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Isolation and production of polyhydroxybutyrate (PHB) from isolated strain *Bacillus* sp. using crude glycerol as a carbon source

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Abstract

Polyhydroxybutyrate (PHB) is polyester produced by a range of microbes under unfavorable growth conditions and stored as an intracellular carbon and energy material. PHB production is more expensive than petrochemical polymer production. The main production cost is the cost of carbon substrate. The aim of this study is to produce PHB using a cheap carbon source, crude glycerol, which is a major byproduct in the biodiesel manufacturing process. PHB producing bacteria species were isolated from the soil collected around biodiesel plants and screened by Nile Red staining method. The effects of sources and concentrations of crude glycerol on the biosynthesis of PHB were investigated. The maximum PHB content obtained after 24 hours of batch cultivation was 23.59 % of cell dry weight at initial crude glycerol of 10 g/L and C:N ratio of 1:0.4.
Keywords : *Bacillus*, biodiesel, crude glycerol, polyhydroxybutyrate, production

1. Introduction

Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic, there are a class of bacterial polyesters collectively called polyhydroxyalkanoates (PHAs), accumulated intracellularly as reserve granules by many bacteria in harsh environmental conditions. PHB is synthesized as an intracellular storage material and accumulates as distinct white granules during unbalanced growth in the cell, these are clearly visible in the cytoplasm of the cell. Many bacteria including those in the soil, are capable of PHB production and breakdown. These biodegradable polyesters display a special interest due to their possible use as substitutes of common plastics because they are completely degraded by the microorganisms present in the environment and they can be produced from regenerable carbon sources.

The main by-product of biodiesel production is crude glycerol, generated from the transesterification of vegetable or animal fats and oils. For an annual production of 150 million gallons per year, an amount of 50 million kg of crude glycerol is generated. Crude glycerol from biodiesel has a relativly low value due to the presence of impurities (João et al., 2009). Crude glycerol principally consists of residual ethanol or methanol, glycerol, fatty acid ethyl (or methyl) esters, and residual fatty acids. In regard to converting this resource to a commodity, these carbon sources are direct precursors for the bacterial synthesis of PHAs (Zachary et al., 2011). PHAs can be produced from renewable resources through a fermentation process under restricted growth condition for nitrogen, phosphorus, sulfur and/or oxygen in the presence of an excess carbon source, and they can also be completely biodegraded by many microorganisms. PHB were the first types of PHAs discovered and the most widely studied. PHB has similar properties to conventional plastics like polypropylene or polyethylene, and it can be extruded, molded, spun into fibers, made into films, and used to make heteropolymers with other synthetic polymers (John et al., 2011), but its production costs are higher than those of petrochemical plastics. The carbon source could account for 25-45% of the total production cost (Javier et al., 2013). Wider use of PHB requires a less expensive product; hence, the improvements of fermentation strategies, low-cost media, and easier downstream recovery methods are needed (Enrico et al., 1999). The study focused on the producing of PHB granules by strain isolated from soil sample in biodiesel plants of Samutsakorn, Thailand. There were screening, isolation and optimization conditions. The PHB granules production was tested by using various sources of carbon, concentrations of carbon used, C:N ratios and comparison between crude glycerol and glycerol commercial.

2. Methodology

2.1 Soil collection sites

Soil samples were collected from biodiesel plants of Samutsakorn, Thailand. The surface soil (top 5-10 cm depth) was collected in sterilized sampling tubes and stored at -20 °C until use.

2.2 Isolations of bacteria from the soil samples

The soil suspensions in sterilized distilled water were prepared and plated on Nutrient agar (NA) plates containing 5 g peptone, 3 g beef extract and 15 g bacteriological agar per litre of culture medium. The agar plates were then incubated at 37 °C for 24 h until bacterial colonies were observed. All single colonies were picked from the plates and maintained at 4 °C.

2.3 Screening of bacterial isolates for PHB production

Their ability to synthesize PHB was determined using NA plates containing Nile red (0.5 µg/mL) (Spiekermann et al., 1999). The plates were incubated at 37 °C for 24 h, and viewed under UV light to detect the PHB production based on fluorescence emitted by the bacterial colonies. The purified bacterial isolates were screened for PHB production by culturing them in NA plate. Each isolate was first grown in 5 mL Nutrient broth (NB) medium (containing 5 g crude glycerol, 5 g peptone, 3 g beef extract per litre of culture medium). It was incubated at 37 °C with

shaking rate at 150 rpm for 24 h. The 10% (v/v) (OD_{600} = 0.5) of seed cultures were inoculated into 50 mL NB medium. It was incubated at 37 °C with shaking rate at 150 rpm for 24 h to allow for PHB accumulation in their cells. In order to study the effects of crude glycerol concentration on growth and PHB accumulation, different concentrations of crude glycerol (0-15 g/L) were added to NB medium. The carbon to nitrogen ratio (C:N ratio) using glycerol as carbon source and beef extract and peptone as nitrogen source were investigated vary from 1:0.4 to 1:1.6 by weight.

2.4 Estimations of total biomass, PHB concentration and PHB content

The cells with PHB accumulations were harvested by centrifugation at 4000 × g for 15 min. The cells were washed twice with distilled water before drying in an oven at 80 °C until constant mass. This cell dry weight (CDW) gave a measure of the total biomass generated. The PHB was extracted from cells by 1 mL NaOCl at 37 °C for 1 h. The mixture was added with 4 mL distilled water. The released PHB was extracted into 5 ml chloroform at 100 °C for 30 second. These result showed in three separate phases. The PHB was recovered from the bottom phase that contained PHB dissolved in chloroform (Javier et al., 2013). After chloroform evaporation, the precipitated form was used to analyses for 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. The PHB concentration was calculated by comparing with the standard curve using the PHB standard from Sigma (eq (1)). The PHB content was defined as the percentage of PHB mass in cell dry weight.

$$y = 0.0542x \quad (1)$$

Where, y is OD 235 nm, x is PHB standard concentration (μg/mL).

3. Results

3.1 Isolation of PHB from different cultures and screening for PHB producting bacteria

In this study, The total 8 isolated bacteria (C1, C3, C4, C5, C7, C9 and C13) isolated from soil sample in biodiesel plants of Samutsakorn, Thailand were screened for PHB accumulation. Figure 1 shows bacterial cells (C5) containing PHB fluoresced when grown on NA plates containing Nile Red (0.5 μg/mL) and crude glycerol as a carbon source for 24 h at 37 °C.

Figure 1. Bacterial cells (C5) containing PHB fluoresced when grown on NA plates containing Nile Red.

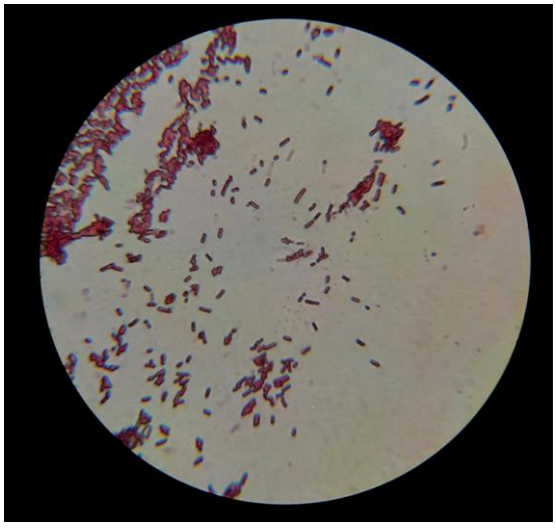

Figure 2. The strain C5 under microscope by Gram strain method.

Figure 2 shows the strain C5 under microscope by Gram strain method. Strain C5 is gram negative rod. The 16S rRNA gene sequence of the isolated strain C5 was *Bacillus* sp with 99% similarity. The cell growth was determined by CDW. The PHB concentration was determined by the above method, and the total PHB contents, determined as the proportion of bacterial CDW and PHB concentration were calculated. Figure 3 shows PHB concentrations (µg/mL) by PHB producing bacteria (C1, C3, C4, C5, C7, C9 and C13) grown on NB medium in rotary shaker at 150 rpm and 37 °C for 24 h. Strains C5 showed the high potential for PHB synthesis with the PHB concentration at 47.92 µg/mL.

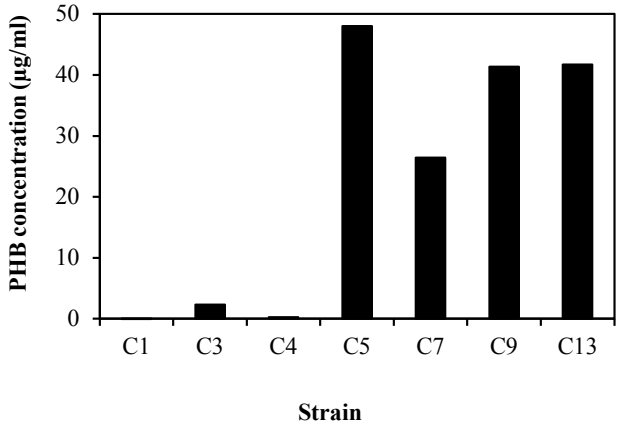

Figure 3. The PHB concentration by PHB producing bacteria (C1, C3, C4, C5, C7, C9 and C13) grown on NB medium (containing 5 g crude glycerol, 5 g peptone, 3 g beef extract per litre of culture medium) in rotary shaker at 150 rpm and 37 °C for 24 h.

3.2 Effect of crude glycerol concentration on PHB production

To estimate the effect of crude glycerol on growth and PHB accumulation by strain C5, different concentrations of crude glycerol were added to NB medium. Analyses were carried out after 24 h incubation. Crude glycerol at a concentration of 10 g/L enhanced both PHB concentration (µg/mL) and %PHB content of the cells. In comparison to a control culture (5 g/L crude glycerol), PHB concentration and PHB content increased from 47.92 to 62.34 µg/mL and from 7.04 to 11.09%, respectively, after 24 h of incubation (Figure 4).

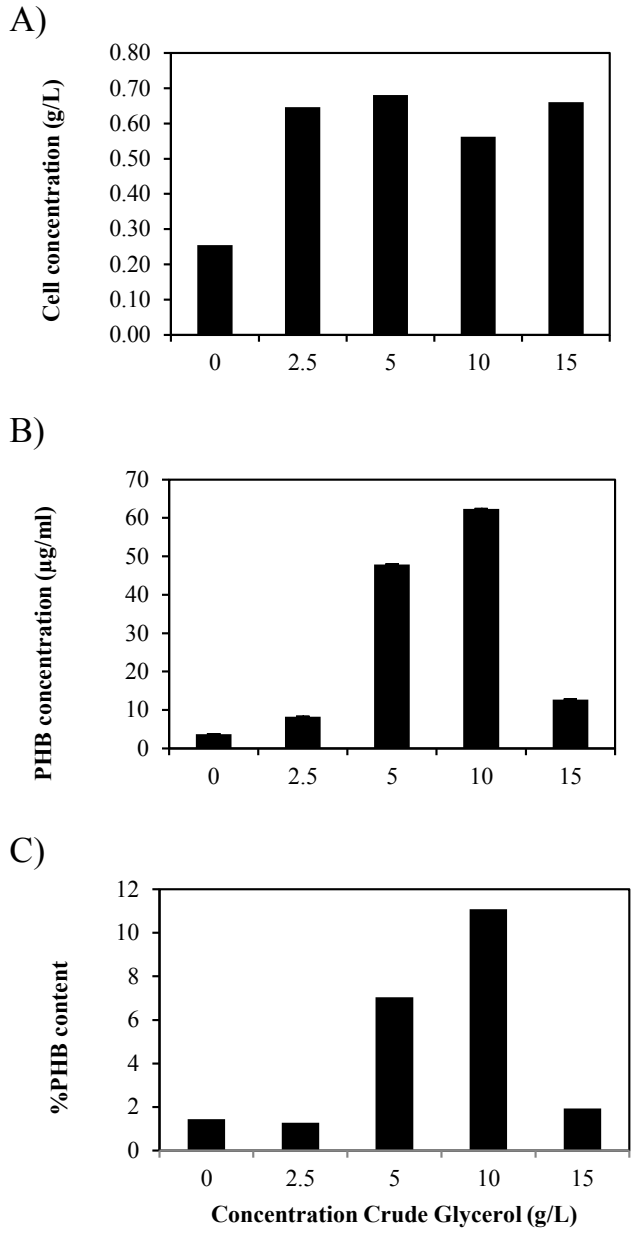

Figure 4. Production of PHB by strain C5 grown on NB medium in rotary shaker at 150 rpm and 37 °C for 24 h. (A) Cell dry weight (CDW, g/L), (B) PHB concentration (µg/mL) and (C) PHB content (%).

3.3 Effect of C:N ratios on PHB production

The bacterial isolates were grown in 250 ml in flask containing 50 mL NB medium with different C:N ratios of 1:0.625 to 1:2.5 using beef extract and peptone and incubated on a rotary shaker (150 rpm) at 37 °C. After 24 h, PHB yields were quantified. Beef extract (0-6 g/L) and peptone (0-10 g/L) were used to vary the

C:N ratio in separate experiments. Other medium components were as listed for NB medium. The results are shown in Figure 5. Both PHB concentration (75.50 µg/mL) and PHB content (23.59 %) showed maxima value at 1.5 g/L beef extract and 2.5 g/L peptone (C:N ratio = 1:0.4). Beef extract and peptone are chemically undefined medium and composed of peptides and amino acids that used in the cell culture. However, PHB can be accumulated as a response to limitation of nitrogen.

3.3 The comparison PHB production using crude glycerol and glycerol commercial as a carbon source.

Table 1 shows the results of experiments comparison between crude and commercial glycerol. The concentration of PHB and PHB content by using commercial glycerol as a carbon source was greater than crude glycerol.

Table 1. Comparison of cell dry weight, PHB concentration and PHB content from strain C5 with concentration of crude glycerol and commercial glycerol (10 g/L).

Carbon source	Cell dry weight (g/L)	PHB concentration (µg/mL)	%PHB content
Crude glycerol	0.32	75.50	23.59
Commercial glycerol	0.28	85.76	30.63

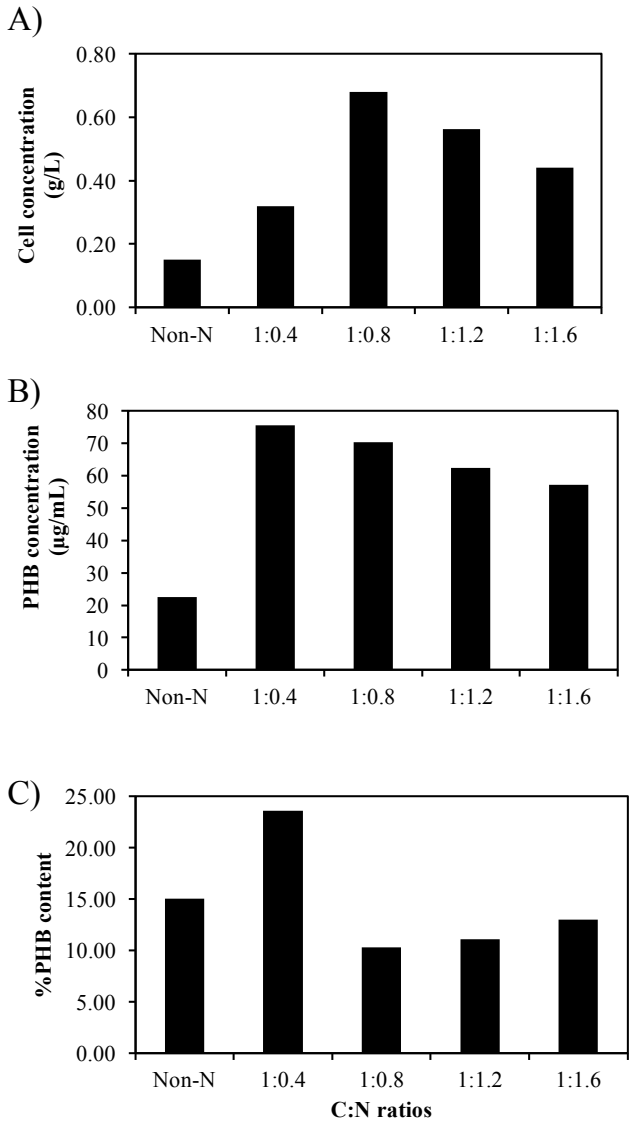

Figure 5. Production of PHB by strain C5 grown in 250 ml in flask containing 50 ml NB medium with different C:N ratios in rotary shaker at 150 rpm and 37 °C for 24 h. (A) Cell dry weight (CDW, g/L), (B) PHB concentration (µg/mL) and (C) PHB content (%).

4. Conclusion

In this work, a PHB-producing bacteria *Bacillus* sp. from around biodiesel plants of Samutsakorn, Thailand was successfully isolated. It is capable of accumulating PHB up to 23.59 % of cell dry weight at initial crude glycerol of 10 g/L and C:N ratio of 1:0.4 which may be of interest as potential industrial organism under optimized conditions.

5. Acknowledgement

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6. References

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