Influence of carbon and nitrogen sources on the performance of Polyhydoxyalkanoates (PHA) production using *Novosphingobium* sp. THA_AIK7 in batch operation

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Abstract

The effect of various carbon and nitrogen sources on the growth and Polyhydoxyalkanoates (PHA) production of *Novosphingobium* sp. THA_AIK7 were investigated. *Novosphingobium* sp. THA_AIK7 was grown on Mineral salt medium (MSM) supplemented with four different carbon (molasses, starch hydrolysate, glucose and crude glycerol) and nitrogen sources (ammonium sulfate, ammonium chloride, urea and monosodium glutamate (MSG)). Crude glycerol with MSG was chosen as the best source for PHA production as it produced a flexible and white PHA casted film. This condition gave PHA concentration, PHA content and Yp/s of 0.407 g/L, 14.59% of dry cell weight and 0.298 g PHA/g crude glycerol after 96 h, respectively.

Keywords: carbon and nitrogen sources, *Novosphingobium* sp., Polyhydroxyalkanoates

Introduction

Petroleum based plastic is a very useful material as it can be used in a very wide range of applications such as clothing, housing, automobiles, packaging, recreation items and medical implants (Varsha and Savitha, 2011). This kind of plastic is a synthetic polymer which means that it does not biodegrade when disposed of in the environment, resulting in the accumulation of a large amount of waste in landfills and sea water (Abid et al., 2016). Data from 1950 to 2008 shows that the global plastic production dramatically increased from 1.5 million tons to 245 million tons, with an annual growth rate of 9% (Chanprateep, 2010).

Research is attempted in order to replace conventional plastic with bioplastic in order to lower the harmful effect to nature. Bioplastic is a material capable of biodegradation by microorganisms, such as fungi and bacteria. Polyhydroxyalkanoates (PHA) are among the most well-known bioplastics. PHA has been recognized as completely biosynthetic, producing zero toxic waste and completely recyclable into organic waste (Chanprateep, 2010). Approximately 150 different monomer units have been identified as constituents of the PHA family (Steinbüchel, 2001; Steinbüchel and Valentin, 1995). Among the PHA family, Poly-β-hydroxybutyrate (PHB) homopolymer is the most abundant in nature (Anderson and Dawes, 1990). However, PHA could still not compete with conventional plastic due to the higher price of PHA compared to synthetics. The main reason is the high cost of the carbon source. Researchers, therefore, are trying to explore inexpensive sources for production, for example sweet sorghum juice, waste frying oils, sugar cane juice, sweet sorghum, sugarcane molasses and palm oil mill effluent, to reduce the cost associated with PHA production (Gouda et al., 2001; Alias and Tan, 2005; Kaewkannetra et al., 2008; Verlinden et al., 2011; Suwannasing et al., 2011; de Castro et al., 2014; Muangwong et al., 2016).

Novosphingobium sp. THA_AIK7 is a gram negative bacterium which was isolated from biodiesel-contaminated wastewater (Teeka et al., 2012). It could produce PHA from crude glycerol and has been reported as a naturally endotoxin-free PHA (Teeka et al., 2012). In this present work, crude glycerol, molasses and starch hydrolysate from waste streams in Thailand were used to compare with the most used carbon source, glucose, in order to investigate an appropriate source for PHA production from *Novosphingobium* sp. THA_AIK7.

Materials and Methods

1. Preparation of inoculum

Inoculum of *Novosphingobium* sp. THA_AIK7 was prepared in Nutrient broth (NB) and cultured at 30°C, 150 rpm for 24 h (Teeka et al., 2012).

2. PHA production medium

One liter of Mineral salt medium (MSM) for PHA production consists of $Na_2HPO_4{\cdot}7H_2O$ 6.7 g, KH_2PO_4 1.50 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, Ferrous ammonium citrate 60 g, $CaCl_2{\cdot}2H_2O$ 10 g and trace element solution 100 ml. Trace element 100 ml. includes H_3BO_3 0.03 g, $CoCl_2{\cdot}6H_2O$ 0.02 g, $ZnSO_4{\cdot}7H_2O$ 0.01 g, $MnCl_2{\cdot}7H_2O$ 3 mg, $NaMoO_4{\cdot}7H_2O$ 3 mg, $NiCl_2{\cdot}6H_2O$ 2 mg and $CuSO_4{\cdot}5H_2O$ 1 mg. Various carbon sources including crude glycerol, starch hydrolysate, molasses and glucose at 10 g/L were added into the medium. Various nitrogen sources including ammonium sulfate, ammonium chloride, urea and monosodium glutamate (MSG) at 1 g/L were used in the medium (modified from Ramsay *et al.*, 1990). The initial pH of the medium was 7.

3. The study of carbon and nitrogen sources for PHA production

Ten percent of *Novosphingobium* sp. THA_AIK7 inoculum was added into 100 ml MSM broth with various carbon and nitrogen sources. The culture was incubated at 30°C with an agitation rate of 150 rpm for 120 h and samples were taken every 12 h throughout the fermentation period. The cultivation was done in triplicate. Cell suspensions were centrifuged at 10,000 rpm for 10 min at 15°C. Cell pellets were used to analyze the dry cell weight (DCW) and PHA concentration (modified from Shi *et al.*, 1997). Supernatants were subjected to analyzed total sugar by Phenol-sulfuric acid method (Dubois et al., 1956) , crude glycerol by spectrophotometric method (Bondioli and Della Bella, 2005) and pH.

Results

Novosphingobium sp. THA_AIK7 cultured in MSM containing molasses plus various types of nitrogen gave PHA concentration, PHA content and Yp/s in the range of 0.034 – 0.036 g/L, 0.98- 1.67% of DCW and 0.012-0.015, respectively. Among these conditions, molasses and $(NH_4)_2SO_4$ gave the highest PHA content of 1.67% of DCW with 0.035 g/L of PHA concentration. The product formed per substrate consumed (Yp/s) was 0.013 g PHA/g total sugar. However, yields of PHA product on molasses among different nitrogen sources tested were not significantly different (p-value>0.05) (Table 1). Different from Gouda *et al.* (2001), they used various kinds of nitrogen sources with 2% sugarcane molasses in medium testing for growth and PHA production from *Bacillus megaterium* and they found that NH_4Cl gave the best results of PHA content of 40.1% /mg CDM and dry weight of 1.27 g/L. These results revealed that *Novosphingobium* sp. THA_AIK7 inefficiently used molasses for product formation compared to that of other carbons, since molasses contains sucrose as the main element (Ghazi *et al.*, 2006). Therefore, it might not be suitable carbon for this bacterium.

Table 1 Influence of molasses with various nitrogen sources on PHA production.

Nitrogen source	PHA concentration (g/L)	PHA content (% of DCW)	$Y_{p/s}$ (g PHA/g substrate)
$(NH_4)_2SO_4$	0.035±0.002	1.67±0.084	0.013±0.001
NH_4Cl	0.036±0.002	1.28±0.064	0.012±0.001
Urea	0.034±0.002	0.98±0.050	0.014±0.001
MSG	0.034±0.002	1.29±0.065	0.015±0.001

Starch hydrolysate added to the medium with various types of nitrogen gave PHA concentration, PHA content and Yp/s in the range of 0.032 – 0.052 g/L, 2.60 – 3.29 % of DCW and 0.020 – 0.026, respectively. The highest PHA content was achieved from the culture containing NH_4Cl with 3.29% of DCW, 0.035 g/L of PHA concentration and Yp/s of 0.026 g PHA/g total sugar (Table 2). Wei *et al*. (2009) reported that starch hydrolysate comprising of main three kinds of sugar; glucose, maltose and maltotriose with their relative percentages of 80.65, 16.13 and 3.23%, respectively. Poomipuk et al. (2014) used 20 g/L of cassava starch hydrolysate plus 3.5 g/L $NaNH_4HPO_4{\cdot}4H_2O$ in E2 medium for PHA production from *Cupriavidus* sp. KKU38 and this achieved 4.42 g/L of DCW, 2.81 g/L of PHA with Yp/s of 0.19. From these results, it can be seen that *Novosphingobium* sp. THA_AIK7 could not gain a high yield of polymer from this carbon source.

Table 2 Influence of starch hydrolysate with various nitrogen sources on PHA production.

Nitrogen source	PHA concentration (g/L)	PHA content (% of DCW)	$Y_{p/s}$ (g PHA/g substrate)
$(NH_4)_2SO_4$	0.034±0.001	2.60±0.130	0.020±0.001
NH_4Cl	0.035±0.002	3.29±0.165	0.026±0.001
Urea	0.032±0.002	2.70±0.135	0.020±0.001
MSG	0.052±0.003	2.86±0.143	0.022±0.001

Glucose, a widely used carbon source, gave PHA concentration, PHA content and Yp/s in the range of 0.048 – 0.193 g/L, 0.90 – 10.13 % of DCW and 0.014- 0.034, respectively. The combination of glucose and $(NH_4)_2SO_4$ achieved the highest PHA content of 10.13% of DCW, PHA concentration of 0.193 g/L and Yp/s of 0.034 g PHA/g total sugar. A yield of PHA product on glucose plus $(NH_4)_2SO_4$ was found to be higher significantly (p-value <0.05) than other nitrogen sources (Table 3). *Novosphingobium* sp. THA_AIK7 cultured in the medium containing glucose as a carbon source gave a better result for PHA production than molasses and starch hydrolysate, especially when combined with $(NH_4)_2SO_4$.

Table 3 Influence of glucose with various nitrogen sources on PHA production.

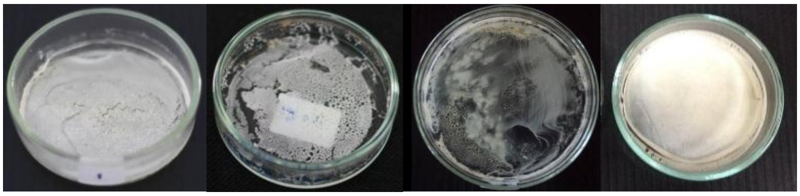
Nitrogen source	PHA concentration (g/L)	PHA content (% of DCW)	$Y_{p/s}$ (g PHA/g substrate)
$(NH_4)_2SO_4$	0.193±0.010	10.13±0.384	0.034±0.002
NH_4Cl	0.133±0.010	4.46±0.223	0.014±0.001
Urea	0.048±0.002	1.42±0.071	0.015±0.001
MSG	0.085±0.004	0.90±0.045	0.028±0.001

Among all sources of carbon in this study, crude glycerol gave the best results of product formation. Actually, *Novosphingobium* sp. THA_AIK7 was originally isolated by using crude glycerol as a sole carbon source in previous work (Teeka et al., 2012). However, the overall production from crude glycerol in this study was lower than 45% of DCW as previously reported. This could be because the crude glycerol was provided by a different company. In this recent work, crude glycerol gave PHA concentration, PHA content and Yp/s in the range of 0.398 – 0.407 g/L, 14.07 – 15.63 % of DCW and 0.182- 0.303 g PHA/g crude glycerol, respectively (Table 4). The highest PHA content was given from culture in which crude glycerol plus NH_4Cl was used, with 15.63% of DCW, 0.398 g/L PHA concentration and Yp/s of 0.303 g PHA/g crude glycerol.

Table 4 Influence of crude glycerol with various nitrogen sources on PHA production.

Nitrogen source	PHA concentration (g/L)	PHA content (% of DCW)	$Y_{p/s}$ (g PHA/g substrate)
$(NH_4)_2SO_4$	0.398±0.020	15.29±0.765	0.182±0.001
NH_4Cl	0.398±0.020	15.63±0.782	0.303±0.002
Urea	0.402±0.020	14.07±0.704	0.182±0.001
MSG	0.407±0.020	14.59±0.630	0.298±0.001

The appearance of polymer films extracted from each condition was also investigated. Unfortunately the best condition, crude glycerol plus NH_4Cl, could not form a film (Figure 1b). Among all conditions, the best film appearance was casted from crude glycerol plus MSG. It showed flexible and white features (Figure 1d). Therefore, crude glycerol and MSG was chosen as the optimal source for PHA production. PHA content of 14.59% of DCW, PHA concentration of 0.407 g/L and Yp/s of 0.298 g PHA/g crude glycerol were obtained from this condition.



(a) (b) (c) (d)

Figure 1 PHA film derived from MSM containing crude glycerol plus (a) $(NH_4)_2SO_4$ (b) NH_4Cl (c) urea and (d) MSG.

Quillaguaman et al. (2008) concluded that replacing the less expensive MSG instead of glutamine in the medium showed notable change in the final cell density of *Halomonas boliviensis* and it could gain DCW of 44 g/L, PHB content of 81 wt% and PHB productivity of 1.1 g/L/h in fed-batch culture. Between all sources of nitrogen used, urea seems to be less suitable for both growth and product formation as it achieved only 0.98, 2.70, 1.42 and 14.07 % of DCW from molasses, starch hydrolysate, glucose and crude glycerol, respectively.

Compared to the batch culture of crude glycerol-based PHA production from other works, Kumar *et al.* (2015) could efficiently produce PHA from *Bacillus thuringiensis* at 72.7 % of DCW with 3.585 g/L PHA concentration from non-nitrogen limiting conditions (Table 5). In order to improve the production efficiency, the scale-up of PHA production from *Novosphingobium* sp. THA_AIK7 in a bioreactor will be performed in the next study.

Table 5 Summary of crude glycerol-based PHA production from microorganism.

[a]MSM = mineral salt medium, CSBP = co-product stream from soy-based biodiesel production

Bacterium	Medium [a]	culture condition	Cultivation time (h)	Biomass (g/L)	PHA (g/L)	Content (%)	Yield (g PHA/g glycerol)	Reference
Pseudomonas corrugate 388	medium E + 3% (w/v) CSBP (6.3 g glycerol)	Batch, 500 ml at 30°C, 250 rpm	72	1.7	0.7	42	-	Ashby *et al*. 2004
Halomonas hydrothermalis	MSM + 2% (w/v) Jatropha biodiesel byproduct (95% glycerol)	Batch, 100 ml at 37°C	96	4	3	76	-	Shrivastav *et al.* 2010
Halomonas sp. KM1	SOT medium + 3% waste glycerol (60 wt% glycerol)	Batch, 20 ml at 30°C, 150 rpm	47	-	1.6	39	0.089	Kawata and Aiba, 2010
Halomonas sp. SA8	MSM + 2 g/L of $(NH_4)_2SO_4$ + 2% (v/v) waste glycerol	Batch, 100 ml at 30°C, 150 rpm	96	2.88	0.95	39	-	de Castro *et al*. 2014
Bacillus thuringiensis	Nutrient broth + 5% (v/v) crude glycerol	Batch, 125 ml at 37°C, 200 rpm	48	4.93	3.58 5	72.7	-	Kumar *et al*. 2015
Novosphingobium sp. THA_AIK7	MSM + 1 g/L MSG + 10 g/L crude glycerol	Batch, 100 ml at 30°C, 150 rpm	96	3.7	0.40 7	14.59	0.298	This study

Conclusion

In this work, various types of carbon and nitrogen sources used in MSM medium were investigated for PHA production. Glucose showed better results of PHA production from *Novosphingobium* sp. THA_AIK7 than molasses and starch hydrolysate. However, crude glycerol was the optimal carbon source for *Novosphingobium* sp. THA_AIK7. Crude glycerol combined with MSG was chosen as it produced a good character of PHA film. PHA content of 14.59% of DCW, PHA concentration of 0.407 g/L, and Yp/s of 0.298 g PHA/g crude glycerol were achieved from this condition.

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