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Nutritional factors influence polyhydroxybutyrate in microalgae growing on palm oil mill effluent

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Abstract
Bioplastic made of microalgae is attractive since it does not consume high amounts of substrate compared to bacteria. However, to make the production more feasible, growth needs to be done in wastewater to replace the commercial medium. This research was done to study the ability of microalgae growing on palm oil mill effluent (POME) to produce a bioplastic, polyhydroxybutirate (PHB). The experiment was conducted in a three step approach: (i) screening the microalgae strain, (ii) investigating a different kind of organic carbon source, and (iii) investigating the interactive effect of Fe-EDTA, POME fractions, and organic carbon concentrations. Results showed that *Botryococcus braunii* produced the highest PHB content compared to *Arthrospira platensis* and *Haematococcus pluvialis*. Glucose and glycerol stimulated the PHB productivity up to 20 mg L^{-1} day^{-1}. The interaction of glycerol-POME and glycerol Fe-EDTA influenced the growth. For PHB accumulation, no interaction between factors was found. The optimal condition to obtain 33% PHB was found on 50% POME supplemented with high glycerol and high Fe-EDTA concentration.

Keywords POME; Microalgae · PHB · *Botryococcus braunii* · Mixotrophic · Glycerol · Fe-EDTA

Introduction

Bioplastic made from microalgae has attracted researchers since it does not consume high amounts of substrate like glucose, while bioplastic produced from bacteria is still costly due to its high fermentation cost (Costa et al. 2019). Microalgae can produce bioplastic by using carbon dioxide and light in autotrophic condition (Yashavanth et al. 2021). However, the production of bioplastic from microalgae still costly compared to conventional plastic, due to low productivity and high energy demand in the downstream process. By using wastewater medium for growth, it is estimated that the production cost of bioplastic from microalgae could be reduced.

Indonesia is the largest crude palm oil (CPO) producer worldwide. However, high CPO production generates high byproducts such as palm oil mill effluent (POME) which threatens the environment due to its high chemical and biological oxygen demand. Each tonne of palm oil produces 50% of palm oil mill effluent (POME). Digested POME contains high contents of organic acids and micronutrients which are suitable as a medium growth for microalgae (Nur and Buma 2019).

Bioplastic from microalgae is mostly in the form of the polyhydroxyalkanoate, polyhydroxybutyrate (PHB), which is used as storing energy when the growth is disturbed due to unfavorable environmental and nutritional conditions. Previous studies have found that microalgae grown in mixotrophic condition could produce higher levels of bioplastic, by utilizing organic and inorganic substrates in the presence of light (Afreen et al. 2021). Environmental and nutritional conditions influenced the production of PHB in microalgae (Onen et al. 2020). Nutritional conditions such as nutrient depletion, and organic carbon addition increased the PHB accumulation in some microalgae strains. Not only cyanobacteria such as *Nostoc* and *Arthrospira* can accumulate PHB, but also green algae such as *Haematococcus* and also *Bortyococcus braunii* (Kavitha et al. 2016a; Abdo and Ali 2019) . In this study, we investigated and compared the potency of *Arthrospira platensis*, *Haematococcus pluvialis*, and *Botryococcus braunii* growing on POME fractions in varied nutritional conditions and investigated the interactive

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effect of the selected factors by using factorial design to the growth, PHB content, and PHB productivity.

Materials and methods

Material pretreatment

Sample POME was collected from a small factory in Sumatra after being discharged from an open facultative aerobic treatment pond. POME was filtered using GF/C filters to remove particles. The filtrate was then sterilized at 121 °C for 15 min. POME was stored in a freezer to prevent degradation. Sample POME was analyzed as according to Nur et al. (2019). Based on the analysis, COD of POME was 1230 mg L^{-1}, total dissolved nitrogen was 230 mg L^{-1}, and total dissolved phosphate was 20 mg L^{-1}.

Experimental setup

Stock cultures of *Arthrospira platensis* (SAG 21.99) were grown on Zarrouk medium (Zarrouk 1966) with salinity adjusted to 15 PSU using commercial salt. For *Haematococcus pluvialis* (SAG 49.94), and *Botryococcus braunii* (SAG 807–1), the cultures were grown in BG-11 medium (Rippka et al. 1979) at salinity 1 PSU. The cultures were adapted to the experimental conditions for at least two weeks. The cultures were cultured in a 12:12 light to dark cycle. The culture medium was diluted with fresh medium if growth reached the stationary phase. Three experiments were carried out. In experiment 1, the best strain was screened. In experiment 2, the best organic carbon source was selected. In experiment 3, the optimal conditions of organic carbon concentration, POME fractions, and iron-EDTA were obtained.

Screening microalgae

The algae samples were grown on a 50 mL culture flask with a working volume of 40 mL. The was algae were cultured on 30% POME and mixed with commercial medium (BG-11 or Zarrouk medium) and inoculated using 2% of the strain (initial optical density 0.01 at 680 nm). The salinity was adjusted to the optimal condition using commercial salt and pH was adjusted to the optimal condition with 1 N NaOH or 1 N HCl. The culture flasks were placed in a chamber equipped with a water bath and lamps. The temperature was set to 30 °C, and light intensity was set to 70 µmol photons m^{-2} s^{-1} at the surface of the culture flasks. Each day, the sample was manually stirred to mix the culture medium. The growth of the sample was monitored by using a spectrophotometer (Perkin Elmer MBA 2000). Two milliliters of sample medium was centrifuged at 4000×*g* for 10 min. The supernatant was discarded and 2 mL milli-Q (1% NaCl) was added and the mixture vortexed for 10 s. The absorbance of the sample was measured at 680 nm. The cultivation was stopped at the end of the exponential stage. At the end of the cultivation, the medium was collected and harvested by using centrifugation at 4000×*g* for 15 min to measure PBH content of the final biomass.

Effect of different organic source addition on growth and PHB accumulation

The best strain obtained from experiment 1 (*Botryococcus braunii*) was grown on 30% POME mixed with BG-11 medium. The cultures were placed in 50 mL culture flasks (Grenier bio one) with 40 mL working volume. The sample was inoculated with 2% of the strain (initial optical density 0.01 at 680 nm) and adjusted the salinity to 1 PSU by using commercial salt and pH to 7.5 by using 0.1 N NaOH or HCl. Organic carbon with different sources (sodium acetate, D-glucose, glycerol) was added with the concentration of 1 g L^{-1}. The culture flasks were placed in a chamber equipped with a water bath and lamps. The temperature was set to 30 °C, and light intensity was set to 70 µmol photons m^{-2} s^{-1} at the surface of the culture flasks. Each day, the culture was manually shaken to mix the culture medium. At the end of the exponential stage, the cultures were harvested by centrifuging at 4000×*g* in 15 min to measure PBH of the final biomass as explained below (the "Analyses" section).

Effects of Fe-EDTA, organic carbon, and POME on growth and PHB accumulation

The effect of Fe-EDTA, organic carbon, and POME was evaluated by using factorial design. The best strain obtained from experiment 1 (*Botryococcus braunii*) was grown on different POME fractions and mixed with BG-11 medium. The cultures were placed in 50 mL culture flasks (Grenier bio one) with 40 mL working volume. The sample was inoculated with 2% of the strain (initial optical density 0.01 at 680 nm) and adjusted the salinity to 1 PSU by using a commercial salt and pH to 7.5 by using 0.1 N NaOH or HCl. The best organic carbon source obtained from experiment 2 (glycerol) was added with the concentration of 1 to 3 g L^{-1}, Fe-EDTA 5 to 10 mg L^{-1}, and POME fraction from 30 to 50%. The cultures were grown and the cells harvested as outlined in the "Effect of different organic source addition on growth and PHB accumulation" section.

Analyses

Biomass and growth rate

The growth of microalgae was monitored indirectly by measuring the absorbance at 680 nm. The correlation between absorbance and dry biomass was determined by diluting the

microalgae culture using Milli-Q by five serial dilutions. The samples were harvested using GF/C filter paper and dried at 70 °C until constant weight. The biomass was determined by gravimetry. The growth rate of the microalgae was calculated from the linear regression of the natural logarithm of the optical density versus time.

PHB analysis

The culture sample (15 mL) was centrifuged at 4000 × *g* for 30 min. 5 mL of NaCOl (10% v/v) was immediately added to the wet pellet and sonicated at 40 °C for 30 min. The sample was centrifuged at 4000 × *g* for 15 min, the supernatant was discarded, and the pellet was used to determine PHB. The sample pellet was placed in a small glass tube and 3 mL concentrated H_2SO_4 added. After heating in a water bath at 100 °C for 15 min to convert PHB into crotonic acid, the absorbance was measured at 245 nm. A correlation between crotonic acid and PHB concentration was introduced as demonstrated previously (Rajankar et al. 2018).

The productivity of PHB was calculated based on Eq. 1.

$$PPHB = CPHB(P_t - P_0)/(t_x - t_0) \quad (1)$$

where P_{PHB} is the productivity of PHB (mg L^{-1} day^{-1}), C_{PHB} is PHB content, P_t is biomass at time t_x, and P_0 is biomass at time t_0.

Statistical analysis

Minitab ver 18 (demo version) was used for statistical analysis. Differences between treatments were analyzed with analysis of variance (ANOVA) with a *P* value of 0.05. The experimental results were obtained for a minimum of two replicates and expressed as averages and standard deviations (SD).

Results

Strain selection

Two green algae (*H. pluvialis,* and *B. braunii*) and a cyanobacterium (*A. platensis*) were grown on commercial media. The highest final biomass was found on *A. platensis* ($P < 0.05$), while the lowest final biomass was found on *B.braunii* which resulted 66.07 mg L^{-1} ($P < 0.05$) (Table 1). *Haematococcus pluvialis* produced 172.7 mg L^{-1} which was 1.2-fold times lower compared to *A. platensis*. For the PHB content to dry weight, the highest content was found on *B.braunii* which accumulated PHB up to 17.45% ($P < 0.05$). *Haemartococcus pluvialis* and *A. platensis* only produced 3.83, and 0.57% PHB, respectively.

When 30% POME was supplemented to the commercial medium (BG-11 or Zarrouk), the final biomass was enhanced for *A. platensis* only ($P < 0.05$). The final biomass of *B. braunii* and *H. pluvialis* was reduced to 47 and 137 mg L^{-1}, respectively.

Effect of organic carbon sources

To investigate the effect of mixotrophic condition, different organic carbon sources were added to BG-11 supplemented with 30% of POME. *Botryococcus braunii* was selected as the test strain since it had the highest PHB content (Table 1). The addition of glucose and glycerol enhanced the specific growth rate up to 0.5 day^{-1}, which is almost 1.3-fold times higher than the control without the addition of the external organic carbon source. The addition of Na-acetate did not influence the growth ($P = 0.065$) (Table 2).

The addition of organic carbon sources (glucose, glycerol, and Na-acetate) significantly enhanced PHB content and productivity compared to control medium ($P < 0.05$). The addition of glucose and glycerol produced higher PHB productivity, a fourfold increase, compared to the control medium, which only produced 5.32 mg L^{-1} day^{-1}. The addition of Na-acetate produced lower PHB productivity compared to glucose and glycerol, but this difference was not significant ($P = 0.064$) (Table 2).

Interaction effect of iron-EDTA, organic carbon, and POME

Factorial design was done to understand the interaction of iron (Fe-EDTA), glycerol, and POME fractions to PHB accumulation and growth rate. Figure 1a and Fig. 1b show

Table 1 Biomass and PHB production from various strain in control and with the addition of 30% POME. Average values are shown. SD is shown after ± symbol. Values that do not share a letter in the same column are significantly different ($P < 0.05$)

Strain	Control media				30% POME + media			
	Final biomass (mg L^{-1})		PHB content (%)		Final biomass (mg L^{-1})		PHB content (%)	
B. braunii	66.07	±5.50[a]	17.45[a]	±0.95	47.29[a]	±5.73	21.50[a]	±1.50
H. pluvialis	172.70	±24.50[b]	3.83[b]	±0.41	137.53[b]	±5.70	6.25[b]	±0.95
A. platensis	211.75	±36.00[c]	0.57[c]	±0.33	269.07[c]	±0.90	0.80[c]	±0.40

Table 2 Effect of different organic source on growth rate, PHB content, and PHB productivity of *B.braunii* growing on 30% POME + BG-11 (without Fe-EDTA). Average values are shown. SD is shown after ± symbol. Values that do not share a letter in the same column are significantly different ($P < 0.05$)

Organic carbon	Growth rate (day^{-1})		PHB content (%)		PHB productivity (mg L^{-1} day^{-1})	
D-glucose	0.49	$\pm 0.02^{a}$	26.97	$\pm 0.41^{a}$	20.25	±0.51a
Glycerol	0.48	$\pm 0.01^{a}$	25.97	$\pm 0.50^{a}$	19.17	±0.64a
Na-acetate	0.41	$\pm 0.01^{b}$	24.20	$\pm 0.08^{b}$	12.72	±1.00a
Control POME	0.38	$\pm 0.01^{b}$	21.50	$\pm 2.12^{b}$	5.32	±0.53b

the significance of the effects on growth rate and PHB content, respectively. The interaction of iron, glycerol, and POME fraction is the most influencing factor for growth ($P < 0.05$), followed by the interaction of iron and glycerol, the interaction of POME and glycerol, and glycerol alone (Fig. 1a). When high glycerol and low iron were supplemented to the medium, growth rate could reach up to 0.5 day^{-1}. High glycerol and high POME fraction (50%) also enhanced the growth rate up to 0.5 day^{-1} (Fig. 2).

For PHB accumulation, iron is the most influencing factor, followed by glycerol addition (Fig. 1b). The addition of iron (10 mg L^{-1}) to the medium enhanced the PHB accumulation up to 33% when using 30% POME and 2 g L^{-1} glycerol (Fig. 3). The addition of glycerol (3 g L^{-1}) enhanced the PHB accumulation to 35% when using 10 mg L^{-1} iron and 40% POME. The addition of POME fraction alone did not significantly enhance PHB accumulation ($P = 0.072$).

To obtain high glycerol and PHB content, the nutritional condition should be set to 10 mg L^{-1} Fe-EDTA, 3 g L^{-1} glycerol, and 50% POME to obtain 33% PHB and growth rate up to 0.58 day^{-1} (Supplementary 1). The results were higher compared to the addition of D-glucose to 30% POME fractions (without the addition of Fe-EDTA) which resulted 27% PHB content, and growth rate 0.49 day^{-1} (Table 2).

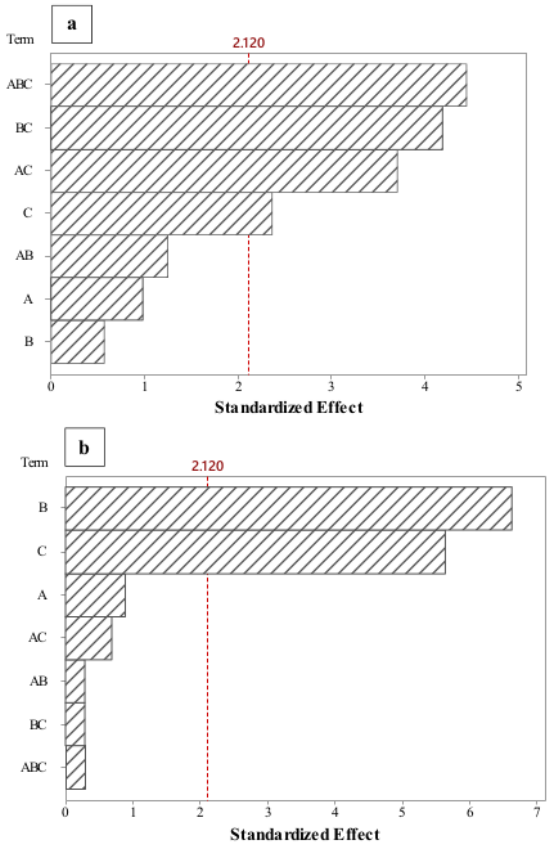

Fig. 1 Pareto chart of **a** growth rate, and **b** PHB content of *Botryococcus braunii*. A is POME fraction (%), B is Fe-EDTA concentration (mg L^{-1}), C is glycerol concentration (mg L^{-1})

Discussion

It is well known that *B. braunii* has a low growth rate compared to other green algae (Zhang et al. 2011), while *H. pluvialis* could grow well on optimized medium (Fábregas et al. 2000). Previous research reported that *B. braunii* could accumulate PHB up to 20% by using Chu-13 medium (Kavitha et al. 2016a). Furthermore, *B. braunii* could accumulate a high hydrocarbon content on the surface of the cells in a matrix composed of aldehyde polymers (Weiss et al. 2010). Furthermore, it is reported that the synthesis of PHB in microalgae utilizes energy which is stored as lipid or carbohydrate (Costa et al. 2018).

In this study, the addition of POME to commercial medium enhanced the biomass of *A. platensis* only. This indicated that *A. platensis* could tolerate the phenolic compounds found in POME (Nur et al. 2021) as well as utilize the ammonium in the high POME fraction (Nur et al. 2019). The addition of POME to the medium enhanced PHB content for all strains ($P < 0.05$). This indicated that POME has a positive effect on PHB accumulation. Kavitha et al. (2016b) reported the accumulation of PHB was enhanced when sewage wastewater was supplemented to the medium

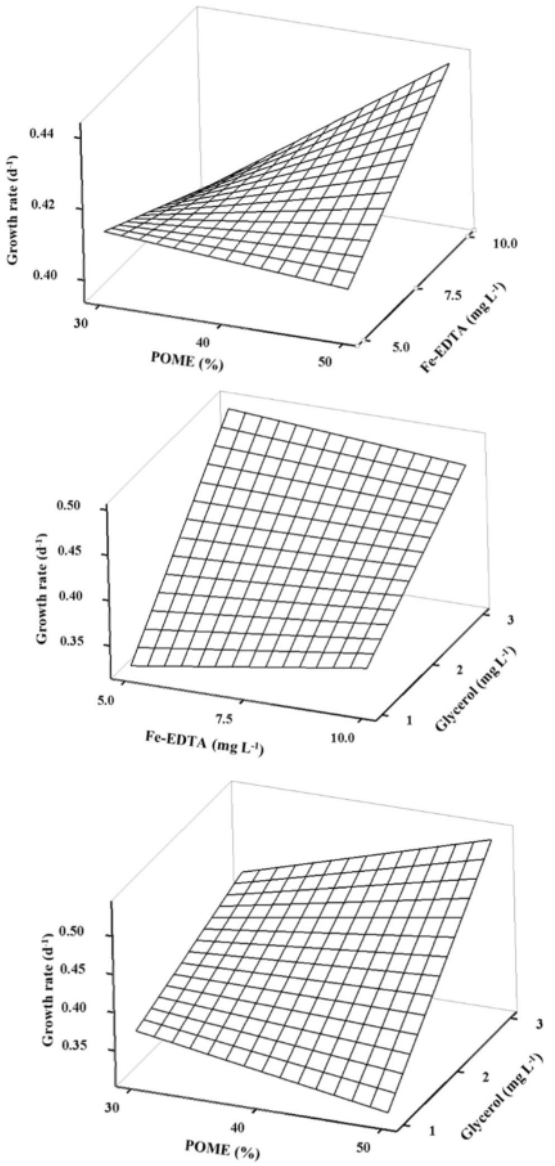

Fig. 2 Response surface showing the effect of POME, glycerol, and Fe-EDTA addition on growth rate of *Botryococcus braunii*

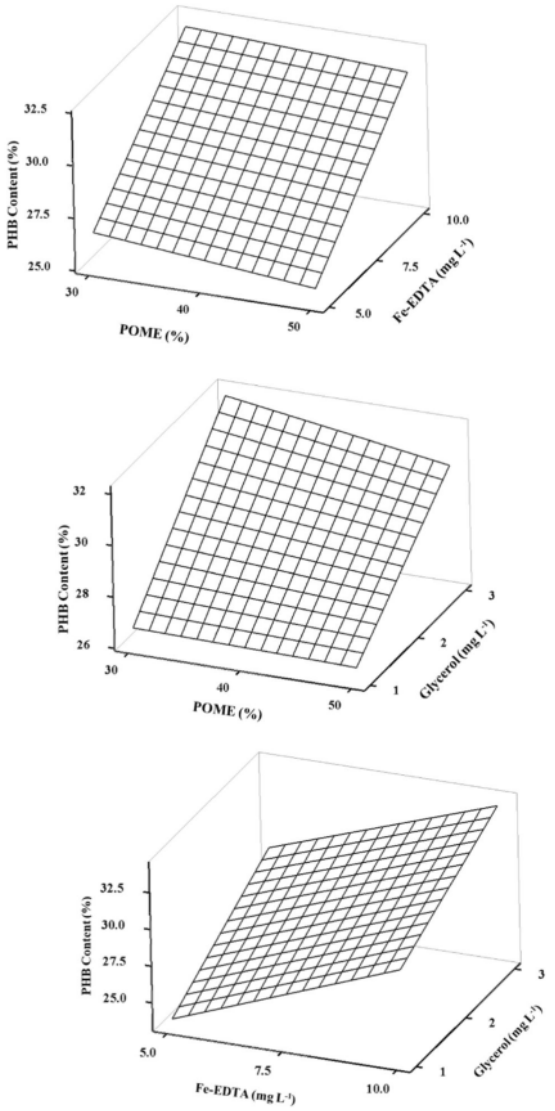

Fig. 3 Response surface showing the effect of POME, glycerol, and Fe-EDTA addition on PHB content of *Botryococcus braunii*

resulting in 247 mg L^{-1} PHB. Some wastewaters contain organic carbon sources which could be utilized by microalgae mixotrophically. Sharma and Mallick (2005) reported that in mixotrophic condition, the PHB content of *Nostoc muscorum* could be improved up to 35% by adding 0.4% glucose and acetate as organic carbon sources. As reported previously, digested POME contains organic acids which could be utilized by microalgae for the growth in mixotrophic condition (Nur et al. 2021).

In this research, the addition of external organic carbon (glucose, glycero, Na-acetate) to the medium significantly enhanced PHB accumulation. The control medium consisting of BG-11 supplemented with 30% POME only (without the addition of organic carbon) produced the lowest PHB accumulation. Previous studies also have found that lipid accumulation of *B. braunii* was high under mixotrophic condition, which is associated to PHB accumulation (Yeesang

and Cheirsilp 2014; Costa et al. 2018). When an external organic source was added, *B. braunii* seems to utilize the substrate and accumulate PHB due to excess of energy provided by this source.

Here the addition of glycerol did not differ significantly compared to glucose. Choi and Yu (2015) found that the addition of glycerol could enhance growth rate and lipid of *B. braunii*. However, Corrêa and Teixeira (2021) found that the addition of glycerol in xenic *A. platensis* culture enhanced final biomass and PHB accumulation. Glycerol was selected as the source of organic carbon for further investigation since the price is lower compared to glucose.

In the third experiment, the interactive effect of iron, POME, and glycerol concentration was investigated showing that the addition of iron has a positive effect on PHB accumulation. This finding agrees with García et al. (2021) who found that the addition of iron to BG11 medium has a positive effect on PHB accumulation in *Scenedesmus* sp. which accumulated PHB up to 30%, while in normal conditions, the PHB content was around 10%. Iron plays important role in microalgal cell metabolism. When Fe-EDTA was presented for *Dunaliella tertiolecta*, the cells could accumulate higher lipid to store it as energy stock (Rizwan et al. 2017). As mentioned previously, PHB synthesis requires organic carbon which is stored as lipid in the cells (Costa et al. 2018).

In this experiment, POME fractions did not statistically influence PHB accumulation. Higher POME contributes to higher nitrogen, phosphorus, and organic carbon. Since carbon, nitrogen, and phosphorus ratio did not change in POME fractions, thus, it did not contribute to nitrogen or phosphorus limitation. This indicated that *B. braunii* could still accumulate high PHB content when glycerol was present. By modifying simple nutrients, the carbon-to-nitrogen ratio in POME could be changed, while the addition of iron could stimulate higher PHB content. By using higher POME fraction, the cost of commercial medium (BG-11) could be reduced.

Conclusions

Growing microalgae on POME is a potential strategy to lower the production cost of bioplastic. By screening three strains it was found that *B. braunii* had the highest PHB content compared to *A. platensis* and *H. pluvialis*. This work, for the first time, reported the potential of *B. braunii* growing on POME mixotrophically. D-glucose and glycerol are potential organic carbon sources for microalgae to enhance growth rate, PHB accumulation, and PHB productivity. Based on factorial design, the interaction of POME-glycerol and glycerol-Fe EDTA influenced the growth rate, while glycerol and Fe-EDTA enhance the PHB content. The optimal condition determined was 10 mg L^{-1} Fe-EDTA, 3 g L^{-1} glycerol, and 50% POME to obtain 33% PHB and growth rate up to 0.58 day^{-1}.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-021-02654-2.

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Data availability The data generated during and/or analyzed during the current study is available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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