Biopolymer Synthesis and Detection by Soil Bacteria and Yeast

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Abstract
Biopolymers have emerged as potential alternatives to synthetic plastics over the last decades because they share very similar characteristics, are biodegradable and thus can reduce the global pollution caused by synthetic plastics. The primary focus of this work was to explore PHB synthesizing ability of indigenous soil bacteria and yeast during a single-phase growth condition. Three bacterial (two soil isolates and Bacillus subtilis) and one yeast species were used for the study. PHB synthesis study was carried out under batch experimental setup at incubation temperatures of 25 and 37°C. Evidence of PHB synthesis were confirmed by Sudan Black B staining for bacterial and yeast cells isolates. The highest PHB granules recovered was estimated to be 87 mg/100ml at an optimal temperature of 37°C, with none of the test microbial species showing PHB production at 25 oC. Generally, the test isolates showed distinct strategies of PHB accumulation at different incubation periods; some throughout the growth period and others only when growth is at the stationary stage. In the majority of the isolates, growth rate of the test isolates was however directly proportional to the quantity of PHB produced. The PHB synthesizing potential of the test microbial species could be optimized by growing mixed cultures of both exponential and stationary PHB synthesizing organisms in a single fermentation process step, where PHB production is guaranteed to occur throughout the incubation process. This optimization strategy along with its ideal process conditions can lend to the sustainability of this technology.

Keywords: PHB, Sudan Black B, Yeast strains, Bacterial isolates, Bacillus subtilis

1. Introduction
Within the last five decades, the production of plastics has seen a drastic rise to almost 200-fold, globally [1]. This increase can be attributed to low production cost, its versatility and lightness [2]. Though, indispensable to humans, plastics are prominent universal pollutant, recording a global waste of about 275 million tonnes in 2010, compared to its global primary production in the same year of 270 million tonnes (waste accumulation from previous years) [3]. This poses severe damage to flora and fauna in form of entanglements, ingestion and low nutrient assimilation due to its non-biodegradability leading to toxicity of marine organism and the ecosystems contributing to the global oceanic pollution [4], [5]. Plastic debris are also a habitual breeding ground for disease causing organisms and does not in any way contribute to the aesthetics of the environment [6]. This calls for a critical redress of the way we use plastics, its recyclability and most especially degradability.

As such a comparative material with very similar beneficial properties, such as low-cost productivity and a plethora of different mechanical properties and most especially biodegradable would revolutionize the world of plastic. Such alternative plastic has been sourced from bio products, such as food and agricultural wastes and also occur naturally in certain microorganisms [7]. Biopolymers (classified as Polyhydroxyalkanoate – PHA) are slowly paving their way into the world of plastics because of their ability to degrade over time
aerobically or anaerobically in the environment, eco-friendly, excellent flexibility and tensile strength, high melting point, making them alike to synthetic plastics [8], [9], [10]. Polyhydroxybutyrate (PHB), a polyester class of PHA [11], has found a place in medical advancement as a choice plastic for many medical implantations such as sutures and implants [12] because of its biocompatibility with human body, posing no toxicity, sensitivity or haemolysing activity, degrading within 2 years, stable under humid conditions, posing no inflammatory challenges and biodurability [12], [13], [14], [15]. It is a high melting crystalline thermoplastic that exists as carbon and energy reserve compounds in the cytoplasm of certain microorganism [16], [17].

Optimization strategies for bulk production of PHB is at the forefront of advancing this technology. Current research has laid heavily on exploiting microbial feeding approaches as many organisms accumulate PHB in a unique combination of the excess carbon source and a limited nutritional source, which could be nitrogen, oxygen, phosphorus, sulphur etc. [17]. This translates into a two-step fermentation process in a bioreactor [18], which would invariably add to the cost of production. A single step fermentation process could be beneficial if organisms can synthesize large quantities of PHB during normal cell growth [19]. Therefore, unearthing organisms with the capability of synthesizing PHB during a single phase of growth, identifying their optimal cultivation conditions in terms of temperature and incubation period would be a great addition to the sustainability of this technology, which is the subject of this investigation.

2. Methodology

Collection of Samples and microbial isolation

Soil samples were collected from various locations at Landmark University Teaching and Research Farms for microbial isolation, using standard procedures. A total of two bacteria and one yeast was isolated from the soil samples for PHB production while Bacillus subtilis (a known PHB-producing bacteria) was obtained as laboratory stock in the Department of Microbiology, Landmark University, Nigeria. The bacteria and yeast species were re-streaked on nutrient agar and sabourand dextrose agar plates, respectively to obtain pure cultures. Agar slants of all pure isolates were preserved at 4 °C for later use.

Screening of Microbes for PHB Biopolymer

Pure cultures of each isolate grown on agar plate were stained with Sudan black B. Sudan black B test was performed by preparing a solution containing 0.3% of the dye in 100ml of 70% ethanol. The plates were flooded with the Sudan black B solution and left for 30 minutes. The plates were then rinsed using 96% ethanol solution.

Optimization of Culture conditions for PHB Production

The culture media used in this study was comprised of peptone (5g/L), sodium chloride (5 g/L), Yeast extract (3 g/L) and glucose (5 g/L). 200 mL of the prepared media was added to 250 mL conical flasks which were sterilized using an autoclave at 15 psi and 121°C for 15 min before allowing to cool.

After cooling, the media were inoculated with 1 mL of the respective isolates, with the media containing Bacillus subtilis serving as a control in the setup and incubated 37 °C for 3 d. Growth of the isolate was measured using optical density at 600nm while synthesis of PHB was verified with Sudan Black B stain test. Growth rate of the test microbes and PHB production in the respective flasks was determined every 24 h for the 3 d incubation period. The growth rate was estimated as:
\[
Growth\ rate\,(d^{-1}) = \frac{\ln(C_1) - \ln(C_0)}{t_1 - t_0}
\]

Where
C₀ is initial absorbance and C₁ final absorbance respectively

t₀ and t₁ represent initial and final time.

**Extraction, Purification and Quantification of PHB at Different Incubation Temperature**

Following the procedures listed above, PHB production was investigated across two different incubation temperatures 37 °C and 25 °C for 72 h respectively and subsequent PHB extraction and quantification analysis were performed. After the cultivation at the respective temperatures for 72 h, the constituents of the various cultures were separated by centrifuging at 10,000 rpm for 10 min. Resulting cell pellets were washed using equal volumes of acetone and ethanol mixture and centrifuged again. Cell lysis was carried out by resuspending the pellets in 4% sodium hypochlorite solution at 37 °C for one hour in a water bath. They were further centrifuged, supernatant discarded and rinsed again with acetone and ethanol mixture. To the lysed pellets, chloroform was added and they were placed in a water bath at 50 °C overnight. Subsequently, methanol was added to precipitate the PHB crystals before evaporating the solution to obtain PHB crystals. The weight of the crystals in each sample was noted.

**Confirmation of PHB Produced**

During the extraction process, the biopolymer eluted in the chloroform solution was precipitated by methanol, the resulting granules were confirmed as PHB granules by the addition of concentrated sulphuric acid. To the 50ml of the warm filtered solution in a test tube, 98% concentrated sulphuric acid was dispensed dropwise while gently stirring sulphuric acid. The formation of a brown coloration, crotonic acid, confirmed that PHB pellets are in the solution; which PHB is known to form when in contact with concentrated sulphuric acid [20]. Quantification of PHB present in the solution was determined gravimetrically by the weight of recovered precipitate. All experimental setups were in triplicate.

**3. Results and Discussion**

**Screening of Microbes and Confirmation of PHB Biopolymer**

Samples that were positive to the test incorporated a black stain in the cells (dark colonies) after rinsing with 96% ethanol, while negative samples did not incorporate the black stain - colonies remain as before, though the agar may be dark (Fig. 1).

In cells grown at incubation temperature of 25±2 °C, all the test microbial species were negative to Sudan Black B stain, thus there was no detection of PHB. However, at temperature 37 °C, isolates ISO 2 and Bacillus subtilis were positive to the test after a day of incubation while the other samples were negative. As the incubation time increased, similar results were obtained at 48 h. At 72 h of cultivation, ISO 1 indicated accumulation of PHA granules while ISO 3 remained negative (Table 1) [21]. A positive reaction to the Sudan black B test implies that PHB is present intercellularly in the organisms.

The success of Sudan Black B stain as a highly sensitive investigative technique in probing the presence of PHB in microbes can be attributed to it being a positive indicator for the presence of lipids in bacterial cells [22] and PHB is known to reside within the lipid bilayers or lipid vesicles of microbes [23].
Figure 1: Sudan Black B test on culture colonies on plates with positive (A) and negative (B) colonies.

Table 1: PHB detection via Sudan Black B stain at different incubation periods for the test organisms

| Isolates    | Incubation Period (h) | 25 °C | 37 °C |
|-------------|-----------------------|-------|-------|
| ISO 1       | 24                    | -VE   | -VE   |
|             | 48                    | -VE   | -VE   |
|             | 72                    | -VE   | +VE   |
| ISO 2       | 24                    | -VE   | +VE   |
|             | 48                    | -VE   | +VE   |
|             | 72                    | -VE   | +VE   |
| ISO 3       | 24                    | -VE   | -VE   |
|             | 48                    | -VE   | -VE   |
|             | 72                    | -VE   | -VE   |
| *Bacillus subtilis* | 24            | -VE   | +VE   |
|             | 48                    | -VE   | +VE   |
|             | 72                    | -VE   | +VE   |

+VE and -VE denoting PHB detected and not detected, respectively.

With the success of Sudan Black B test in detecting PHB intracellularly within PHB synthesizing organisms and bearing in mind that other biological products are likely present during extraction of PHB, we deemed it necessary to further verify PHB granules after recovery from the cells. Concentrated sulphuric acid is known to react with PHB to form Crotonic acid, whose brown coloration is a positive indicative of the presence of PHB in the solution. Samples BS, ISO 1 and ISO 2 were positive to the test while ISO 3 was negative, which is a further confirmation of the result obtained from Sudan Black B staining test (Fig. 2).

Figure 2: PHB in extracted solution. The brown colouration is a positive test for the presence of PHB (Crotonic acid) and the colourless shows absence of PHB.
Growth and PHB production by test isolates

On the basis of the results generated from the preliminary investigation with Sudan Black B, subsequent experiments were conducted at 37 °C. The detection and quantification of PHB production was observed to show progressive increase with incubation time. There was evidence of PHB synthesis during the course of the incubation period for all test organisms, except ISO 3, where no PHB synthesis was observed. The most PHB recovered was 0.87 mg/ml for ISO 1 while Bacillus subtilis had the lowest at 0.73 mg/ml. PHB synthesis for ISO 1 only began during the stationary phase contrary to ISO 2 and Bacillus subtilis, which began synthesizing PHB even during exponential phase (Fig. 3).

The trend in PHB production as observed in this study corroborates the findings of Ojha and Das [24]. In their study of PHB production in yeasts cells isolated from soil and domestic waste over a wide range of temperature from 27 to 67 ± 5 °C, 37 °C was the ideal temperature. Also, many other researchers have found temperature to have great influences on PHB production [25] with 37 °C being the optimum for PHB production in Bacillus subtilis [26] and Enterobacter aerogenes [27].

Also, it was observed that though PHB accumulation for ISO 1 occurred during stationary phase of growth, it recorded the highest estimate of PHB granules, which agrees with other studies of Bacillus sp. having PHB associated growth, with maximum PHA accumulation occurring during early stationary phase [28]. This potential could be optimized by growing mixed cultures of both exponential and stationary PHB synthesizing organisms in a single fermentation process step, where PHB production is guaranteed to occur throughout the incubation process. This optimization strategy along with its ideal process conditions can lend to the sustainability of this technology.
4. Conclusion
Screening of the soil isolates by Sudan Black B staining confirmed potential of PHB accumulation by yeast and bacterial cells. The optimal temperature for the accumulation of PHB occurred at 37 °C rather than 25 ± 2 °C. Monitoring the rate of PHB production in synthesizing organisms during fermentation is a good indicator of organisms with the potential to produce PHB either at stationary and/or exponential phase of growth. The biopolymer accumulating organisms at stationary phase produced higher PHB granules than those at exponential phase for the incubation periods tested.

5. Recommendation
In conclusion, further optimization studies of PHB production process variables in the areas of rapid cell growth, mixed cultures of PHB producing organisms and simple and efficient recovery processes would be key in the reduction of its production cost, which is the subject of future investigation.

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