Screening of PHA-Producing Bacteria Using Biodiesel-Derived Waste Glycerol as a Sole Carbon Source

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ABSTRACT

Different sources of wastewater and soil were used to screen for PHA-producing bacteria using biodiesel-derived waste glycerol as a sole carbon source by the Nile red staining method together with polymer determination. Twelve out of twenty-six isolates from biodiesel-contaminated wastewater consortium were screened for their PHA accumulation ability by cultivation in mineral salt medium supplemented with waste glycerol. The AIK7 isolate was chosen as a potential PHA producer. The PHA production on waste glycerol was examined using pure glycerol as a control substrate. The PHA content of AIK7 isolate cultivated in 10 g/L glycerol could reach 35% cell dry weight in 72 hours from waste glycerol and 33% cell dry weight in 120 hours from pure glycerol cultivation. It can be seen that at this content of waste glycerol, AIK7 isolate is effectively capable of biotransforming glycerol into polymer from low-grade glycerol.

Keywords: biodiesel, isolation, polyhydroxyalkanoates (PHA), waste glycerol.

INTRODUCTION

Polyhydroxyalkanoic acids (PHA) are intracellular granules that are normally found in prokaryotes. The biosynthesis and accumulation of PHA are common phenomena in approximately 300 different microorganisms (Lee, 1996a). Usually, bacteria enter into the PHA accumulation phase when a growth-limiting condition has occurred by the depletion of an essential component such as nitrogen, phosphorus, sulfur and oxygen in the presence of excess carbon source (Anderson and Dawes, 1990; Lee, 1996b). Polyhydroxyalkanoic acids act as a form of carbon and energy reserve (Dawes and Senior, 1973) or as an electron sink (Page and Knosp, 1989). Polyhydroxyalkanoic acids are materials of interest because they have some properties similar to polypropylene. They can completely degrade in the environment because of their very short half-life and they can be produced from renewable resources (Madison and Huisman, 1999; Shimao, 2001). Compared to conventional petroleum-based plastics, PHAs have a smaller environmental effect and a reduced reliance on nonrenewable fossil fuels (Akiyama *et al.*, 2003). They can also be used in many fields, and new potential applications are still emerging (Philip *et al.*, 2007). Nevertheless, the production of biodegradable plastics on a large scale is limited mainly because of the relative expense of the substrate. It has been estimated that substrate costs account for about 40% of the total PHA production costs (Choi and Lee, 1997) and makes the difference in price of poly-3-hydroxybutyrate (P3HB) from Biomer about 12 times the cost of polypropylene (Castilho *et al.*, 2009).

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Biodiesel, one of the best choices among alternative fuels has the potential to completely displace its petroleum counterpart (Johnson and Taconi, 2007). Thailand biodiesel development strategy is to increase the production capacity from 365 million liters in 2007 to 3100 million liters by 2012 (Siriwardhana *et al.*, 2009). It is estimated that about 250 million kilograms of waste glycerol byproduct will be produced annually. The byproduct composition, in addition to glycerol, varies from plant to plant depending on biodiesel feedstock. In general, it contains methanol and soap as two major impurities with various elements such as sodium, potassium, phosphorus, magnesium, sulfur, calcium (Pyle *et al.*, 2008), carbon, nitrogen, glycerides, fatty acid soaps (FFA) and fatty acid methyl ester (FAME) (Ashby *et al.*, 2004; Thompson and He, 2006). Thompson and He (2006) analyzed the waste vegetable oil (WVO) feedstock used in the biodiesel transesterification process and found that the WVO feedstock contained 18.8 wt% palmitic, 6.3 wt% stearic, 40.5 wt% oleic, 28 wt% linoleic and 1.5 wt% linolenic acids. The demand for these byproducts in the glycerol market was limited by their impurities (Johnson and Taconi, 2007) because the purification cost is imperative before using them in other industries. The excess glycerol generated may become an environmental problem, since it cannot be disposed of in the environment (G. P. da Silva *et al.*, 2009).

Recently, much work has been done using biodiesel-derived waste glycerol for PHA production by *Cupriavidus necator* JMP134, *Paracoccus denitrificans* (Mothes *et al.*, 2007), *Cupriavidus necator* DSM 545 (Cavalheiro *et al.*, 2009), *Bacillus sonorensis*, *Halomonas hydrothermalis* (Shrivastav *et al.*, 2010), *Halomonas* sp. KM-1 (Kawata and Aiba, 2010), osmophilic organism (Koller *et al.*, 2005), *Pseudomonas oleovorans* NRRL B-14682 and *Pseudomonas corrugata* 388 (Ashby *et al.*, 2004) from different sources of biodiesel feedstock. Among these, in small scale cultivation, *H. hydrothermalis* could accumulate PHB content up to 75.8% of the cell dry weight (CDW) with 3 g/L PHB concentration by using mineral salt medium (MSM) supplemented with 2% (w/v) of 95% glycerol content from *Jatropha* biodiesel byproduct (Shrivastav *et al.*, 2010)

In Thailand, as the biodiesel industry grows, research study for biodiesel-derived waste glycerol conversion into useful product should be done in order to minimize the environmental problem caused by this waste stream. This gives way to ascertain PHA-producing organisms from this unpurified carbon source.

MATERIALS AND METHODS

Materials

Waste vegetable oil-based biodiesel-derived waste glycerol (WG) with 50% purity was kindly provided by Assoc. Prof. Dr. Alissara Reungsang. Different sources of microorganisms from domestic wastewater, biodiesel-contaminated wastewater, sewage and biodiesel-contaminated soil were designated as WW1, WW2, WW3 and Soil, respectively. All bacterial sources were collected from Khon Kaen Province, Thailand. Each liter of MSM contained 6.7 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g of KH_2PO_4 , 1.0 g of $(\text{NH}_4)_2\text{SO}_4$, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 60 mg of ferrous ammonium citrate, 10 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1 mL of trace-element solution. Each liter of trace-element solution contained 0.3 g of H_3BO_3 , 0.2 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 30 mg of

MnCl₂·4H₂O, 30 mg of NaMoO₄·2H₂O, 20 mg of NiCl₂·6H₂O, and 10 mg of CuSO₄·5H₂O (Ramsay *et al.*, 1990). Nile red solution 0.025% (w/v) was prepared in dimethylsulfoxide (DMSO).

Quantitative analysis of PHA

Extraction and estimation of PHA were performed according to Shi *et al.* (1997) modified method as follows: one milliliter cell was centrifuged and resuspended in 0.5 mL sodium hypochlorite solution and then incubated in a water bath at 37°C for 1 hour. The lysed cell pellet was centrifuged and then successively washed with water, acetone, and alcohol. One milliliter of chloroform was added and incubated at 65°C for 2 hours. Chloroform solution of 0.1 - 0.5 mL volume was transferred to a clean test tube, was evaporated and then 5 mL of concentrated sulfuric acid was added. The sample was boiled for 10 minutes. An absorbance measurement was made at 235 nm using a UV-spectrophotometer against concentrated sulfuric acid.

Free glycerol determination

Substrate utilization was measured by the Bondioli and Della Bella (2005) method. The working reagents prepared for the experiment were 1.6 M acetic acid stock solution and 4.0 M ammonium acetate stock solution. The 0.2 M Acetylacetone solution and 10 mM sodium periodate solution were prepared in 1:1 ratio of acetic acid stock solution and ammonium acetate stock solution. Extraction solvent or working solvent was prepared by mixing an equal volume of distilled water with 95% ethanol. A mixture of hexane and extraction solvent was added to the sample. Upon mixing, two layers were formed and the lower layer was transferred into a new test tube. Then, 1.5 mL of the working solvent and 1.2 mL of sodium periodate were added. Afterwards, 1.2 mL of acetylacetone solution was added and the mixture was placed in a water bath at 70°C for 1 min. An absorbance measurement was made at 410 nm against a blank sample.

Batch culture

The experiment was carried out in a batch culture using wastewater and soil as inoculum. Bacterial source was pre-grown in nutrient broth (NB) for 24 hours and 5% of inoculum was inoculated in MSM supplemented with 5% (w/v) of WG with an initial pH of 7. Aerobic condition was maintained by shaking at 30°C and 150 rpm. Fluorescence of lipid-accumulating bacteria was determined on MSM agar supplemented with 2% (w/v) WG containing 0.5 µg/mL Nile red. Viable culture was directly exposed to ultraviolet light monitoring fluorescent emission compared to a control, i.e. a negative plate without microbial culture. Gram staining was done with twelve selected isolates. The best selected isolate was pre-grown on nutrient agar (NA) and was inoculated in NB for 24 hours. Five percent of inoculum was inoculated into MSM supplemented with 10 g/L glycerol concentration of WG or pure glycerol (PG) with an initial pH of 7. Samples were taken for protein assay by the Lowry method, PHA determination and chemical oxygen demand (COD) analysis by standard methods. Total nitrogen and total phosphorus analyses were done by following the standard methods for the examination of sewage wastewater (Japan Sewage Works Association, 1997). Sodium element was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Optima 3300 DV, Perkin Elmer, USA).

RESULTS AND DISCUSSION

Screening of the potential source of PHA-producing bacteria using waste glycerol as carbon source

In order to screen the potential source of PHA-producer from four kinds of microbial sources, bacterial cultivation in MSM supplemented with 5% (w/v) of WG together with fluorescence investigation of lipid-accumulating consortium were examined. It was found that all bacterial consortiums spread on MSM agar supplemented with 2% (w/v) WG containing Nile red showed weak yellow fluorescence under long wavelength ultraviolet irradiation (Fig. 1).

A lipophilic dye, Nile red stains not only PHA but also many kinds of lipids. Spiekermann *et al.* (1999) proved that triacylglycerols (TAG) accumulated strain *Acinetobacter calcoaceticus* could exhibit a bright fluorescence when grown on mineral salt medium in the presence of 0.5 $\mu\text{g/mL}$ Nile red. In this study, polymer determination was done as a confirmation method. Results showed that at 96 hours, PHA content of WW2 consortium culture reached 33% CDW with overall 53% soluble COD (SCOD) removal whereas Soil consortium reached 15% CDW and showed 56% SCOD removal efficiency (Fig. 2). The result of PHA content indicated that WW2 consortium contained highly potential PHA-producer.

Fig. 1 - Fluorescence of three-day incubation of viable WW1 (a) WW2 (b) WW3 (c) soil (d) and negative control plate (e) incubated at 30°C illuminated with long wavelength ultraviolet

Fig. 2 - PHA content (a) and soluble COD (b) from four consortiums cultivated in MSM supplemented with 5% (w/v) WG under aerobic condition at 150 rpm and 30°C

Isolation of PHA-producing bacteria

Bacterial isolation was done by ten-fold dilution of seven-day-old culture of wastewater consortium spread on NA plates. Twenty six bacterial isolates of different morphology colonies were obtained from WW2 consortium. They were then preliminarily selected by Nile red staining. Twelve selected isolates, appearing as gram-negative, were further examined for their own PHA production ability. PHA biosynthesis of twelve isolates was individually investigated by batch culture in MSM supplemented with 5% (w/v) WG. PHA concentrations from AIK6, AIK7, AIK11 and AIK16 isolates were 219, 662, 330 and 263 mg/L respectively, indicating that all four isolates could utilize and efficiently biotransform WG into PHA inside their cell better than others (Table 1). When colonies of AIK7 and AIK11 isolates were grown in the presence of Nile red, their cells exhibited a bright yellow fluorescence on the first three days of cultivation as described by Spiekermann *et al.* (1999) and then fluorescence intensity decreased relative to the amount of lipid polymer inside their cell. Among twelve isolates, AIK7 isolate was chosen as a highly potential PHA-producing strain for further experiment.

PHA production on pure glycerol (PG) and waste glycerol (WG)

PHA production efficiency of AIK7 isolate on WG was examined by using PG as a control substrate at 10 g/L of glycerol concentration. Nutrient analysis of MSM containing 10 g/L glycerol showed that the medium contained 1.44 g/L and 1.25 g/L of total nitrogen, and 1.28 and 1.26 g/L of total phosphorus in WG and PG medium, respectively. It was interesting to find that when WG was used as carbon source, PHA production could reach to 1.22 g/L in 84 hours which was higher than 0.85 g/L in 120 hours when PG was used (Fig. 3a). The PHA content of AIK7 isolate cultivated in WG reached to 35% in 72 hours whereas in PG cultivation the highest concentration was 33% in 120 hours (Fig. 3b). Optimum pH for PHA accumulation in this condition was 6 - 6.5 (data not shown). The viable colony number of WG cultivation of 6.8×10^8 cfu/mL in 36 hours indicated that AIK7 isolate is able to grow well in this glycerol content. The maximum product yield coefficient 0.22 and 0.09 (g PHA/g glycerol) were attained from WG and PG, respectively. At the end of cultivation, glycerol content in

Table 1- Result summary of twelve isolates from WW2 cultivated in MSM supplemented with 5% (w/v) WG

Isolate	PHA concentration (mg/L)	SCOD removal (%)	g PHA/g COD consumed
AIK3	113	23.18	0.008
AIK4	117	9.82	0.020
AIK6	219	16.50	0.022
AIK7	662	19.84	0.055
AIK10	150	19.84	0.012
AIK11	330	12.48	0.043
AIK12	116	13.16	0.014
AIK15	109	8.14	0.022
AIK16	263	13.16	0.033
AIK17	108	6.48	0.027
AIK21	112	26.52	0.007
AIK22	126	26.52	0.008

each culture medium was almost depleted (Fig. 3c) whereas SCOD removal was 50% in WG and 80% in PG (data not shown). It can be seen that AIK7 isolate not only can utilize glycerol but also has high capability for waste utilization. However, in this experiment, it was observed that AIK7 isolate could not grow well when a higher concentration of WG (25 g/L glycerol) was applied. It also made the colony characteristics change from the original form. This worse effect may be caused by a very high COD (approximately 60 g COD/L) contained in the medium.

Several researchers have reported that the growth and product formation of microorganisms cultivated in biodiesel-derived WG were inhibited resulting from sodium salt and other impurities in WG (Johnson and Taconi, 2007; Mothes *et al.*, 2007; Cavaleiro *et al.*, 2009). In the present study, WG pretreatment was done to remove the residual methanol. Mothes *et al.* (2007) concluded that the sodium quantity which caused PHB biosynthesis reduction is greater than 5 g/L. ICP-AES analysis showed that MSM supplemented with WG contained 1.384 g/L sodium. Therefore, this quantity should not inhibit bacterial growth or polymer production.

The results in Table 2 show that, at 72 hours of cultivation, AIK7 isolate cultivated in WG consumed less glycerol substrate while growing and accumulating PHA much better than in PG cultivation. Ashby *et al.* (2004) studied the PHA production from biodiesel co-product stream (CSBP) by *P.oleovorans* and *P.corrugata*, and they found that the carbon source utilization patterns for both strains were slightly different. They

Fig. 3 - PHA concentration (a) PHA content (b) and residual glycerol (c) of AIK7 isolate cultivated in MSM supplemented with 10 g/L glycerol

Table 2- Result summary of AIK7 isolate cultivated in MSM supplemented with 10 g/L glycerol at 72 hours

Carbon source	PHA (g/L)	cell dry weight (g/L)	PHA content (%CDW)	substrate consumed (g/L)	initial COD (g/L)	COD consumed (g/L)	SCOD removal (%)
Waste glycerol	1.05	3.01	35.00	4.74	27.50	7.01	25.50
Pure glycerol	0.54	2.30	23.50	6.75	16.00	9.05	56.55

explained that glycerol could be used as a growth substrate while the FFA and FAME, also contained in CSBP, could undergo β -oxidation to form the precursor of PHA biosynthesis. From the results obtained in this study, it might be speculated that AIK7 isolate is capable of using FFA or FAME which might reside in waste glycerol for polymer biosynthesis. Compared to PG cultivation, the medium contained only one kind of carbon source which must be used for growth and polymer biosynthesis. This could be the reason why a higher maximum yield coefficient and a higher PHA concentration were obtained in WG cultivation.

There are many factors affecting PHA production cost and they include carbon substrate cost, product yield, and the product recovery method (Choi and Lee, 1999). The cost of the substrate accounts for about 40% of the total PHA production cost (Choi and Lee, 1997). The cost of glucose, the most commonly used substrate, is about \$0.23/lb (\$0.50/kg) (Choi and Lee, 1999). Johnson and Taconi (2007) mentioned that the market price of WG has fallen to about \$0.05/lb (\$0.011/kg). Considering only the effect of substrate cost, we see that the PHA production cost could be significantly reduced by using WG as the substrate as compared to glucose. The PHA production process efficiency using AIK7 isolate is low compared to a well-developed process. However, the use of AIK7 to convert abundant waste streams into higher value products could help to overcome the cost to refine these streams into higher grade glycerol or the cost associated with their discharge to the environment. The development of an efficient process using AIK7 isolate will be investigated further to decrease the overall economics of PHA production.

CONCLUSIONS

Waste glycerol from the biodiesel process is an excellent carbon source for PHA production. It is a byproduct from sustainable process, has low market price and contains a high amount of glycerol substrate for bacterial growth and PHA biosynthesis. Our prospective study to investigate biodiesel-derived waste glycerol utilization would lower the bioplastic production cost by using unpurified cheap carbon source concomitantly with harnessing disposed waste glycerol. From this research, it can be concluded that AIK7 isolate could be a good candidate as a PHA producer by using this low-grade waste glycerol as a sole carbon source.

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