAs are found to accumulate in varieties of microorganisms as reserve food material for example, Alcaligenes latus, Ralstonia eutropha, Azotobacter beijerinckii, Bacillus megaterium, and Pseudomonas oleovorans, including some fungi and archaea. Among the members of PHA family, polyhydroxybutyrates (PHB) is the most common biodegradable polymer and promising alternative to synthetic nondegradable plastics. These polymers are accumulated intracellular membrane enclosed inclusion up to 90% of the cell dry weight under conditions of nutrient stress and act as energy reserve material. It has similar mechanical properties as those of the oil-derived conventional plastics like polypropylene or polyethylene which can be molded, made into films, spun into monofilaments, and used to make heteropolymers with other synthetic polymers and many more applications in agriculture, packaging, and medical field being biodegradable and also immunologically compatible
with human tissue [3]. Recently, another application of PHAs is reported as biofuel [4].

In spite of these interesting properties, industrial production of PHAs is still not well established. In the 1950s, North-American Company W.R. Grace Co. made the first attempt to produce PHB at commercial level. However, this process was not successful due to low production efficiency and a lack of suitable purification method. Then, in the 1970s, Imperial Chemical Industries (ICI, UK) started producing PHAs by using a mutant stain Cupriavidus necator, NCIB 11599 from various carbon sources such as 1,4-butanediol, 1,6-hexanediol, and butyrolactone. The commercial product was recognized as Biopol. The patents were later sold to Zeneca, then to Monsanto and are currently the property of Metabolix, Inc. (USA). Commercially, some other biopolyester products with different monomer composition are also produced with trade names such as poly(3HB-co-3HV) Nodax, poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) poly(3HB-co-3HA) as Biogreen, and poly(3HB) as Biomer [5]. But the large scale production was halted at commercial level due to the high production cost as compared with the oil-derived plastics [1, 6]. With the aim of commercializing PHA, great efforts have been employed to reduce the production cost by the development of bacterial strains and more efficient fermentation/recovery process [7, 8]. From the literature, it has been found that cost in this biopolymer production is the cost of the substrate [9, 10] which accounts for more than 50% of production cost [5, II] and makes the difference in price of poly-3-hydroxybutyrate (P3HB) from Biomer about 12 times the cost of polypropylene [12]. To solve this problem, inexpensive substrate, renewable substrates, waste material, and waste water are used as nutrient source for microorganisms for PHA production. Various types of waste products have been used for PHB production because it provides dual benefits of utilizing the waste and cost-effective production of biodegradable microbial bioplastic.

Cardboard industry is one of the parts of pulp, paper, and packaging industry. Cardboard industry includes two main industries corrugated cardboard industry and noncorrugated cardboard or paper-board industry. Waste water from chemical and mechanical pulping contains 12–25 kg of BOD/t of ADP, but the BOD discharges are 3 to 10 times higher in chemimechanical pulping as compared to mechanical pulping. Nitrogen and phosphorus are also present in waste waters and released from paperboard by the pulping process of raw material such as wood, agricultural waste, and paper waste. Waste waters released from pulp and paperboard mills are typically rich in carbohydrates but poor in fixed nitrogen. If we consider the scenario of India, pulp and paperboard industry around 905.8 million m$^3$ of water is consumed and around 695.7 million m$^3$ of waste water is discharged annually. The largest part of the fresh water is used in sheet formation on the cardboard machine (200 m$^3$ h$^{-1}$) and the smallest quantity is used in stock preparation (90 m$^3$ h$^{-1}$ for thickening). Furthermore, the treatment of waste stream to purified effluent needs much effort and is very difficult, because the waste stream often contains various organic compounds. So instead of costly treatment, we can exploit the waste water directly for cultivation of PHB accumulating microorganisms. In this study, polyhydroxyalkanoic acids (PHAs) accumulating bacterial strains were isolated and screened using cardboard industry waste water as a sole carbon source with dual benefit of utilizing the waste and cost-effective production of biodegradable microbial bioplastic.

2. Material and Methods

2.1. Isolation of Polyhydroxyalkanoic Acids (PHAs) Producing Bacteria. For the isolation of PHA producing bacteria activated sludge and waste water were collected from pulp, kraft, and cardboard manufacturing industry from Khanna pulp and paper mills at Amrisar and Topara Kurdh, Yamuna Nagar, India, respectively. The samples were stored at room temperature until analysis. In 99 mL sterilized water, 1 gm of sludge sample was dissolved. Then, the sample was serially diluted in sterile distilled water and followed by plating on the nutrient agar medium with 1% glucose. For isolation from waste water sample, 1 mL of water sample was added in 99 mL sterilized water. After serial dilution (10$^{-3}$ to 10$^{-7}$), 1 mL of each dilution was spread on carbon rich nutrient agar plate. For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted and plates were rinsed gently by adding 100% ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black [13].

2.2. Screening for PHA Producing Bacteria. Sudan black B positive isolates were checked for PHA production by Nile blue A staining, a more specific stain for Polyhydroxyalkanoic acids (PHAs) by a more rapid and sensitive, viable colony method [14]. This dye at concentrations of only 0.5 μg/mL was directly included in carbon rich nutrient agar medium (glucose 1%, beef extract 0.3%, peptone 0.5%, sodium chloride 0.8%, and agar 1.5%) and growth of the cells occurred in the presence of the dye. This allowed an estimation of the presence of PHAs in viable colonies at any time during the growth experiment and a powerful discrimination between PHA-negative and PHA-positive strains. The PHA accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with UV light and their fluorescence intensity increased with the increase in PHA content of the bacterial cells. The isolates which showed bright orange fluorescence on irradiation with UV light after Nile blue A staining were selected as PHA accumulators.

2.3. Pretreatment of Cardboard Industry Waste Water. Untreated cardboard industry effluent was collected from the cardboard industry, Topara Kurdh, Yamuna Nagar, Haryana, India, and stored at 4°C until used for analysis. The effluent was first filtered through the muslin cloth and then by rough filter paper to remove the undesired suspended solid materials from waste water. After this pretreatment step,
cardboard industry waste water was used as quantification and production medium for PHA production by selected bacteria.

2.4. Extraction and Quantitative Analysis of PHA. The PHB production was observed in 250 mL Erlenmeyer flask containing 50 mL of treated cardboard industry waste water, as production medium under stationary conditions of growth. After 72 h of incubation at 37°C, culture broth was centrifuged at 8000 rpm for 15 min. The pellet along with 10 mL sodium hypochlorite was incubated at 50°C for 1 h for lyses of cells. The cell extract obtained was centrifuged at 12000 rpm for 30 min and then washed sequentially with distilled water, acetone, and absolute ethanol. After washing, the pellet was dissolved in 10 mL chloroform (AR grade) and incubated overnight at 50°C and was evaporated at room temperature [15]. After evaporation, 10 mL of sulphuric acid was added to it and placed in water bath for 10 min at 100°C. This converts polyhydroxalkanoic acids (PHAs) into crotonic acid, which gives maximum absorbance at 235 nm [16, 17]. PHB (Sigma Aldrich) was used as standard for making standard curve. For quantitative analysis of PHA, cell culture was grown as described earlier and cell pellet was dried to estimate the dry cell weight (DCW) in units of g/L [18]. Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA [19]. This was calculated to determine the cellular weight and accumulation other than PHAs. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight:

\[
\text{Residual biomass (g/L)} = \text{DCW (g/L)} - \text{Dry weight of extracted PHA (g/L)},
\]

\[
\text{PHA accumulation (%) = Dry weight of extracted PHA (g/L)} \times 100/\text{DCW (g/L)}. \tag{1}
\]

2.5. Morphological Characterization and Biochemical Identification of PHA Producing Bacteria. Microscope Stereo Olympus was used to observe the morphology of bacterial colonies grown on nutrient agar. The growth characteristics such as structure, shape, color, margin, surface characteristics, surface upwards, smell, elevation, opacity, end of cells, cell's arrangement, and Gram-staining of the bacterial colonies were observed to characterize the bacterial colonies. Various biochemical tests were performed in selected PHB producing bacteria, namely, indole production test, methyl red and Voges-Proskauer, citrate utilization test, and H2S production for their biochemical characterization. The fermentative utilization of various carbohydrates was also followed for 48 hrs at 37°C by inoculating the isolates separately in the defined medium to which various sugars like xylose, mannose, maltose, sucrose, raffinose, dextrose, trehalose, fructose, glucose, ribose, lactose, rhamnose, esculin, inulin, mannitol, arabinose, sorbitol, and melibiose were added.

2.6. Polymer Analysis

2.6.1. 1H-NMR Spectroscopy and Thermal Gravimetric Analysis (TGA). The identity of individual monomer unit was confirmed by proton nuclear magnetic resonance (1H-NMR) spectroscopy. 1H-NMR spectra were acquired by dissolving the polymer in deuterochloroform (CDCl3) at a concentration of 10 mg/mL and analyzed on a Bruker Avance II 500 spectrometer at 22°C with 7.4 ms pulse width (30° pulse angle), 1 s pulse repetition, 10,330 Hz spectral width, and 65,536 data points. Tetramethysilane was used as an internal shift standard. Thermal gravimetric analysis (TGA) was performed using a TGA instrument (Mettler-Toledo, TGA/SDTA 851, USA) calibrated with indium. The temperature was ramped at a heating rate of 10°C/min under nitrogen to a temperature (700°C) well above the degradation temperature of the polymers.

2.7. FT-IR Analysis. FT-IR analysis of the polymer sample was carried out on MB-3000, ABB FTIR spectrophotometer in the range 4000–600 cm−1.

2.8. GC-MS Analysis. Purified polymer, prepared as described before, was dissolved in 2 mL of chloroform and then 2 mL of methanol was added and acidified with 3% (v/v) H2SO4 and heated at 100°C for 3.5 h for depolymerization and methanolysis of polyesters and 3 μL was injected into GCMS-QP 2010 Plus model. The samples were injected in the splitless mode and the injection temperature was 260°C and column oven temperature was 100°C.

3. Result and Discussion

3.1. Isolation and Selection of PHA Producing Bacteria. A wide variety of bacteria are known to accumulate PHA. Today, approximately 150 different hydroxyalkanoic acids are known to be incorporated into polyhydroxyalkanoates [20], with microbial species from over 90 genera being reported to accumulate these polyesters [21]. These bacteria have been reported from various environments, but only a few from the waste water and sludge ecosystems. For the rapid detection and isolation of PHB producing bacteria, 0.02% alcoholic solution of Sudan black B and Nile blue A staining viable colony method [14] was used. The isolation of PHA producing bacteria was done from cardboard manufacturing industry waste water and pulp cardboard and kraft industry sludge. A large proportion about 35% of isolated bacteria produced PHA as energy reserve material. A total of 42 isolates showed black-blue coloration when stained with Sudan black B, a preliminary screening agent for lipophilic compounds, and a total of 15 isolates showed positive result with Nile blue A staining (Figure 1), a specific dye for the of PHA granules. Both gram-positive and gram-negative bacteria showed PHA production, but gram-positive bacteria dominated the waste material microflora of pulp, kraft, and cardboard manufacturing industry. Teeka et al. [22] used this method to screen the potential PHA producing bacteria from soil, and Ramachandran and Abdullah [23] also observed

The PHA-positive isolates selected after Nile blue A staining were grown in 50 mL cardboard industry waste water in Erlenmeyer flasks and were employed to extract PHA after 2 days of incubation under stationary conditions of growth. The PHA from the isolates was extracted by the hypochlorite and chloroform method [15] as described earlier. The isolates NAPII and NACI showed maximum PHA production 79.27% and 77.63% (Table 1) with cardboard industry waste water and were selected for further biochemical identification and chemical characterization.

Organic matter from wastes and waste waters has high BOD and COD values, and hence microorganisms can grow, utilizing the nutrient present in waste water, and can convert them into valuable compounds and polymers. Based on this idea, many researchers reported the PHA production from various industrial waste materials. PHA production by A. vinelandii from swine waste liquor was studied by [25]. The raw liquor supported the production of only 0.43 g/L PHA, at a polymer content of 37% w/w, whereas twofold dilution and supplementation with 30 g glucose/L allowed a PHA concentration of 5.5 g/L at a 58.3% w/w polymer content. Few researchers have proposed coupling PHA production to biological waste water treatment [26–28]. Ceyhan and Ozdemir [29] reported polyhydroxybutyrate (PHB) production from domestic waste water using Enterobacter aerogenes 12Bi strain with good yield ranging from 16.66 to 96.25% (w/w). The use of pure C. necator cultures to produce PHAs from waste waters has been explored by Ganzeveld et al. [30]. They used a supernatant, obtained by centrifuging fermented organic waste, as the sole carbon source for the production of P(3HBco-3HV), and obtained a maximum polymer concentration of 1.13 g/L at a polymer content of 40.8% in 45 h. Cardboard industry waste water is typically rich in carbohydrates but poor in fixed nitrogen, due to the high C/N ratio. This high carbon-nitrogen ratio favors the growth of PHA producing bacteria. It is the first time that cardboard industry waste water is used for the isolation, screening, and production of polyhydroxyalkanoates. This waste has high BOD and COD values 680–1250 mg/L and 3400–5780 mg/L and COD/BOD ratio between 3.9 and 5 [31], which is suitable for microbial growth.

Extracted PHA of selected isolates was quantified and its efficiencies were compared with the standard. Standard pure culture of Ralstonia eutrophus MTCC no. 1473 was used for PHA production with cardboard waste water producing a polymer concentration of 2.974 g/L and PHB content up to 41.30% with cardboard industry waste water. The selected isolates NAPII from pulp sludge have produced 79.27% w/w PHA with polymer concentration of 5.236 g/L using cardboard waste water which are 37% higher as compared to standard stain of Ralstonia eutrophus (Figure 2). The other NACI isolates showed PHA production up to 77.63% with polymer concentration of 4.042 g/L under stationary conditions of growth.

3.3. Morphological and Biochemical Characterization of Selected Isolates. By using Bergey's Manual of Determinative Bacteriology [32] and by ABIS Online-Advanced Bacterial Identification Software, both isolates were classified up to genus level using the morphological and biochemical characteristics (Table 2). NAPII and NACI were found to be
### Table 1: List of PHA accumulating bacteria with source of isolation.

<table>
<thead>
<tr>
<th>Name of isolate</th>
<th>Source of isolate</th>
<th>Gram reaction</th>
<th>PHA concentration (g/L)</th>
<th>PHA content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAP11</td>
<td>Pulp industry sludge</td>
<td>+ve</td>
<td>5.236</td>
<td>79.27 ± 0.3</td>
</tr>
<tr>
<td>NAP4</td>
<td>Pulp industry sludge</td>
<td>+ve</td>
<td>3.682</td>
<td>65.75 ± 0.2</td>
</tr>
<tr>
<td>NAW2</td>
<td>Waste water from cardboard manufacturing industry</td>
<td>−ve</td>
<td>4.012</td>
<td>76.26 ± 0.26</td>
</tr>
<tr>
<td>NAW24</td>
<td>Waste water from cardboard manufacturing industry</td>
<td>+ve</td>
<td>4.018</td>
<td>62.90 ± 0.11</td>
</tr>
<tr>
<td>NAW19</td>
<td>Waste water from cardboard manufacturing industry</td>
<td>+ve</td>
<td>3.824</td>
<td>59.32 ± 0.34</td>
</tr>
<tr>
<td>NAW23</td>
<td>Waste water from cardboard manufacturing industry</td>
<td>+ve</td>
<td>3.63</td>
<td>58.54 ± 0.27</td>
</tr>
<tr>
<td>NAW27</td>
<td>Waste water from cardboard manufacturing industry</td>
<td>−ve</td>
<td>2.966</td>
<td>51.13 ± 0.16</td>
</tr>
<tr>
<td>NAW34</td>
<td>Waste water from cardboard manufacturing industry</td>
<td>+ve</td>
<td>1.61</td>
<td>40.25 ± 0.18</td>
</tr>
<tr>
<td>NAC1</td>
<td>Waste sludge from cardboard manufacturing industry</td>
<td>−ve</td>
<td>4.042</td>
<td>77.63 ± 0.3</td>
</tr>
<tr>
<td>NAC24</td>
<td>Waste sludge from cardboard manufacturing industry</td>
<td>+ve</td>
<td>4.006</td>
<td>65.75 ± 0.12</td>
</tr>
<tr>
<td>NAC10</td>
<td>Waste sludge from cardboard manufacturing industry</td>
<td>+ve</td>
<td>3.97</td>
<td>64.80 ± 0.16</td>
</tr>
<tr>
<td>NAC9</td>
<td>Waste sludge from cardboard manufacturing industry</td>
<td>+ve</td>
<td>3.802</td>
<td>70.92 ± 0.13</td>
</tr>
<tr>
<td>NAC12</td>
<td>Waste sludge from cardboard manufacturing industry</td>
<td>+ve</td>
<td>3.682</td>
<td>68.28 ± 0.04</td>
</tr>
<tr>
<td>NAK8</td>
<td>Kraft industry sludge</td>
<td>+ve</td>
<td>3.83</td>
<td>71.53 ± 0.05</td>
</tr>
<tr>
<td>NAK31</td>
<td>Kraft industry sludge</td>
<td>+ve</td>
<td>3.366</td>
<td>62.33 ± 0.13</td>
</tr>
<tr>
<td>NAK17</td>
<td>Kraft industry sludge</td>
<td>+ve</td>
<td>3.184</td>
<td>63.68 ± 0.18</td>
</tr>
<tr>
<td>Ralstonia eutropha</td>
<td>MTCC no. 1473</td>
<td>−ve</td>
<td>2.974</td>
<td>41.86 ± 0.1</td>
</tr>
</tbody>
</table>

**Figure 2:** Comparison of PHB production from selected isolates with Ralstonia eutropha.

**Enterococcus sp., and Brevundimonas sp., respectively.** Other researchers also reported these genuses from the waste effluents. Silva et al. [33] studied the ecology of *Enterococci* and related bacteria in raw and treated waste water from a treatment plant receiving domestic and pretreated industrial effluents. The predominant species found in the raw waste water were *Enterococcus hirae, Enterococcus faecium, and Enterococcus faecalis*. Jiang et al. [34] isolated 3,851 in total *Enterococci* isolates from eight individual source categories including feces from animals and birds, soil, and sewage water samples to establish antibiotic resistance analysis (ARA). Reddy and Mohan [35] also reported the *Enterococcus italicus* sp. in mixed consortia in waste water treatment and produced PHA up to 71.4%. During their study of influence of substrate load and nutrient concentration (nitrogen and phosphorous) on PHA production using waste water as substrate and mixed culture as biocatalyst, they found that PHA accumulation was high at higher substrate load (40.3% of dry cell weight (DCW)), low nitrogen (45.1% DCW), and low phosphorous (54.2% DCW) conditions by mixed consortia containing in *Enterococcus sp.* this paper confirms that Rani et al. [36] reported *Brevundimonas* with other bacteria as the dominant cultured bacteria in microbial diversity in functional pesticide effluent treatment plants (ETPs). *Brevundimonas aveniformis sp*. A stalked species, was isolated from activated sludge by Ryu et al. [37]. *Brevundimonas sp. MIFC* and *Brevundimonas diminuta* was isolated from refinery active sludge and olive mill waste water, respectively, [38] and a *Brevundimonas sp.* were isolated from tannery waste treatment plant [39]. PHA production also reported up to 64% from the acid hydrolyzed saw dust (hydrolyzed wood) by *Brevundimonas vesicularis*. They also optimized the C:N ratio for PHA production in *Brevundimonas vesicularis sp.* and found that C:N proportion of 100 : 3.5 yielded maximum PHA up to 64% of cell dry weight. Thus, they concluded that acid hydrolyzed saw dust can be used as substrate by *Brevundimonas vesicularis sp.* for PHA production [40].

3.4. Polymer Analysis by ¹H-NMR Spectroscopy. Based on the characterization of the PHA produced by NAP11 and NAC1 through NMR comparison with the standard PHB (Sigma), it was observed that the PHA obtained from NAP11 and NAC1 is having properties similar to that of the standard PHB (Sigma) (Figure 3(a)), so the PHA produced by both bacteria is polyhydroxybutyrate (PHB). The structures of polyesters

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were investigated by $^1$H NMR. The $^1$H NMR spectra of the PHAs extracted from Enterococcus sp. NAPII show the following resonance signals: HC=CH bond at 5.25 ppm, CH$_2$O–COOH bond at 2.580 ppm, a high signal at 1.26 ppm that belongs to the hydrogen of methylene in the saturated lateral chain, and a terminal –CH$_3$ group at 0.8 ppm; the $^1$H NMR spectra (Figure 3(b)) of the PHAs extracted from Brevundimonas sp. NAC1 (Figure 3(c)) show the following resonance signals: HC=CH bond at 5.30 ppm, CH$_2$O–COOH bond at 2.574 ppm, a high signal at 1.30 ppm that belongs to the hydrogen of methylene in the saturated lateral chain, and a terminal –CH$_3$ group at 0.857 ppm [15]. The $^1$H NMR spectra of the samples and the standard are almost identical, conferring that extracted intracellular compounds are polyhydroxybutyrates (PHBs).

3.5. Fourier Transform Infrared Spectroscopy (FTIR). Polymer extracted from NAPII and NAC1 was used for recording IR spectra in the range 4000–600 cm$^{-1}$. IR spectra (Figure 4) showed two intense absorption bands at 1720 and 1281 cm$^{-1}$ of NAPII and at 1720 and 1273 of NAC1 specific for C=O and C–O stretching vibrations, respectively. The absorption bands at 2932 and 2954 cm$^{-1}$ are due to C–H stretching vibrations of methyl, methylene groups. These prominent absorption bands confirm the structure of poly-$\beta$-hydroxybutyrate.

3.6. Thermogravimetric Analysis (TGA). TGA results of NAM5 showed that the $T_m$ is 171.33$^\circ$C and the enthalpy of PHA fusion is 85.56 J/g. The result showed similarity with the data obtained from standard PHB (176.29$^\circ$C and 86.49 J/g) [41] and from other studies from the literature also [42, 43].

3.7. GC-MS Analysis of Extracted PHA. In this study, the PHB was methanolyzed in the presence of sulphuric acid and methanol, and the methanolyzed 3HB was then analyzed...
Table 2: Morphological and biochemical characters of selected isolates.

<table>
<thead>
<tr>
<th>Morphological characters</th>
<th>NAPII</th>
<th>NAC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony color</td>
<td>White</td>
<td>Cream</td>
</tr>
<tr>
<td>Colony texture</td>
<td>Smooth</td>
<td>Smooth-elevated</td>
</tr>
<tr>
<td>Gram reaction</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Cocci-shaped</td>
<td>Rod-shaped</td>
</tr>
<tr>
<td>Cell arrangement</td>
<td>Chain</td>
<td>Chain</td>
</tr>
<tr>
<td>Spore formation</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate utilization test</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Indole test</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>V-P test</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>H₂S production test</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Amylase production test</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Dextrose</td>
<td>+ve</td>
<td>+ve</td>
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<td>Ramanose</td>
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by GC-MS. Figures 5(a) and 5(b) showed that a common molecular fragment of the 3HB methyl ester ion chromatogram of the PHB was produced. A predominant peak corresponding to the dimer 3HB methyl ester was noted at 13.63 to 13.667 min, respectively, in GC purified product from NAPII and NAC1, while 3 other major peaks were observed at 11.5, 12.2, and 12.3 min. The retention times and ion fragment patterns of the peaks at 11.6 and 12.2 min were identical to those of the dimer methyl esters of 3HV and 3HBV, respectively, but in very low percentage up to 5% in isolate NAPII and 20% and 11% in NAC1 isolate, respectively. From the data obtained by GC-MS, the molecular weight of PHB obtained from isolate NAPII is 256 kDa and from isolate NAC1 is 242 kDa.

4. Conclusion

In this study, inexpensive cardboard industry waste water was tried as a carbon source to produce PHA. Different bacterial strains were isolated from cardboard industry waste water and pulp, kraft, and cardboard manufacturing industry sludge and screened for polyhydroxyalkanoate production using cardboard manufacturing industry waste water as a carbon source. The bacterial isolates NAPII and NAC1 can be regarded as potential strains for the conversion of cardboard industry waste water into PHB. Both of the selected isolates utilized cardboard industry waste water as sole carbon source for growth and PHB biosynthesis, accumulating PHB up to 79.27% and 77.63% of the cell dry mass, respectively. As a conclusion, isolates NAPII and NAC1 can be considered as good candidates for industrial production of PHB from cardboard industry waste water. Based on the morphological and biochemical characterization, NAPII and NAC1 were identified up to genus level as Enterococcus sp. and Brevundimonas sp., respectively. Currently, these bacterial strains are further investigated to increase the productivity of PHB by the optimization of the process parameters and making the whole process more cost-effective.

Conflict of Interests

The authors declare that they have no competing interests.
**Authors’ Contribution**

All authors have made extensive contribution into the design, experiments and data analysis, paper preparation, and the review and finalization of this paper. All authors read and approved the final paper.

**Acknowledgments**

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**References**


A. Yezza, A. Halas, W. Levadoux, and J. Hawari, “Production of poly-β-hydroxybutyrate (PHB) by Alcaligenes latus from maple

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Poly-β-hydroxybutyrate production and management of cardboard industry effluent by new Bacillus sp. NA10

Anish Kumari Bhuwal1, Gulab Singh1, Neeraj Kumar Aggarwal1, Varsha Goyal1 and Anita Yadav2

Abstract

Background: In the present study, we aim to utilize the ecological diversity of soil for the isolation and screening for poly-β-hydroxybutyrate (PHB)-accumulating bacteria and production of cost-effective bioplastic using cardboard industry effluent.

Results: A total of 120 isolates were isolated from different soil samples and a total of 62 isolates showed positive results with Nile blue A staining, a specific dye for PHB granules and 27 isolates produced PHB using cardboard industry effluent. The selected isolate NA10 was identified as Bacillus sp. NA10 by studying its morphological, biochemical, and molecular characteristics. The growth pattern for the microorganism was studied by logistic model and exactly fitted in the model. A maximum cell dry weight (CDW) of 7.8 g l⁻¹ with a PHB concentration of 5.202 g l⁻¹ was obtained when batch cultivation was conducted at 37°C for 72 h, and the PHB content was up to 66.6% and productivity was 0.072 g l⁻¹ h⁻¹ in 2.0 L fermentor. Chemical characterization of the extracted PHB was done by H1NMR, Fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA), Gas chromatography–mass spectrometry (GC-MS) analysis to determine the structure, melting point, and molecular mass of the purified PHB. The polymer sheet of extracted polymer was prepared by blending the polymer with starch for packaging applications.

Conclusions: The isolate NA10 can be a good candidate for industrial production of PHB from cardboard industry waste water cost-effectively and ecofriendly.

Keywords: Polyhydroxybutyrate (PHB); Bioplastic; Cardboard industry waste water; Bacillus sp.

Background

Today, plastics have become a necessary part of contemporary life due to their durability and resistance to degradation. Worldwide production of petroleum-based synthetic polymer was approximately 270.0 million tons in 2007 [1], and these synthetic polymers are found to be recalcitrant to microbial degradation [2]. Problems related to solid waste management of these petrochemical-derived plastics pose a serious threat to global environment. Therefore, current concern about the environmental fate of polymeric materials have created much interest in the development of biodegradable plastic (bioplastic), such as starch derivatives, polylactic acid, cellulose polymers and polyhydroxyalkanoates, which plays an important role. In addition to being biodegradable, they have further advantage of being produced from renewable resources [3]. Among the various biodegradable polymer materials, polyhydroxyalkanoates (PHAs) provide a good fully degradable alternative to petrochemical plastics [4,5]. Polyhydroxybutyrate (PHB) was the first PHA to be discovered and is also the most widely studied and best characterized PHA. It is accumulated as a membrane enclosed inclusion in many bacteria at up to 80% of the dry cell weight and nearly 90% in recombinant E. coli [6]. In addition to the easy biodegradability and biocompatibility, it has mechanical properties that are very similar to conventional plastics like polypropylene or polyethylene and have many domestic and commercial applications such as food packing films, biodegradable carriers for medicines and insecticides, disposable cosmetic products, absorbable surgical devices and being immunologically compatible.
with human tissue can form microspheres and microcapsules [7]. Recently, PHA has been found useful as a new type of biofuel [8]. Besides all these properties and applications, wider use of PHAs is prevented mainly due to their high production cost compared with the oil-derived plastics [9]. High production cost of PHB production is mainly devoted to the expensive carbon substrates and tedious production procedures [10]. Due to the large impact of the high price of carbon sources on production costs, one of the most important approaches to reduce costs is to use wastes and by-products as raw material for the fermentation process. Novel technologies have been developed to produce PHAs from organic matters in wastewater [12-14], industrial wastes [15,16], municipal waste [17], food wastes [18], and activated sludge of paper and pulp mills [19]. Hence, replacement of non-biodegradable with biodegradable plastic from organic waste can provide multiple benefits to the environment and contribute to sustainable development [20]. Therefore, organic waste from cardboard industry waste water could be a good approach for cost effective production PHA.

**Methods**

**Isolation of PHA-producing bacteria**

For the isolation of PHA-producing bacteria, various soil samples were collected from different ecological niches. The samples were stored at room temperature until analysis. In 99 mL sterilized water, 1 g of soil sample was mixed. Then, the sample was serially diluted in sterile distilled water and followed by plating on the carbon-rich nutrient agar medium (beef extract 0.3%, peptone 0.5%, sodium chloride 0.5%, glucose 1%, and agar 2%). For the rapid detection and isolation of PHB-producing bacteria, 0.02% alcoholic solution of Sudan black B was applied to stain bacterial colonies and the plates were kept undisturbed for 30 min. The excess dye was then decanted, and plates were rinsed gently by adding 100% ethanol. Colonies unable to incorporate the Sudan black B appeared white, while PHB producers appeared bluish black [21].

**Rapid screening for PHA-producing bacteria**

The Sudan black B-positive isolates were further screened by Nile blue A, a more specific stain for PHA by a more rapid and sensitive, viable colony method [22]. This dye at concentrations of only 0.5 μg/mL was directly included in carbon-rich nutrient agar medium, and growth of the cells occurred in the presence of the dye. The PHA-accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with UV light and their fluorescence intensity increased with the increase in PHA content of the bacterial cells. The isolates which showed bright orange fluorescence on irradiation with UV light after Nile blue A staining were selected as PHA accumulators. The selected PHA accumulators after Nile blue A staining were checked for growth and PHA production in both nutrient broth (beef extract 0.3%, peptone 0.5%, sodium chloride 0.5%) and cardboard industry effluent.

**Pretreatment of cardboard industry waste water**

Untreated cardboard industry effluent was collected from the cardboard industry, Yamunanagar (Haryana), India, and stored at 4°C until used for analysis. The effluent was first filtered through the muslin cloth and then through rough filter paper to remove the undesired suspended solid materials from waste water. After this pretreatment step, cardboard industry waste water was used as quantification and production medium for PHA production by selected bacterial isolate.

**Morphological and biochemical characterization-selected bacteria**

Microscope Stereo Olympus (America) was used to observe the morphology of bacterial colonies grown on nutrient agar. The growth characteristics such as structure, shape, color, margin, surface characteristics, elevation, cell's arrangement, and Gram staining of the bacterial colonies were observed to characterize the bacterial colonies. Various biochemical tests were performed in selected PHB-producing bacteria, namely, indole production test, methyl red and Voges-Proskauer, citrate utilization test, and H2S production for their biochemical characterization. The fermentative utilization of various carbohydrates (xylose, mannose, maltose, sucrose, raffinose, dextrose, trehalose, fructose, glucose, ribose, lactose, rhamnose, esculin, inulin, mannitol, arabinose, sorbitol, and melibiose) were also followed for 48 h at 37°C by inoculating the selected isolate separately in the defined medium to which various sugars were added.

**Molecular identification**

**Colony PCR (16S rRNA gene amplification)**

Colony polymerase chain reaction (Colony PCR) of the isolate was performed according to Gen Elute™ Bacterial genomic DNA kit (Sigma, St. Louis, MO, USA), and the colonies (approximately 1 mm in diameter) were picked up with a sterilized toothpick and directly transferred to the PCR tubes as DNA templates. The PCR amplification of 16S rRNA was done at 94°C for 4 min, 94°C for 20 s, 52°C for 30 s, 72°C for 2 min, and 72°C for 7 min with hold at 4°C. The universal primer 16sF-5′ AGA GTT TGA TCC TGG CTC AGA 3′ and 16sR-5′ ACG GCT ACC TTG TTA CGA CTG 3′ were used.

**Detection of PCR products**

PCR-amplified DNA fragments were observed by agarose gel electrophoresis in 1% ± agarose gels (FMC). Ten microliters of each amplified mixture and the molecular mass marker were subjected to agarose gel electrophoresis.
and ethidium bromide staining and tracked by 0.25% of bromophenol blue. The amplified DNA fragments were visualized by gel documentation box (Genie, Redmond, WA, USA).

**Sequencing and analysis of 16S rRNA gene**

After that, the PCR product was purified by GenElute™ gel extraction kit (Sigma) method and sequencing of PCR product was done by Sanger sequencing method at 96°C for 1 min, at 96°C for 15 s, at 52°C for 30 s, and at 60°C for 4 min for 25 cycles with a hold at 4°C. The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the gene sequence. Samples were run on an ABI Prism 3130x1 Genetic Analyzer (Applied Biosystems). The chromatogram was made by Chromas 2.4. software. The obtained sequence was subjected to search for closest possible species by BLAST tools and distance matrix tree tool available at National Centre for Biotechnology Information (NCBI). Phylogenetic tree was constructed by MEGA5.2 phylogenetic tree analysis software.

**Optimization of various growth parameters for PHB production in shake flask culture**

The optimization for maximum PHA production by selected isolate was carried in 250-ml Erlenmeyer flask using preprocessed cardboard industry waste water as production medium at 100 rpm. Several cultural parameters were evaluated to determine their effect on biomass and PHB production using cardboard industry waste water. The optimized value for each parameter was selected and kept constant for further experiments. Several cultural parameters like production media concentration (25%–100%) time of incubation (24–96 h), temperature of incubation (25°C–55°C), and effect of pH (5.5–8) were evaluated to determine their effect on biomass accumulation and PHA production.

**Scale up of poly-β-hydroxybutyrate production and growth kinetics**

The production of PHAs was carried out in a 2.0-L fermenter (Minifors CH 4103, Switzerland) with a working volume of 1.25 L using the optimized parameters and production medium. Initially, the production medium (preprocessed undiluted cardboard industry waste water) was added into the fermenter, the pH was adjusted to 7.0, and the medium was sterilized in situ. Dissolved oxygen was maintained at 80%–100% air saturation; at the start of the process, 1% (v/v) of overnight culture (at log phase) was inoculated aseptically and the impeller speed was maintained at 100 rpm and temperature at 37°C. The pH was maintained at 7.0 using 1 N NaOH and 1 N HCl. Antifoam (silicone oil) at a concentration of 1:10 (v/v) in water was added after 36th and 54th hour.

The growth curves (DCW vs. time) were prepared to determine the start and end point of exponential phase and doubling time (τd) were determined according to the method provided by Painter and Marr [24] and Levasseur et al. [25].

\[ \mu' = \frac{\ln w_{t2}/w_{t1}}{t_2 - t_1}; \ t_2 > t_1 \]

where \( w_{t2} \) and \( w_{t1} \) are the dry cell weight at the different time points (\( t_1 \) and \( t_2 \), respectively).

\[ T_d = \ln 2/\mu_m \]

where \( T_d \) is the doubling time and \( \mu_m \) is the maximum specific growth rate.

Cell dry weight (CDW) and PHB yield coefficient relative to cell dry weight (Yp/x, g/g, defined as gram PHB produced per gram dry cell mass produced) [26], PHB concentration (g/L, defined as g PHB measured in 1 L culture), PHB content (g/g, defined as the ratio of PHB concentration (g/L) to dry cell concentration (g/L)), and PHB productivity (g/L/h) [10,11] were measured and calculated per definition during the fermentation process.

All experiments were performed in triplicate to check the reproducibility. The results were analyzed statistically by determining standard deviations values and performing analysis of variance (ANOVA) test.

**Extraction and quantitative analysis of PHB**

For extraction and quantitative analysis of PHB, culture broth was centrifuged at 8,000 rpm for 15 min after 72 h of incubation at 37°C. The pellet dissolved in 10 mL sodium hypochlorite was incubated at 50°C for 1 h for lyses of cells. The cell extract obtained was centrifuged at 12,000 rpm for 30 min and then washed sequentially with distilled water, acetone, and absolute ethanol. After washing, the pellet was dissolved in 10 mL chloroform (AR grade) and incubated overnight at 50°C [23]. After evaporation at 50°C, 10 mL of sulfuric acid was added to it and placed in water bath for 10 min at 100°C. This converts PHB into crotonic acid, which gives absorbance maximum at 235 nm [24,25]. PHB (Sigma Aldrich) was used as standard for making standard curve.

**Polymer analysis**

\( ^1H\)-NMR spectroscopy and FTIR analysis

The identity of individual monomer unit was confirmed by proton nuclear magnetic resonance \((^1H\text{-NMR})\) spectroscopy. \( ^1H\)-NMR spectra were acquired by dissolving the polymer in deuterated chloroform (CDCl₃) at a concentration of 10 mg/ml and analyzed on a Bruker Avance
II 500 spectrometer (Madison, WI, USA) at 22°C with 7.4 ms pulse width (30° pulse angle), 1 s pulse repetition, 10,330 Hz spectral width, and 65,536 data points. Tetramethylsilane was used as an internal shift standard. FTIR analysis of the polymer sample was carried out on MB-3000, ABB Fourier transform infrared (FTIR) spectrophotometer in the range 4,000 to 600 cm⁻¹.

**TGA**

Thermal gravimetric analysis (TGA) was performed using a TGA instrument (Perkin Elmer, Diamond TG/DTA analyzer, USA) calibrated with indium. The temperature was ramped at a heating rate of 10°C/min in nitrogenous environment to a temperature (700°C) well above the degradation temperature of the polymers.

**GC-MS analysis**

For molecular analysis of purified polymer, a coupled Gas chromatography–mass spectrometry (GC-MS) was performed using a GC-MS-QP 2010 Plus model, with capillary Column-Rtx-5 MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The samples were injected (3 μL) in the splitless mode, and the injection temperature was 260°C and column oven temperature was 100°C. The mass spectra obtained were compared with the Nist-08 and Willey-08 mass spectral library.

**Results and discussion**

**Isolation and selection of PHA-producing bacteria**

A wide variety of bacteria are known to accumulate PHA granules intracellularly as an energy reserve material. Microbial species from over 90 genera have been reported to accumulate approximately 150 different hydroxyalkanoic acids as polyhydroxyalkanoate polyesters granules [27,28]. These bacteria have been reported from various environments. For the rapid detection and isolation of PHB-producing bacteria, 0.02% alcoholic solution of Sudan black B and Nile blue A, viable colony method [20] was used. The isolation of PHA-producing bacteria was done from various ecological niches. A total of 120 isolates showed black-blue coloration when stained with Sudan black B, a preliminary screening agent for lipophilic compounds, and a total of 62 isolates showed positive result with Nile blue A staining, a specific dye for the presence of PHA granules. Teeka et al. [29] used this method to screen the potential PHA-producing bacteria from soil, and Ramachandra and Abdullah [30] also observed the colonies formed on nutrient-rich medium under ultraviolet light (UV) to screen for the pink fluorescence which indicated the presence of PHA producers. Kitamura and Doi [31] first demonstrated this viable colony method on agar plates; they induced the isolates to accumulate PHA by culturing in E₂ medium, containing 2% (w/v) glucose before Nile blue A staining. The PHA-accumulating colonies, after Nile blue A staining, showed bright orange fluorescence on irradiation with UV light and their fluorescence intensity increased with increase in PHA content of the bacterial cells.

**Production of PHB in nutrient broth and cardboard industry waste water**

The PHA-positive isolates selected after Nile blue A staining were screened in nutrient broth and best 27 producers were grown in processed cardboard industry waste water in 250-mL Erlenmeyer flasks and were employed to extract PHB after 72 h of incubation under stationary conditions of growth. The PHB from the isolates was extracted by the hypochlorite and chloroform method [21] as described earlier. The isolate NA10 showed maximum PHB production in both nutrient broth and cardboard industry waste water (3.951 g/L) (Table 1) and were selected for further optimization of PHB production.

Organic matter from waste and wastewater have high biological oxygen demand (BOD) and carbon oxygen demand (COD) values and, hence, microorganisms can grow, utilizing the nutrient present in waste water and can convert them into valuable compounds and polymers. Based on this idea, many researchers reported the PHA production from various industrial waste materials. Many researchers have proposed coupling PHA production to biological wastewater treatment [32-34]. Rebah et al. [14] utilized two types of industrial wastewater (starch and slaughterhouse wastewater from a plant located around Quebec region) and a secondary sludge (Quebec municipal waste water treatment plant) for PHB production by cultivating fast-growing *Rhizobia* but the PHB production was very low up to 10%. In an another study, Mockos et al. [35] reported that selectively enriched pulp mill waste activated sludge can serve as an inoculum for PHA production from methanol-rich pulp mill effluents, and the enriched cultures accumulated nearly 14% PHA on a dry weight basis under nitrogen-limited conditions.

Cardboard industry waste water is typically rich in carbohydrates but poor in fixed nitrogen due to the high C/N ratio. This high carbon-nitrogen ratio favors the growth of PHA-producing bacteria. This waste have high BOD and COD values 680–1,250 mg/L and 3,400–5,780 mg/L and COD/BOD ratio between 3.9–5 [36], which is suitable for microbial growth.

**Biochemical and molecular characterization of selected isolate**

By using Bergey’s manual of determinative bacteriology [37] and by ABIS online, advanced bacterial identification software, the selected isolate was classified up to genus level using the morphological and biochemical characteristics (Tables 2 and 3) and was identified as *Bacillus sp.* For further characterization, almost complete 16S rRNA
gene sequences were determined. The obtained sequences were aligned and compared with the bacterial sequences available in the GenBank database. The phylogenetic analysis (Figure 1) was done using MEGA 5.2 software by neighbor-joining tree and distance matrix-based nucleotide sequence homology which revealed that isolate NA10 is Bacillus sp. Yilmaz et al. [38] isolated 29 strains of the genus Bacillus from different soil samples which were taken from grasslands of Ankara, Turkey, and the highest PHB production and productivity percentage was found in Bacillus brevis M6 (41.67% w/v).

Growth and production of PHA in nutrient broth and preprocessed cardboard industry waste water

As such, there is no report available from literature for PHB accumulation by microbial cells using cardboard industry waste water. The growth of Bacillus NA10 was recorded in different concentration of cardboard industry waste water (Figure 2), and the maximum growth was noted in undiluted (100% v/v) cardboard industry waste water after 72 h of incubation at 37°C. Dilution of waste water not only affected the microbial growth but also the PHB accumulation. This may be due to the fact that with increasing dilution of waste water, the carbon and other nutrient got diluted and was not able to support microbial growth [38]. By submerged fermentation, Bacillus sp. NA10 accumulated highest PHB (3.952 g/L) from undiluted processed cardboard industry waste water in unoptimized cultural conditions. Quagliano et al. [39] studied the effect of molasses concentration on PHB by increasing the molasses concentration from 1% to 5% (w/v), and a maximum of 54% PHA was obtained with 5% molasses concentration with highest Mw 640 kDa. Pozo et al.

<table>
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<th>Table 1 List of best PHA accumulating bacteria screened in nutrient broth and cardboard industry effluent</th>
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<td><strong>Isolate</strong></td>
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Growth pattern and PHB production in cardboard industry waste water

As such, there is no report available from literature for PHB accumulation by microbial cells using cardboard industry waste water. The growth of Bacillus NA10 was recorded in different concentration of cardboard industry waste water (Figure 2), and the maximum growth was noted in undiluted (100% v/v) cardboard industry waste water after 72 h of incubation at 37°C. Dilution of waste water not only affected the microbial growth but also the PHB accumulation. This may be due to the fact that with increasing dilution of waste water, the carbon and other nutrient got diluted and was not able to support microbial growth [38]. By submerged fermentation, Bacillus sp. NA10 accumulated highest PHB (3.952 g/L) from undiluted processed cardboard industry waste water in unoptimized cultural conditions. Quagliano et al. [39] studied the effect of molasses concentration on PHB by increasing the molasses concentration from 1% to 5% (w/v), and a maximum of 54% PHA was obtained with 5% molasses concentration with highest Mw 640 kDa. Pozo et al.

<table>
<thead>
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<th>Table 2 Morphological characteristics of selected isolate NA10</th>
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<td>Gram reaction</td>
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<th>Table 3 Biochemical characteristics and carbohydrate tests of selected isolate NA10</th>
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<td><strong>Biochemical/carbohydrate test</strong></td>
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<td>Citrate utilization test</td>
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<tr>
<td>Methyl red test</td>
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<tr>
<td>V-P Test</td>
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<tr>
<td>H2S production test</td>
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<td>Amylase production test</td>
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<td>Sucrose</td>
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<td>Dextrose</td>
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<td>Mannitol</td>
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<td>D-Arabinose</td>
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found that NH4 medium amended with 60% alpechin showed the maximum PHA production and concluded that alpechin was tolerated by *A. chroococcum* strain H23 at high concentrations and that it acts as a substrate to support the growth of the microorganisms.

**Optimization of growth parameters**

**PHB production versus incubation time**

The PHB production in cardboard industry waste water was followed for 120 h by submerged fermentation in 250 mL Erlenmeyer flask at an interval of 24 h. The production of PHB increased up to 72 h (4.456 g/L) and, thereafter, got reduced (3.26 g/L after 72 h) (Figure 3a). This reduction in PHB production after 72 h may be due to lack of micronutrients as well as increase in metabolites that might have negative effect on the PHB production. The observation was supported by Yüksekdağ et al. [41] *Alcaligenes eutrophus* [42] and *A. vinelandii* [43]. Klüttermann et al. [44] also reported that the highest PHB level in *Agrobacterium radiobacter* was achieved after 96 h. After this time, the PHB contents in fermentation broth decreased which might indicate that the bacteria used PHB as a nutrient source due to inadequate nitrogen and carbon sources in the medium.

**Effect of incubation temperature on PHB production**

The maximum PHB production of 4.274 g/L was recorded at 35°C after 72 h. The increase of temperature beyond 35°C has negative impact on PHB production (Figure 3b). This decrease in PHB production at high temperature is also supported by studies of Grothe et al. [10] and Yüksekdağ et al. [41]. The decrease in PHB production at high temperature could be due to low PHB polymerase enzyme activity [45]. Tamoğlan and Sidal [46] also reported that higher and lower temperatures than 30 C lead to decrease in cell biomass and PHB synthesis by *Bacillus subtilis* ATCC6633. This result coincides with that represented by Aslim et al. [47], Hamieh et al. [48], who reported that optimum incubation temperature for PHB production by *Bacillus subtilis*, *Bacillus pumilis* and *Bacillus thuringiensis* was 37 C.

**Effect of pH of the production medium on PHB production**

Typically, metabolic process was highly susceptible to even slight changes in pH. Therefore, proper control of pH was critical. The experimental results showed that highest PHB content (4.492 g L⁻¹) was obtained at pH 7.5 by isolate NA10 (Figure 3c). The current observation was in agreement with Sindhu et al. [49] who observed that the maximum PHB was produced (0.01 to 0.5 g/L) at
pH 7–7.5 by Bacillus sphaericus NII 0838 from crude glycerol. Flora et al. [50] revealed that the maximum PHB production (25%) by B. sphaericus was at pH range from 6.5–7.5, and the reduction of polymer accumulation at higher pH values is due to the effect on the degradative enzymes of polymer breakdown, so that PHB is utilized at a rate almost equal to the rate of its synthesis. Shivakumar [51] also reported that pH 6.8 to 8.0 was optimum for PHB production by A. eutrophus.

Scale up study and growth kinetics
Growth kinetics of the culture was studied in 2.0 L lab scale bioreactor. The profiles of PHB accumulation were analyzed in batch cultivation by growing on the cardboard industry waste water under optimized growth conditions obtained at flask level (Figure 4). The growth pattern for the microorganism was studied by logistic model and exactly fitted as presented in Figure 5. The bacteria NA10 was found to accumulate cell dry weight and specific
product yield after the initial logarithmic growth (24 h) and reached a maximum 7.8 g/l at a rate 0.028 h\(^{-1}\) in stationary growth phase at 72 h. Similarly specific product yield (Yp/x) increased by about 50% from initial exponential growth to stationary phase, suggesting that cells accumulated PHB while growing and aging; hence, any strategy that prolongs the stationary phase is likely to further enhance the PHB yield per unit biomass. A maximum CDW of 7.8 g l\(^{-1}\) with a PHB concentration of 5.202 g l\(^{-1}\) was obtained when batch cultivation was conducted at 37°C after 72 h, and the PHB content was up to 66% and productivity was 0.072 g l\(^{-1}\) h\(^{-1}\). Tanamool et al. \[52\] reported that maximum 0.920 g/l of cell dry mass and 0.034 g/l PHB using sweet sorghum juice by \textit{Ralstonia eutropha} with yield and productivity of 0.037 g PHB/g dry cell and 0.0019 g/(l h), respectively. Similarly, El-Sayed et al. \[53\] found specific growth rate of 0.055 h\(^{-1}\) on sucrose media while a specific growth rate of 0.053 h\(^{-1}\) was obtained with \textit{R. eutropha} on glucose medium.

**Polymer analysis**

\textbf{\(^1^H\)-NMR spectroscopy}

The structures of polyesters were investigated by \(^1^H\) NMR (Figure 6a). The \(^1^H\) NMR spectra of the PHAs extracted from \textit{Bacillus sp.} NA10 shows a doublet at 1.26 ppm which is attributed to the methyl group coupled to one proton, a doublet of quadruplet at 2.580 ppm which is attributed to a methylene group adjacent to an asymmetric carbon atom bearing a single proton, and a multiplet at 5.25 ppm characteristic of the methyne group. Two other signals are also observed, the broad one at 1.04 ppm which is due to water, and another one at 7.27 ppm which is attributed to chloroform. From these results, it can be concluded that \textit{Bacillus sp.} NA10 cells grown with cardboard industry effluent as the carbon source produce PHA exclusively in the form of PHB.

\textbf{FTIR}

Polymer extracted from NA10 was used for recording IR spectra in the range 4,000 to 600 cm\(^{-1}\). IR spectra (Figure 6b) showed two intense absorption band at 1,720 and 1,273 cm\(^{-1}\) which are specific for C = O and C–O stretching vibrations, respectively. The absorption bands at 2,932 and 2,962 cm\(^{-1}\) are due to C-H stretching vibrations of methyl and methylene groups. These prominent absorption bands confirm the structure of poly-β-hydroxybutyrate.

\textbf{TGA}

TGA curves were obtained in the temperature range of 30°C to 700°C for PHB. TGA results of \textit{Bacillus sp.} NA10 showed that the T\(_m\) is 182.34°C, and the enthalpy of PHA fusion is 83.62 J/g. The result showed similarity with the data obtained from standard PHB (174.29°C and 86.49 J/g) and from other works \[54,55\]. The PHA gave a rapid thermal degradation between 250°C and 290°C while
the standard PHB represented at between 250°C and 295°C. It is indicated that biopolymeric material obtained can possibly be further used in a large-scale processing of bioplastic [56,57].

**GC-MS analysis of extracted PHB**

GC-MS analysis helps in elucidating the structure of components. The key compounds of concern were identified based on their retention peak. PHA from *Bacillus sp.* NA10 cultured in processed cardboard industry waste significantly contained hexadecanoic acid, methyl ester (56%), and tetradecanoic acid (32%). These compounds signify that the monomer chains were of biodegradable polyester family [8]. Figure 6c,d shows that a common molecular fragment of the 3HB ion chromatogram of the PHB was produced. A predominant peak corresponding to the tetramer of 3HB (hexadecanoic acid) was noted at 13.639 to 13.88 min, respectively, in GC-purified product from *Bacillus sp.* NA10. The retention times and ion fragment patterns of the peaks at 11.6, 12.24, and 15.73 min were identical to those of the dimer methyl esters of 3HV and 3HBV, respectively, but in low percentage up to 32% and 11%, respectively. The similar results of GC-MS were observed by He et al. [58] with 3-hydroxydecanoate (HD or C10) 63% and 3-hydroxyoctanoate (HO or C8) 21% with other medium chain length (mcl) monomers. From the data obtained by GC-MS, the molecular weight of PHB obtained from isolate *Bacillus sp.* NA10 is 242 kDa while, the commercial PHB have a molecular weight of 275 kDa. Galego et al. [59] extracted the PHB with a molecular weight of about 177 kDa. This lowering in molecular weight is due to the presence of other polyhydroxyalkanoic acids e.g. 3HV and 3HBV up to a low percentage (32%) with major polymer 3HB.
Preparation of polymer blended (PHB-starch) sheet
The PHB produced was found to be brittle and breaks easily. Blending PHB with other polymers is an economic way to improve its mechanical properties. The PHB blend sheet (Figure 7) was prepared by mixing extracted polymer with soluble starch following the conventional solvent cast technique. Starch is used for blending because it has few advantages of being biodegradable, biocompatible, cheap, and also readily available while all other biodegradable polymer such as polyethylene glycol and polyactic acid are costly. Starch-blended sheet shows better mechanical and thermal properties making it more reliable for packaging industry. As PHB and starch was not completely miscible, the blend showed insoluble particle aggregation on the surface. Identical observations were reported by Choi et al. [60] in which they prepared PHB sheet with EPB blends. The blends showed a higher thermal stability compared to PHB sheet. Parra et al. [61] reported PHB blend preparation with polyethylene-glycol (PEG) with improved properties.

Conclusions
In this study, inexpensive cardboard industry waste water was tried as a carbon source to produce PHB. Different bacterial strains were isolated from soil and screened for polyhydroxybutyrate production using cardboard manufacturing industry waste water as a carbon source. The bacterial isolate Bacillus sp. NA10 can be regarded as potential strain for conversion of cardboard industry waste water into PHB. The selected isolate efficiently utilized cardboard industry waste water as sole carbon source for growth and PHB biosynthesis, accumulating PHB up to 66.6% of the cell dry mass with 0.072 g l\(^{-1}\) growth and PHB biosynthesis, accumulating PHB up to cardboard industry waste water as sole carbon source for water into PHB. The selected isolate efficiently utilized potential strain for conversion of cardboard industry waste water. The authors express their sincere gratitude to the Rana Cardboard industry, Kurukshetra, for providing the necessary facilities for NMR, FTIR, and TGA analysis of the polymer.

Authors' contributions
AKB carried out all the experimental work such as isolation, screening, fermentation studies, chemical, and statistical analysis and drafted the manuscript. GS (Gulab Singh) collected the substrate and helped in fermentor running and chemical and statistical analysis. NKA guided all the research work and participated in the design of the study. VG and AV helped in isolation and screening and provided the moral support in the whole study. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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