High-throughput approaches to screen the effective bioplastic producing bacteria by using biodiesel waste by-product as carbon source

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ABSTRACT: With dramatic use of biodiesel in world-wild consequently increases the accumulation the huge amounts of by-products, mainly crude glycerol via transesterification reaction. Therefore, use of the biotechnological strategies for biotransformation of crude glycerol to the other valuable products would be advantageous. In this research, we have attempted to use biodiesel waste by-product to produce an environmentally friendly bioplastic of polyhydroxyalkanoates (PHAs) by newly isolated strain from different bacterial sources. The primary isolation was carried out by using the standard morphological and their colonial appearance. 47 positive isolates were screened and selected from 250 isolates by Nile red staining-colony under UV-light. Then, spectrofluorometric technique was used to evaluate the high potential PHAs-producing strain. The results showed that 11 PHAs-producing strains were detected with high folds intensity and effective growth. The accumulation of PHAs was further investigated in cells which were cultured in the medium containing 20 % (w/v) glycerol waste by-product without optimization process and revealed that isolate NK14 showed the highest dry cell weight (6.74 g/L) and PHAs production (3.07 g/L). Therefore, this research results highlight the high potential of microbe that might be the exploitable application for the industrial PHAs production from biodiesel waste by-product.

Keywords: crude glycerol, bioplastic, polyhydroxyalkanoate

Introduction

The synthetic plastics mainly derived from petroleum have been used daily for a long time. With a slow degradation and subsequently high accumulation in the environment cause them as one of a serious pollutant. The discovered of biopolymer (polyhydroxyalkanoates; PHAs) by Lemoigne (1926) led to widely study of bioplastic production. PHAs are defined as a kind of biodegradable plastic and biocompatibility, which can be produced from a numbers of microorganisms such as yeast, fungi and mostly bacteria. Typically, PHAs are intracellular and accumulated as energy storage when the microbial cells encountered under nutrient imbalance condition such as a limitations of nitrogen and phosphorus meanwhile also in the presence of excess carbon source (Anderson and Dawes, 1990). Previous studies reported that there were various bacterial strains such as *Ralstronia eutropha* (Kaewkannetra et al., 2008; Chakraborty et al., 2012), *Hydrogenofaga* sp. *(*Tanamool et al., 2011*)* and *Bacillus spp*. *(*Tanamool et al., 2013*)* that can be accumulated PHAs in their cells as energy reserved source. Moreover, various soil and ma-

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rine environments were also found as promising PHAs-producing bacteria. Extensive study has been focus on isolation the effective soil or marine bacteria to produce PHAs from inexpensive raw material such as, sweet sorghum juice (Tanamool et al., 2013), molasses, starch and jatropha biodiesel waste, etc (Shrivastav et al., 2010).

Biodiesel is a promising an alternative renewable bioenergy, which would be overcome a limitation of a fossil fuel and environmental concerns. Trend to increase exponential utilization of biodiesel in world wild results in consequently accumulation of crude glycerol by product from transesterification of lipids on biodiesel processing (with approximately 10% by weight of the product) (Yang et al., 2012). Moreover, With being an inexpensive feed stock and available in huge amount, it has been considered to be a promising carbon source for various microbes to produce added value products such as hydrogen, unsaturated fatty acids likes docosahexaenic acid (DHA) and PHAs (Hermann-Krauss et al., 2013). However, the productions of value added products from crude glycerol were still limited resulting in the composition as well as the variety and concentration of impurities found in crude glycerol such as methanol, alkaline or acid and others fatty acid.

Hence, in this study, we attempted to isolate utilizing crude glycerol bacteria from different environments and to investigate a potential use of the biodiesel waste by-product as a sole carbon source by spectrofluorometric method and produce PHAs from isolated bacteria by batch fermentation.

Materials and Methods

Collection of microbial resource samples

The samples were collected from different 10 soil sources nearby Khon Kaen University, Nong Khai campus Nong Khai province, Thailand. After collection, those samples were preserved at 4°C before isolation.

Preparation of biodieselbyproduct

Crude glycerol was kindly provided from biodiesel plant, Faculty of Engineering, Khon Kaen University, Thailand. It was filtered through cheesecloth in order to remove solid contaminants and kept at room temperature prior to use as a sole carbon source.

Isolation of bioplastic-producing bacteria

Samples from different 10 soil sources were added into 250 mL flask containing 100 mL nutrient broth (NB) and incubated at 30 °C under orbital shaking at 200 rpm for 24 hrs. The culture supernatant was then diluted by 10-folds in sterile distilled water before spreading on mineral salt agar (MSA) medium containing 20 % (w/v) crude glycerol and incubated at 30 °C for 48 h. The different bacterial colonies were picked and re-streaked on mineral salt medium containing 20 % (w/v) pure glycerol which was supplemented with a solution of 0.5 mg Nile red dye in dimethyl sulfoxide (DMSO) at final concentration of 0.5µg/mL (Spiekermann et al., 1999). The plates were incubated at 30 °C for 24 hrs. Several PHA-producing colonies were observed under UV light. The strong bright colonies were selected for further study (**Figure 1**).

The evaluation PHAs-producing bacteria by spectrofluorometer

The selected strains from above were cultured in 250 mL flask containing 50 mL crude glycerol medium which were supplemented with Nile red dye in dimethyl sulfoxide (DMSO) to give a final concentration of 0.5µg/mL in media. Each strain was incubated in shaking incubator at 30 °C, 200 rpm for 5 days. The microbial cell was collected by centrifugation at 10,000 g for 5 mins. and the supernatant was discarded. Distilled water (1 mL) was added and the resulting pellet was vigorously vortexed. The fluorescence exposure from microbial cell was then read at excitation and emission wavelength 543 and 598 nm (Berlanga et al., 2006). The fold intensity of Nile red fluorescence was estimated by the ratio of fluorescence intensity and optical cell density at 600 nm (OD_{600nm}) to evaluate PHA accumulation capacity.

PHAs production from crude glycerol

The fermentative utilization of crude glycerol was studied by inoculating the selected isolate in culture media containing crude glycerol. All inoculums were adjusted the final optical density at 600 nm ($OD\ 600_{nm}$) to 0.1, in culture media consisting of (unit per liter); 20 % crude glycerol; 1.5 g KH_2PO_4, 3.57 g Na_2HPO_4, 0.2 g $MgSO_4 7 H_2O$; 1 g $(NH_4)_2SO_4$ and 1 mL trace element solution which prepared according to the method described by Tanamool et al [7]. In addition, dry cell weight (DCW) was considered, 1 mL the broth was withdrawn and centrifuged at 10,000 g for 5 mins. After centrifugation, cell pellets were washed twice with distilled water, dried at 90 °C until constant weight. PHAs concentration was the determined by modification method of Law and Slepeky (1961).

Results and Discussion

The isolation of crude glycerol utilizing bacteria from various sources

Various bacteria from different sample sources (**Figure 1 a-j**) were able to grow on solid crude glycerol medium. Nile red dyes could be diffused into the bacterial cytoplasm during growth and subsequently into the PHAs inclusions (Berlang et al., 2006). The positive PHAs-producing colonies can be exposed under UV illuminator at the wavelength 280 - 360 nm and showed in brighten colonies. In this study, 47 positive isolates were screened and selected from 250 isolates by Nile red staining-colony under UV-light (Fig 1.). The positive isolate showed strong bright fluorescence intensity and differences in the accumulation of PHAs were correlated with fluorescence intensity. With the use of this test, screening of PHA producer would be facilitated for further study to observe the PHAs accumulation ability by spectrofluorometer.

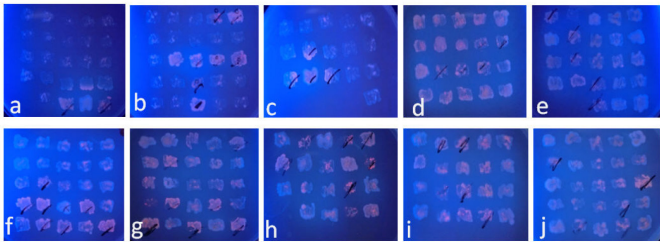

Figure 1 Comparison of the accumulation of PHAs in isolates bacterial from various soils samples under UV exposure. The bacterial colonies obtained from MSA plate were replica to the agar medium containing Nile Red. After 2 days of incubation the cells were exposed under UV light. The bright orange light observed was speculated to be the PHAs producing colony. (a-j indicates the soil sample collected from different positions around Nong Khai campus)

Spectrofluorometric monitoring of PHA accumulation

47 positive isolates were cultured in crude glycerol medium with Nile red stain. After 3 days, PHAs accumulation was measured as fluorescence intensity by spectrofluorometrically in cells grown under the same conditions as described above. Cell growth was monitored as optical density by spectrophotometer at 600 nm

The fluorescence fold intensity and optical densities of bacterial cultures were shown in **Figure 2**. Among them, 11 PHAs-producing isolates (NK08, NK10, NK12, NK13, NK14, NK18, NK22, NK23, NK27 and NK40) were detected with high folds intensity, suggested that those isolates presented higher cell density with higher accumulation of PHAs in microbial cell as well. The accumulation of PHAs by 11 selected isolates was further investigated in cells which were cultured in the medium containing 20 % (w/v) crude glycerol.

Berlanga et al. (2006) reports that fluorescence intensity of Nile red stained cells depends on the PHA concentration. Bacteria that did not produce PHA are only lightly fluorescent because they contain almost no lipids. Hence, spectrofluorometric method combined with Nile red staining can be efficiently applied for rapid detecting of PHA production.

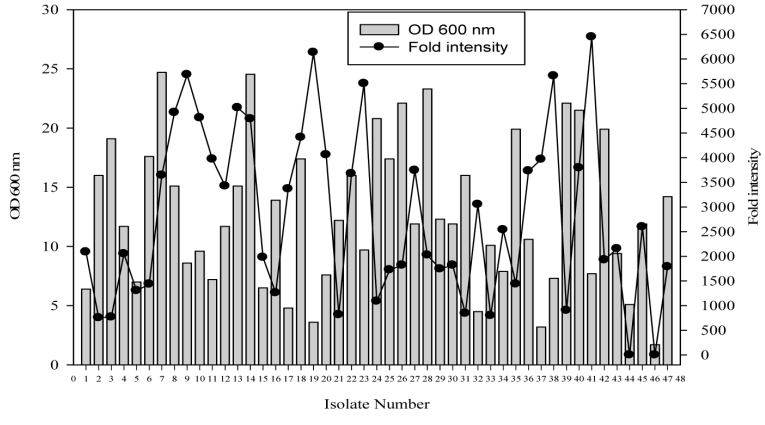

Figure 2 Cell growth and fluorescence fold intensities emitted by Nile red stained intracellular PHA granules assayed using spectrofluorometer.

PHAs production from crude glycerol

From the results shown in **Table 1**, all isolates could be produced PHAs and grown in crude glycerol. The isolate NK14 was showed as promising PHAs-producing bacteria. The PHAs productions from those isolates were 3.07 g/L with PHAs contents of 45.55 % by DCW. As compared with, Teeka et al. (2012) isolates PHA producer from biodiesel contaminated wastewater by using crude glycerol. It was identified as *Novospingobium capsulatum*. PHA accumulation was 45 % by DCW. Palmeri et al. (2012) reported that PHAs were produced from crude glycerol by *P. mediterranea* 9.1, were obtained at 2.93 g/L with PHAs contents of 61.43 % by DCW. The results indicated that isolate NK14 would be a candidate for the effective PHA producer even though the PHA content was not obviously high but it was able to grow reasonable on low grade crude glycerol without any purification.

Table 1 PHAs accumulation in cells of the isolates grown on crude glycerol containing medium

Isolate number	Dry cell weight (g/L)	PHA (g/L)	PHA content (%w/w)
NK07	5.31	1.39	26.26
NK08	3.43	1.63	47.38
NK10	1.35	0.62	46.25
NK12	1.68	0.39	23.24
NK13	3.52	1.92	54.48
NK14	6.74	3.07	45.55
NK18	3.84	1.47	38.27
NK22	3.41	0.96	28.06
NK23	1.52	0.84	55.42
NK27	1.62	0.49	30.21
NK40	5.25	1.58	30.12

Conclusions

In this study, we demonstrated a rapid and reliable procedure for screening the PHAs producing microbes using crude glycerol as a sole carbon source. Using Nile red staining subsequently exposing under UV light and measuring by spectrofluorometer could be obtained the effective isolate of these microorganisms, the isolate NK14 showed the highest efficiency on bioconversion of crude glycerol into PHAs. Moreover, the potential use of biodiesel wastes byproduct as an inexpensive raw material might be economic feasibility on production of PHAs. However, this isolate still not be identified and the optimal condition by using response surface methods (RSM) for this isolate will be further carried out in large scale fermentation.

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