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Utilization of Water Hyacinth Weed as a Low Cost Substrate for Polyhydroxyalkanoates (PHAs) Biopolymer Production

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Abstract

Polyhydroxyalkanoates (PHAs) is a biopolymer, synthesized and accumulated in some microorganisms as storage granules for carbon and energy reserve. The material properties of PHAs are of great interest due to the inherent biodegradability and excellent biocompatibility. However, the PHAs production cost is still too high, one approach to reduce the cost is the use of inexpensive carbon source such as lignocellulosic biomass. This study aimed to investigate the PHAs production using water hyacinth hydrolysate (WHH) obtained by alkaline pretreatment and enzymatic hydrolysis. After pretreatment reaction, the highest reducing sugar concentration of 25.2 g/L was produced in 2%(v/v) NaOH with steam followed by cellulase hydrolysis, then the pretreated 1%(v/v) of total reducing sugar (TRS) in WHH was utilized as a carbon source for PHAs production from Pseudomonas mendocina PSU. The results showed a maximum cell growth and PHA content reached to 3.86 g/L of biomass and 1.66 g/L of PHA (43.02%DCW) at 48 h when cultivated the cells in 1%(v/v)TRS in WHH and also higher than 1%(w/v) glucose. In addition, the PHAs yield and volumetric productivity was found to be 0.18 g/g and 0.035 g/L/h, respectively. The produced PHAs was further characterized for monomer compositions using GC-MS analysis and revealed that it composed of only 3-hydroxybutyrate (3HB) homopolymer. Therefore, this work indicated that WHH is promising potential substrate for PHAs production.

Keywords: Polyhydroxyalkanoate (PHA), water hyacinth, hydrolysate, pretreatment, total reducing sugar (TRS)

Introduction

Polyhydroxyalkanoates (PHAs) is a biopolymer, synthesized and accumulated intracellular in a wide range of microorganisms especially bacteria as storage granules for carbon and energy reserve. The production of PHAs is enhanced by limitation of some essential nutrients such as nitrogen but carbon source is excess (Zhu et al., 2013; Muhammadi et al., 2015; Desouky et al., 2017) The material properties of PHAs are of great interest due to the inherent biodegradability and excellent biocompatibility beside its physical and mechanical properties that resemble to those of petrochemical thermoplastics. PHAs is biodegraded within 5-6 weeks releasing carbon dioxide and water, which is non-toxic to environments. PHAs has several applications e.g. packaging, moulded goods, paper coatings, non-woven fabrics, adhesives, and films (Bugnicourt et al., 2014; Muhammadi et al., 2015) PHAs are commonly grouped into two major categories: short-chain-length (scl-) and the medium-chain-length (mcl-) PHAs. The repeat units of scl-PHAs are composed of three to five carbon atoms whereas mcl-PHAs contains six or more carbon atoms (Bugnicourt et al., 2014). The monomer compositions of the synthesized PHAs is influenced by the bacterial strain, type and quantity of carbon sources supplied to the culture medium (Preethi et al., 2015) However, the major obstacles for using PHAs in commercial is their high production cost, a significant portion of total cost is attributed to carbon substrate. In sustainable PHAs production using non-edible, abundant and renewable carbon sources such as waste biomass or agricultural residues will not only ensure zero waste but also enable up-cycling of the generated wastes to high value products.

Eichhornia crassipes (Mart.) Solms, commonly known as water hyacinth (WH), is a rapid growing perennial aquatic noxious weed widely distributed in tropical and subtropical areas worldwide. Because of a higher growth rate and can tolerate environmental conditions, it forms a large mat on the water surface and severely deteriorates the aquatic ecosystem. WH has been considered as sustainable biomass resource for economic perspectives of biopolymer production due to high cellulose content (40–70% of dry matter) but lower amount of lignin (Preethi et al., 2015; Saratale et al., 2020). However, WH biomass requires pretreatment process to break down the structure and delignification of biomass. Alkaline pretreatment is well-known for the effective delignification of biomass by cleaving the bonds between hemicelluloses and lignin, then the depolymerized lignin fragments are soluble in water and make cellulose easily accessible for enzymatic hydrolysis in order to generate some fermentable sugars (Saratale et al., 2015; 2016; 2020)

The objectives of this study are exploring the suitable pretreatments of WH biomass by determine the optimal conditions for better saccharification before enzymatic hydrolysis. Then, the resulting water hyacinth hydrolysate (WHH) was used as a sole carbon source for PHAs production from *Pseudomonas mendocina* PSU. Finally, the extracted PHAs was further analyzed the monomer compositions by GC-MS technique.

Purposes

1) To produce water hyacinth hydrolysate (WHH) by alkaline pretreatment and enzymatic hydrolysis to convert complex sugars in WHH to easily fermentable reducing sugars e.g. glucose
2) To produce PHAs from Pseudomonas mendocina PSU using WHH as a low-cost carbon source

Research Methodology

1. Collection of raw material

Water hyacinth (WH) leaves were collected from 'Thale Noi Lake, Khuan Khanun, Phatthalung,Thailand. It was washed several times with water and was dried in hot air oven at 70˚C for 48h and then grinded into fine powder with 40 mesh sieve strainer and stored in air tight container in room temperature until further analysis.

2. Pretreatment of water hyacinth (WH) biomass for fermentation

To hydrolyze and to achieve better saccharification before enzymatic hydrolysis, the method used was modified from Saratale et.al. (2020). WH biomass was pretreated with NaOH at different concentration of NaOH e.g. 1%(w/v), 2%(w/v) and 3%(w/v). Initially, WH biomass was subjected to NaOH solution and steam exploded in an autoclave at 121°C, 15 psi, for 30 min. The water hyacinth hydrolysate (WHH) was then recovered by filtration with gauze and were then adjusted with HCl to pH 7.0 and the precipitate was removed by filtration with Whatman filter paper No.1. The pretreated WHH was further hydrolyzed by enzymatic hydrolysis with Cellulase from *Trichoderma reesei* (Celluclast® 1.5L) and the dosage in the reaction was 16 FPU/g-substrate (Tsai and Meyer, 2014).

3. PHA production

3.1 Bacterial strain

Pseudomonas mendocina PSU was isolated from soil environment and had been reported that it accumulated relatively high amount of PHA content (Chanasit et. al., 2016).

3.2 Fermentation conditions

P. mendocina PSU was cultivated in nutrient rich (NR) medium containing 10.0 g/L peptone, 10.0 g/L meat extract, 2.0 g/L yeast extract (Lau et al., 2012) at an initial pH of 7.0 and 35 °C with a shaking of 200 rpm until mid-log phase was reached. Then, 10% (v/v) inoculum suspension (OD_{600} =0.5) was added to the nitrogen-limiting mineral salts medium (MSM) containing in g/L: $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 2.0; Na_2HPO_4, 0.6; $MgSO_4 \cdot 7H_2O$, 1.0; and a trace element solution 1 mL containing in g/L:$CaCl_2 \cdot 2H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 1.30; $FeSO_4 \cdot 7H_2O$, 2.0; $(NH_4)_2MoO_4$, 0.6; and boric acid, 0.6 (Kulpreecha et al., 2009).These MSM was supplemented with 10 g/L of carbon source e.g. water hyacinth hydrolysate (WHH) or 1% (w/v) glucose.

4. Analytical procedures

4.1 Dry cell weight

The cells were harvested by centrifugation (7,155*xg*, 20 min at 4°C) followed by lyophilization of the pellets until constant cell weights were obtained. The dry cell weight (DCW) was calculated in g/L unit.

4.2 PHA assay

Approximately 20 mg sample of freeze dried cells of *P. mendocina* PSU was added into two mL each of chloroform and acidified methanol [15% (v/v) H2SO4]. The mixture was then heated at 100°C for 3 h. After cooling to room temperature, 2 mL of distilled water was added followed by vigorous shaking and then the reaction was left overnight for phase separation. The chloroform portion containing the PHA methyl esters were then analyzed by gas chromatography-flame ionization detector (GC-FID) for PHA quantitation and gas chromatography-mass spectrometry (GC-MS) in the total-ion scan mode at a mass-to-charge ratio (*m/z*) = 45–600 for detection of monomer compositions. Standard monomers of methyl hydroxyalkanoates (Larodan, Sweden) was used as a reference peak and benzoic acid was used as an internal standard (Sun et al., 2007).

4.3 Total reducing sugar (TRS)

The total reducing sugar concentration was measured by dinitrosalicylic (DNS) (Miller, 1959). Briefly, 500 µl of cell-free supernatant was added to 500 µl of the color reagent. These solutions were heated in

boiling water for 10 min and immediately transferred on ice and the absorbance was measured at 540 nm when calibration curve is glucose at 0 to 1.0 g/L

5. Fermentation kinetics

Production kinetics of PHA and DCW were calculated in the product yield of PHA with respect to sugar consumption $Y_{P/S}$ (g/g), the product yield of PHA with respect to biomass $Y_{P/X}$ (g/g), biomass yield related to sugar consumption $Y_{X/S}$ (g/g) and volumetric productivity of PHA (g/L/h) of the culture media (Desouky et al., 2017)

Results

1. Analysis of Water hyacinth hydrolysate (WHH)

Dried powder of WH biomass was prepared by alkaline pretreatment in steam exploded at 121°C, 15 psi, for 30 min followed by Cellulase hydrolysis reaction **(Figure 1)**.

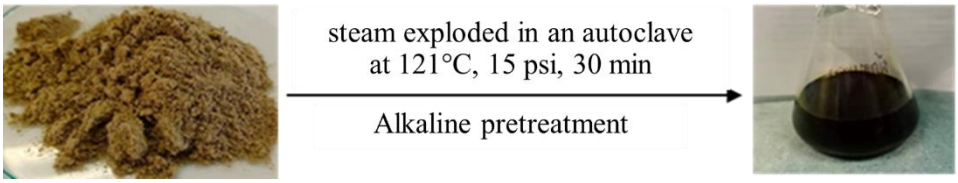

Figure 1 Water hyacinth hydrolysate (WHH) after alkaline pretreatment

The WHH was then determined the amount of total reducing sugar (TRS) concentration after pretreated with NaOH at different concentration at 1%(w/v), 2%(w/v) and 3%(w/v) followed by Cellulase hydrolysis for saccharification to generate fermentable sugar e.g. glucose. The results clearly showed that at 2%(w/v) NaOH produced a maximum total reducing sugar about 25.20 g/L **(Figure 2)**.

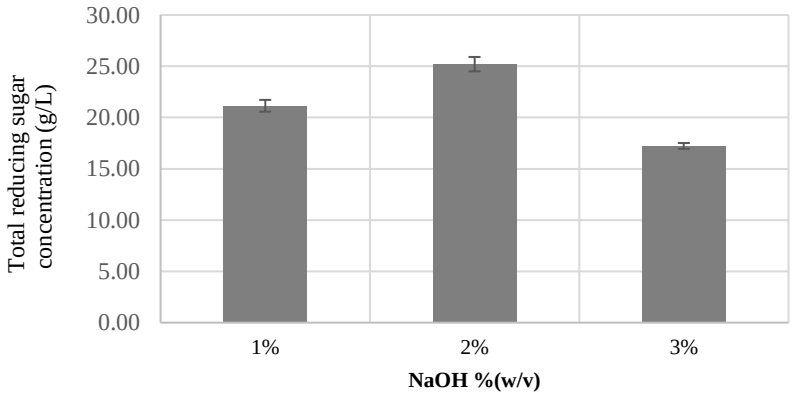

Figure 2 Total reducing sugar concentration of water hyacinth hydrolysate (WHH) after pretreatment by alkaline (NaOH) with steam exploded followed by hydrolyzed with Cellulase. Error bar represents the mean of triplicates ± standard deviation

2. Cell growth and PHA production from WHH

A maximum biomass concentration of 3.86 g/L and PHB concentration of 1.66 g/L (accounted for 43.02%DCW of PHA content) were produced at 48 h when *Pseudomonas mendocina* PSU grown in MSM production medium supplemented with 1% (v/v) TRS in WWH as a carbon source whereas in 1% (w/v) glucose, the bacterial growth and PHA production were less **(Table 1)**. In addition, the comparison of fermentation kinetics e.g. $Y_{P/X}$, $Y_{P/S}$, $Y_{X/S}$, and PHB productivity between 1% (v/v) TRS in WWH and 1% (w/v) glucose, the results showed that all those fermentation kinetics in 1% (v/v) TRS in WWH had slightly higher than in 1% (w/v) glucose. These was also corresponded to the sugar consumption **(Table 2)**.

Table 1 Comparison of cell growth and PHA production when grown *P. mendocina* PSU in MSM supplemented with 1% (v/v) TRS in WWH or 1% (w/v) glucose as a sole carbon source

Cultivation time (h)	1% (v/v) TRS in WWH			1% (w/v) Glucose		
	DCW (g/L)	PHA conc. (g/L)	PHA content (%)	DCW (g/L)	PHA conc. (g/L)	PHA content (%)
0	0.55±0.09	0	0	0.58±0.04	0	0
12	1.82±0.11	0.25±0.10	13.73	1.18±0.11	0.24±0.04	21.08
24	2.05±0.08	0.60±0.15	29.37	1.84±0.12	0.52±0.11	28.11
36	2.94±0.11	1.17±0.22	39.75	3.38±0.05	1.37±0.20	40.47
48	3.86±0.11	1.66±0.15	43.02	2.56±0.08	0.91±0.05	35.64
60	2.60±0.18	0.65±0.08	24.96	2.44±0.05	0.58±0.04	23.66
72	2.45±0.09	0.52±0.02	21.08	1.18±0.05	0.17±0.02	15.52

Data represents the mean of triplicates ± standard deviation

Table 2 Fermentation kinetic parameters when grown *P. mendocina* PSU in MSM containing 1% (v/v) TRS in WHH or 1% (w/v) glucose as a sole carbon source

Carbon source	Sugar Consumption (g/L)	$Y_{P/X}$	$Y_{P/S}$	$Y_{X/S}$	PHB productivity (g/L/h)
1% (v/v) TRS in WWH	9.25±0.12	0.43	0.18	0.42	0.035
1% (w/v) Glucose	8.86±0.10	0.41	0.15	0.38	0.038

Data represents the mean of triplicates ± standard deviation

3. PHA monomer compositions

The monomer compositions of the produced PHA from *P. mendocina* PSU when grown the cell in MSM containing 1% (v/v) WWH as a sole carbon source was identified retention time of 10.81 min as butanoic acid, 3-hydroxy-methyl ester, and 13.84 min was referred to benzoic acid methyl ester (internal standard). The compound identifications were usually achieved by matching query spectra to spectra present in a reference library ; NIST 20 Mass Spectral Library& Search Software (NIST 2020/2017/EPA/NIH) **(Figure 3)**.

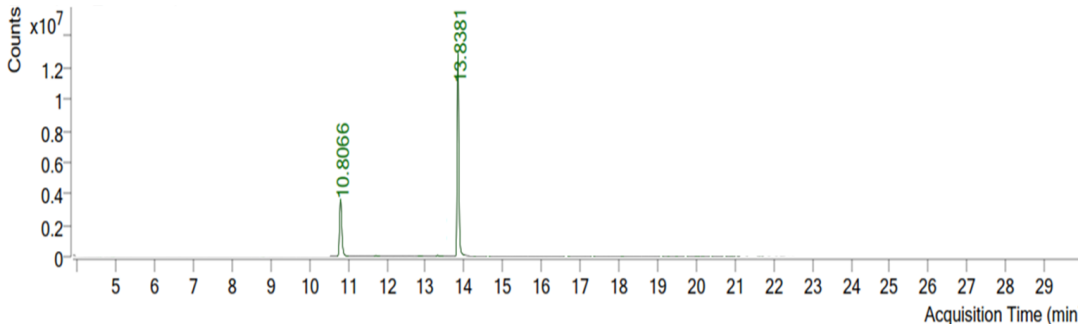

Figure 3 GC-MS chromatogram of the produced PHA from *Pseudomonas mendocina* PSU when grown in MSM containing WWH as a sole carbon source. Only 3HB was detected.

Discussion

Eichhornia crassipes (Mart.) Solms, commonly known as water hyacinth (WH) is aquatic noxious weed containing high cellulose content but lower amount of lignin (Preethi et al., 2015; Saratale et al., 2020) thus WH seems to be an alternative low cost substrate for microbial fermentation such as biopolymer production. However, WH biomass requires pretreatment process to break down the structure and delignification of biomass. In this study, alkaline pretreatment and enzymatic hydrolysis were carried out. Alkaline pretreatment is well-known for the effective delignification of biomass by cleaving the bonds between hemicelluloses and lignin, thereby increasing the porosity of biomass and facilitating the accessibility of biomass to hydrolytic enzymes (Saratale et al., 2015; 2016; 2020).In this study, the suitable concentration of 2%(w/v) NaOH showed the highest reducing

sugar production. These results corresponded with Saratale et. al. (2020) reported that at 2% NaOH could remove more than 50% of lignin and produced a maximum of 75% glucose yield and a similar result was obtained by Wititsuwankul and Jaturapiree (2021) indicated that the optimal pretreatment condition which had 2% (w/v) NaOH with autoclaving at 121°C 15 psi for 30 min increased the enzymatic hydrolysis efficiency up to 5.47-fold. Generally, proper pre-treatment of lignocellulose prior to its enzymatic hydrolysis by cellulases significantly improves glucose yields (Obruca et al., 2015). There are several studies on PHA production from water hyacinth hydrolysate (WWH), for example, the PHB production from WHH by *Ralstonia eutropha* NCIMB 11599 at C/N ratio of 20:4 in a bioreactor gave the maximum PHB concentration of 1.22 g/L after 32 h of cultivation, and reached the PHB yield and volumetric productivity of 0.366 g/g and 0.038 g/l/h, respectively (Wititsuwankul and Jaturapiree,2021) whereas in *R. eutropha* ATCC 17699 was able to accumulate about 7.3 g/L of DCW and up to 73% PHA content when grown in WHH supplemented with corn steep liquor (Saratale et al., 2020). In addition, Radhika and Murugesan (2012) reported that when grown *R. eutropha* MTCC 1472 in 35 g/L of reducing sugar contained WHH and 1.5 g /L of $(NH_4)_2SO_4$ produced the highest biomass of 12 g/L and 7.0 g/L of PHB (58.0 %DCW). Moreover, Omara et al. (2019) produced a maximum PHA yield of 61.3% and reached the highest DCW of 4.9 g/L from *Bacillus megaterium* using acid-catalysed WH biomass while *Pseudomonas aeruginosa* accumulated up to 65.51%(w/w) of PHB with 4.2 g/L of biomass at 72 h when cultivated in WH medium (Preethi et al., 2015). Upadhayay et. al (2019) suggested that the optimum substrate concentration of WWH at 10%(v/v) could produce the highest PHB of 132 µg/mL, the further increase substrate concentration resulted in a decrease of PHB content. Finally, the monomer compositions in the extracted PHA produced from *P. mendocina* PSU when grown in WWH as a sole carbon source found that only 3-hydroxybutyrate (3HB) was detected. These may due to this bacteria has *phbC* gene encoding for SCL-PHA synthase that prefers three to five carbon substrates such as 3HB(Matsusaki et al.1998; Chanasit et al. 2016).

Conclusions

After water hyacinth hydrolysate (WHH) was pretreated by alkaline pretreatment in steam exploded and enzymatic hydrolysis for breaking down complex sugars in WHH to easily fermentable reducing sugars, the highest reducing sugar concentration of 25.2 g/L was obtained in 2%(v/v) NaOH followed by hydrolyzed with cellulase, then the pretreated 1%(v/v) of TRS in WHH was used as a low cost carbon substrate for PHAs production from *Pseudomonas mendocina* PSU. A maximum biomass concentration of approximately 3.86 g/L of DCW and PHA content about 43%DCW were produced when grown the cells in MSM supplemented with 1%(v/v) TRS in WHH. Moreover, the PHAs yield and volumetric productivity was found to be 0.18 g/g and 0.035 g/L/h, respectively. Finally, the produced PHAs from *P. mendocina* PSU when grown in WWH was then characterized and the results revealed that it contained only 3HB. Therefore, the water hyacinth hydrolysate seems to be a potential substrate for PHAs production which was supposed to reduce the PHAs production cost and at the same time may able to minimize the waste disposal problem.

Recommendations

The nutritive parameters of the water hyacinth leaves and the sugar compositions in hydrolysate will be determined. In addition, the optimal substrate concentration e.g. C/N ratios for bacterial growth and PHA production will be further investigated to obtain the higher PHA biosynthesis.

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