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Polyhydroxybutyrate (PHB) production by *Alcaligenes eutrophus* NCIMB 11599 from low-cost substrate as carbon source

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Abstract

Poly-3-hydroxybutyrate (PHB), homopolymers of hydroxybutarate, are wildly applied as biodegradable substitutes of the conventional petrochemical-based plastic due to its similar properties to polypropylene. The problem of the commercial application of PHB is its high production cost due to the cost of substrate (mainly carbon source). In this work, the effect of different cheap carbon sources (coconut juice, sugar cane juice, palmyra palm sugar, brown sugar, palm sugar and pineapple peel juice) on the production of PHB was assessed. *Alcaligenes eutrophus* NCIMB 11599 was cultivated in 250 ml Erlenmeyer flasks at 37^{o}C, 120 rpm for 24 h. Plam sugar and brown sugar showed the high potential for PHB synthesis with the PHB content at 1.93 and 1.90 %, respectively. The effect of carbon and nitrogen sources on PHB production was also investigated. The maximum PHB content (5%) was obtained from 2 g/l palm sugar but ammonium sulphate was not added in the medium. A maximum specific growth rate (0.175 h^{-1}) and PHB productivity (0.0035 g/(l•h)) were calculated in a 5 l fermenter.

Keywords: PHB, PHAs

1. Introduction

In the last decade, synthetic non-biodegradable polymers produced from petrochemical sources are an essential part of our everyday life due to their excellent physical and chemical properties. It replaces glass and paper in packing. However, accumulation of non-biodegradable plastics in the environment at the rate of 25 million tons per year (1) has become a large problem. Biodegradable plastics such as different kinds of polysaccharides, polyester-polyhydroxyalcanoates (PHAs), polylactide and aliphatic polyester offer the best solution to the environmental problem. PHAs, the only 100% biodegradable plastics, are synthesized and accumulated by several microorganisms as a carbon and energy material under unbalanced growth condition (2). Polyhydroxybutyrate (PHB) from *Bacillus megaterium*, a homopolymer of β-hydroxybutyrate monomer unit, was the first PHA

discovered by a French microbiologist M. Lemoignein 1962. Its physical properties is similar to polypropylene (PP) and also could be used in a wide range of application including the packing industry, food industry, medicine, pharmacy and agriculture. Nevertheless, an industrial production of PHB has been hampered due to PHB are 5-10 times more expensive than petrochemical plastic. The high production cost of PHB (up to 45%) is mainly from the cost of raw material (3-5). Thus, several scientists have studied to use low cost substrates based on agro-industrial wastes and by-products such as starch (6), molasses (7), methanol (8), whey (9) or glycerol (10) in order to reduce this cost. Therefore, the aim of this study was to investigate the effect of different cheap carbon sources on the production of PHB.

2. Material and Methods

2.1 Microoganisms and culture condition

A PHB producer *Acaligenes eutrophus* NCIMB 11599 was used in this study. Culture of *A. eutrophus* was maintained on slant of nutrient agar (NA) at 4 ^{o}C. Prior to each fermentation, a single colony of *A. eutrophus* was transferred from NA slant to 250 mL Erlenmeyer flask containing 50 mL sterile nutrient broth (NB). Flasks were then incubated at 37 ^{o}C and 120 rpm for 24 h and used for inoculation of PHB production and 10% (v/v) of culture was added into a production medium.

A mineral salt (MS) medium (11) was used for the production of PHB. The medium contained in g/l: 1.0 $(NH_4)_2SO_4$, 1.5 KH_2PO_4, 9.0 $Na_2HPO_4 \cdot 12H_2O$, 0.2 $MgSO_4 \cdot 7H_2O$ and 1.0 ml of trace element solution. Each liter of trace element solution contained (g): 2.25 $ZnSO_4 \cdot 7H_2O$, 2.0 $CaCl_2 \cdot H_2O$, 10.0 $FeSO_4 \cdot 7H_2O$, 0.1 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.0 $CuSO_4 \cdot 5H_2O$, 0.5 $MnSO_4 \cdot 5H_2O$ and 10.0 ml of 35% HCl After inoculation, flasks were incubated at 37 ^{o}C for 24 h on a rotary shaker at 120 rpm. In order to evaluate the effect of different cheap carbon sources on the production of PHB, a variety of carbon sources with 2 g/l total sugar were added to the MS medium (as describe above). To investigate the effect of carbon sources concentration upon PHB concentration, different carbon source concentration was supplemented. The effects of carbon to nitrogen concentration ratio were also investigated. The ranges of ammonium sulphate concentration (0-2 g/l) were added to the medium. Samples were analyzed for cell dry weight, total sugar, PHB concentration and PHB content. All the experiments were carried out in duplicate and the average values are reported.

2.2 Fermentation study

In order to study the growth curve and PHB accumulation during the fermentation time, batch fermentation was carried out in a 5 l (Biostat B. Plus) fermenter with a working volume of 3 l. The ferment mineral salt (FMS) medium (12) consists of (g/l): 2 palm sugar, 4.0 $(NH_4)_2SO_4$, 13.3 KH_2PO_4, 1.2 $MgSO_4 \cdot 7H_2O$, 1.7 citric acid and 10.0 ml of trace element solution. The temperature and pH were controlled at 37 ^{o}C and 7.0, respectively. Air flow rate and agitation speed in the fermenter were set at 1.0 vvm and 120 rpm, respectively. Samples were withdraw at time interval and were used for analytical method.

2.3 Analytical method

The culture broth was centrifuged at 4,000 rpm for 15 min. The cell growth was determined by measuring the absorbance with a spectrophotometer at 600 nm. The cell dry weight (CDW) was calculated from the standard curve between absorbance against CDW.

$Y = 0.132X$

Where, Y is OD 600 nm, X is dry cell weight (g/l).

The supernatant that was obtained by centrifugation was used for total sugar analysis. Total sugar was assayed by the Phenol-H_2SO_4 method (13). Biomass included PHB was broken using 1 ml NaOCl and incubated at 37 ^{o}C for 1 h. The mixture was added with 4 ml distilled water. The release PHB was extracted into 5 ml chloroform at 100 ^{o}C for 30 second. PHB was recov-

ered after chloroform evaporation and the precipitated form was used to analyse the PHB concentration by adding 3 ml of concentrated H_2SO_4 and heated in a boiling water bath for 10 min to oxidize PHB to chrotonic acid and cooled. The chrotonic acid formed was quantified by measuring the absorbance at 235 nm using a spectrophotometer (14). The PHB concentration was calculated by comparing with the standard curve using PHB standard from Sigma. The PHB content was defined as the percentage of PHB mass in cell dry weight.

3. Results and Discussion

Six different carbon sources based on the local availability and cheap cost (coconut juice, sugar cane juice, palmyra palm sugar, brown sugar, palm sugar and pineapple peel juice) were used for the PHB accumulation by *A.eutrophus*. Palm sugar was the best carbon sources for PHB production with PHB content of 1.93% and followed by brown sugar with PHB content of 1.90%. However, the PHB content of 1.58, 1.04, 0.64, 0.572 and 1.48% were obtained from coconut juice, pineapple peel juice, palmyra palm sugar, sugar cane juice and glucose, respectively (Figure 1). Therefore, brown sugar and palm sugar were used as carbon sources for further studies.

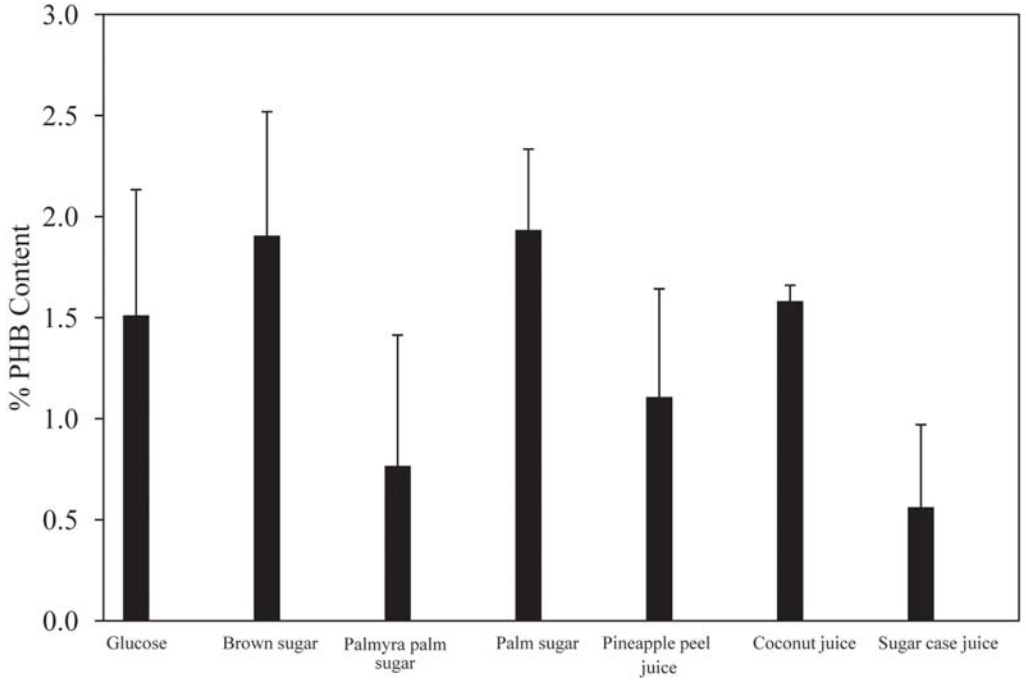

Figure 1. Effect of different carbon sources on PHB synthesis by *A. eutrophus* NCIMB 11599 grown on MS medium in a rotary shaker at 120 rpm and 37 ^{o}C for 24 h.

The influence of carbon sources on the production of PHB by *A. eutrophus* was reported by Linko et. al. (15). They indicated that fructose was the best carbon source. Khanna (16) obtained the maximum PHB concentration (1.4 g/l) by *Rastonia eutropha* NRRL B14690 with fructose as a carbon source. Kim et. al. (17) reported the maximum accumulation of PHB when glucose was used as suitable source by *A. eutrophus*. Arun et. al. (18) also reported PHB production by *A. eutrophus* MTCC 1285 using various industrial waste (malts, soya, sesame, molasses, bagasse and pharmaceutical waste) as carbon source. Maximum PHB production was observed in 40% sesame oil medium. *Bacillus megaterium* produced PHB in the cheap carbon source (date syrup and beet molasses) (19). Similar result was reported by Kulpreecha et al. (20). Cheap carbon source (molasses) was able to produce PHB by *B.megaterium* BA-019. Lactose, a waste product from cheese industry, was an economic carbon source for PHB production by *A. eutrophus* H16 (9).

Various concentration of carbon sources (1-8 g/l) were added to the MS medium. The results indicated that the amount of carbon source affected the PHB production (Figure 2). The maximum PHB content was obtained from 2 g/l palm sugar (2.28%) and followed by 1 g/l brown sugar (2.25%). Therefore, 2 g/l palm sugar was selected for further experiments.

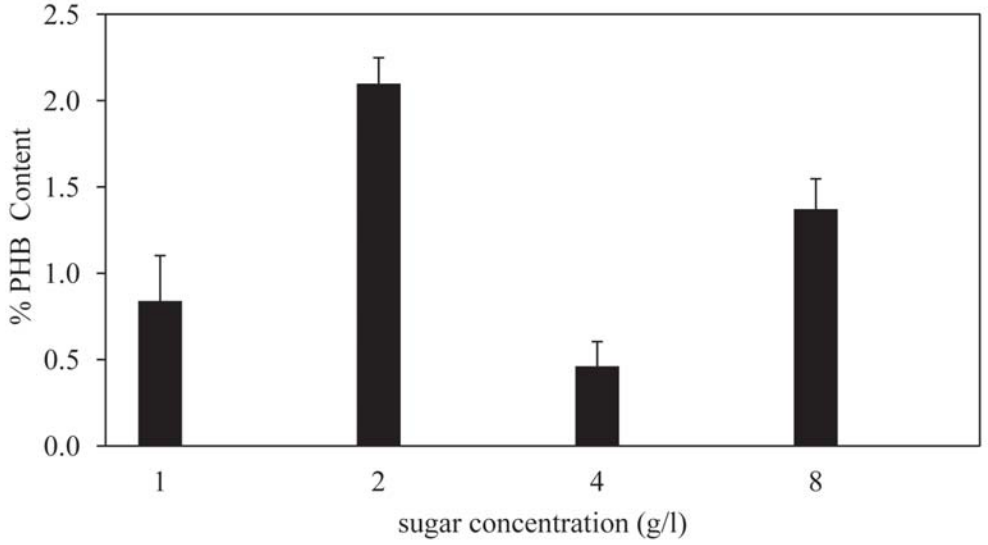

Figure 2. The effects of palm sugar concentration on the PHB production by *A. eutrophus* NCIMB 11599 grown on MS medium in a rotary shaker at 120 rpm and 37 ^{o}C for 24 h.

Increasing the amount of carbon source concentration significantly increased the cell growth. Gouda (7) reported the effect of different sugar cane molasses indicating that the maximum PHB production by *B.megaterium* was obtained from 2% molasses. Beauliet et. al. (21) also pointed out the influence of cane molasses concentration on the production of PHB. *Azotobacter vinelandii* UWD exhibited the maximum PHB with 5% can molasses.

The ratio of carbon to nitrogrn concentration is also an important factor of the accumulation of PHB. Thus, the effect of carbon to nitrogen concentration ratio was also assessed. The ranges of ammonium sulphate concentration (0-2 g/l) were supplemented to the MS medium with 2 g/l palm sugar (ratio of carbon to nitrogen concentration (w/w); 2:0, 2:0.5, 2:1 and 2:2). Imbalanced growth conditions, the limitation of nutrients such as nitrogen, phosphate, or oxygen in the presence of an excess of carbon source, are desirable for PHB biosynthesis and

accumulation (2). The best PHB content (5%) was obtained when ammonium sulphate was not added in the medium (Figure 3). Higher carbon to nitrogen concentration ratio would promote the production of PHB.

Figure 4 shows the time course of batch experiment with respect to biomass and PHB production in a Biostat B. Plus fermenter. *A. eutrophus* growth increased steadily and attained its maximum value (1.02 g/l) within 24 h of fermentation. During the growth, PHB was synthesized and accumulated in the cell. PHB accumulation reached the maximum of 0.09 g/l with PHB content of 12% at 26 h of growth. The cell and PHB yield were 0.5 g-CDW/ g-sugar consumed and 0.045 g-PHB/g- sugar consumed, respectively. The kinetics parameters, a maximum specific growth rate and PHB productivity were 0.175 h^{-1} and 0.0035 g/(l•h), respectively.

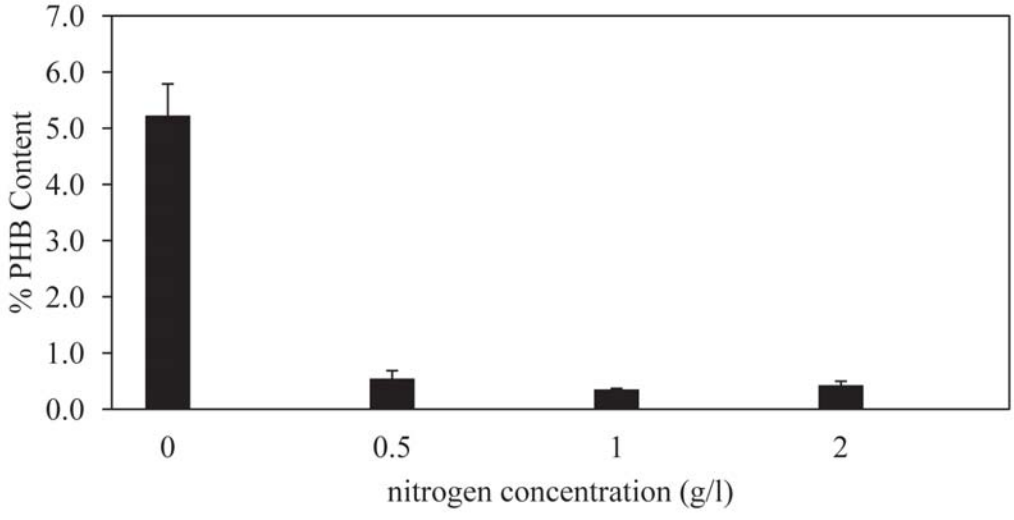

Figure 3. The effects of nitrogen concentration on the PHB production by *A. eutrophus* NCIMB 11599 grown on MS medium in a rotary shaker at 120 rpm and 37 ^{o}C for 24 h.

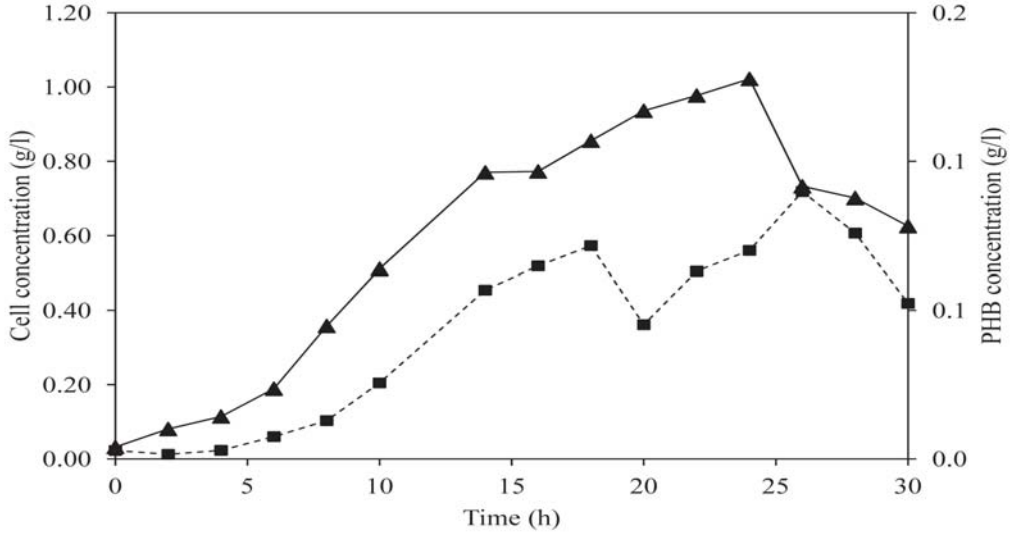

Figure 4. Growth and PHB production by *A. eutrophus* NCIMB 11599 in a Biostat B. Plus fermentor. (●) cell concentration, (■) PHB concentration

4. Conclusion

A. eutrophus NCIMB 11599 can be used as a low cost substrate for the production of PHB. This result indicated the possible way to reduce the PHB production cost. Further work on different cultivation strategies to improve the PHB production will be investigated.

5. Acknowledgement

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