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Substrate Concentration and Thermal Effects During Polyhydroxyalkanoate Bioproduction

***[1]Asafa-Adedimeji, K. O., [1]Jimoh, S. O., [1]Badmus-Oladapo, R. B., and [2]Anjous-Alao, R. M.**

[1]Industrial Microbiology Research Group, Department of Biological Sciences, College of Natural and Applied Sciences, Fountain University Osogbo, Osun State, Nigeria.
[2]Department of Chemical Sciences, College of Natural and Applied Sciences, Fountain University Osogbo, Osun State, Nigeria.

Abstract

Polyhydroxyalkanoates (PHAs) have long been thought to have the potential to replace petrochemical-based polymers because their production cost has been a challenging factor hindering their production processes. Previously characterized quantities of *Micrococcus flavus* SS21B screened for polyhydroxyalkanoates (PHAs) were utilized. PHAs were then synthesized using corn cobs as a carbon source. Proximate analysis of corn cobs undertaken during this research reveals the presence of moisture and ash contents, crude fibre and protein, and carbohydrates. Optimization studies reveal an optimal PHA production at 8% substrate concentration and 37°C. This study shows that the carbon source has a more significant effect on the production of PHAs; i.e., the higher the carbon source in the production, the higher the PHAs synthesized, and the thermal effect required is average and not in excess.

Keywords: Polymers, Polyhydroxyalkanoates (PHA), Bio-polyesters, *Micrococcus flavus* SS21B, Optimization.

Introduction

Biodegradable polymers, which occur naturally, belong to the family of bio-polyesters, and their physical properties depend upon the type of monomer in the polymers (Sneha *et al*., 2017). Polyhydroxyalkanoates (PHA) are polyesters synthesized in nature by various microorganisms, including fermentation of lipids and sugar by bacteria (Songyuan *et al*.,2019). Hydroxy fatty acids are components of PHAs, representing an intricate class of intracellular storage polymers produced by different bacteria and archaea. PHAs are generated in the presence of excess carbon sources, while growth is inhibited due to limited nutrient availability (Jimoh *et al*., 2018). The usage of plastic materials, which seems to be very important to human life, is now causing severe environmental problems due to their non-biodegradability thus an alternative polymers attracting considerable interest is the PHAs, which may be utilized in similar applications as the existing synthetic polymers (Muhammad *et al*., 2015).

*Corresponding author:
Email address: asafakifayahadedimeji@gmail.com

Materials and Methods

Processing of corn cobs

Corn cobs obtained during the harvesting period from Osogbo metropolis, Osun state, Nigeria, were air-dried at 30°C for two weeks and processed into smooth powdery form. The corn cobs powder was stored in an air-tight container and kept at 4°C in a refrigerator for further analysis.

Proximate Analysis of Corn Cobs Powder

The proximate analysis was determined using the standard analysis methods of the Association of Official Analytical Chemists (2019).

Optimization Studies for PHA Production

Sterile PHA production medium [potassium hydrogen phosphate (0.4 g/L), di-potassium hydrogen phosphate (1.6 g/L), magnesium sulphate (0.4 g), calcium sulphate (0.2 g/L), ferric chloride (0.004 g/L), yeast extract (1.18 g/L) and corn cob (10 g/L) was inoculated with 0.1 ml of 1.2 x 102 cells/ml of 24 hours old *Micrococcus flavus* SS21B culture and incubated on a thermostatic shaking incubator (250 rpm) at 37ºC for five days. Environmental conditions such as substrate concentration (2%, 4%, 6% and 8%) and temperature (25ºC, 37ºC, 40ºC) were optimized; thus, biomass yield, reducing sugar concentration, and PHA yield was determined at 24 hours intervals using JENWAY 6300 UV- Vis spectrophotometer according to the methodology of Jimoh *et al*., (2018).

Estimation of cell dry weight

Estimating dry cell weight was done according to the modified procedure of Mascarenhas and Aruna (2019). The culture (10 ml) was transferred onto a filter paper of known weight; air-dried, and reweighed at 24-hour intervals until a constant cell dry weight was obtained using the formulae below:

Cell dry weight (g/l) = weight of the dried cell in a petri dish – the weight of the empty petri dish.

Biomass Yield

The cultured medium (2 ml) was withdrawn at 24-hours intervals to determine its biomass yield using UV-vis spectrophotometer at 650 nm. (Jimoh *et al*., 2018).

Estimation of Reducing Sugar Concentration

Cell free-culture medium (1 mL) withdrawn at 24-hour intervals was boiled in a water bath at 100 ºC for 5 minutes. Subsequently, 10 ml of distilled water was added, and the absorbance of each sample was determined at 540 nm using JENWAY 6300 spectrophotometer. The corresponding reducing sugar concentration values were extrapolated from the glucose standard curve (Jimoh *et al*., 2013).

Extraction and Preliminary Quantification of PHAs

PHAs were extracted according to Mascarenhas and Aruna's modified method (2019). The culture medium (10 ml) was centrifuged after 72 hours at 10,000 rpm for 20 minutes. Cell pellets were washed with sterile phosphate-buffered saline (pH 7.2) and recentrifuged at 8000 rpm for 15 minutes. The supernatant was disposed of, and the pellet was suspended in 10 ml of chloroform, vortexed and incubated at 37°C for 24 hours; then further centrifuged at 8000 rpm for 12 minutes (Jimoh *et al*., 2018). After centrifugation, the supernatant was transferred onto sterile Petri dishes at 30°C for evaporation of chloroform and to obtain polymer granule composition. The polymer granule obtained was dissolved in concentrated H_2SO4 and heated in a water bath at 100°C for 10 minutes; thus, depolymerization of PHA when heated with concentrated H_2SO4 leads to the formation of brownish-coloured crotonic acid. After cooling, the absorbance was read at 235 nm against a concentrated H_2SO4 blank using a spectrophotometer. A standard curve was busked with Pure PHA (20-100μg/ml) (Sigma, Aldrich); thus, PHA concentration and percentage yield were determined using the formulae below:

Cell dry weight (g/l) = weight of the dried cell in a petri dish – the weight of the empty petri dish.

PHA yield % = (Weight of PHA/ Cell dry weight) × 100.

Results and Discussion

Proximate Analysis of Corn cobs

The proximate composition of corn cobs utilized in this research work was determined during PHA production. The corn cobs' high moisture content (0.037 ± 0.014 %) shows its support for microbial growth, which eventually increases microbial activity (Aletan and Kwazo, 2019). Ash content (3.550 ± 0.054%) expresses the mineral content of the corn cobs, which indicates the availability of low mineral concentration (especially the macro elements) and vice versa (Otunola and Afolayan, 2019). The crude lipid content (45.995 ± 1.068%) indicates that corn cobs are an excellent source of fat-soluble vitamins and contribute significantly to the energy content of the PHA product that is synthesized from such agricultural waste (Der *et al*., 2012). Corn cobs contain the essential component of the diet required for microbial survival through the availability of crude protein (0.085 ± 0.007%). The carbohydrate content of 13.740 ± 0.651% shows that corn cobs can serve as a good energy and carbon source during microbial metabolic activity (Lu-Ji *et al.*, 2011). The two-way ANOVA results reveal a significant difference between corn cobs' mean proximate composition (Table 1).

Table 1: Proximate composition of Corn cobs

Parameters	Corn cobs
Moisture content	0.37 ± 0.014
Ash content	3.550 ± 0.054
Crude lipid	45.995 ± 1.068
Crude fibre	37.270 ± 0.665
Crude protein	0.085 ± 0.007
Nitrogen–free extracts (Digestible carbohydrate)	13.740 ± 0.651

where the recorded values were significantly different at $p< 0.05$. Data are mean ± SD (standard deviation), n= 2. The statistical level of significance analyzed by two-way ANOVA is followed by the Tukey post hoc pairwise multiple comparisons test.

Osmotolerance and Thermotolerance Effect during PHA production

The biomass yield, reducing sugar concentration, and PHA yield obtained during PHA production varied due to varying environmental conditions (substrate concentration and temperature).

Biomass yield and Reducing sugar concentration

Maximum biomass yield was obtained at 8%, as shown in (Figure 1). The reducing sugar concentration increases as the substrate concentration increases, indicating the utilization of glucose by the organisms (Figure 2). The highest biomass yield and reducing sugar concentration were also obtained at 37°C, thus, supporting *Micrococcus flavus* growth and activity (Figures 4 and 5). Based on the results acquired in this research, corn cobs are economically, and environmentally valuable raw materials for industrial-scale PHA production complemented with metabolically active *Micrococcus flavus SS21B strain.*

PHA concentration and PHA yield

In this study, PHA yield obtained using corn cobs as carbon sources made it easier for *Micrococcus flavus* to absorb nutrients (Rodrigues and Druzian, 2018). Variations in PHA yield obtained using varying substrate concentrations and temperature (figure 3) indicated the potential of *Micrococcus flavus* SS21B to synthesize and accumulate PHA as carbon and energy storage materials or as storage for redundant reducing power under the condition of limiting nutrients in the presence of excess carbon source (Nwinyi and Owolabi 2019). The accumulated PHA can be degraded by intracellular depolymerases and metabolized as a carbon and energy source instantly the supply of the limiting nutrient is re-established. The effect of temperature (37°C) during the synthesis of PHA is significant, and the percentage yield and concentration of PHA increase as the substrate concentration increases (Figure 3). These environmental conditions support *Micrococcus flavus SS21B* strain activity during PHA production (Figure 6). Thus, the availability of PHA through microbial synthesis using corn cobs would replace petrochemically-synthesized materials (plastic materials, packages, absorbents, biomedical materials, tissue engineering materials) due to the availability of agricultural residues.

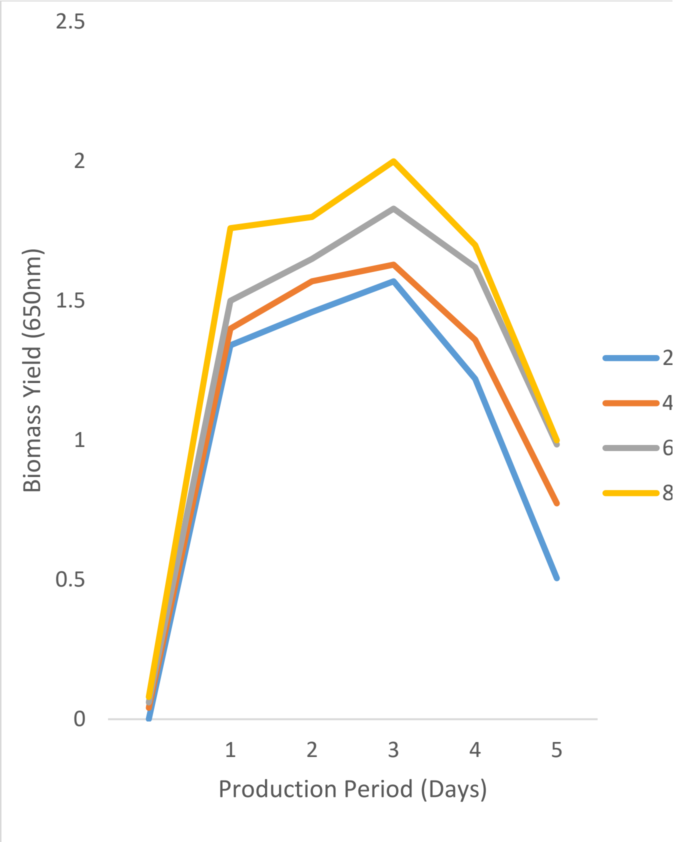

Figure 1: Biomass Yield at varying Substrate Concentrations

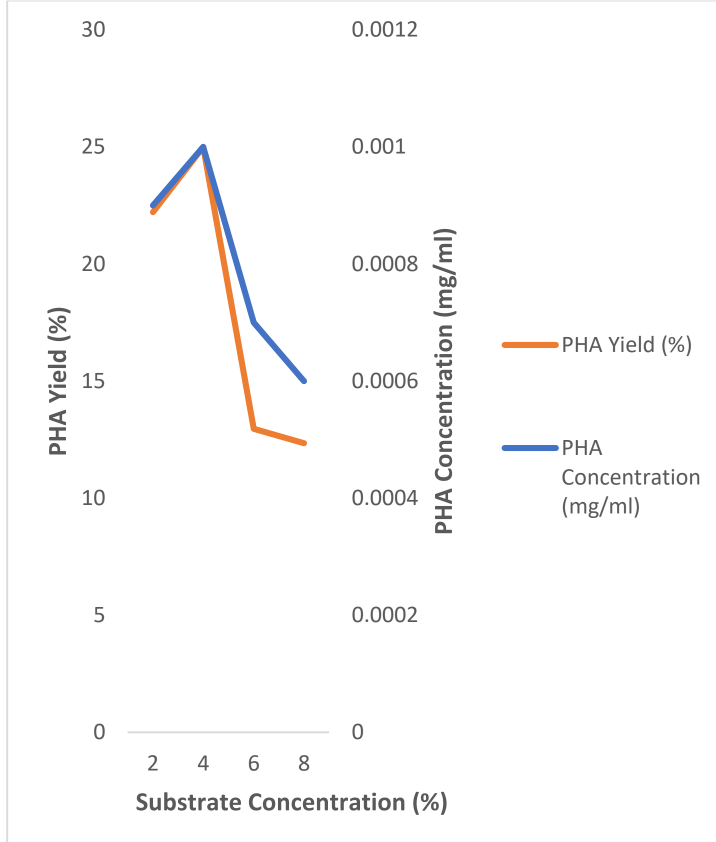

Figure 3: PHA yield and PHA concentration at varying Substrate concentrations

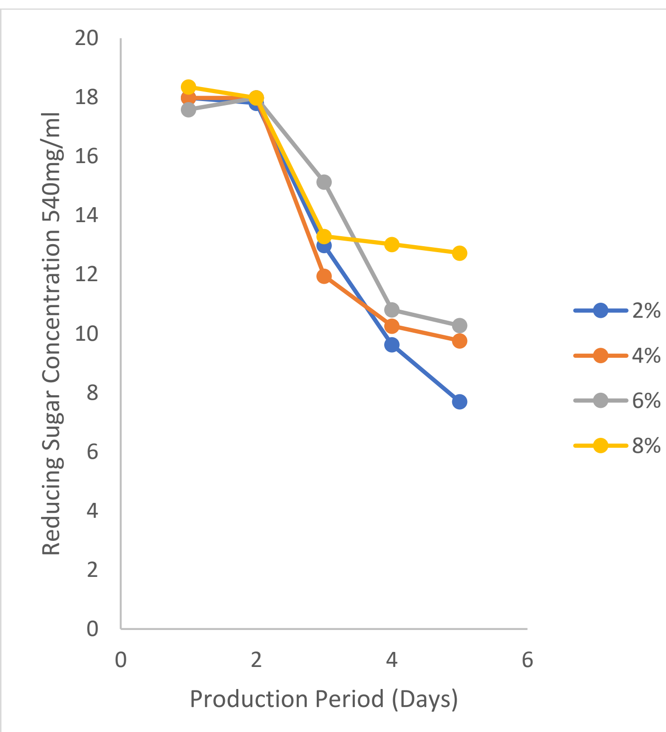

Figure 2: Reducing Sugar Concentration at varying Substrate Concentrations

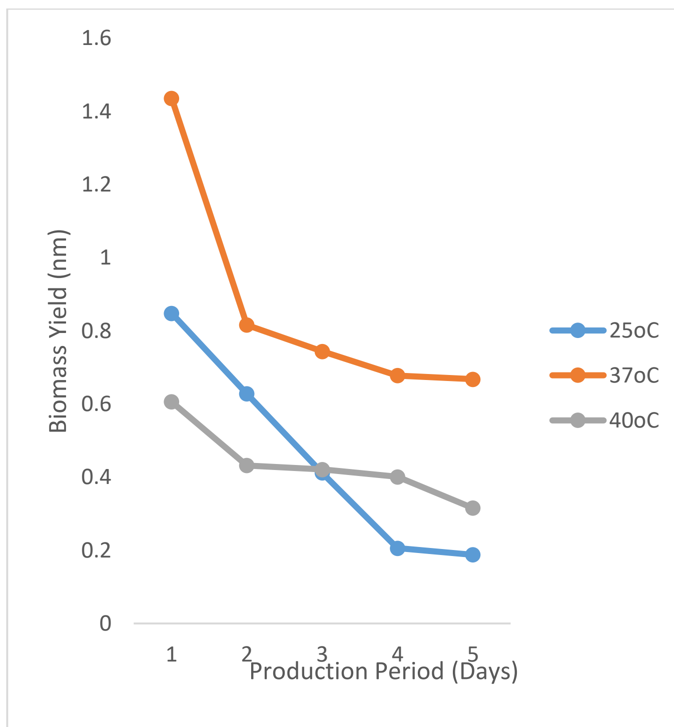

Figure 4: Biomass Yield at Varying Temperature (°C)

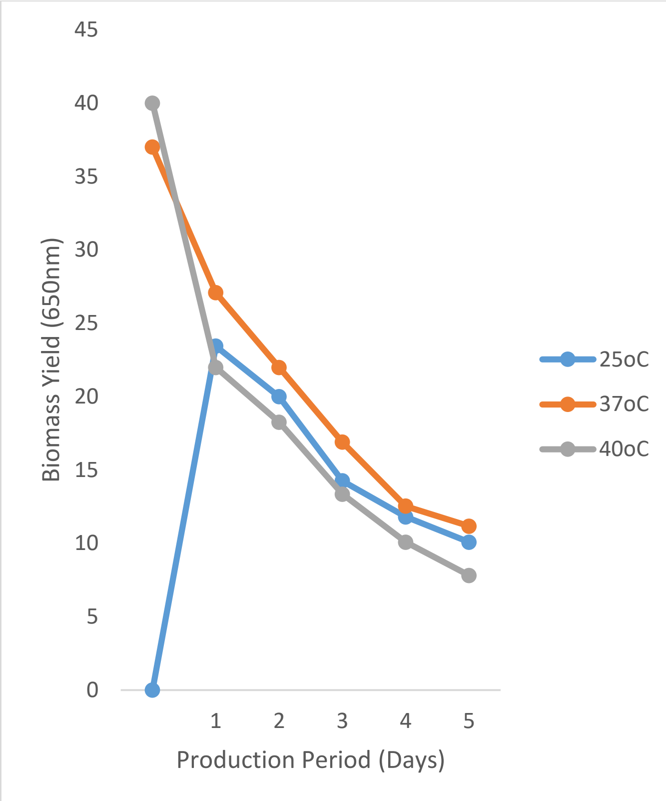

Figure 5: Reducing Sugar Concentration at Varying Temperature

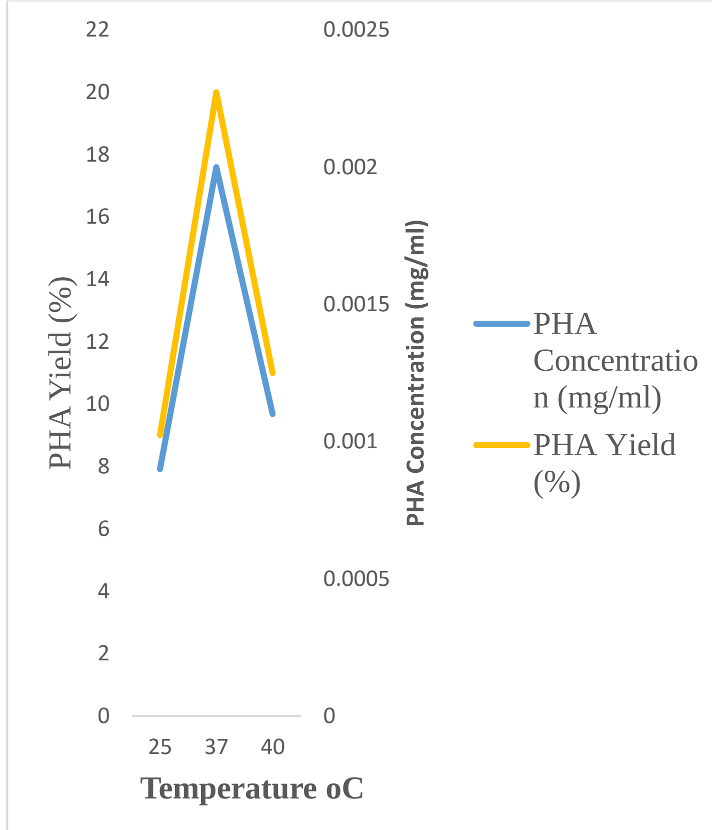

Figure 6: PHA Yield and Concentration at Varying Temperature

Conclusion

Microbial synthesis of PHA at 8% and 37°C will increase the quantitative and qualitative characteristics of PHA-synthesized products since PHAs are produced from biodegradable renewable sources. Its utilization would also improve the state of the environment by solving the problems of solid waste management that result from the accumulation of corn cobs in the environment, thus preventing air pollution and flooding caused by the recycling of corn cobs (Jimoh *et al*., 2018).

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