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Short Communication: Isolation and screening of polyhydroxylalkanoates producing microorganisms from Thailand

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Abstract. *Pungsungvorn N, Wisetsing A. 2021. Short Communication: Isolation and screening of polyhydroxylalkanoates producing microorganisms from Thailand. Biodiversitas 22: 4963-4967.* Polyhydroxylalkanotes (PHAs) are polyesters produced in nature by numerous microorganisms. They are biodegradable and are used in the production of bioplastics. In this study, one hundred twenty samples from different regions of Thailand were collected and screened for polyhydroxylalkanoates (PHAs) producing microorganisms. The samples were screened on YM agar containing 0.5 µg Nile-red/mL (YM-NR). Only one isolate of yeast (Y1) gave a positive result on PHA accumulation. The yeast isolate (Y1) was identified as *Candida tropicalis* by API 20C AUX kit and 18S rRNA nucleotide comparison. The yeast isolate Y1 produced 2.62% PHA when grown in synthetic N-limiting medium using rice straw hydrolysate as carbon source. The selected four bacteria (B1, B2, B3 and B4) were identified by BlastN of 16S rRNA as *Enterobacter cloacae*, *Enterobacter carcerogenus*, *Escherichia coli* and *Klebsiella pneumonia*, respectively. The selected yeast and bacterial strains gave PHA content of 2.62, 2.76, 5.38, 3.66 and 0.44%, respectively, in synthetic N-limiting medium using rice straw hydrolysate. Hence, these microorganisms could be used in PHA production from biomass in the future.

Keywords: Biodiversity, bioplastic, PHA, polyester, rice straw

INTRODUCTION

Plastics and pollution are two deeply connected terms. Millions tons of nondegradable plastics accumulate in the environment per year. It can cause serious environmental problems to the world, especially green house effect. Currently, the use of bioplastics is continuously increasing around the world. Bioplastics are plastics that are naturally biodegradable and are used as a substitute for plastics made from petrochemicals. Scientists pay attention to the development of natural and environmentally friendly materials for bioplastics applications. Among these materials, polyhydroxyalkanoic acids or polyhydroxyl-alkanoates (PHAs) are very interesting because PHAs are similar to petrochemical polyester with good formability. PHAs have interesting properties of bioplastics production since PHAs are thermoplastic, gas barrier, UV resistant, biocompatible, elastic, rigid and hydrophobic. PHAs are eco-friendly, 100% biodegradable, recyclable, non-toxic, biocompatible, and biodegradable (Anjum et al. 2016). PHAs are biodegradable in both soil and water environments and can be composted by biological methods which are found to be completely decomposed within 49 days (Folino et al. 2020; Karan et al. 2019). PHAs can be used for both medical and industrial applications.

PHAs are biopolyesters found in microorganisms, such as bacteria (*Alcaligenes latus*, *Ralstonia eutropha*, *Azotobacter beijerinckii*, *Bacillus megaterium*, *Aeromonas hydrophila*, *Cupriavidus necator* and *Pseudomonas oleovorans*), yeast (*Arxula adeninivorans*, *Rhodotorula minuta*) and blue-green algae (*Chlorogloea fritschii*, *Gloeocapsa* strain 6501) (Anjum et al. 2016; Tan et al. 2019). PHAs are accumulated in cells as granules in the cytoplasm. PHAs are composed of hydroxyalkanoate units and can be classified according to the number of carbon atoms present in the monomer units into two groups: medium chain length PHAs consist of 6-14 carbon atoms, e.g. poly-3-hydroxybutyrate-co-3-hydroxyvalerate copolymer [P(3HB-co-3HV)], and short chain length PHAs consist of 3-5 carbon atoms, e.g. poly-3-hydroxybutyrate [P(3HB)] (Ciesielski et al. 2015). The composition of PHA is clearly affected by the choice of the microorganism and the carbon source (Anjum et al. 2016). PHAs consist of several types of hydroxycarboxylic acid polyesters which are produced by large number of bacteria. It is a hydrophobic aggregation that occurs in bacterial cells with excess carbon and other nutrient restrictions such as N, P, S or Mg. They are used as carbon reserves and energy (Anjum et al. 2016). PolyFerm Canada, a Canadian bioplastics company, uses *Aeromonas hydrophila* and *Pseudomonas putida* for industrial production of PHA. Tianan Biologic, a Chinese company, also uses *Cupriavidus necator* for industrial production (Pakalapati et al. 2018). However, the cost of production is 5-10 times more expensive than conventional plastic (Anjum et al. 2016). Raw material, carbon sources cost more than 50% of the process and therefore tend to switch to cheap, waste and sustainable substrates that are easily processed or require no processing (Pakalapati et al. 2018). The carbon source used in the production of PHA can be divided into 6

groups namely, starch-based media, sugar-based media, cellulosic and hemicellulosic media, whey-based media, oil-based media and glycerol-based media (Amache et al. 2013). Various types of waste products are used for PHB production because it provides dual benefits of utilizing the waste and cost-effective production of biodegradable microbial bioplastic (Anjum et al. 2016). In this study, PHAs producing microorganisms were isolated and screened using rice straw hydrolysate as a sole carbon source with the dual benefit of utilizing the waste and cost-effective production of biodegradable microbial bioplastic.

MATERIALS AND METHODS

Samples

One hundred twenty samples of soils, leaves, fruits, flowers and agricultural wastes (10 samples from each province: Chonburi, Chachoengsao, Rayong, Chanthaburi, Trat, Prachinburi, Sa Kaeo, Lampang, Phuket, Phra Nakhon Si Ayutthaya, Khon Kaen, and Ratchaburi, Thailand) were collected and used as source for isolation of PHA producing microorganisms. The samples were kept in refrigerator for further analysis.

Isolation and screening

The sample, 1 g of solid sample was suspended in 5 mL YM broth (containing (in g/L) glucose 10, malt extract 3, peptone 5, yeast extract 3) and incubated at 30°C, 18-24 hr for enrichment (Abd-El-Haleem 2009). The enriched broth was spread on YM agar and incubated at 30°C, 18-24 hr. The selected colonies were maintained in YM slant and 15% glycerol for further study. The isolates were screened for PHA production on YM agar containing 0.5µg Nile-red/mL (YM-NR) at 30°C (Abd-El-Haleem et al. 2007b). The PHA accumulation was observed under ultraviolet light every 24 h, for 6 days (Spiekermann et al. 1999).

Morphological and molecular identification

The morphology of the selected strains were observed under microscope. The selected strains were cultured in YM broth and genomic DNA was extracted using Wizard Genomic DNA Purification kit (Promega). The 18S rRNA and 16S rRNA were amplified and cloned from yeast isolate and bacteria isolates, respectively (Abd-El-Haleem 2009; Martı´nez-Gutie´rrez et al. 2018; Tan et al. 2019). The 18 S rRNA and 16S rRNA gene were sequenced and subjected to BlastN search at www.ncbi.nlmnih.gov/blast for identification.

Rice straw hydrolysate preparation

Rice straw was pretreated by drying in hot air oven at 60°C for 12 h and grinded with blender. The rice straw powder was soaked in 2% hydrogenperoxide, pH 11.5 at ratio 1 g: 10 mL at 35°C for 24 h. The powder was filtered, washed with distilled water and dried at 60°C for 12 h. The rice straw powder was kept in cool and dry place for further study.

For hydrolysis, rice straw powder was hydrolyzed with 2% sulfuric acid at ratio 1 g:10 mL at 121°C for 15 min. The hydrolysate was separated by filtration and pH was adjusted to 7 with 10M sodium hydroxide. The activated carbon was mixed with hydrolysate at ratio 1.5 g:100 mL for 30 min. The rice straw hydrolysate was obtained after filtration. The concentration of glucose, xylose and arabinose were determined using HPLC (Sritrakul et al. 2017). The HPLC system was equipped with an Aminex HPX-87P (Bio-Rad Labs; Hercules, CA, USA) and a refractive index detector (Waters; Mildford, MA, USA). The column was operated at 80°C and 0.60 mL/min using a mobile phase of filtered deionized. Furfural and HMF were measured using HPLC with an ultraviolet/visible detector (Waters; Mildford, MA, USA). The Aminex HPX-87H operating at 60°C with 5 mM H_2SO_4 as a mobile phase (0.6 mL/min), was used for separation. Detection was performed at 280 nm. Acetic acid was analyzed using HPLC with a refractive index detector and the Aminex HPX-87H column maintained at 60°C with a flow rate of 0.6 mL /min and H_2SO_4 as the mobile phase.

Reducing sugar was analyzed by DNS method (Miller 1959). 2 mL of the sample was mixed with 2 mL of 3,5-Dinitrosalicylic acid (DNS) reagent, boiled for 5 min in boiling water, cooled and measured the absorbance at a wave-length of 540 nm. Glucose was used as standard for calculation.

Phenolic compound was measured by Folin ciocalteau method (Singleton and Rossi 1965). 0.05 mL of the sample was mixed with 2 mL of saturated sodium carbonate solution and 2.5 mL of 1:10 diluted Folin ciocalteau reagent, incubated at 45°C for 30 min. The absorbance was measured at 765 nm, and the phenolic content was calculated using vanillic acid as standard solution.

PHA production on glucose and rice straw hydrolysate

The selected isolates were evaluated for PHA production using 2% glucose or 10% rice straw hydrolysate (0.2% sugar hydrolysate) as carbon sources. The test was carried out using synthetic N-limiting medium (SNL) (containing (g/L) KH_2PO_4 1.0, $MgSO_47H_2O$ 0.5, $(NH_4)_2SO_4$ 2.0, yeast extract 0.5, fatty acid (oleic acid) 1.0, carbon source) in shaking flask at 30°C, for 6 days (Abd-El-Haleem et al. 2007b; Pirozzi et al. 2014).

Extraction and quantitative analysis of PHA

The cell pellet was centrifuged and washed twice with distilled water. PHA was extracted from cell mass by chloroform-soxhlet extraction method (Pirozzi et al. 2014). The cell was ruptured by toluene solution by vigorous mixing and then centrifuged at 12,000 rpm for 15 min. The pellet was washed with distilled water, acetone and ethanol, respectively. Then the pellet was dissolved in chloroform, and incubated at 50°C for 16 h. PHA was extracted by chloroform in soxhlet apparatus for 2 h and chloroform was vacuum evaporated.

PHA content was analyzed by crotonic acid assay (Law and Slepecky 1961). PHA was dissolved in sulfuric acid and incubated at 100°C for 10 min. The absorbance was measured at 235 nm, and PHA content was calculated using 3-hydroxybutyrate (PHB) as standard (Thammasittirong et al. 2017).

$$\text{PHA content (\%)} = \frac{\text{PHA (g/L)}}{\text{CDW (g/L)}} \times 100$$

RESULTS AND DISCUSSION

Isolation and screening

Results revealed that a total of 84 isolates were obtained from different regions of Thailand (Table 1). Screening results of PHAs accumulation using Nile-red assay showed that 37 isolates gave positive results (1 yeast isolate and 36 bacterial isolates). Under the UV transilluminator, five isolates (Y1, B1, B2, B3 and B4) exhibited strong fluorescence signals in comparison to other isolates (Figure 1). The red fluorescence of Nile red is shown to be strongly positive only with hydrophobic compounds such as PHA and lipids. Nile-red is intended to express intracellular lipid particles. It does not react with any tissue component except by solution and can be detected by fluorescence spectroscopy. This fluorescence depends on cell age and the amount of PHA accumulated inside the cell (Spiekermann et al. 1999).

Morphological identification

The results showed that isolate Y1 was identified as *Candida tropicalis* with 94% identity (Table 2). The colony and cell morphology of bacterial isolates were observed under microscope (Figure 1) and subjected to BlastN analysis of 16S ribosomal RNA nucleotide sequence. The results exhibited that the isolates B1, B2, B3, and B4 were identified as *Enterobacter cloacae*, *Enterobacter carcerogenus*, *Escherichia coli* and *Klebsiella pneumonia* with 99% identity, respectively (Table 2).

Rice straw hydrolysate preparation

Rice straw hydrolysate was prepared by digesting 100 g of rice straw with 2% sulfuric acid, containing 22.03 g/L of reducing sugar, 2.67 g/L glucose, > 10 g/L xylose, 3.59 g/L arabinose, 0.47 g/L phenolic compounds, 0.07 g/L acetic acid, 0.0096 g/L HMF and 0.4285 g/L furfural (Table 3). The results obtained were similar to the rice straw hydrolysate in the other experiments of Brazilian researchers (Fonseca et al. 2013). When detoxifying rice straw hydrolysate with activated carbon, it was found that the amount of reducing sugar, glucose, arabinose, phenolic compound, HMF and furfural decreased by 7.4, 5.24, 5.57, 100, 95.8 and 99.95%, respectively. This revealed that detoxification with activated carbon showed negative effect on sugar concentration but it was effective in removing toxic compounds. Similar results were also observed by Lanka et al. (2011) and Yadav et al. (2011). The detoxified rice straw hydrolysate contained 20.04 g/L of reducing sugar, 2.53 g/L glucose, >10 g/L xylose, 3.39 g/L arabinose, 0.06 g/L acetic acid, <0.0004 g/L HMF and 0.0002 g/L furfural (Table 3). The result clearly showed that detoxified rice straw hydrolysate can be used in the PHA production. The detoxified rice straw hydrolysate was clear liquid, without any impurities. It can be used in PHA production because the lower impurity makes it easier to separate/purify PHA from fermentation broth than using contaminated waste. This could reduce the cost of PHA purification in process (Anjum et al. 2016; Pakalapati et al. 2018).

Table 1. Sources and number of PHA producing isolates

Provinces	Number of isolates	Number of PHA producing yeast	Number of PHA producing bacteria
Chonburi	7	-	6
Chachoengsao	5	-	2
Rayong	4	-	3
Chanthaburi	6	-	-
Trat	7	-	5
Prachinburi	4	-	3
Sa Kaeo	7	-	-
Lampang	8	-	2
Phuket	13	-	11
Phra Nakhon Si Ayutthaya	8	-	-
Khon Kaen	12	1	4
Ratchaburi	3	-	-
Total	84	1	36

Table 2. BlastN result of the selected isolates

Isolates	BlastN results	Identities (%)
Y1	KC597824.1 *Candida tropicalis* strain KY7	94
B1	CP010384.1 *Enterobacter cloacae* strain 34399	99
B2	AB776827.1 *Enterobacter carcerogenus* MB18-2	99
B3	KC013977.1 *Escherichia coli* strain moh1	99
B4	CP010361.1 *Klebsiella pneumonia* strain 32192	99

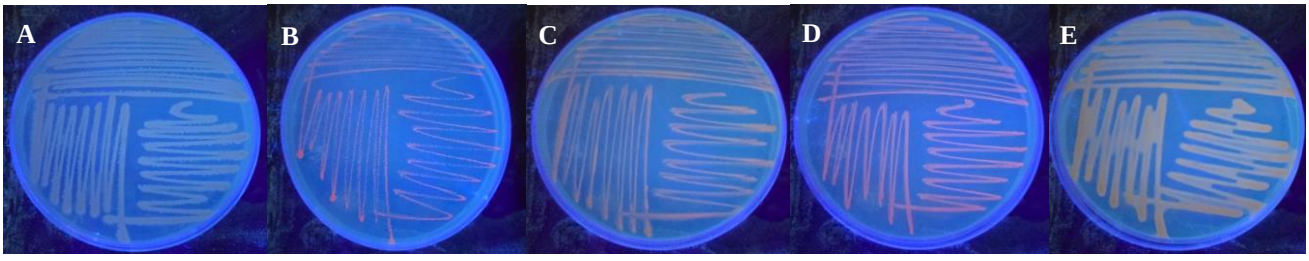

Figure 1. Pink/orange fluorescence under UV light indicates PHA producer isolates Y1, B1, B2, B3, and B4. A: Isolate Y1 (*Candida tropicalis*), B: Isolate B1 (*Enterobacter cloacae*), C: Isolate B2 (*Enterobacter carcerogenus*), D: Isolate B3 (*Escherichia coli*), E: Isolate B4 (*Klebsiella pneumonia*)

Table 3. Chemical analysis of rice straw hydrolysate

Processes	Reducing sugar (g/L)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Phenolic compounds (g/L)	Acetic acid (g/L)	HMF(g/L)	Furfural(g/L)
Acid hydrolysis	22.03	2.67± 0.01	> 10	3.59± 0.05	0.47	0.07± 0.00	0.0096± 0.0001	0.4285± 0.0005
Activated carbon treatment	20.04	2.53± 0.11	> 10	3.39± 0.28	0	0.06± 0.00	<0.0004	0.0002± 0.0000

Table 4. Production of PHA by selected isolates

Isolates	PHA content (%)	
	SNL with 2% glucose	SNL with 10% rice straw hydrolysate
Candida tropicalis (Y1)	0.64	2.62
Enterobacter cloacae (B1)	1.30	2.76
Enterobacter carcerogenus (B2)	0.72	5.38
Escherichia coli (B3)	1.65	3.66
Klebsiella pneumonia (B4)	0.38	0.44

PHA production on glucose and rice straw hydrolysate

The PHA production by *Candida tropicalis* (Y1), *Enterobacter cloacae* (B1), *Enterobacter carcerogenus* (B2), *Escherichia coli* (B3), *Klebsiella pneumonia* (B4) were 0.64, 1.30, 0.72, 1.65, 0.38% on SNL with 2% glucose and 2.62, 2.76, 5.38, 3.66, 0.44% on SNL with 10% rice straw hydrolysate. The isolate B3 and B2 showed the highest PHA production in SNL with 2% glucose and 10% rice straw hydrolysate, respectively. Interestingly, all isolates showed higher PHA production in 10% rice straw hydrolysate than 2% glucose (Table 4) but the rice straw hydrolysate contained 10 times less reducing sugar than SNL with 2% glucose. It was observed that rice straw hydrolysate contained other nutrients that could promote PHA formation in microorganisms. Silva et al. (2013) reported that rice straw hydrolysate obtained by digesting rice straw with sulfuric acid contained xylose, glucose, arabinose and minerals such as potassium, iron, manganese, chromium, aluminum, sodium, zinc and copper. These nutrients could help to promote the production of PHA in microorganisms. The result was comparable with the previous reports that *Candida norvegensis* C-Y8 (Aba-El-Haleem et al. 2007a), *Enterobacter cloacae* SU-1 (Samrot et al. 2011), *Klebsiella pneumonia* (Feng et al. 2015) and *Escherichia coli* (Qi et al. 1998) can produce PHA at different level depending on the culture condition. However, the result was very promising for biotechnological application when using rice straw hydrolysate as the sole carbon source. The PHA content of the selected strains grown on rice straw hydrolysate were higher than that on glucose. Rice straw hydrolysate has more nutrients than glucose only, and hence microorganisms can grow, utilizing the nutrients present in rice straw hydrolysate, and convert them into valuable compounds and polymers. Ahn et al. (2015) reported that rice straw hydrolysate has carbon to nitrogen ratio (C/N ratio) which could stimulate the PHA accumulation in *Cupriavidus necator*. With this concept, researchers have reported PHA production from biomass or various wastes. Polyhydroxybutyrate (PHB) is produced from community wastewater with *Enterobacter aerogens* 12Bi strain (Ceyhan and Ozdemir 2011). Javaid et al. (2020) reported PHA production from waste, such as wood chips, cardboard cutouts, plastic bottle cutouts, shredded polystyrene cups and plastic bags, using *Stenotrophomonas maltophilia* HA-16 with good yield. The bacterial isolates B2 (*Enterobacter carcerogenus*) could be considered as potential strain for the conversion of rice straw hydrolysate into PHA. The selected isolate utilized rice straw hydrolysate as sole carbon source for growth, PHA biosynthesis, and accumulating PHA 5.38%. However, other isolates also showed a promising result in PHA production.

In conclusion, these isolates could be considered good candidates for industrial production of PHA from lignocellulosic biomass. Currently, these strains need to be further investigated for optimization of the fermentation parameters to increase the productivity of PHA and make the whole process more cost-effective.

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