JOBIMB, 2024, Vol 12, No 2, 50-54
https://doi.org/10.54987/jobimb.v12i2.1065

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index

JOBIMB VOL 12 NO 2 2024
Bacterial acrylamidase

# Optimization and Production of Polyhydroxybutyrate from Potato Peel Waste using *Bacillus subtilis* Isolated from Soils

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**HISTORY**

Received: 19th Sep 2024
Received in revised form: 21st Nov 2024
Accepted: 20th Dec 2024

**KEYWORDS**

Polyhydroxybutyrates
*Bacillus subtilis*
Potato peels
FTIR
Bio-plastic

**ABSTRACT**

Polyhydroxybutyrates (PHB) are bio-plastics accumulated by some bacteria living in environments where the carbon source is in excess and other nutrients are limited. The aim of this study was to screen *Bacillus subtilis* for accumulation of PHB, using potato peel as a carbon source. A total of 100 g of potato peels were processed and analyzed for proximate composition. A total of 25 g from 12 soil samples, including four samples from three different locations, were assayed using microbiological techniques to isolate and characterize *B. subtilis*. PHB accumulation was determined using Sudan Black B dye, and quantified with a spectrophotometer to identify the best-producing isolates. The production conditions, consisting of pH, NaCl concentrations, and nitrogenous sources were optimized and employed in the PHB production, with the processed peel as the sole carbon source cultured with the best isolates. The PHB was analyzed using Fourier Transform Infrared Spectroscopy (FTIR). The proximate compositions of the carbohydrate and protein in the potato peel were 55.11% and 20.38%, respectively. The occurrence rate of *B. subtilis* was 58.3% (7/12), with 57.14% (4/7) of these isolates being PHB producers, and 4 of them were the best producers. The optimized conditions were pH 7, 3% NaCl and $NH_4Cl$ as the nitrogen source. The PHB yield from the three best producers under the optimized conditions was three times higher. FTIR analysis confirmed the polymer as PHB. Garden soil was the best source for isolating PHB-producing *B. subtilis,* and potato peels were converted from waste to wealth (PHB) with a moderate yield.

## INTRODUCTION

Plastics are polymer molecules of petroleum origin, moldable materials with high durability, and are among the most needed products in the world [1]. They have numerous applications in households, buildings, industries, and medicine [2]. However, they are non-biodegradable, so they are considered pollutants in land and aquatic environments [3,4,5]. Incineration of these plastics generates toxic molecules, and recycling can only be done a limited number of times before they lose their mechanical properties [5]. This limitation necessitates a search for new alternatives. Bioplastics are derived from renewable sources, such as vegetable fats and oils, corn starch, or microorganisms surviving in environmental or laboratory conditions with excessive carbon and depletion of other nutrients [6,7]. Polyhydroxybutyrate (PHB) is a bioplastic that is considered a better replacement for petroleum-derived plastic due to its good physical properties [4,6,7]. *Bacillus subtilis* is known to biosynthesize PHB polymers and accumulate them in their cytoplasm as granules to serve as carbon and energy sources [8,9]. The major problem with the commercial production of PHB is its high production cost compared to plastics derived from petrochemicals. Recently, many efforts have been made to reduce production costs by using strategies such as cheap or dumped materials as substrates. This research aimed to optimize the production of polyhydroxybutyrate from potato peel using *Bacillus subtilis* isolated from soil. The PHB produced was then confirmed using Fourier Transform Infrared Spectroscopy (FTIR).

## MATERIALS AND METHODS

### Preparation of Potato and Cassava Peels

The potato peel samples were collected from a domestic garbage dump (Samaru area), washed with distilled water, and air-dried for 48 hours at room temperature. The peels were then

milled into powder using a blender and dried overnight at 55ºC (328 K) in a hot-air oven.

**Acid Pre-treatment of Potato Peels using Sulfuric Acid ($H_2S0_4$)**

The pre-treatment was carried out by suspending 100 g of the potato peel powder in 900 mL of 0.2 M sulphuric acid ($H_2SO_4$) and heating at 121 ˚C (394 K) for 15 min in an autoclave [10]. The solid residues were collected, neutralized with 2 M NaOH, and washed extensively with tap water until neutral pH was achieved. The material was then subjected to enzymatic hydrolysis, allowed to settle, and the starch sediment was separated from the slurry [11, 12].

**Determination of the Proximate Compositions of the Peels**

The solid residues of the potato peels were subjected to proximate analysis to determine nutritional compositions such as moisture, ash, crude fiber, fat, carbohydrate, and protein at the Institute of Agricultural Research (IAR) Samaru, Zaria, according to a Standard Operating Procedure [13]. The protein content was quantified by combustion using a micro elemental analyzer (Leco Corporation, USA). The combustion of samples was conducted under calibrated conditions with a temperature of 1050 °C (1323 K) and within a pure oxygen atmosphere. This combustion process effectively converts nitrogen (N), sulfur (S), carbon (C), and hydrogen (H) into their respective gaseous states, facilitating quantitative assessment. The nitrogen content within the sample was ascertained and subsequently multiplied by a factor of 6.25 [11], as per established convention. Concurrently, the determination of lipid, moisture, and ash content followed the standardized protocols outlined by the AOAC [13]

**Collection of Soil Samples and Isolation of *Bacillus subtilis***

Soil samples were collected from three locations, with four samples each from Ribadu hostel, A.B.U Dam, and Botanical Garden, all within Samaru Campus, Ahmadu Bello University, Zaria. From each location, 25 g of the sample was collected and dispensed into 225 mL of sterile normal saline. The mixture was heat-shocked in a water bath at 60°C (333 K) for 2 hours. A serial dilution was carried out using the mixture as stock from $10^{-1}$ to $10^{-7}$. Using the spread plate method, a 0.1 mL sample from the $10^{-6}$ and $10^{-7}$ dilutions was plated onto Tryptic Soy Agar plates. The plates were incubated at 37˚C (310 K) for 48 hours. Colonies typical of *Bacillus subtilis* were purified by sub-culturing on fresh Tryptic Soy Agar plates and incubating at 37˚C (310 K) for 48 hours. The pure cultures were stored at 4°C (277 K) on nutrient agar slants for further analysis.

**Characterization of the Isolates**

The isolates were characterized using conventional microbiological techniques. Gram and endospore staining were carried out on slides and analyzed under the oil immersion objective. A catalase test was carried out on a slide emulsified with a colony of the isolate, mixed with 4% $H_2O_2$, and observed for air bubbles. A citrate utilization test was carried out using Simmon's Citrate Agar slants cultured with the colony of the suspected isolate and observed for a colour change after overnight incubation at 37°C (310 K). Methyl Red-Voges-Proskauer broth was inoculated with a pinch from the colony of the suspected isolate for MR-VP tests.

Appropriate reagents were added after the overnight incubation at 37°C (310 K) and were observed for colour appearance. Sulfide-indole-motility (SIM) medium was used to test for hydrogen sulfide production, indole, and motility tests. A starch hydrolysis test was carried out using starch agar plates.

The plates were streaked with the suspected isolates and incubated at 37°C (310 K) for 24 hours, after which they were flooded with iodine reagent and examined for the presence of clear halos around the colonies. [14].

**Screening of the Isolates for PHB Granules Accumulation**

The isolates were streaked on nutrient agar plates and incubated at 37°C (310 K) for 24 hours. The colonies were then smeared on slides, flooded with Sudan black B solution, and allowed to stand for 30 minutes. The smears were flooded with 96% ethanol for 30 minutes and observed for retention or loss of colour (blue-black) [15]. The PHB accumulation capacity of the isolates was evaluated based on the colour retention ability and confirmed by quantification using a spectrophotometer.

**Extraction of PHB Granules**

From each PHB granule-positive culture, 10 mL was prepared in normal saline solution, adjusted to a 0.5 MacFarland standard, and centrifuged at 11000 rpm for 20 minutes. The supernatant was discarded. 2.5 mL each of 4% sodium hypochlorite and chloroform were added to the pellets (for digestion) and incubated at 37˚C (310 K) for 1 hour. The suspension was centrifuged at 15000rpm for 10 minutes, after which both the upper and the middle phases were discarded. The bottom phase containing PHB was precipitated with acetone-ethanol (1:1) solution and allowed to evaporate to dryness [16].

**Quantification of the PHB Extracted**

The extracted PHB was first converted to crotonic acid by adding 5 mL of sulfuric acid ($H_2SO_4$) to a test tube containing the extracted PHB. The solution was heated for 10 minutes until it turned brown (crotonic acid assay). Absorption was measured at 235 nm using a UV-VIS spectrophotometer. The relative PHB accumulation by different isolates was compared and recorded to help identify the best PHB producers. Quantification was used to confirm the rate of PHB accumulation [12].

**Formulation of Salt Medium Used in the Production and Optimization of PHB**

The Mineral Salt Medium (MSM) used in the production and optimization of PHB was composed of the following (concentration in g/L): potato starch (1), $KH_2PO_4$ (1.5), $Na_2HPO_4$ (3.5) and $MgSO_4.7H_2O$ (0.2). In addition, trace elements composed of (g/L): $FeSO_4.H_2O$ (0.02), $CaCl_2.H_2O$ (0.1), $MnSO_4.4H2O$ (0.02), ZnCl (0.1) were added to the medium [17].

**Optimization of some Cultural Parameters**

The parameters optimized in the study were pH, sodium chloride (NaCl), and nitrogen sources, and the three best PHB producers were used [18].

**Optimization of pH**

A set of three flasks containing 100 mL of MSM broth was prepared. One flask was left at pH 7.0, one was adjusted to the pH of 6.0 by adding 1N HCl and the third was adjusted to pH 8.0 by adding 1N NaOH. Each was inoculated with a PHB-producing isolate and incubated at 37°C (310 K) for 48 hours. The PHB produced was quantified using a spectrophotometer [18].

**Optimization of the concentrationof Sodium chloride**

0.1 mL of the standardized (0.5 McFarland) inoculum of the PHB-producing isolate was inoculated into each of the 3 MSM flasks containing 1%, 3%, and 5% NaCl concentrations,

respectively. The PHB produced at different NaCl concentrations was quantified using a spectrophotometer [19].

**Optimization of Nitrogenous Sources**

Different sources of nitrogen (1%) were added to MSM containing a PHB-positive isolate. The sources were ammonium chloride, yeast extract, and ammonium nitrate. The PHB produced was quantified using a spectrophotometer as described by [20].

**Production of PHB under Optimized Conditions**

A 100 mL MSM was prepared in a 250 mL Erlenmeyer flask based on the standardized parameters. In addition, 5 g of the potato peel residue was supplemented and inoculated with a PHB-producing *B. subtilis* and incubated at 37°C (310 K) for 48 hours.

**Characterization of PHB Produced**

The polymer was extracted from the highest-producing isolates and qualitatively analyzed using FT-IR spectroscopy in the spectral range of 4000–500 $cm^{-1}$ to confirm the structure and functional groups of the polymer. The functional groups were characterized using the Infrared (IR) spectra.

## RESULTS AND DISCUSSION

The proximate composition of the potato peels revealed high concentrations of carbohydrates (55.11%) and proteins (20.38%), while moisture (6.97%), ash (10.90%), lipids (6.64%), and fiber (6.28%) were found in lower concentrations. The creamy and opaque colonies with lobate/irregular margins were suspected to be *Bacillus subtilis*.They were tentatively confirmed as Gram-positive, endospore-positive, motile, indole-negative, VP-positive, with the ability to hydrolyze starch and utilize citrate. A total of 7 isolates (7/12, 58.3%) were identified as *B. subtilis*; 4 (4/4, 100%) from the Botanical Garden, 2 (2/4, 50%) from the ABU Dam, and 1 (1/4, 25%) from the students' hostel soil (Table 2). Only 4 out of the 7 (57.14%) *B. subtilis* were PHB producers; comprising 3 (3/4, 75%) from the Botanical Garden, 1 from (1/2, 50%) the ABU Dam, and none (0%) from Ribadu hostel (**Table 1**).

Three isolates, coded ABG1, ABG4, and ADM6, were identified as the best PHB producers, with PHB quantities of 0.341 mg/mL, 0.391 mg/mL, and 0.37 mg/mL, respectively. Additionally, a pH of 7 (**Fig.1**), 3% NaCl (**Fig.2**), and $NH_4Cl$ nitrogen source (**Fig.3**) was found to be optimal for production. Under optimized conditions, the PHB produced was highest in ABG4 (1.75 mg/mL), lowest in ABG1 (1.27 mg/mL), and 1.59 mg/mL in ADM6 (**Fig.4**). The peak values of the bands revealed by FTIR analysis of the extracted polymer (indicated by the wavy line in **Fig.5)** were characterized as PHB based on the Infrared (IR) spectra.

**Table 1.**Distribution of PHB-positive *Bacillus subtilis* according to the sampling location.

| Location | Samples collected | No. positive for *B. subtilis* | No. positive for PHB (%) |
|---|---|---|---|
| Ribadu Hostel | 4 | 1 | 0 (0) |
| A.B.U Dam | 4 | 2 | 1 (50) |
| Botanical Garden | 4 | 4 | 3 (75) |
| Total | 12 | 7 | 4 (57.14) |

Key: A.B.U = Ahmadu Bello University

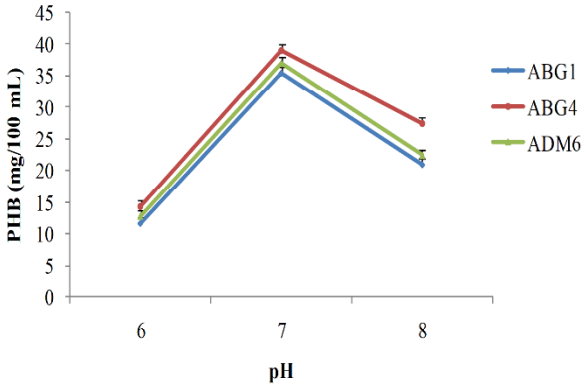


**Fig. 1.** PHB yield of the best producing isolates under different pH Conditions. Error bars represent ± standard deviation of three independent experiments, each conducted in triplicate.

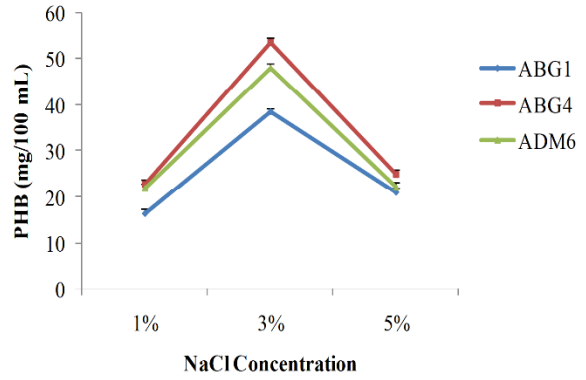


**Fig. 2.** Effect of NaCl concentrations on PHB yield. Error bars represent ± standard deviation of three independent experiments, each conducted in triplicate.

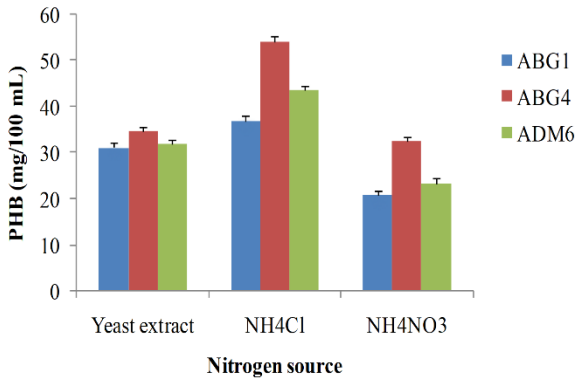


**Fig. 3.**Effect of nitrogen sources on PHB production in the isolates. Error bars represent ± standard deviation of three independent experiments, each conducted in triplicate.

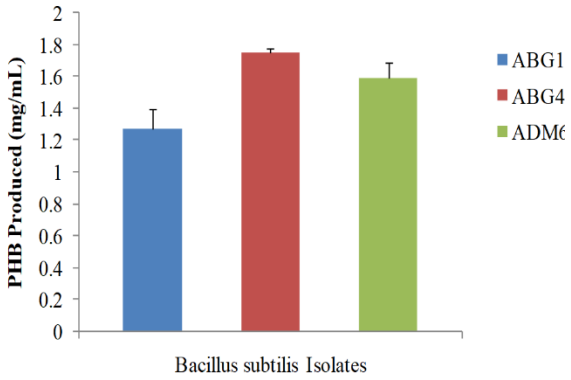


**Fig 4.** PHB Produced (mg/100 mL) under the optimized condition.Error bars represent ± standard deviation of three independent experiments, each conducted in triplicate.

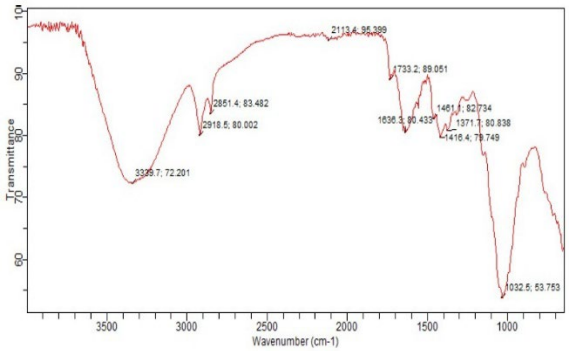


**Fig 5.** FT-IR Spectrum of PHB produced by isolate ABG4 using potato peel as substrate.

The proximate composition of potato peel revealed 55.11% carbohydrates and 20.38% protein, suggesting that the peel could support microbial growth, particularly for PHB production. The higher carbohydrate content compared to protein suggests its potential as a substrate for PHB biosynthesis. This finding is consistent with previous research, which reported 41% carbohydrates and 17% protein in Kenyan potato cultivars [21]. The highest prevalence of *Bacillus subtilis* (100%) was observed in the garden, compared to the water dam (50%) and student hostel (25%). This may be attributed to the presence of an ideal proportion of organic materials in the garden, which promotes the growth of *B. subtilis*. These findings are consistent with previous studies, where the garden showed the highest occurrence of *B. subtilis* (58.5%) among other sampling locations [22].

Our study also found that 66.7% of *B. subtilis* from the garden were PHB producers. This may be due to the higher carbohydrate and lower protein concentrations in the garden soil, which are optimal for PHB production. Gardens typically contain decomposed organic materials, with carbohydrates being the predominant nutritional component. Similarly, *B. subtilis* isolated from gardens were reported to have the highest PHB production, compared to the isolates from other sources [22].

The optimal pH for PHB production was found to be neutral, indicating that *B. subtilis* is a neutrophile. Furthermore, *B. subtilis* showed a tendency to accumulate more PHB at low salt concentrations, but this accumulation decreased as salt concentration increased. This pattern indicates that either *B. subtilis* is non-halophile (survives in environments with low salt concentration) or the PHB is unstable at higher salt concentrations. Also, higher PHB accumulation was observed when NH4Cl was used as the nitrogen source, supporting the idea that lower nitrogen concentrations promote PHB production.

These findings agree with previous research showing that neutral pH [23], $NH_4Cl$ [24], and 3% NaCl are optimal for PHB accumulation. Interestingly, PHB accumulation increased by at least threefold in all the PHB-producing isolates under the optimized conditions (pH 7, $NH_4Cl$, and 3% NaCl), using potato peel as the sole carbon source. This indicates the potential of potato peel as a substrate for bio-plastic production, offering a cost-effective alternative that could enhance production profitability. For every 5 g of potato peel used as the substrate, at least 1.27mg of PHB could be produced. FTIR analysis of the PHB produced by *B. subtilis* confirmed the polymer's identity, with carbon atom peaks matching the standard peaks of PHB [25].

## ACKNOWLEDGMENT

We would like to express our sincere appreciation to the Department of Microbiology, Faculty of Life Sciences, and the Chemistry Multipurpose Laboratory at Ahmadu Bello University Zaria for their valuable support during the laboratory work.

## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

## FUNDING

This research received no grants from any source.

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