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A Potential Strain RB36 of Polyhydroxyalkanoates-Producing Bacterium Isolated from Rice Barn Located in Nakhon Ratchasima Province, Thailand

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Abstract: Polyhydroxyalkanoates (PHAs) are groups of biodegradable thermoplastic polymers synthesized by various microorganisms. PHA producing bacteria accumulate PHA in their cells as carbon and energy storage compounds. This research objective was to acquire a strain of PHA producing bacterium isolated from rice barn. Rice barn samples were collected from rice mills at Nakhon Ratchasima Province, Thailand. Bacterial PHA production was isolated on Tryptic Soy Agar (TSA), cultured on complex and minimal medium agar and then screened on minimal medium agar supplemented with Nile Red. PHA accumulation in bacterial cells was detected by Transmission Electron Microscopy (TEM) and fluorescent microscopy on samples stained with Nile Blue A. The 40 of 214 isolates produce PHAs in their cell at rates from 5.1-76%. Bacterial strain RB36 produced PHAs in as much as 76% of cell areas. The strain was sequenced based on 16S ribosomal RNA gene and then submitted in GenBank with Accession No. MG745383 and identified as 99.8-100% similarity to *Enterococcus faecium*. This is the first report of a potential strain of PHAs-producing bacterium isolated from rice barn from Thailand.

Key words: Potential strain, polyhydroxyalkanoates and rice barn

INTRODUCTION

The extensive production of conventional plastics and their use in different commercial applications poses a significant threat to fossil fuels sources and to the environment. Alternatives called bioplastics evolved during development of renewable resources (Emadian *et al*., 2017). Biodegradable Polyesters of Polyhydroxyalkanoates (PHAs) synthesized by microorganisms are viable candidates for the gradual substitution of synthetic plastics (Boyandin *et al*., 2012). Bacterial PHAs are polyesters accumulated as carbon and energy storage materials under limited growth conditions in the presence of excess carbon sources (Mozejko-Ciesielska and Kiewisz, 2016; Kosseva and Rusbandi, 2017). However, commercialization of PHAs is impeded by its high total production cost, half of which is from the cost of pure carbon source feedstock. Thus, it is desirable to find cheap alternative carbon feedstocks in order to improve sustainability of PHAs production while obtaining commercial viability through fermentation. Inexpensive carbon sources such as agro-industrial by-products and agricultural residues have been identified as potential alternative substrates (Aslan *et al*., 2016). PHAs can also be produced by fermentation using inexpensive raw materials, making its commercial production economically feasible and contribute to a 40-50% reduction in the overall production cost (Gowda and Shivakumar, 2014).

Asia is the world's primary rice-growing region, producing nearly 90% of total world rice output. Currently, the major rice-exporting countries are Thailand, India and Vietnam. In Thailand, rice plantation area is nearly 60 million Rai which yields annual output of 30-32 million tons. Rice bran is a byproduct of the milling of rice with the bran containing protein-rich (about 12% by weight of rough rice), sugars, minerals, vitamins and fatty acids. This product is low-cost and used as animal feed (Shih, 2003; Oh *et al*., 2015).

Researchers have used rice barn as a cheap carbon source of PHAs-producing bacteria cultivation (Huang *et al*., 2006; Shamala *et al*., 2012; Khandpur *et al*., 2012; Oh *et al*., 2015; Nagamani *et al*., 2015). The utilization of rice barn is an inexpensive carbon source and yields the high production of PHAs, however, none of research group isolated PHAs producing bacteria this product. Preferred bacteria contained in rice barn could be used as the carbon source which would create a product that contains high amounts of PHAs. The research objective was to achieve a strain of potential PHAs-producing bacterium isolated from rice barn.

MATERIALS AND METHODS

Sample collection: Rice barns were collected from rice mills at Suranaree Sub-District, Muaeang Nakhon Ratchasima District, Nakhon Ratchasima Province, Thailand. The samples were placed in to sterile glass

bottles then transported to Microbiology Laboratory, Faculty of Medical Science, Nakhonratchasima College.

Bacterial isolation: The 25 g of rice barn were added to 225 mL of Tryptic Soy Broth (TSB) (Difco, USA) and mixed until homogenous. Tenfold dilution series were prepared from 10^{-1}-10^{-7} in 9 mL of phosphate buffer pH 7.2. Each dilution was spread on Tryptic Soy Agar; TSA (TSB with 15 g agar/L) (Difco, USA) with duplication plates then incubated at 30°C for 24 h. Bacterial isolates obtained from TSA were purified and investigated for PHAs-accumulation in their cells.

Bacterial cultivation: Two-steps of bacterial cultivation were cultured on complex and minimal media agar. Bacterial isolates obtained from TSA were purified then streaked on complex medium agar (5 g yeast extract, 5 g polypeptone, 5 g tryptone, 2.5 g NaCl, 10 g glucose and 15 g agar/L) and incubated at 30°C for 24 h. Bacterial colonies from complex medium agar were streaked on minimal medium agar and incubated at 30°C for 24-48 h. The minimal medium agar was composed of 0.01 g $CaCl_2.2H_2O$, 0.05 g ferrous ammonium citrate, 10 g glucose, 1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 3 g Na_2HPO_4, 1 g (NH_4) $2SO_4$, 1 mL of trace element solution (0.2 g $CoCl_2.6H_2O$, 0.01 g $CuSO_4.5H_2O$, 5.56 g $FeSO_4.7H_2O$, 0.3 g H_3BO_3, 0.03 g $MnCl_2.4H_2O$, 0.03 g $NaMoO_4.2H_2O$, 0.02 g $NiCl_2.6H_2O$, 0.1 g $ZnSO_4.7H_2O$) and 15 g agar/L. Both complex and minimal medium agars were developed by Chansatein *et al*. (2012).

Screening of PHAS-producing bacteria: The purified isolates were cultured on complex medium agar and incubated at 30°C for 24 h, then transferred to minimal medium agar which was supplemented with 0.5 mg/L Nile red (dissolved in 1 mL of dimethylsulfoxide) (Berlanga *et al*., 2006) then incubated at 30°C for 24-48 h. Development of pink colonies under UV light (280 nm of wave length) after cultivation on minimal medium agar were selected for microscopic detection of PHAs. *Alcaligenes eutrophus* TISTR 1095 was used as the positive control of PHAs-producing strain. *Escherichia coli* TISTR 527 was utilized for negative control.

Detection of PHAs inclusions in bacterial cells: The detection of PHAs inclusions in bacterial cells used fluorescent and electron microscopy. PHAs-producing bacteria isolated from rice barn were detected by Nile blue A dyeing technique and observed under fluorescence microscope (Olympus Model BX51 TFR, Olympus Optical Co. Ltd., Japan) with an excitation wavelength of approximately 650 nm. Bacterial isolates produced PHAs in their cells emitted bright orange. The cell and PHAs areas were measured by Image-Pro Plus Version 6.0 Program (Media Cybernetics, Bethesda, MD, USA). Transmission Electron Microscope (TEM) was performed as the selected of bacterial isolates isolated from rice barn were fixed in fixative solution (5% glutaraldehyde, 1% OsO_4 and 0.2 M phosphate buffer) and placed in the secondary fixative solution (1% osmium) (Tian *et al*., 2005). The ratio of the first fixative solution was 1:1:1 at 4°C. Dehydration solution was used acetone at series 20, 40, 60, 80 and 100%. Epon viscosity embedding resin with incubating at 60°C for 24 h. was used for TEM polymerization. After sectioning, copper grids were strained with uranyl acetate and lead acetate. The PHAs granules in bacterial cells were observed under TEM (JEOL JEM-1230, JEOL, Japan).

Bacterial identification: PHAs-producing isolates were identified by cell morphological characteristics and molecular methods. Cell morphology was Gram's stained and observed under light microscope (Olympus Model BX51 TFR, Olympus Optical Co., Ltd., Japan). Potential isolates were examined for bacterial identification including genomic DNA extraction, Polymerase Chain Reaction (PCR), PCR amplification, PCR products purification and DNA sequencing based on 16S ribosomal RNA gene analysis according to Macrogen Inc., Seoul, Korea. Genomic DNA samples were extracted using a QIAamp DNA mini kit (QIAgen, Germany). Target gene specific primer pairs (27F 5' AGA GTT TGA TCM TGG CTC AG 3' and 1492R 5' TAC GGY TAC CTT GTT ACG ACT T 3') and Dr. MAX DNA Polymerase (Doctor Protein INC, Korea) were utilized for the PCR reactions. PCR amplification conditions were as follows: 94°C for 5 min, 94°C for 30 sec variable temperature for 30 sec, 72°C for 40 sec for 35 cycles and 72°C for 7 min (Engine Tetrad 2 Peltier Thermal Cycler, BIO-RAD). PCR products were purified using Millipore plate MSNU030 (Millipore SAS, Molsheim, France). The purified PCR products were then Sanger-sequenced with the BigDye terminator V 3.1 sequencing kit and a 3730 xl automated sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

Phylogenetic tree construction: Bacterial data of 16S ribosomal RNA gene analysis were constructed using National Center for Biotechnology Information (NCBI, USA) database for the reference sequences. BioEdit 7.2, MEGA7 and ClustalX programs were used for blasted, aligned and constructed of the tree.

RESULTS AND DISCUSSION

The 214 bacterial rice barn isolates were screened for PHAs-producing bacteria. All of their cell morphology was Gram-positive when Gram's stained and observed

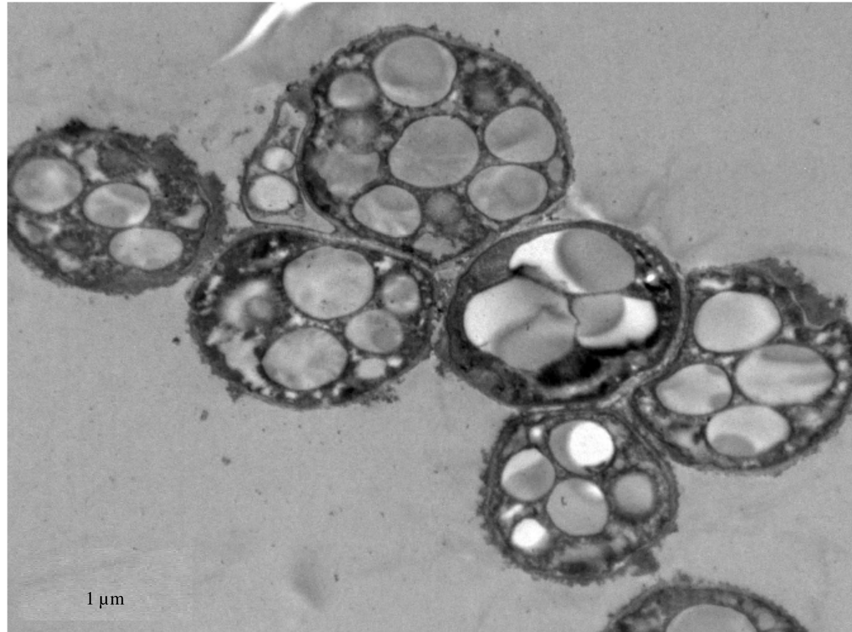

Fig. 1: TEM micrographs of granules in cells of bacterials strain RB36

Table 1: The 16S rRNA gene sequence similarity of isolate RB36 and related species

Bacterial isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	100																		
2	99.9	100																	
3	99.9	100	100																
4	100	99.9	99.9	100															
5	99.9	99.8	99.8	99.9	100														
6	99.9	99.8	99.8	99.9	100	100													
7	100	99.5	99.9	100	99.9	99.9	100												
8	100	99.9	99.9	100	99.9	99.9	100	100											
9	99.9	100	100	99.9	99.8	99.8	99.9	99.9	100										
10	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100									
11	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100								
12	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100	100							
13	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100	100	100						
14	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100	100	100	100					
15	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100	100	100	100	100				
16	99.9	99.8	99.8	99.9	99.8	99.8	99.9	99.8	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100			
17	99.8	99.9	99.9	99.8	99.7	99.8	99.8	99.8	99.9	99.8	99.8	99.8	99.8	99.8	98.8	99.7	100		
18	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100	100	100	100	100	99.9	99.8	100	
19	100	99.9	99.9	100	99.9	99.9	100	100	99.9	100	100	100	100	100	100	99.9	99.8	100	100

under microscope. Cell size of Gram-positive and cocci was about 0.18-0.28×0.18-0.28 to 0.4-1.2×0.4-1.2 μm. The 40 bacterial isolates gave the pink colony on minimal medium agar supplemented with Nile Red were investigated for PHAs accumulation in their cells. The isolates accumulated PHAs granules in their cells which were detected by fluorescence microscopy, producing a bright orange hue. The PHA accumulation was 5.1-76% in their cell areas when estimated by Image-Pro plus Version 6.0 program. A potential isolate RB36 was shown as the highest of PHAs accumulation when detected by TEM (Fig. 1). Isolate RB36 was selected for 16S ribosomal RNA gene analysis. Nucleotide sequences of the gene were obtained and submitted in GenBank, USA with Accession No. MG745383. When compared the sequence of strain RB36 to sequences from GenBank database, it was found that the strain had 99.8, 99.9 and 100% similarity to *Enterococcus faecium* (*E. faecium*) strain P1 (Accession No. JQ837456), *E. faecium* strain gp 80 (Accession No. KM495943) and *E. faecium* strain C-7 (Accession No. KY348701), respectively. Eighteen strains were selected from GenBank using for similarity comparing with bacterial strain RB36, the data shown in Table 1. Phylogenetic tree of strain RB36 based on 16S ribosomal RNA gene sequence data was constructed as Fig. 2.

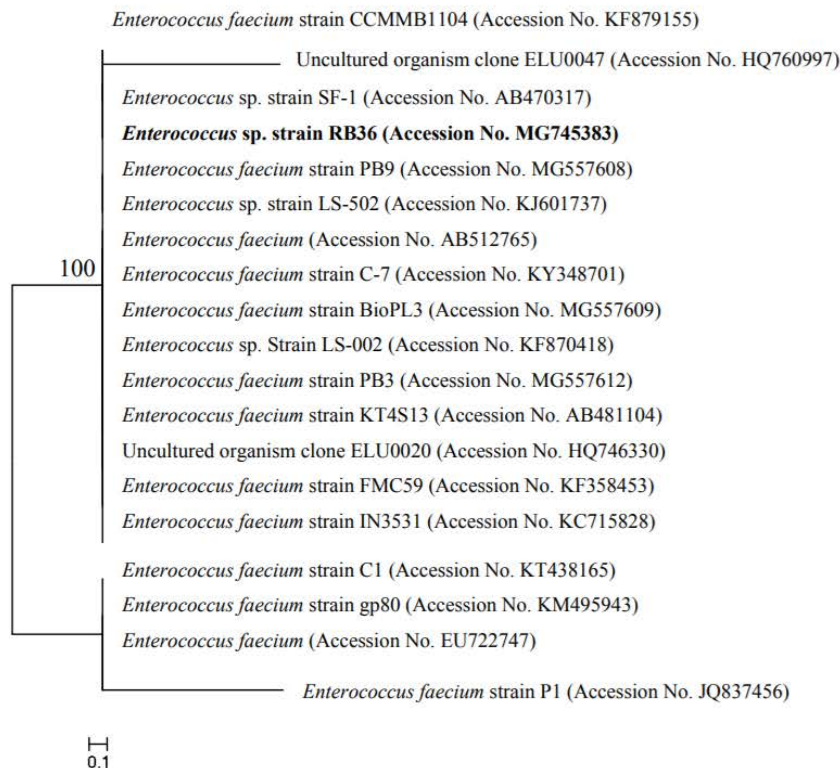

Fig. 2: Phylogenetic tree of strain RB36 based on 16S ribosomal RNA gene sequence data was using the maximum parsimony method. The unrooted tree was derived by using clustal X, BioEdit and Mega7 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance

Some research groups have used rice barn as a cheap carbon source of PHAs-producing bacteria cultivation (Aslan *et al*., 2016). Oh *et al.* (2015) used 10 mL/L of rice barn hydrolysate solution for the synthesis of PHAs. Bacteria could produce polymer contents of 90.1-97.2% by weight. Hydrolyzed rice barn was cultured of *Brevundimonas* sp. OU6T. The culture is able to produce 1.72 g/L of polymer after 40 h at 30°C (Nagamani *et al*., 2015). Mixture of hydrolyzed wheat barn and rice barn with ration 1:1 can produce PHA 5.9 g/L by *Bacillus* sp. CFR-67 (Shamala *et al*., 2012) whereas extruded rice barn and cornstarch 1:8 obtained PHAs concentration of 77.8 g/L by *Haloferax mediterranei* (Huang *et al*., 2006). *Pseudomonas aeruginosa* in combination with rice barn produced 48% of PHAs (Khandpur *et al*., 2012). The utilization of rice barn as an inexpensive carbon source of PHAs-producing bacteria cultivation could decrease the cost of PHAs production. Especially, the bacteria that had a habitat in rice barn, they could bring nutrients from the source for their cells metabolisms effectively. This is the first report on PHAs-producing bacterium isolated from rice barn. Bacterial strain RB36 could have potential biopolymer production.

CONCLUSION

The 40 of 214 bacterial isolates isolated from rice barn can produce PHAs between 5.1 and 76% in their cell areas when detected by microscopes. All isolates are Gram positive cocci.

A potential strain RB36 was identified as 99.8-100% similar to *Enterococcus faecium* when sequenced based on 16S rRNA gene and submitted in GenBank with Acession No. MG745383. This is the first report on PHAs-producing bacterium isolated from rice barn from Thailand.

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