Production of polyhydroxyalkanoate from nipa sap using *Cupriavidus necator* DSM545

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Abstract. Polyhydroxyalkanoate or PHA is produced through microbial fermentation of sugar and lipid where in nature it is stored as energy and carbon source for microorganisms. Most of the carbon feedstock used for PHA production are derived from biomass as they remain the cheapest carbon source to date. In this study, nipa palm sap was proposed as an alternative carbon feedstock since it is underutilized and widely distributed along river estuaries in Malaysia. This nipa sap contains high sugar content and essential nutrients which are required for PHA production. The aim of this work was to explore potential of nipa sap as a carbon source and to investigate effect of added and non-added nutrient nipa media on PHA production during batch fermentation using *Cupriavidus necator* DSM545. Extraction of PHA using chloroform showed that 2 g/L of PHA was recovered when nipa added with nutrient was used while nipa only media produced 2.16 g/L of PHA. All the samples showed presence of PHA carbonyl band (C=O) from extracted PHA granules by using FTIR while peak obtained using UV Spectrophotometer confirmed the presence of PHA. These findings proved that nipa palm sap as a novel and alternative carbon source for PHA production.
Keywords: Nipa palm sap; Polyhydroxyalkanoate; PHA; *Cupriavidus necator* DSM545; FTIR

1. Introduction

Global plastic waste is increasing rapidly and most plastic types are resistant to degradation and release toxic substances which adversely affects the environment. As sustainable alternative to conventional plastic, polyhydroxyalkanoates (PHAs) can be used since it has capability to mimic petroleum-based plastic and biodegradable. PHA is produced and stored by various microorganism under stress conditions such as nutrient deprivation, excess of carbon, limited supply of oxygen and non- optimum pH culture medium [1].

Among main applications for PHA polymers include food packaging, disposable items like cups, bottles and containers, drinking straw and compostable bags [2]. It also has wide range of potential medical applications such as surgical materials, as a slow-release carrier for long-term drug delivery and tissue engineering [3]. However, major challenges in sustainable production of PHA include cost

for the fermentation substrate and extraction of polymers from the cells [4]. In conventional production of PHA, common carbon sources used are carbohydrates, fatty acids, sugars and alkanes which imposed high cost productions for the substrate [5].

For the past few decades, research on renewable and inexpensive carbon sources has been explored in which oil palm industry is recognized as suitable carbon source for PHA production [6,7]. However, overreliance on one feedstock source will not ensure sustainability in bioplastic industry. In Malaysia, *Nypa fruticans* or nipa palm can be found abundantly in the coastal regions of Malaysia or in plantations. Biomass from nipa palm, especially the nipa sap which is rich in sugar can be a potential alternative feedstock for production of PHA through fermentation. In the past two decades, there has been renewed interest in nipa palm due to its potential for producing ethanol yields equivalent to sugarcane in Brazil [8]. However, nipa sap is still underutilized and either used as seasonal beverage or marketed as healthy food products.

The aim of this research was to evaluate the potential use of nipa palm sap as a carbon source and fermentation medium for PHA production as well as to characterize extracted PHA using FTIR Spectroscopy. This study hopes to find a potential in utilizing new sugar carbon feedstock in producing PHA, as nipa palm has certain unique characteristics that may aid in the fermentation process without the need for additional nutrient thus able to reduce cost from extra raw materials.

2. Methodology

2.1. Collection and preparation of nipa sap

Nipa sap was obtained from a local nipa plantation in Tanjung Manis, Sarawak. In order to avoid degradation of sugar content, the sap was processed under heat to form solid brown sugar. The sugar was further freeze dried to remove excess water, kept in a sealed bag and stored at 4°C in a dark environment until further utilized.

2.2. Bacteria and media preparation

Cupriavidus necator DSM545 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) and maintained on nutrient agar plates at 4°C. The bacteria were grown in 250 mL conical flask containing 100 mL of autoclaved mineral salt media (MSM) [9]. The MSM includes 6.47g/L Na_2HPO_4 x $2H_2O$, 0.1 g/L $NH_4(SO_4)_2$, 0.2 g/L $MgSO_4$ x $7H_2O$, 1.5 g /L KH_2PO_4, 0.06 g/L $(NH_4)_5[Fe(C_6H_4O_7)_2]$, 0.01 g/L $CaCl_2.2H_2O$ and 1 ml/L trace element solution. All flasks were incubated at 30°C for 48 hours on an orbital shaker at 150 rpm.

2.3. PHA production

PHA production was conducted in 500 mL Schott bottle with 400 mL working volume. Three bottles were varied as i) mineral salt media with pure glucose, ii) mineral salt media with nipa sap, iii) nipa sap only. All the bottles were incubated with 5% (v/v) inoculum of *C. necator* DSM545 and the fermentation process was done at 33°C on a rotary shaker at 150rpm for 28 hours.

2.4. PHA extraction

Extraction of PHA was done using chloroform in which 0.2g of the freeze dried biomass was dissolved in 10ml of chloroform, heated and stirred at 60°C for 2 hours to disrupt the cell [10,11]. The mixture was then centrifuged at 6000rpm for 15min to separate non PHA cell debris from the PHA extracted, followed by precipitation and purification using a mixture of methanol and water (7:3, v/v) at 4°C for 3 hours. The mixture was then vacuum filtered using 0.2µm cellulose acetate filter paper for measurement of CDW. Residual biomass was estimated as the difference between CDW and dry weight of the extracted PHA. PHA accumulation was estimated as the percentage composition of PHA present in the dry cell weight where it can be calculated as in Equation 1 [12]:

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA (g/L) x 100}}{\text{CDW (g/L)}} \quad (1)$$

2.5. Quantification of extracted PHAs using crotonic acid assay

The amount of PHA in the sample can be determined using UV-Vis spectrophotometer. Extracted PHA was dissolved in boiling chloroform and evaporated. Later, 10 mL of sulfuric acid (H_2SO_4) was added to the polymer and heated on a water bath at 100ºC for 10 min. Crotonic acid standard solution were prepared with different increasing concentrations of 10-40 µg and measured at wavelength 235nm [13]. The standard curve was extrapolated to determine the PHA concentrations and all the diluted samples were measured by UV spectrophotometer.

2.6. Analytical method

Sugar concentrations were determined using high performance liquid chromatography (HPLC) and ash content from nipa sap was determined as described in method Laboratory Analytical Procedure (LAP) of National Renewable Energy Laboratory (NREL) [14]. The ash produced were then subjected for elemental analysis using elemental dispersive X-ray (EDX) spectroscopy.

2.7. FTIR analysis

Functional group presence in the extracted PHA were confirmed and compared with PHA standard from Sigma Aldrich using Fourier Transform Infrared Spectroscopy (FTIR) Nicolet 6700 from Thermo Fisher equipped with Smart iTR™ in the range of 4000-500 cm^{-1}.

3. Results and discussion

In this study, the carbon source of interest was derived from nipa palm. Sap from the palm was extracted traditionally and processed via heating to produce brownish nipa sugar. Sugar content composed of sucrose, glucose and fructose was analysed in weight percentage (wt%) as well as total ash content as tabulated in Table 1. Sugarcane sugar was also analysed as comparison to nipa sugar since it is widely used for raw material for polymer production in countries with sugarcane industries such as Thailand and Brazil [15].

Table 1. Comparison of sugar content and total ash content for nipa sugar and commercial sugarcane based sugar [15].

Sample	Sucrose content (wt%)	Glucose content (wt%)	Fructose content (wt%)	Total ash content (wt%)
Nipa sugar	65.6	0.6	0.1	0.1
Sugarcane sugar	67.5	0.1	n.d*	0.0

n.d* - Not detected

From the results, it can be seen that nipa sap has significant amount of sugar as sugarcane. Other than that, nipa sap also contains natural inorganic elements such as sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) which are known micronutrient essential for metabolism and growth of bacteria and may further aid in fermentation process. These was proven by using energy dispersive X-ray (EDX) spectroscopy of the nipa sugar ash where trace elements such as sodium (Na), magnesium (Mg), silicon (Si), phosphorus (P), sulphur (S), chlorine (Cl), potassium (K) and calcium (Ca) were detected as shown in Figure 1.

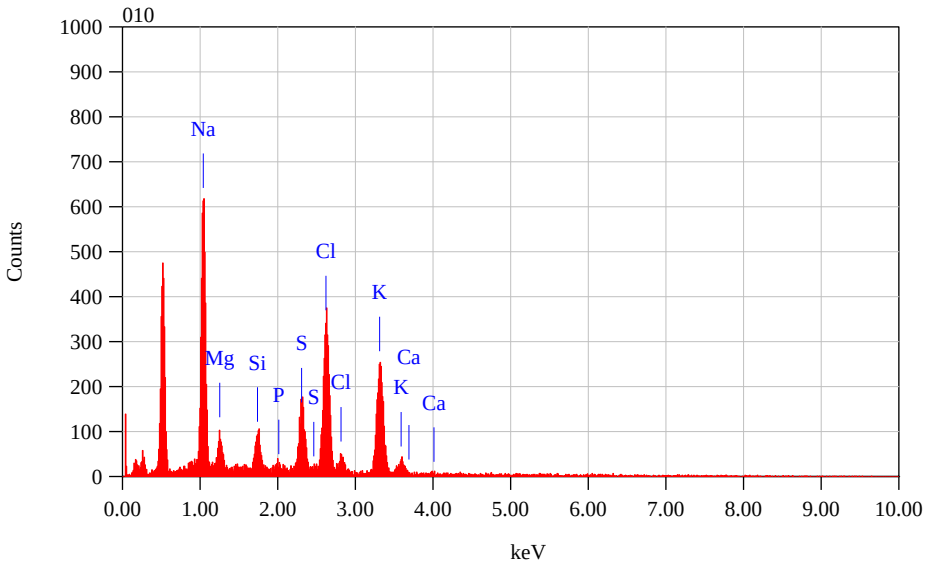

Figure 1. EDX spectrum of nipa sugar ash.

During batch fermentation process, *C. necator* DSM545 was grown in three different carbon sources which were, i) mineral salt media with glucose (A28), ii) mineral salt media with nipa sap (N+), iii) nipa sap with no added nutrients (N-). Table 2 tabulated the data obtained for CDW, PHA concentration and PHA accumulation for each treatments.

Table 2. Effect of different carbon sources on CDW, PHA yield and PHA accumulation.

Treatment	CDW (g/L)	PHA concentration (g/L)	PHA accumulation (%)
C. necator (A28)	3.99	2.13	41.8
C. necator (N+)	2.22	2	89.9
C. necator (N-)	2.86	2.16	75.6

Among all the treatments, *C. necator* DSM545 supplied with glucose produced highest CDW compared to nipa sap media and nipa sap only. This indicates that glucose promotes faster growth rate compared to other sugars while nipa sap rich in sucrose were not fully degraded and consumed by the bacteria. High cell biomass could be produced but not in PHA yield and accumulation. This was caused by nutrient depletion within 24h due to rapid cell growth and the culture has reached stationary growth phase [16]. However high PHA concentration was achieved when nipa sap only was used indicating excess carbon with limited nutrients trigger accumulation of PHAs within the cell.

Quantification of PHA can be further determined by using UV-Vis spectrophotometer [12]. Extracted PHA were measured at absorbance from 190-300nm where presence of PHA were indicated at peak wavelength of 230-240nm as shown in Figure 2 [17,18].

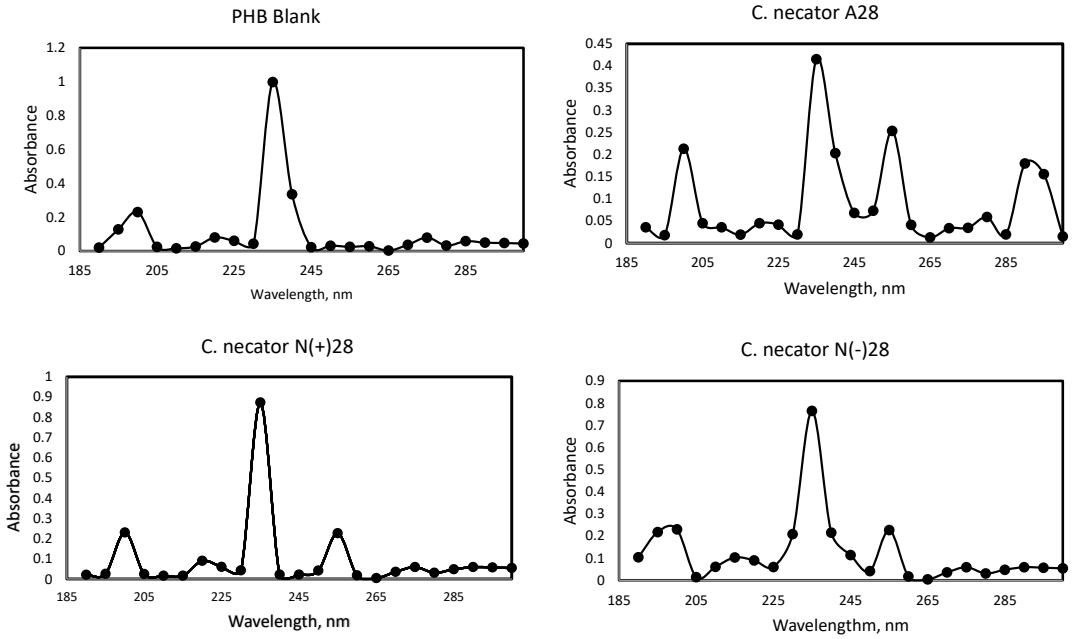

Figure 2. Data of *C. necator* DSM545 under three different treatments in comparison to standard PHB from Sigma Aldrich.

After extraction of PHA using chloroform, presence of polymers was identified using FTIR. From Figure 3, all the FTIR spectrum revealed the presence of typical functional peaks band of a PHA polymer from *C.necator* DSM545 grow with pure sugar, nipa with nutrient (N+) and nipa only (N-) media. Similar peak values obtained as commercial standard PHBs can be detected such as presence of stretching vibration of C-O bond found in the region of 1000 to 1300 cm^{-1}, C-O-C stretch between 1185 to 1228 cm^{-1}, peak observed at 1600 to 1850 cm^{-1} represent stretching of carbonyl group (C=O), peak in the region of 2922 to 929 cm^{-1} were assigned to C-H linkages and the presence of hydroxyl group (OH-) can be observed in the region of 3200 to 3500 cm^{-1}. For FTIR analysis, PHA marker band generated by carbonyl ester stretching C=O bond are indicated by peaks of 1728 and 1740 cm^{-1} which are denotation for short chain length (scl)-PHA and medium chain length (mcl)-PHA, respectively [19]. In this study, the PHA specific peaks are only observed in samples of *C. necator* DSM545 grown in N+ and N- at 1731 and 1733 cm^{-1}, respectively. This findings indicates the presence of PHB+PHA co polymers [20] which have more potential applications compared to homo PHA polymers such as PHB. On another note, peaks recorded at 1641 cm^{-1} from *C. necator* DSM545 grown in glucose indicated weak C=O bond generated by conjugated carbonyl or amide group. Similar result has been shown in PHA production from bean curd waste and sugarcane bagasse [19,21].

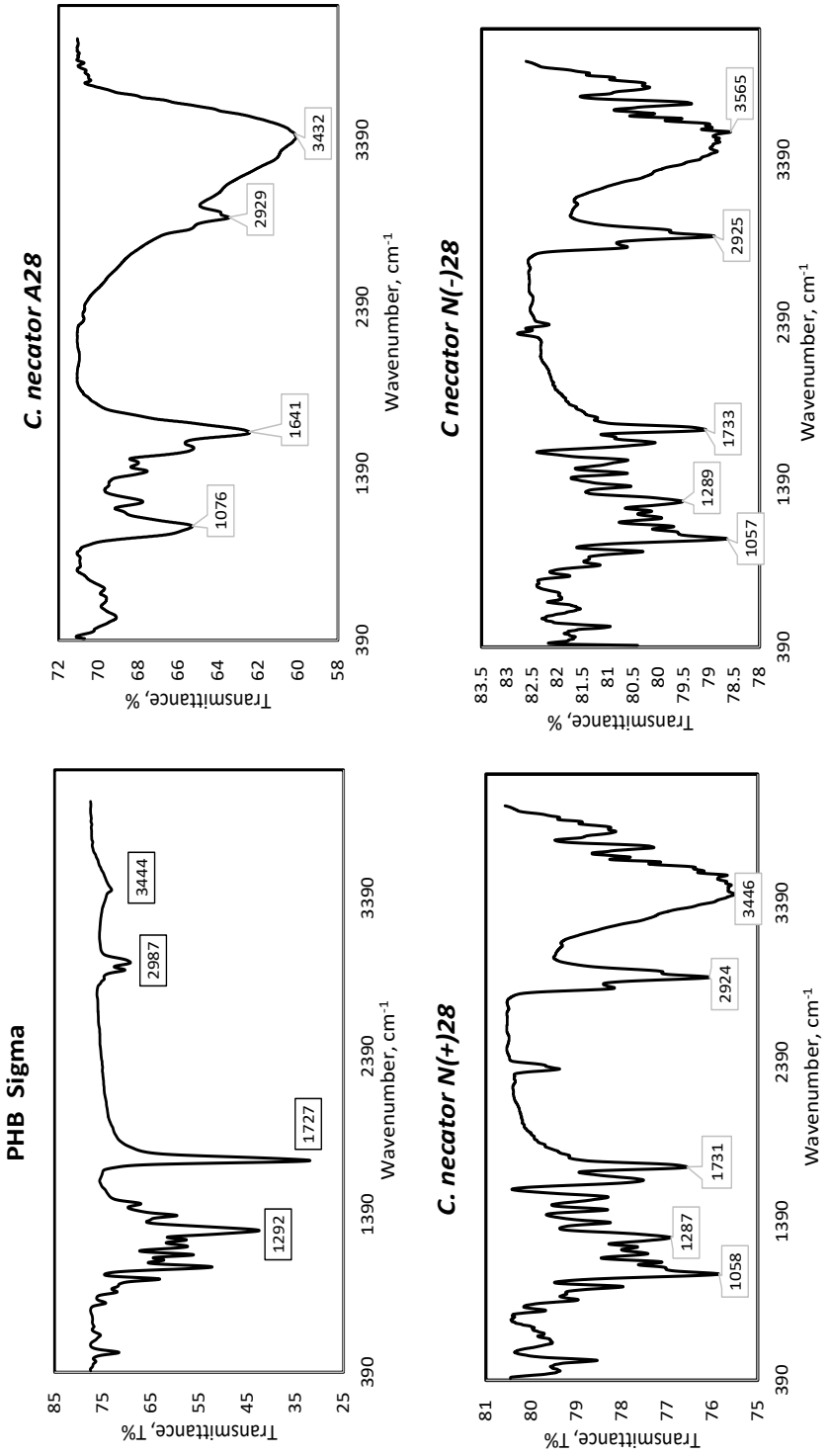

Figure 3. FTIR analysis of extracted polymer from *C. necator* DSM545 grown in pure sugar, nipa based and nipa with no added nutrients extracted using chloroform

4. Conclusions

This study successfully proved the potential of nipa sap as novel and alternative carbon source for production of polyhydroxyalkanoate via bacterial fermentation by employing *Cupriavidus necator* DSM545. Nipa sap at 10 g/L with addition of nutrient can produce PHA up to 2 g/L while 10 g/L of nipa sap without nutrients capable to produce 2.16 g/L of PHA. This shows the ability to reduce cost for additional nutrients in fermentation process while at the same time producing PHA in high yield by utilizing nipa sap as carbon source.

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