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Discovery of a new polyhydroxyalkanoate synthase from limestone soil through metagenomic approach

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PHA synthase (PhaC) is the key enzyme in the production of biodegradable plastics known as polyhydroxyalkanoate (PHA). Nevertheless, most of these enzymes are isolated from cultivable bacteria using traditional isolation method. Most of the microorganisms found in nature could not be successfully cultivated due to the lack of knowledge on their growth conditions. In this study, a culture-independent approach was applied. The presence of *phaC* genes in limestone soil was screened using primers targeting the class I and II PHA synthases. Based on the partial gene sequences, a total of 19 gene clusters have been identified and 7 clones were selected for full length amplification through genome walking. The complete *phaC* gene sequence of one of the clones (SC8) was obtained and it revealed 81% nucleotide identity to the PHA synthase gene of *Chromobacterium violaceum* ATCC 12472. This gene obtained from uncultured bacterium was successfully cloned and expressed in a *Cupriavidus necator* PHB^{-}4 PHA-negative mutant resulting in the accumulation of significant amount of PHA. The PHA synthase activity of this transformant was 64 ± 12 U/g proteins. This paper presents a pioneering study on the discovery of *phaC* in a limestone area using metagenomic approach. Through this study, a new functional *phaC* was discovered from uncultured bacterium. Phylogenetic classification for all the *phaCs* isolated from this study has revealed that limestone hill harbors a great diversity of PhaCs with activities that have not yet been investigated.

[**Key words:** Polyhydroxyalkanoate; PHA synthase gene; Metagenomic; Genome walking; Limestone; Uncultured bacterium]

In this modern era, plastics play a vital role in many aspects of our lives. Plastics have been widely used in daily products ranging from food packages, containers, furniture, toys, shoes, clothes, electronic devices and even medical devices. However, they are produced from petroleum, an extract of non-renewable fossil fuels. They pose several setbacks such as waste accumulation due to their non-biodegradability. As such, the widespread use of plastics has caused global environmental issues due to its non-renewability and long-term non-sustainability. An increasing cost of production due to depletion of oil reserves is another discouraging factor on the petrochemical-based plastics.

In the USA, Europe and Japan, about 55 million tons of post-consumer plastic wastes were generated and ended up in landfill sites (1). In Malaysia, plastics also contribute to a large amount of the solid wastes in Kuala Lumpur and an increase in the volume of such waste has been observed year on year (2). In order to overcome problems of plastics waste management, bioplastics offer a good alternative to replace some of these petrochemical-based plastics. Hence, polyhydroxyalkanoate (PHA) is a promising substitute due to its biodegradability in the environment.

PHA is a type of naturally occurring polyester that can be produced from renewable resources such as agricultural wastes, sugar and vegetable oils. It can be synthesized by a wide range of gram-positive and gram-negative bacteria, for instance *Cupriavidus necator* (previously known as *Ralstonia eutropha*), *Pseudomonas oleovorans*, *Bacillus* sp., *Chromobacterium* sp. and *Burkholderia* sp. (3–7). PHA is stored as intracellular storage materials in the cytoplasm of bacteria under stress conditions such as in the event of limitation of nitrogen, phosphorus or magnesium although carbon is in excess (8).

In order to synthesize and accumulate PHA in the bacterial cell cytoplasm, several enzymes are involved. One of the key enzymes is the PHA synthase (PhaC), which catalyzes the stereo-selective conversion of (*R*)-3-hydroxyacyl-CoA substrates to PHA with the concomitant release of CoA. There are four classes of PHA synthase enzymes based on their subunit composition, representative species and substrate specificity. The type of PHA produced is strongly dependent on the classes of PHA synthase in the bacteria. However, most of these enzymes have been isolated from cultivable bacteria because the classical method of enzyme discovery involves isolating, culturing and growing of microorganisms under different conditions. Unfortunately, 99% of the microorganisms found in nature are still uncultivable (9).

Metagenomics, sometimes referred to as community genomics or environmental genomics, is the sequencing and analysis of DNA of microorganisms recovered from an environment, without the need of culturing. By generating metagenomic libraries from different environmental niches, different types of novel genes or enzymes have been successfully isolated. Previous study showed

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that less than 1% of all bacterial species have been cultured due to the lack of knowledge of their growth conditions. For example, the commonly used culture medium may be biased towards certain types of bacteria (9,10). Therefore, metagenomics enables the study of various communities of microorganisms by deciphering their genetic information from DNA that is extracted directly and thus sidestepping the need for culturing or isolation. Due to its ability to uncover hidden diversity of microbial communities, metagenomics offers a powerful approach.

Many studies have been performed on metagenomic samples from different niches such as canine oral cavity, sewage sludge and bovine rumen (11–13). Besides that, there are also reports on soil metagenomes yielding new knowledge of the uncultured world (14–17). Since Malaysia is well known as one of the twelve mega biodiversity countries in the world, its natural diversity serves as a natural gene bank of biodiversity. There are an enormous number of unknown PHA-producing microorganisms that are yet to be explored in these natural environments.

The presence of PHA producing bacteria in different environments including a limestone area was previously reported (18). Limestone hills and outcrops are common in Malaysia especially in the northern region. Ipoh, also known as Hill City is surrounded by limestone outcrops and caves making it a top tourist attraction site in Malaysia. As these caves are abundant in calcium carbonate, organisms in these niches are able to endure highly alkaline environment. Besides that, these organisms are able to withstand the severity of exceedingly arid conditions during the dry season. Thus, limestone was chosen in this study because it provides a unique periodically stressful habitat to bacteria. It is believed that there will be an enormous amount of PHA producing bacteria in this area due to such stressful conditions that favor the accumulation of PHA.

This is the first attempt to explore PhaC diversity from limestone using a culture-independent approach. In this study, screening of class I and II PHA synthase genes was carried out from limestone soil metagenomic samples of Gunung Lang, Ipoh, Perak. The aim was to identify new PHA synthase genes from uncultivable bacteria. Subsequently, the substrate specificity and enzyme activity of the PHA synthase was determined by heterologous expression of the identified gene in a PHA negative mutant strain.

MATERIALS AND METHODS

Bacterial strains, plasmids and maintenance Bacterial strains and plasmids used and constructed in this study are shown in Table S1. *C. necator* was routinely cultivated at 30°C in a nutrient rich (NR) medium composed of 10 g L^{-1} of meat extract, 10 g L^{-1} of peptone, and 2 g L^{-1} of yeast extract (19). Cultures of *Escherichia coli* JM109 and S17-1 were grown at 37°C in a Luria Bertani (LB) medium for general cloning and transconjugation experiments, respectively. Kanamycin (50 μg mL^{-1}) or ampicillin (100 μg mL^{-1}) was added to the media when necessary. Plasmid pGEM-T Easy (Promega, USA) and pBBR1MCS-2 (20) were used for cloning purpose.

Sample collection and soil pretreatment Soil sampling was carried out at Gunung Lang, Ipoh, Perak (GPS coordinate: 4.622N, 101.0888E) in May, 2013. The location of the soil was underneath a limestone hill. The soil sample was collected from about 5 cm deep from the top layer. Rock sample was also collected from the limestone hill. The materials were then transported to the laboratory under sterile conditions. In the laboratory, the plant roots and humus were removed from the soil samples in a sterile condition. The samples were then stored in a freezer at a temperature of −20°C.

Rock analysis The limestone sample was sent to the Archeology Centre, Universiti Sains Malaysia (USM) for X-Ray Fluorescence (XRF) analysis to confirm the identity of the rock and at the same time, determine the major elements present.

DNA extraction and purification from limestone soil The DNA was extracted using a sodium dodecyl sulphate (SDS)-based method for DNA recovery from soil with some modifications (21). Hexadecylmethylammonium bromide (CTAB) was used to reduce humic acids contamination. Approximately 5 g of soil samples were weighed in sterile 50 mL centrifuge tubes. Subsequently, 13.5 mL of DNA extraction buffer (100 mmol l^{-1} Tris–HCl [pH 8.0], 100 mmol l^{-1} sodium EDTA [pH 8.0], 100 mmol l^{-1} sodium phosphate [pH 8.0], 1.5 mol l^{-1} NaCl, 1% CTAB) were added together with 100 μL of proteinase K (10 $mgmL^{-1}$). The soil samples were incubated at 37°C for 30 min by horizontal shaking at 225 rpm. After that, 3 mL of 10% SDS solution was added to each sample and incubated at 65°C for 2 h with gentle end-over-end inversions every 15–20 min.

Following incubation, each suspension of soil samples was centrifuged at 6000 ×*g* for 10 min at room temperature and was transferred into a new sterile 50 mL centrifuge tube. The soil pellets were extracted again by adding 4.5 mL of the DNA extraction buffer and 1 mL of 10% SDS, vortexing for 10 s and incubating at 65°C for 10 min. The soil samples were once again centrifuged and the supernatants from the two cycles of extractions were combined. Subsequently, an equal volume of chloroform isoamyl alcohol (24:1, vol/vol) was added in. The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at −20°C overnight. On the following day, the soil samples were centrifuged at 16,000 ×*g* for 20 min at room temperature. Subsequently, the pellet was washed with cold 70% ethanol and resuspended in sterile deionized water to give a final volume of 200 μL. Gel electrophoresis was carried out on 0.8% agarose gel using Mupid-exU System (Takara, Japan) with 50 V for 1 h to observe the quality of the extracted DNA. The agarose gel containing DNA band of approximately 20 kb in size was excised and subjected to gel purification with Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's protocol.

Screening of PHA synthase genes from limestone soil PCR was performed using class I and II degenerate primers (G-D and G-1R) to amplify the partial PHA synthase (*phaC*) gene from the soil genomic DNA (22) (Table S2). The optimized PCR reaction mixture contained 1× Green GoTaq Flexi Buffer, 2.5 mmol l^{-1} $MgCl_2$ Solution, 0.2 mmol l^{-1} dNTP Mix, 1 μmol l^{-1} of upstream and downstream primers, 1.25 U of GoTaq DNA Polymerase (Promega, USA) and 100 ng of purified soil DNA. The PCR run was performed in an MJ Mini Gradient Thermal Cycler (Bio-Rad, USA) and consisted of one cycle of 95°C for 2 min and 39 cycles of 95°C for 30 s, 55.5°C for 1 min, 72°C for 2 min; one cycle at 72°C for 5 min. The PCR product was purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA).

The PCR product was ligated into pGEM-T Easy Vector (Promega, USA) and transformed into *E. coli* JM109 High Efficiency Competent Cells (Promega, USA) according to the manufacturer's protocols. The transformation culture was plated onto LB/ampicillin/IPTG/X-Gal plates and the plates were incubated at 37°C for 16 h. White colonies were sub-cultured onto LB/ampicillin plates and incubated overnight at 37°C.

Sequence analyses All of the positive transformants were subjected to plasmid DNA extraction using QIAprep Plasmid Miniprep Kit (Qiagen, German). The plasmids were sent to Genomics Bioscience, Kuala Lumpur for DNA sequencing. The sequence analyses were carried out according to previous reports with modifications (23,24). Each of the obtained sequence was trimmed by removing the pGEM-T Easy Vector sequence using BioEdit (25). After that, the sequences were used to check for open reading frame through Expasy (http://web.expasy.org/translate/). Sequences without proper open reading frame were removed from further analysis.

The identity of each clone was determined by using the Basic Local Alignment Search Tool (BLAST) analysis of the translated amino acid sequence. Sequence similarities among the clones were identified using MegAlign (DNASTAR, USA) and those with more than 90% sequence similarities were classified under the same gene group. All the gene groups were then further analyzed by MEGA 5.2 (26) using CLUSTAL W and a phylogenetic tree was drawn using neighbor-joining method with bootstrap value of 1000.

Full length PHA synthase gene amplification through genome walking The full length *phaC* gene amplification was carried out using inverse affinity nested-PCR (IAN-PCR) as described by previous studies (24,27). Primers used are listed in Table S2. The extracted genomic DNA (5 μg) was digested using *Apa*I, *Bam*HI, *Hind*III and *Not*I restriction enzymes (REs) respectively (Thermo Scientific, USA) and then self-ligated using DNA Ligation Kit Ver 2.1 (TaKaRa, Japan) at 16°C for 45 min. Since the size of complete PHA synthase gene is approximately 2 kb and thus, the genomic DNA cannot be over-digested (size of fragments < 2 kb) in order to obtain the complete gene. The self-ligated DNA was used as a template for the first round inverse PCR. A PCR mixture consisted of 2.5 U *LA Taq* DNA polymerase (TaKaRa, Japan), 0.4 mmol l^{-1} each dNTP, 1× GC buffer I, and 0.4 μmol l^{-1} each primer in a total volume of 50 μL. The PCR conditions were as follows: 94°C for 1 min, followed by 40 cycles of 98°C for 20 s, 68°C for 20 s, and a final step at 72°C for 10 min. After that, the PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Germany) and subjected to affinity purification using Dynal Dynabeads Kilobase Binder kit (Invitrogen, USA). Then, 2 μL of the product was used as a template for second round nested PCR. The PCR mixture and conditions used were similar as described earlier (T_a = 50°C). Purified PCR products were cloned into vector pGEM-T Easy and JM109 High Efficiency Competent Cells (Promega, USA). The plasmids were extracted and sent for DNA sequencing. Each clone was identified with the BLAST using translated amino acid sequence. Full length PHA synthase gene was determined by assembling the sequenced DNA fragments using SeqMan (DNASTAR, USA).

Construction of *C. necator* PHB^{-}4 transformant Forward and reverse primers (SC8F and SC8R) were designed based on the complete *phaC* gene sequence with each primer containing additional restriction sites at the 5′-end (Table S2). PCR was carried out using the enzyme *LA Taq* (TaKaRa, Japan) with the following conditions: 94°C for 1 min followed by 35 cycles of 98°C for 20 s, 63°C for 20 s