PHAs Producing Bacteria Isolated from Waste Cooking Oil: Isolation and Characterization

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Abstract
Polyhydroxyalkanoates (PHAs) are a family of biodegradable thermoplastic polyesters that have been proposed as potential substitutes for synthetic plastics. PHAs, biopolymers can be used in many fields. This study aims to search for bacterial strains which able to produce PHA. Thus, isolation was conducted from waste cooking oil from Restaurant Ayub in Taman Universiti, Johor Bahru, Johor, Malaysia. The isolates were then cultured in nitrogen limiting media (PHA production media) in purpose of PHA accumulation. Sudan Black Staining was conducted to detect PHA granules. Positive result indicates the present of PHA granule in the bacteria. Biochemical tests were also conducted to characterize the isolate. The 16S rRNA analysis revealed that bacterium F had 98% similarity to *Bacillus sp*. All the biochemical test results for the isolated bacteria were same as to *Bacillus cereus* except Triple Sugar Iron (TSI) test. All the tests conducted shows the bacterium may belong to *Bacillus cereus* species. Fourier Transform-infrared Spectroscopy (FT-IR analysis) were conducted to characterize the PHAs produced by the bacterium.
Keywords: PHAs; Thermoplastic; Waste cooking oil; 16S rRNA gene sequencing; FTIR

Introduction

Polyhydroxyalkanoates (PHAs) are biocompatible and biodegradable polyesters that are synthesized by several types of bacteria. PHAs are stored in the bacterial cytoplasm as inclusion bodies and intracellularly accumulated as granules [1]. They are also accumulated as energy and carbon reserves under unfavourable nutrient conditions in the presence of extra carbon source [2] and limiting supplies sulphur, phosphate, nitrogen, or oxygen [3].

These polymers are naturally synthesized biopolymers and decomposed by some microbial metabolisms [2]. Microorganisms transform fatty acids sugars to PHAs through different metabolic pathways, which involve as intermediate either acyl-CoA or acetyl-CoA, and end with monomer polymerization by PHA synthases [4]. PHAs can be divided into 3 groups by the number of carbon atoms in their side chain: short chain length is composed of 3–5 carbon atoms (e.g. polyhydroxybutyrate (PHB) and hydroxyvalerate (PHV)), while medium chain length consists of 6–18 carbon atoms and long chain length comprises 18 or more carbon atoms [5].

PHAs are naturally accumulated by several bacteria such as *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Cupriavidu* [6]. PHA producer bacteria can be classified into two groups based on the culture conditions which favour PHA accumulation: the bacteria that need limitation of essential nutrients and the bacteria that do not need nutrient limitation for PHA synthesis [7].

PHAs can be produced by bacteria from renewable resources such as lipids, carbohydrates, organic acids and alcohols under unfavourable growth conditions owing to an imbalance in the nutrient supply such as limitations of oxygen, nitrogen or phosphorus and the presence of an excess carbon source [8]. Moreover, relevant substrates for the production of PHA are renewable resources, carbon dioxide fossil resources, waste materials, specialty chemicals [9].

Some of wastes contain carbon-rich substrates which may be useful for PHA production. Production of PHA from wastes has double benefits. Firstly, polluting waste derived from environment can be converted into biodegradable polymer or environmental friendly pollutants. Secondly, as a major problem in production of PHAs in industrial is their high production cost compared to petrochemical plastics, using waste products as a substrate can decrease the cost of the carbon source as the most important factor to the overall production costs [10].

In response to the harmful effects and problems of plastic wastes on the environment, there has been considerable interest in the development of biodegradable plastic materials. PHA is an attractive substitutes for petrochemical plastics among the various biodegradable polymer materials because they have similar material properties to various thermoplastics and elastomers, and complete biodegradability upon disposal under various environments [10].

Several researchers are trying to isolate PHA producing-microorganisms from different sources since the potential to discover and identify novel species with much higher production capacity remains untouched. Isolation, identification and genetic manipulation of natural microorganisms that produce new polymers containing different constituents, properties and applications direct a promising future for industrialization of bioplastics [6]. In this study, waste cooking oil was used as a source for isolation of PHA producer bacteria.

Materials and methods

Waste cooking oil (WCO) used throughout the study was obtained from Restaurant Ayub at Taman Universiti, Skudai, Johor, Malaysia. Sudan Black-B was purchased from Sigma-Aldrich Co. DNA extraction kit and Primers were purchased from Promega Co.

Nutrient broth medium was used for the preparation of the bacterial inoculums. Approximately 8 g/L of nutrient broth powder was added into a 1L Schott bottle containing 1L of distilled water. They were mixed using heat shaker and sterilized via autoclaving at 121 °C for 15 min. The broth was left to cool to 50 °C prior to pour into universal bottle. The universal bottle could be directly used or stored at 4 °C for further using [11].

Nutrient agar was used as culture media for bacterial cells. An amount of 20.0 g/L of the nutrient agar powder was added into 1L of distilled water. They were mixed using heat shaker and sterilized via autoclaving at 121°C for 15 min. The medium was left to cool at 50°C prior to pour into sterile plates. The agar was left to become solid completely. The plates could be used directly or stored at 4°C for further using. [11].

Nutrient-Limiting Media (PHA detection Media) was used to induce bacteria to PHA accumulation [12]. In this study, ammonium sodium phosphate dibasic tetra hydrate, dipotassium phosphate and mono potassium phosphate was mixed using heat shaker and sterilized via autoclaving at 121°C for 15 min. Also, Glucose was sterilized via autoclaving separately. Afterward, magnesium sulphate heptahydrate, and trace element are added to them by filtering.

Isolation of PHA producing bacteria were made by serial dilution and enrichment isolation technique [13]. Sample (1 ml) was added to 10 ml of nutrient broth (NB). Samples were incubated in 37°C at 200 rpm for 24 h. Then, 1 ml of each sample was added to 9 ml sterilized distilled water and diluted 7 times. Then 100 μl/mg of these pre-treated samples were added to nutrient agar (NA) under the aforementioned conditions for 24 h. Pure bacterial isolates were obtained by re-culturing individual colonies several times on fresh agar medium to produce single colonies.

The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan Black-B [14]. All the colonies were grown in 10 ml of nutrient rich medium with shaking for 24 h at 37°C. These cultures were then used as inoculums for a 50 ml nitrogen limiting media for 24 h at 37°C with shaking at 150 rpm. As turbidity increase in the culture medium, a loopful of each of these cultures was smeared on glass slide for staining [13].

After the complete production of PHA under suitable growth conditions, thin smear of strain was fixed on a glass slide by applying heat. This smear was air dried and fixed by immersing in 2% acetic acid for 5min. Then it was stained with a 3% w/v Sudan Black B (0.3 g of the powdered stain in 100 ml of 70 percent ethyl alcohol) solution for 10 min at room temperature and blot the cleared slide dry. The slide was then immersed in xylene until completely decolorized. The sample was counterstained with

safranin (5%, w/v, in distilled water) for 10 s, twice washed with distilled water, and dried afterwards. Once the slide was completely dried, several drops of immersion oil were directly added on cell sample for microscopic examination under optical microscopy. Cellular compartments shown in black blue and pink colour under examination indicated the highly possible PHA containing and PHA-absent granules in cell samples, respectively [15]. Colonies that producing PHA were purified and streaked on fresh NA plate, for further study.

Firstly, a drop of sterile distilled water was added on the slide followed by a loopful of bacteria. The smear is heat-fixed by passing the slides over the flame about few times. Then, the slide was flooded with crystal violet for a minute and rinsed with water. Next, a few drops of gram iodine for one minute and rinsed by distilled water. Subsequently, the smear decolourized using 70% acetone alcohol and washed with distilled water without any delay to prevent decolourization. Safranin was flooded on for 30 seconds and rinsed with water and excess water was removed by using blotting paper. Then, slide was observed under microscope to see the morphology of bacteria.

To collect the cell pellet, 1ml of an overnight culture underwent centrifugation for 2 minutes at 13,000-16,000x g, leading to the removal of the supernatant. The cells were then resuspended in 480μl of 50mM EDTA, followed by the addition of 120μl of lytic enzyme. Subsequent to a 30-60 minute incubation at 37°C, the sample underwent another centrifugation step (2 minutes at 13,000-16,000 x g), and the supernatant was discarded.

For cell lysis, 600μl of Nuclei lysis solution was introduced. The solution was gently mixed and then incubated at 80°C for 5 minutes, followed by cooling to room temperature. The next step involved adding 3μl of RNase solution, which was mixed and incubated at 37°C for 15-60 minutes before cooling to room temperature. Following this, 200μl of protein precipitation solution was added to precipitate the protein, and the mixture was vortexed. After a 5-minute incubation on ice, the solution underwent centrifugation at 13,000-16,000 x g for 3 minutes. The resulting supernatant was transferred to a clean tube containing 600μl of room temperature isopropanol, mixed, and centrifuged for 2 minutes at 13,000-16,000 x g.

Subsequently, 600μl of room temperature 70% ethanol was added and mixed, followed by another round of centrifugation for 2 minutes at 13,000-16,000 x g. The ethanol was then aspirated, and the pellet was air-dried for 10-15 minutes. Finally, the DNA pellet was rehydrated in 100μl of rehydration solution for 1 hour at 65°C (or overnight at 4°C). The extracted DNA was utilized in gel electrophoresis, with the extraction process carried out using the Promega kit A1120.

After DNA extraction, the present of DNA was examined by gel electrophoresis. Nano-Drop instrument was used to determine whether there is enough amounts of pure DNA or not. The DNA purity was estimated at A260nm/A280nm. A ratio of 1.8- 2 shows pure DNA.

The polymerase chain reaction (PCR) was carried out to amplify the target DNA fragments using universal primers, Fd1 (5' - AGA GTT TGA TCC TGG CTC AG - 3') and rP1 (5' - ACG GTC ATA CCT TGT TAC GAC TT - 3'). DNA amplification was performed for 30 cycles and the PCR cycle was set as initial denaturation 94°C for 5 min, followed by cooling, denaturation 94°C for 1 min, annealing 55°C for 1 min, 72 °C for 5 min and final extension 72°C for 10 min.

The product after PCR amplification was purified by gene cleans kit. Firstly, an equal volume of Membrane Binding Solution was added to the PCR amplification. Then, SV Minicolumn was inserted into Collection Tube and dissolved gel mixture or prepared PCR product was transferred to the Minicolumn assembly and was incubated at room temperature for 1 minute. After that, it was centrifuged at 16,000 × g for 1 minute. Then flow through was discarded and Minicolumn was reinserted into Collection Tube. In the washing step, 700μl Membrane Wash Solution (ethanol added) was added and centrifuged at 16,000 × g for 1 minute. Then flow through was discarded and Minicolumn was reinserted into Collection Tube. Also 500μl Membrane Wash Solution was added again and was centrifuge at 16,000 × g for 5 minutes.

The collection tube was emptied and the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. Minicolumn was carefully transferred to a clean 1.5ml micro centrifuge tube. And 50μl of Nuclease-Free Water was added to the Minicolumn. Finally, it was incubated at room temperature for 1 minute and was centrifuged at 16,000 × g for 1 minute. Minicolumn was discarded and DNA was stored at 4°C or –20°C.

Molecular Evolutionary Genetics Analysis version 5 (MEGA5) is a user-friendly software for building sequence alignments, mining online databases and phylogenetic trees and exploiting methods of evolutionary bioinformatics in basic biology, evolution and biomedicine. MEGA5 performs better than other software packages in terms of accuracy of the estimates of phylogenetic trees and computational efficiency [17].

The identity of the sequence were determined by comparing the 16S rRNA sequence with the sequence available in the public nucleotide databases at the National Centre for Biotechnology Information (NCBI) by using world wide web site and the BLAST algorithm. The sequences were aligned and the phylogenetic tree was constructed using the neighbour joining method using MEGA5 [18].

The reconstruction of phylogenetic trees is known as one of the most important and interesting problems of evolutionary study. A computer program package called MEGA5 has been developed for estimating evolutionary distances, reconstructing phylogenetic trees and computing basic statistical quantities from molecular data. In this program, various methods for estimating evolutionary distances from nucleotide and amino acid sequence data, three different methods of phylogenetic inference (UPGMA, neighbour joining and maximum parsimony) and two statistical tests of topological differences are included.

The Molecular Evolutionary Genetics Analysis (abbr. MEGA) software was used to infer evolutionary relationships of homologous sequences, explore basic statistical properties of genes and estimating neutral and selective evolutionary divergence among sequences. Phylogeny tree was constructed based on the results of BLAST. Mega 5.2.1 was used in the construction of phylogenetic tree to estimate the similarity to other bacterial strains and evolutionary pattern.

The isolate was initially inoculated in nutrient-rich medium for 24 h, and then cells were harvested and again re-inoculate into nitrogen-limiting medium containing glucose. The organism was allowed to grow for 48 at 37°C. After that the cells were harvested, washed for PHA extraction and PHA analyse [19].
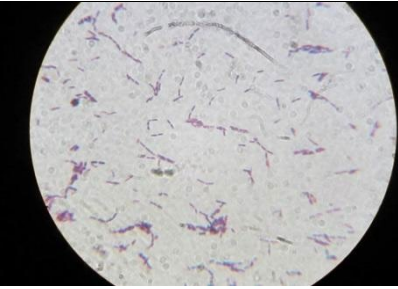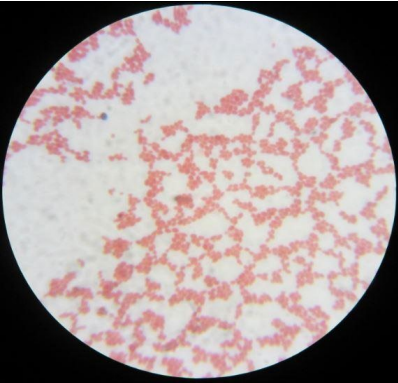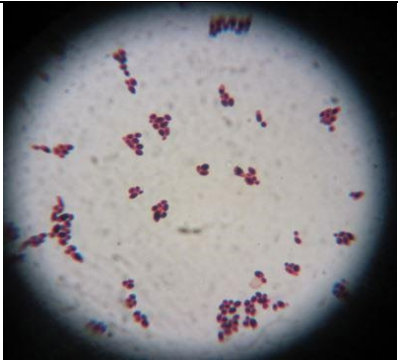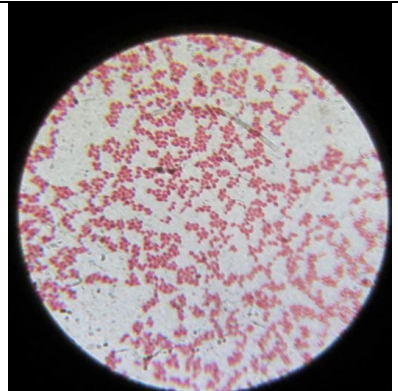
The cells that were grown in the nutrient-limiting media were harvested by centrifugation at 10,000 rpm for 20 min. The pellet was suspended in distilled water and maintained for 24 h at 5°C, conditions which ensure the lysis of the cells. This lysed suspension was centrifuged at 2,000 rpm for 30 min. The pellet was washed 5 to 10 times with the distilled water, showing finally a pure white colour, an indication that there were almost no cells or cell membranes left. The final pellet was dried in an oven at 80°C until constant weight was attained. The white dust resulting from this treatment was dissolved in distilled chloroform. Most of the material dissolved readily, and the undissolved remains were discarded. Finally, the chloroform was evaporated at room temperature and a film was obtained [20].
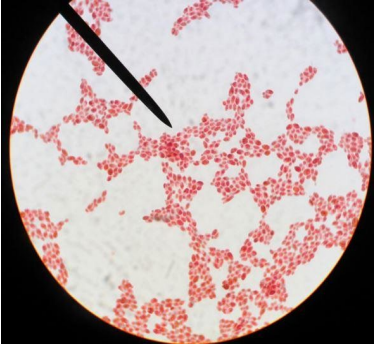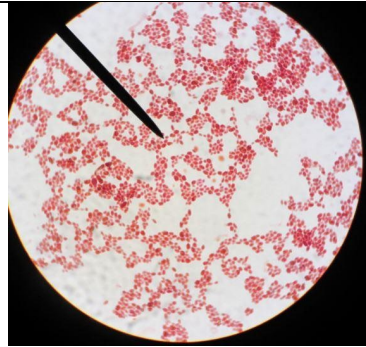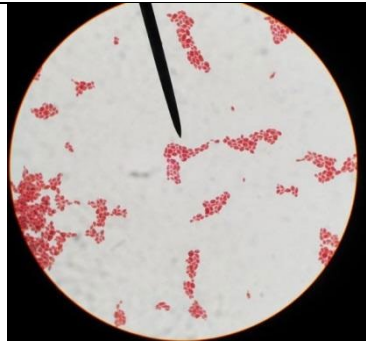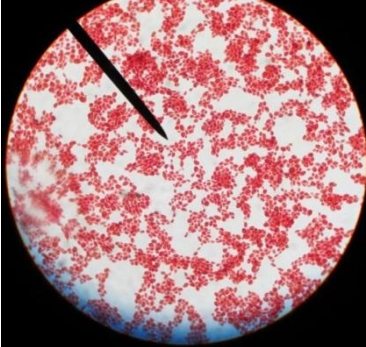
The PHA extracted from the bacteria was analysed by FT-IR spectroscopy. To confirm the functional group of the extracted polymer, it was used under the following conditions: spectral range, 4000-400 cm^{-1} [21].

Results and discussion

From the waste cooking oil which was taken from Restaurant Ayub in Taman Universiti, Johor Bahru, Malaysia, eight strains were isolated by serially diluted samples were spread on nutrient agar plates from the 10^{-7} and 10^{-3} concentration. Pure colonies were obtained by sub-culturing more than three times. The differential staining method, gram-staining has been carried out to classify the isolate strains into two basic group, gram positive and gram negative. Based on the analysed data under 1000x magnification, three strains fall under gram-positive category and other five strains are under gram-negative category. Table 1 summarizes the morphology of the cell structures under light microscope.

Table 1: Morphology of cell culture under light microscope

Colony	Observation under light microscope
F1 1000x magnification Gram positive (Purple colour) Rod shape	
F2 1000x magnification Gram negative (pink colour) spherical shape	
F3 1000x magnification Gram positive (Purple colour) spherical shape	
F4 1000x magnification Gram negative (pink colour) spherical shape	

F5 1000x magnification Gram negative (Pink colour) spherical shape	
F6 1000x magnification Gram negative (pink colour) spherical shape	
F7 1000x magnification Gram negative (pink colour) spherical shape	
F8 1000x magnification Gram negative (pink colour) spherical shape	

All the bacterial isolates were then cultured in a two-stage process. In the first stage, isolates were grown in 10 ml of nutrient rich medium to encourage cell proliferation with shaking for 24 h at 37 °C, and then the cells were transferred to nitrogen-limiting medium containing glucose as sole carbon source, to stimulate PHA accumulation since PHA is accumulated generally under conditions of a nutrient limitation in the presence of excess carbon source. All the isolates were able to grow on nitrogen limiting medium. As turbidity increase in the culture medium, a loopful of each of these cultures was smeared on glass slide [13].

All the bacterial isolates were subjected to an initial Sudan Black B staining to detect the presence of PHA granules in the bacteria. The lipophilic dye Sudan black B used to stain colonies and to

distinguish between PHA-accumulating and non-accumulating strains [22]. However, before the staining with Sudan Black B the isolate was first induced to accumulate PHA.

All strains were negative while PHA granules were found in only one strain. The negatively stained isolates were considered to be unable to produce PHA. The PHA producer bacterium was named "F1" strain. Black blue colour under microscope indicated the PHA granules in cell sample [23]. Figure 1 indicates the bacterium after Sudan Black B staining under the microscope.

Figure 1. Sudan black staining result (1000x magnification)

After isolation and screening the PHA producing bacteria, the genomic material of the bacterium F1 has been isolated by Promega DNA Purification Kit for identification the bacteria. Existence of DNA was identified by nano drop machine. The DNA concentration was 239.36ng/μl and the ration of 260/280 was 2.09. Before Polymerase Chain Reaction, the presence of DNA was checked by gel electrophoresis. The bright band indicates the present of DNA. In DNA extraction the whole genome of bacterium was extracted so the band seen in the top of the gel. Figure 2 shows the DNA extraction outcome on gel electrophoresis. Lane 1 shows the DNA ladder and lane 2 show extracted DNA.
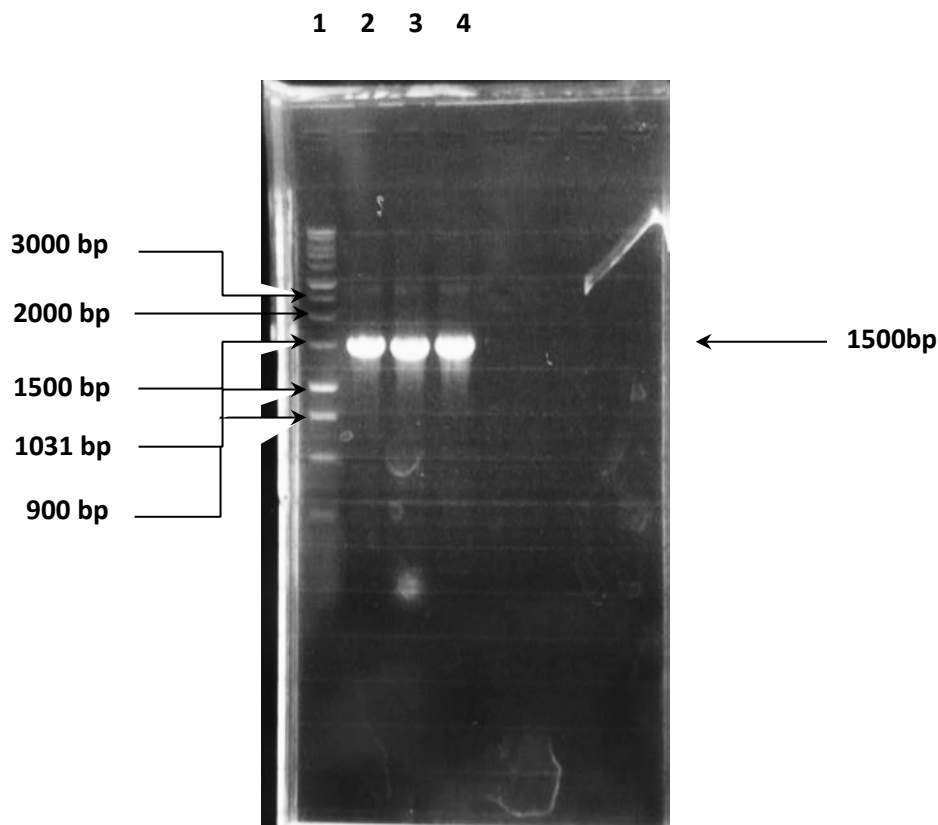
Figure 2 The gel electrophoresis outcome for PCR product of 16S rRNA gene

PCR was performed using pure bacterial DNA. The universal primers Fd1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP1 (5'-ACG GTC ATA CCT TGT TAC GAC TT-3') were used. Product of PCR reaction was checked by gel electrophoresis. Figure 2 shows the PCR outcome on gel electrophoresis. The 1.5 kb of the PCR product of 16S rRNA was amplified. The bright band near 1500bp indicates the 16S rRNA gene amplification. Lane 1 shows the DNA ladder. The other lanes show the 16S rRNA gene amplification. Before sending the PCR product for sequencing, purification was performed for PCR product by Promega PCR clean-up kit. After purification the concentration of DNA was measured by nano drop and the result was 50 ng/µl. After purification, the PCR product was sent to First Base Company for sequencing.

In order to analyse of 16S rRNA gene, the forward and revers primers were aligned by clustalw according to Molecular Evolutionary Genetics Analysis (MEGA 5.2) tutorial. The result was viewed by MEGA 5.2 in FASTA format.

The sequence was BLAST in National Centre of Biotechnology (NCBI) database in order to determine its similarity with other species. Sequence analyses predict that strain F1 belongs to *Bacillus* sp. The isolated bacterium F1 has 98% identical to *Bacillus sp.* Although 16S rRNA gene sequencing is highly useful regarding bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera. The genus *Bacillus* is a good example of this.In the current investigation, the 16S rRNA sequence of PHA producing bacteria shared 98% homology to *Bacillus sp.* Thus the isolated bacterium may tentatively belong to *Bacillus* species. The genera *Bacillus* being identified as one of the first Gram-positive bacteria capable of PHA production [24].

Seven candidates from BLAST analysis were chosen together with sequence of the isolated bacterium to perform alignment using MEGA5 version 5.2 and the Neighbour-Joining bootstrap test phylogeny tree of isolated bacteria (strain F1) was constructed. The details were shown in Figure 3.

The phylogenetic tree shows that strain F is a closer relative of *Bacillus.topicus* strain MCCC 1A01406 and *Bacillus nitratireducens* strain MACC 1A00732.
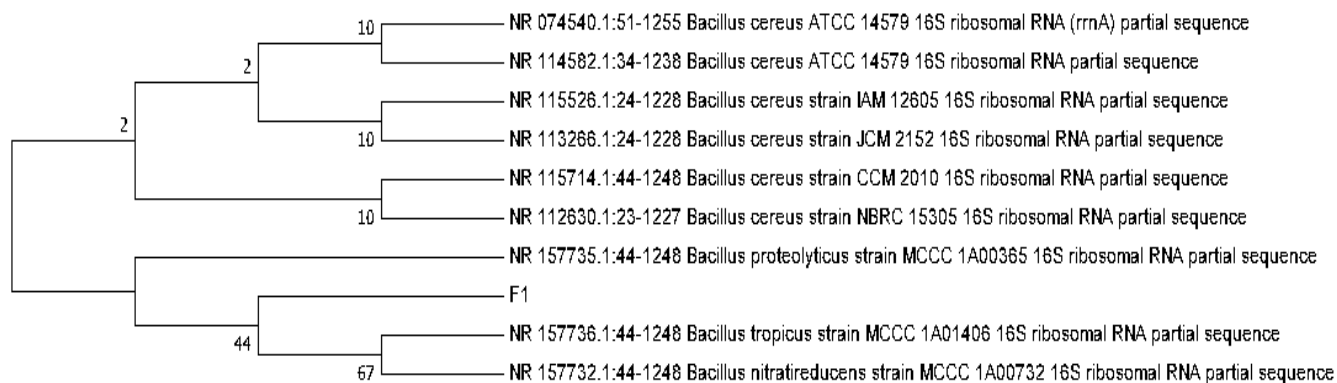

NR 074540.1:51-1255 Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) partial sequence
NR 114582.1:34-1238 Bacillus cereus ATCC 14579 16S ribosomal RNA partial sequence
NR 115526.1:24-1228 Bacillus cereus strain IAM 12605 16S ribosomal RNA partial sequence
NR 113266.1:24-1228 Bacillus cereus strain JCM 2152 16S ribosomal RNA partial sequence
NR 115714.1:44-1248 Bacillus cereus strain CCM 2010 16S ribosomal RNA partial sequence
NR 112630.1:23-1227 Bacillus cereus strain NBRC 15305 16S ribosomal RNA partial sequence
NR 157735.1:44-1248 Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA partial sequence
F1
NR 157736.1:44-1248 Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA partial sequence
NR 157732.1:44-1248 Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA partial sequence

Figure 3 Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [25]. The bootstrap consensus tree inferred from 10000 replicates [26] is taken to represent the evolutionary history of the taxa analyzed [26]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches [27]. The evolutionary distances were computed using the p-distance method [28] and are in the units of the number of base differences per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1205 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [29].

After the fermentation process cells containing bio-polymers (PHA) are separated. Later, when complete evaporation and dryness, a white film was observed. This film was then characterized by FT-IR analysis. Figure 4 shows the result of extraction.

Figure 4 PHA extraction result

In this study, the functional groups of the polymer PHA was confirmed as C=O groups by FT-IR spectroscopy. As is shown in Figure 5, the drastic absorption band located at approximately 1720 cm^{-1} indicates to the stretching vibration of the C=O groups in the PHA polyester. The band around 1378 cm-^{1} corresponds to the symmetrical wagging of terminal CH$_3$ groups and at 1453 cm-^{1} was due to asymmetric deformation of methylene groups [25]. The region from 2800 to 3100 is corresponds to the stretching vibration of C-H bonds [30]. Absorption band at 2927.28 cm-^{1} was assigned to asymmetric CH$_2$ group of the lateral monomeric chains. Series of absorption bands at 1282.21 cm-^{1} to 723.12 cm-^{1} were assigned to asymmetric C-O-C, C-O and C-C stretching vibration [21].

Many species of *Bacillus* are reported to be able to produce PHA. The *Bacillus sp* as PHA producer has been isolated from different sources such as soil [13], palm-oil mill effluent and integrated-farming pond [31], water [32], municipal wastewater, chicken dung, cow dung and pickle waste [33]. Also, in this study, isolation of PHA producer bacteria from waste cooking oil led to find *Bacillus sp. MML1* F1 as PHA producer.

Many studies have been conducted on different environmental conditions, different pHs (5.0–12.0) and different temperatures (30°C and 37°C), for isolation of *Bacillus spices* bacteria. For instance, *B. licheniformis* (GQ478407) in pH 5, *B. licheniformis* (JF798392) in pH 6, *B. cereus* (JF815404) and *B. subtilis* (GQ861469) in pH 7 and *Bacillus sp.* (GQ925365) in pH 10 [6](Sangkharak and Prasertsan, 2012a). In this study *Bacillus sp.MML1* is isolated under culture conditions with pH 7 and temperature equal to 37°C.

The genera *Bacillus,* identified as bacteria capable of PHA production, offers several advantages for PHA fermentation studies. These include chemoorganotrophic features, lack of lipopolysaccharide and secretion of a large number of amylases and proteases. These features of *Bacillus* sp. provide the possibility of utilizing various agricultural raw materials as a carbon source for production of different PHAs [24]. *Bacillus* is also able to utilize glycerol or sucrose [34], glucose [35, 36], starch [24], sugarcane molasses, date syrup and soy molasses [37] as carbon source. Reports suggesting use of *Bacillus* for production of a wide range of different PHAs by using different carbon sources are known. These include incorporation of the tercopolymer of 3HB, HV, 3HHX (3-hydroxy-hexanoate) and P (3HB-co-3HV). Also, *Bacillus* has the potential for production of a new PHA co-polymer utilizing different substrates [24].

Conclusion

Increased growth of the human population has led to a significant increase of non-degradable waste materials; of these, plastic waste is the most harmful pollutant of the environment. Polyhydroxyalkanoates (PHAs) biopolymers can be used in many fields. This study has led to finding of bacterial strains which are able to produce PHA. So, bacteria was isolated from the waste cooking oil from Restaurant Ayub in Taman Universiti, Johor Bahru, Johor, Malaysia, cultured in nitrogen limiting media (PHA production media) in purpose of PHA accumulation. The Sudan Black Staining was done to detect for PHA granules. Positive result indicates the present of PHA granule in the bacteria. Also, biochemical test were done to characterize bacterium. Results of 16S rRNA analysis demonstrated that the bacterium F had 98% similarity to *Bacillus sp*. All the biochemical test results for the isolated bacteria were same as to *Bacillus cereus* except TSI (Triple Sugar Iron) Test. Thus the isolated bacterium may belong to *Bacillus cereus* species. Finally, the PHAs that are produced by the bacterium were characterized by FTIR method based on functional group on PHA. The drastic absorption band located at approximately 1720 is indicated to the stretching vibration of the C=O groups in the PHA polyester.

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