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Polyhydroxyalkanoate (PHA) Production Using Waste Vegetable Oil by *Pseudomonas* sp. Strain DR2

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To produce polyhydroxyalkanoate (PHA) from inexpensive substrates by bacteria, vegetable-oil-degrading bacteria were isolated from a rice field using enrichment cultivation. The isolated *Pseudomonas* sp. strain DR2 showed clear orange or red spots of accumulated PHA granules when grown on phosphate and nitrogen limited medium containing vegetable oil as the sole carbon source and stained with Nile blue A. Up to 37.34% (w/w) of intracellular PHA was produced from corn oil, which consisted of three major 3-hydroxyalkanoates; octanoic (C8:0, 37.75% of the total 3-hydroxyalkanoate content of PHA), decanoic (C10:0, 36.74%), and dodecanoic (C12:0, 11.36%). *Pseudomonas* sp. strain DR2 accumulated up to 23.52% (w/w) of PHA_{MCL} from waste vegetable oil. The proportion of 3-hydroxyalkanoate of the waste vegetable-oil-derived PHA [hexanoic (5.86%), octanoic (45.67%), decanoic (34.88%), tetradecanoic (8.35%), and hexadecanoic (5.24%)] showed a composition ratio different from that of the corn-oil-derived PHA. Strain DR2 used three major fatty acids in the same ratio, and linoleic acid was the major source of PHA production. Interestingly, the production of PHA in *Pseudomonas* sp. strain DR2 could not occur in either acetate- or butyrate-amended media. *Pseudomonas* sp. strain DR2 accumulated a greater amount of PHA than other well-studied strains (*Chromobacterium violaceum* and *Ralstonia eutropha* H16) when grown on vegetable oil. The data showed that *Pseudomonas* sp. strain DR2 was capable of producing PHA from waste vegetable oil.

Keywords: Polyhydroxyalkanoate, inexpensive substrate, corn oil, waste vegetable oil, *Pseudomonas*, *phaC*

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Many environmental bacteria [7] and archaea [6] accumulate excess carbon sources as intracellular polyhydroxyalkanoate (PHA) when exposed to environmental stress, such as nutrient limitation [24]. PHAs, polyesters of 3-(R)-hydroxyalkanoate, which is used for energy source storage, are degraded by microorganisms. PHAs have a potential use as bioplastics owing to their high molecular weight, chiral polymeric structure, and biodegradable aliphatic esters [1]. These materials have properties similar to those of petrochemical-derived thermoplastics and elastomers, but are not toxic to the environment [3]. Bacteria degrade PHAs in the environment using several enzymes, such as PHA depolymerase, lipases, and esterases [13].

PHAs are generally classified as either short or medium chain length PHAs (SCL- and MCL-PHA, respectively). SCL-PHAs contain four to five carbon monomers; polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) contain four carbon monomers [3-(R)-hydroxybutyrate] and five carbon monomers [3-(R)-hydroxyvalerate], respectively. *Ralstonia eutropha*, *Allochromatium vinosum*, and *Bacillus megaterium* are representative SCL-PHA producers. Class I (*phaC*), class III (*phaC*, *phaE*), and class IV (*phaC*, *phaR*) PHA synthases are active toward short chain length 3-hydroxyalkanoate-CoA (SCL-3HA-CoA) containing three to five carbons for the production of SCL-PHA. MCL-PHAs contain six to fourteen carbon monomers. *Pseudomonas putida*, *Pseudomonas oleovorans*, and *Pseudomonas aeruginosa* are representative MCL-PHA producers. Class II PHA synthase *phaC* is active toward medium chain length 3-hydroxyalkanoate-CoA (MCL-3HA-CoA) containing six to fourteen carbons for the production of MCL-PHA [14]. Interestingly, *Aeromonas caviae* FA440 and *Pseudomonas* sp. 61-3 produce copolymers containing both SCL-PHA and MCL-PHA [20].

The production of PHAs has been studied in recombinant *Escherichia coli* and other bacteria using various carbon sources. Recombinant *E. coli* effectively produces PHA_{SCL} and PHA_{MCL} from a substrate mixture containing glucose, xylose, and fatty acid [18]. *Ralstonia eutrophus* accumulates polyhydroxybutyrate (PHB) and its copolymers with polyhydroxyvalerate (PHV) or PHAs from gluconate, fructose, CO_2/H_2, and fatty acid [12]. Moreover, *Pseudomonas* and *Methylobacterium* synthesize various PHA from complex substrates, such as sugar, sugar cane, whey, styrene, and phenyl acetic acid [16]. However, the high cost of production is one of the critical problems for the application of research work; therefore, the cost of the raw material, which accounts for about 40% of the total PHA production cost, needs to be reduced [11].

Several bacteria produce PHA from inexpensive resources, such as palm oil and triglyceride [35]. Palm oil is one of the few vegetable oils that contain relatively high saturated fats [2]. Adhesive PHA_{MCL} consisting of saturated and unsaturated aliphatic fatty acid units have been produced from palm oil [3]. However, the industrial abuse of palm oil has caused the extermination of many endangered species, and the clearing of tropical forests, from which fruit and palm trees are obtained, has resulted in climate change.

The expensive raw material is a critical problem in PHA industrial production. Carbon sources of low cost, such as corn oil, are required to decrease the price of PHAs. Corn oil is less expensive than most other vegetable oils. It typically consists of approximately 87% unsaturated fatty acid, and its high smoke point allows its use as frying oil. Waste frying oils (waste vegetable oil) can be stably supplied as cheap carbon sources, such as other waste oils and whey, and can be used as alternative carbon sources for the production of bacterial bioplastics. In this study, vegetable-oil-degrading bacteria (*Pseudomonas* sp. strain DR2) were isolated from rice field soil for the production of PHA from inexpensive substrates corn oil and waste vegetable oil. Using sugar, glycerol, and fatty acid, PHA_{MCL} with slightly different composition was produced in *Pseudomonas* sp. strain DR2. Strain DR2 produced especially large amounts of gluey PHA_{MCL} from waste vegetable oil and corn oil.

MATERIALS AND METHODS

Isolation and Culture Conditions

Enrichment cultivation was carried out in minimal salt basal medium [MSB, 29], complemented with 1% of defined corn oil, at room temperature for 4 days for the isolation of corn-oil-degrading bacteria from a Korea University rice field. *Ralstonia eutropha* H16 (ATCC 17699) and *Chromobacterium violaceum* (KACC 11542; ATCC 12472) were used to compare the production and growth rates of PHA with the isolates. The bacteria isolates were grown at 30°C in Luria-Bertani (LB) broth or MSB, with vigorous shaking at 220 rpm. The MSB medium was supplemented with various carbon sources [corn oil (1%), waste vegetable oil (1%), diesel (1%), glucose (0.4%), sodium citrate (10 mM), sodium acetate (0.8%, pH 7.0), sodium butyrate (0.8%, pH 7), palmitate (0.4%), glycerol (0.8%)] at 30°C for 72 h. The growth was monitored by measuring OD_{600} of the cultures using a Biophotometer (Eppendorf). Bacteria were cultured in MMSB (modified MSB, phosphate and ammonium limited minimal salt media: 20 mM of phosphate, and 7.6 mM of ammonium sulfate) medium, with the above-mentioned carbon as the sole carbon source for 72 h for the accumulation of PHAs.

16S rRNA and Partial Detection of PHA Synthase Genes

The 16S rRNA genes were PCR amplified using the 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R primer (5'-GGTTACCTTGTTACGACTT-3'), which are complementary to the 5'-end and 3'-end of the prokaryotic 16S rRNA genes, respectively. Partial PHA synthase genes were detected by degenerate primers; forward phaCDP-F2 (5'-GTSTTCRTSRTSWSCTGGCGCAACCC-3') and reverse phaCDP-R3 (5'-AGGTAGTTGTYGACSMMRTA-GKTCCA-3') [26]. Amplifications were performed by 35 cycles as follows; after denaturation for 1.5 min at 94°C, denaturation for 45 sec at 94°C, primer annealing for 45 sec at 55°C, and primer extension for 45 sec at 72°C, with a final extension for 5 min at 72°C. The sequencing of the PCR products was performed with a DNA Sequencer (Bionics, Korea), with nucleotide sequences analyzed using the nucleotide BLAST program within the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences of the 16S rRNA from four vegetable oil degraders have been deposited in GenBank under the following accession numbers [strain CR1 (EU181264), strain CR2 (EU181263), strain DR1 (EF520734), and strain DR2 (EU181265)].

Staining Bacteria Producing PHA

Staining of cultures, grown under PHA accumulation conditions, was performed with Nile blue A using a modified method [21, 28]. To select PHA-accumulating bacteria, colonies of isolates were stained with Nile blue A on agar media using a modified method [28]. Briefly, Nile blue A, dissolved in dimethyl sulfoxide (DMSO), was added at a concentration of 0.5 μg/ml to the agar media for viable-colony staining. Stained cells were shown as bright orange colonies under a 550 nm ultraviolet lamp [28]. Glucose (0.4%) and citrate (10 mM) were added to the MMSB as the carbon sources instead of corn oil because of the lipophilic dye. For confirmation of intracellular granules, fixed cells were treated with 1% ethanolic Nile blue A for 5 min and then washed with 100% ethanol. Stained granules in the cells were shown as bright red or orange (depending on exposure time and white balance) spots at 550 nm excitation wavelength filter [21]. Ethanolic Nile blue A solution (1%) was transformed into Nile pink or red (oxazone form) by spontaneous oxidation, which was used as a more effective stain.

PHA Extraction and Composition Analysis

Cultures grown on different carbon sources were dried under vacuum using a freeze dryer. Polyesters were extracted with hot chloroform to dissolve the intracellular PHAs, in a Pyrex Soxhlet apparatus for 9 h, and the dried cell mass was filtered. The concentrated extract solution was precipitated by rapidly stirring in 10 volumes of cold methanol. The isolated PHAs were purified by

re-precipitation in methanol and dried under vacuum [4]. To cleave the ester bond of the polymers, 1 ml of the mixtures, containing sulfuric acid and methanol (3:17), and 1 ml of chloroform were added on extracted polymers or PHA accumulated within the dry cells, and the mixture was then boiled at 100°C for 3 h. The 3-hydroxyalkanoate (3-HA) monomers were separated by adding 1 ml of distilled water, and isolated from the chloroform layers. The composition of the 3-HA monomer was determined using gas chromatography/mass selective detection (GC/MSD; Agilent). The corn oil composition was determined by chromatography of fatty acid methyl esters. Nothing was detected by mass spectrometry for 2.5 min to avoid the peak of solvent (chloroform); the gas chromatography analysis was performed as follows: column, HP-5MS (Agilent); initial; 80°C for 8 min; heating rate, 8°C/min up to 150°C and 2°C/min up to 176°C; holding, 176°C for 1 min; 1 μl of sample was injected at 250°C; carrier (He) flow rate, 34.1 ml/min; oven temperature, 250; detector temperature; 300°C [6].

Derivatization of Corn Oil for Analysis by GC/MS

The analysis of fatty acid methyl esters in the corn oil was performed using the method in *Application Note* 123 supplied by Sigma-Aldrich Co. Briefly, 20–50 mg of corn oil or sample was measured into a vial, and 1 ml of methanolic HCl, and 1 ml of dichloromethane (DCM) and 2,2-Dimethoxypropane (2,2-DMP) were then added. The samples were heated at 100°C for 3 h and then allowed to cool. One ml of water and 1 ml of DCM were then added, and the phases were allowed to be separated after vigorous shaking. The organic layers were sampled for analysis by GC. The fatty acid was quantified every six hours to measure the amount of a fatty acid remaining in the media during growth of *Pseudomonas* sp. strain DR2, using GC with a FAME standard (Supelco 37 Component FAME Mix).

Results

Vegetable-Oil-Degrading Bacteria from Rice Field Soil Samples

Vegetable-oil-degrading bacteria were isolated by enrichment cultivation, using corn oil as the sole carbon source, from Korea University rice field soil. Four strains were isolated from 0 to 10 cm depth of soil from the ground level, but no vegetable-oil-degrading bacteria were isolated from 10 to 40 cm depth of soil from the ground level; the four strains were named *Enterobacteriaceae* sp. CR1, *Rhodococcus* sp. CR2, *Acinetobacter* sp. DR1, and *Pseudomonas* sp. DR2 [27]. An approximate 1,500 bp of the 16s rRNA genes of the strains were amplified by PCR, and 600 from 1,400 bp of these were sequenced. Then, the partial 16S rRNA gene sequences of the strains were compared with those available from the nucleotide BLAST program within the NCBI database to determine an approximate phylogenetic affiliation [32]. A comparative 16S rRNA gene sequence analysis showed that strain DR2 was related to *Pseudomonas fluorescens* and *Pseudomonas jessenii* with 100% identity, and strains CR1, CR2, and DR1 were most closely related to *Enterobacteriaceae bacterium p4f-1*, *Rhodococcus erythropolis* strain LAM18, and *Acinetobacter calcoaceticus*, with similarities of 100%, 100%, and 99%, respectively.

Identification and Sequencing of *Pseudomonas* sp. Strain DR2 PHA Synthase

Partial PHA synthase genes were amplified using PCR with degenerate primers [26], with a 232 bp gene sequenced. The PCR product of *Acinetobacter* sp. strain DR1 showed 88% similarity to the putative FMN oxidoreductase of *Acinetobacter baumannii* ATCC 17978, and 68% to putative FMN oxidoreductase of *Acinetobacter* sp. ADP1. The nucleotide sequences of the 391 bp fragment of strain DR2 and the partial PHA synthase nucleotide of the sequence showed 93% similarity to that of *Pseudomonas* sp. KBOS 03 *phaC1*, 90% to that of *Pseudomonas fluorescens phaC1*, and 89% to that of *Pseudomonas aureofaciens phaC1*. The PHA synthase gene of *Pseudomonas* sp. strain DR2 was conserved with the MCL-PHA synthase *phaC* of several Pseudomonads in class II. *Pseudomonas* sp. strain DR2 produced only PHA_{MCL} from various carbon sources.

Characterization of PHA-Accumulating *Pseudomonas* sp. Strain DR2 from Corn Oil

PHA granules were stained by Nile pink or red (spontaneous oxidized form of Nile blue A), when PHA-accumulated cultures were washed to remove the lipid substrate from the vegetable oil remaining in the medium, which were observed as bright red or orange spots on fluorescence microscopy. PHA inclusions of strains CR1 and CR2 were not detected on the agar medium (data not shown) or under fluorescence microscopy (Figs. 1e, 1f, 1g, and 1h). Whole cells of strains DR1 and DR2 were stained on the agar medium (data not shown) and slide glasses (Figs. 1a, 1b, 1c, and 1d) by Nile pink or red; however, only the PHA granules of strain DR2 were detected as orange or red spots. *Pseudomonas* sp. strain DR2 grew the most cells in the corn-oil-amended medium in 42 h (Fig. 1i). Strain DR2 grown on corn oil accumulated 37.34% PHA_{MCL} in 72 h, and contained three major 3-hydroxyalkanoates [3-HO (39.63%, 3-hydroxyoctanoate), 3-HD (34.13%), and 3-HDD (11.37%)], together with other minor 3-hydroxyalkanoates [3-HB (3-hydroxybutyrate), 3-HHx (3-hydroxyhexanoate), 3-HTD, and 3-HHxD (3-hydroxyhexadecanoate)], which accounted for less than 15% of the total content (Table 1). The PHA_{MCL} produced in *Pseudomonas* sp. strain DR2 using corn oil was rubbery and gluey [35]. Staining PHA with Nile blue or pink has a drawback in the selection of PHA-accumulating bacteria from vegetable oils: Nile blue or pink does not always directly stain the PHA-producing bacteria in broth or on agar plate supplemented with lipid substrates because these lipophilic dyes stain lipid substrates and Nile blue A stains to both PHA monomers in the cell membrane and PHA granules. Colonies of *Acinetobacter* sp. strain DR1

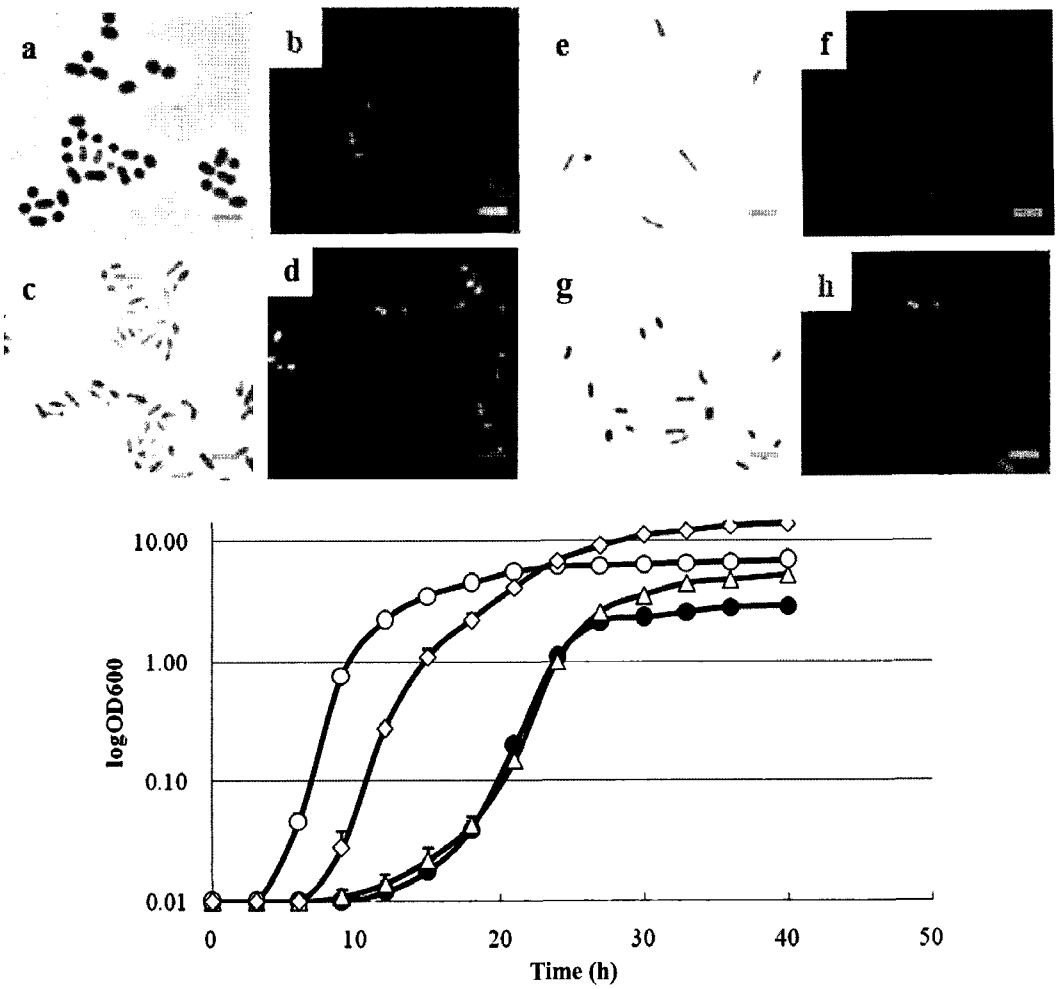

Fig. 1. Selection of vegetable-oil-degrading isolates that are accumulating PHAs in the presence of sodium citrate and glucose.
Isolates were grown in modified minimal salt basal media (MMSB) at 220 rpm and 30°C for 72 h, and stained with 1% ethanolic Nile blue A and Nile pink to confirm granule-formed polymers; (a) visible light and (b) fluorescence photographs of strain DR1, (c) visible light and (d) fluorescence photographs of strain DR2, (e) visible light and (f) fluorescence photographs of strain CR1, (g) visible light and (h) fluorescence photographs of strain CR2; (i) Growth rates of plant-oil-degrading isolates. Corn oil was added to the modified minimal salt basal broth media at 1% concentration. DR1, open circle; DR2, open diamond; CR1, closed circle; CR2, open triangle.

were stained by Nile blue or pink. PHA monomers [39% 3-hydroxydecanoate (3-HD), 52% 3-hydroxydodecanoate (3-HDD), and 9% 3-hydroxytetradecanoate (3-HTD)] were detected with GC/MS in *Acinetobacter* sp. strain DR1 grown on palmitate. However, the PHA granules of strain DR1 were not observed with fluorescence microscopy and not extracted with hot chloroform, indicating that caution should be taken when claiming the presence of PHA-accumulating bacteria by just staining bacteria with these dyes.

PHA Produced with Various Substrates in *Pseudomonas* sp. Strain DR2

Different compositions of PHAs accumulated in *Pseudomonas* sp. strain DR2 when grown on MMSB supplemented with various substrates (Table 1). PHA inclusions were visualized by staining the PHA extracted from strain DR2 with Nile blue A or pink (Fig. 2) and analyzed by GC/MS. PHA was produced in *Pseudomonas* sp. strain DR2 using glucose (5.05%; Figs. 2c, 2d), citrate (3.29%; Figs. 2a, 2b), glycerol (3.59%; Figs. 2k, 2l), palmitate (4.93%; Figs. 2i, 2j), defined corn oil (37.34%; Figs. 2m, 2n), and waste vegetable oil (23.52%; Figs. 2o, 2p). 3-HD or 3-HDD was one of the major components of PHA accumulated from glucose, citrate, and palmitate. 3-HD, 3-HDD, and 3-HTD were the major components in PHA when strain DR2 was grown on glycerol. *Pseudomonas* sp. strain DR2 accumulated PHA_{MCL} from corn oil and waste vegetable oil, and produced PHA_{MCL} up to 37.34% [weight per dried cell weight (DCW)] and 23.52% (weight per DCW), respectively. Interestingly, 2.33% and 0.90% 3-hydroxybutryrate (3-HB) was detected in the strain DR2 using glucose and corn oil, respectively (Table 1). The 3-HA was detected in *Pseudomonas* sp. strain DR2 grown on acetate and butyrate, but PHA was not extracted. The 3-HB was detected in *Pseudomonas* sp. strain DR2 grown on glucose and corn oil, but 3-hydroxybutryate-

Table 1. Composition of PHAs produced from various substrates in *Pseudomonas* sp. strain DR2.

	PHA[a] % (w/w)	CDW[b] (g/l)	3-Hydroxyalkanoate, methyl esters[c] (%)						
			3HB[d]	3HHx[e]	3HO[f]	3HD[g]	3HDD[h]	3HTD[i]	3HHxD[j]
Glucose	5.02	0.89		-	13.20	39.68	42.25	4.87	-
Citrate	3.29	0.84		-	7.740	41.14	51.12	-	-
Glycerol	3.59	0.75		-	11.64	34.42	20.8	33.14	-
Acetate*	-	0.78		-	-	42.09	57.91	-	-
Butyrate*	-	0.83		-	-	43.03	56.97	-	-
Palmitate	4.93	0.76		3.40	18.71	35.6	36.97	-	5.33
Corn oil	37.34	0.96		4.61	37.75	36.74	11.36	7.50	2.04
Fried oil	23.52	0.54		5.86	45.67	34.88		8.35	5.24
Glucose*	-	-	2.33	-	12.89	38.76	41.26	4.76	-
Corn oil*	-	-	0.90	5.24	39.63	34.13	11.37	6.53	2.20

Cells were cultivated in a 1,000-ml Erlenmeyer flask, containing 500 ml of phosphorus and nitrogen limited minimal salt basal media (MMSB), supplemented with various carbon sources (0.4% glucose, 10 mM sodium citrate, 0.8% glycerol, 0.8% sodium acetate adjusted to pH 7.0, 0.8% sodium butyrate adjusted to pH 7.0, 0.4% palmitate, 1% defined corn oil, 1% fried oil) at 30°C for 72 h; [a]polyhydroxyalkanote, [b]cellular dry weight, [c]GC area%; the monomer structures were determined by GC/MS: [d]3-hydroxybutyrate, [e]3-hydroxyhexanoate, [f]3-hydroxyoctanoate, [g]3-hydroxydecanoate, [h]3-hydroxydodecanoate, [i]3-hydroxytetradecanoate, and [j]3-hydroxyhexanoate; *3-hydroxyalkanoate composition in cell lysates without PHA extraction.

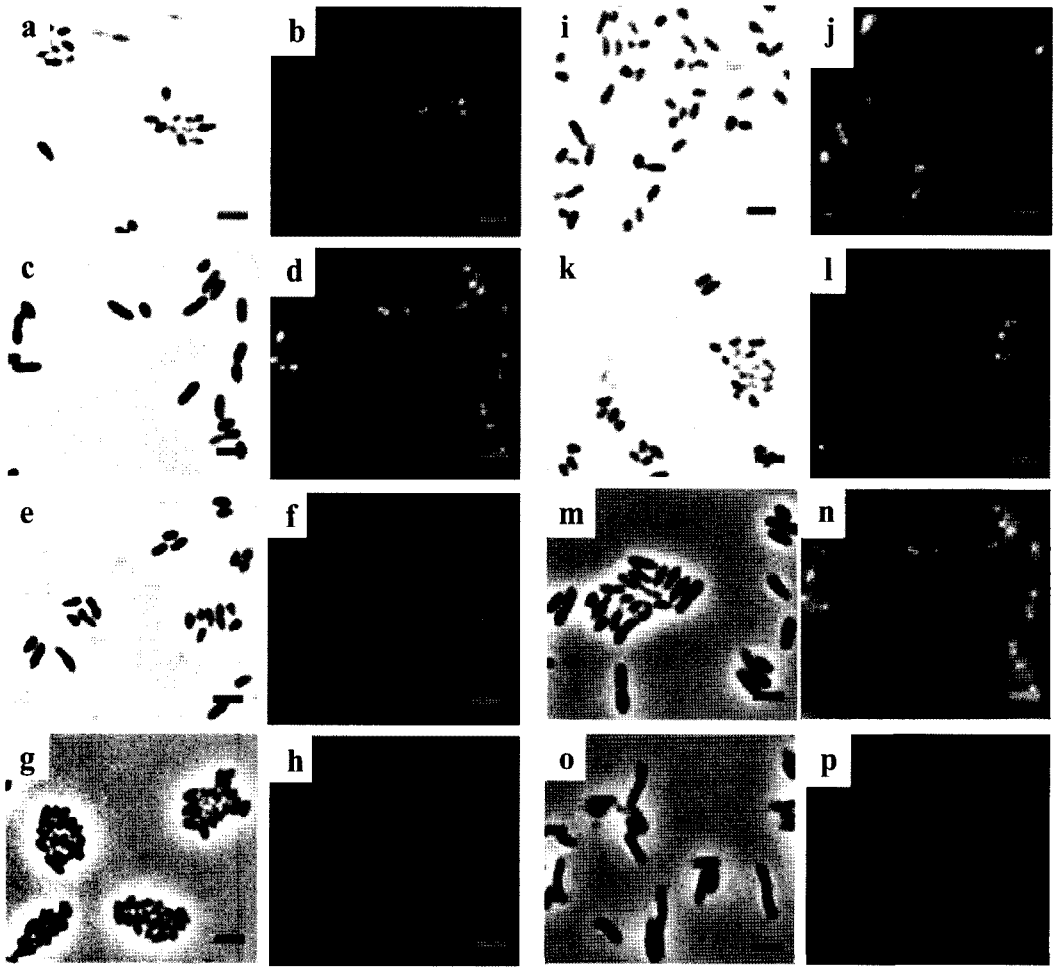

Fig. 2. The effect of polyhydroxyalkanoate accumulation on carbon sources in *Pseudomonas* sp. strain DR2.
Five hundred nm filtered fluorescence photographs of isolates stained with 1% ethanolic Nile blue A and Nile pink to observe the PHA granules formed in *Pseudomonas* sp. strain DR2 grown on various sole carbon sources. A photograph of (a) visible light and (b) ultraviolet ray of grown cells with 10 mM citrate, (c) visible light and (d) ultraviolet ray of cells grown with 0.4% glucose, and (e) visible light and (f) ultraviolet ray of cells grown with 0.8% acetate (pH7.0), (g) visible light and (h) ultraviolet ray of cells grown with 0.8% butyrate (pH 7.0), (i) visible light and (j) ultraviolet ray of cells grown with 0.4% palmitate, (k) visible light and (l) ultraviolet ray of cells grown with 0.8% glycerol, (m) visible light and (n) ultraviolet ray of cells grown with 1% refined corn oil, and (o) visible light and (p) ultraviolet ray of cells grown with 1% chicken fried oil.

polymerized form was not extracted. When the strain DR2 was grown on acetate (Figs. 2e, 2f) or butyrate (Figs. 2g, 2h), no granules were detected; however, the cell membrane was stained. Acetate- and butyrate-derived 3-HA remains in the membrane [10]. The detection of 3-HO indicated an accumulation of PHA_{MCL} when cells were cultivated using sugar, fatty acid, or vegetable oil for 72 h.

Comparison of PHA Accumulation with Well Studied PHA Producers Using Corn Oils

Studies have shown that *Ralstonia eutropha* H16 [23] and *Chromobacterium violaceum* [15, 20] are short chain length PHA producers. Although *C. violaceum* is a well known PHV producer, it accumulates a small amount of PHB (1.55%, w/w), not PHV, in the presence of corn oil as the carbon source. Only PHB was detected in the PHAs extracted from *R. eutropha* H16 and *C. violaceum*. The color of *C. violaceum* was light violet when cultivated in modified minimal basal medium (MMSB) supplemented with corn oil as the sole carbon source, for 72 h. *R. eutropha* H16 accumulated 20.70% (w/w) PHB from corn oil. *Pseudomonas* sp. strain DR2 accumulated more PHA (37.34%) than the other two PHA producers. The PHA_{MCL} extracted from the strain DR2 grown on corn oil had different properties from that of PHB. Both *R. eutropha* H16 and *C. violaceum* showed growth similar to strain DR2; the growth rate of *R. eutropha* H16 was the highest, but the cellular dry weight (CDW) obtained from strain DR2 was as much as *R. eutropha* H16 when grown for sufficient time to accumulate PHA. Model strain *Pseudomonas putida* KT2440 grew on glucose, sodium citrate, and palmitate, and the MCL-PHA could be extracted after 72 h (data not shown). However, *Pseudomonas putida* KT2440 did not grow in media complemented with corn oil or waste vegetable oil (Fig. 3).

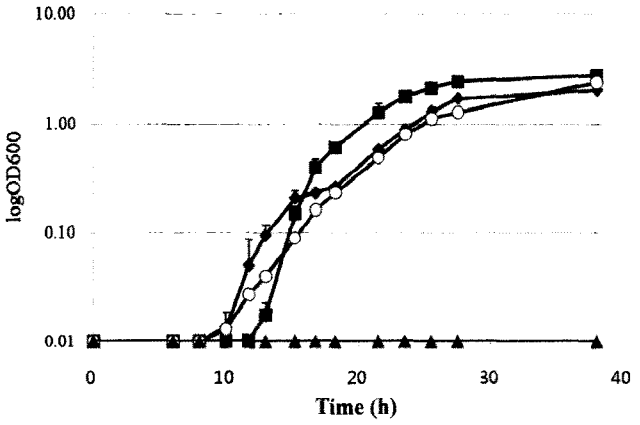

Fig. 3. Growth rate of *Pseudomonas* sp. strain DR2 compared with polyhydroxyalkanoate-accumulating model strains grown on modified minimal salts basal media (MMSB) with corn oil as the sole carbon source.

Chromobacterium violaceum closed diamond, *Ralstonia eutropha* H16, closed square; *Pseudomonas* sp. DR2, open circle; *Pseudomonas putida* KT2440, closed triangle.

Corn Oil Utilization of *Pseudomonas* sp. Strain DR2

The compositions of defined corn oil (http://www.cj.co.kr) and waste vegetable oil were analyzed using GC/MS and GC (FAME standard). The corn oil contained 99% triglyceride with 1.10% other minor fatty acids. Waste vegetable oil consisted of 58.98% linoleic (C18:2), 21.07% palmitic (C16:0), 19.27% oleic (C18:1), 0.41% *cis*-10-pentadecenoic (C15:1), and 0.27% mysteric (C14:0) acids. The strain DR2 (10^{10} cells) were inoculated in MMSB. The concentrations of three major fatty acids in cell-free supernatant were measured every 6 h during the growth of *Pseudomonas* sp. strain DR2 in media complemented with corn oil or waste vegetable oil. The concentrations of the remaining fatty acids were measured 6 times. *Pseudomonas* sp. strain DR2 simultaneously used the three major fatty acids found in waste vegetable oil (Fig. 4). The strain DR2 used a little more palmitic acid than the other fatty acids present in the corn-oil-amended media (Fig. 4a). The relative fatty acid

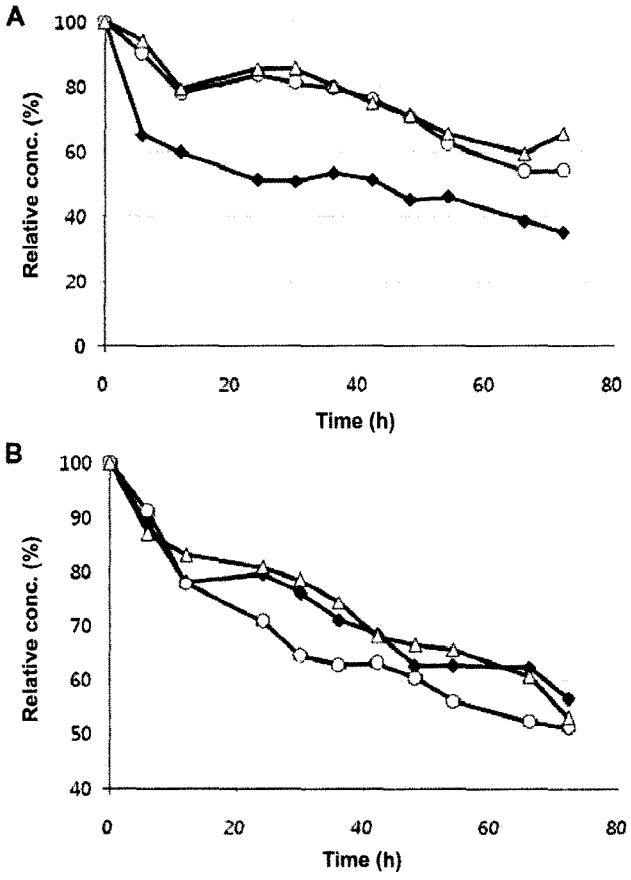

Fig. 4. Fatty acid utilization of *Pseudomonas* sp. strain DR2.

10^{10} cells of *Pseudomonas* sp. strain DR2 were inoculated into MMSB. *Pseudomonas* sp. strain DR2 was grown on 1% (**A**) corn oil and (**B**) waste vegetable oil, and three major fatty acids in the cell-free supernatant were measured every 6 h using GC/MS. Linoleic acid, open circle; oleic acid, open triangle; palmitic acid, closed diamond.

consumptions were similar, but linoleic acid was used at the highest concentration of the fatty acids in the vegetable oils. Linoleic acid (C18:2) was the principal substrate for the production of the PHA_{MCL} monomer (C6-C16).

Discussion

Pseudomonas sp. strain DR2 synthesized only MCL-PHA, comprising C6 to C16, from the various carbon sources. PHA synthase of strain DR2 belongs to the class II MCL-PHA synthase PhaC1 determined [19]. *Pseudomonas* sp. strain DR2 produced PHA_{MCL} from glucose, citrate, glycerol, palmitate, corn oil, and waste vegetable oil (Table 1). PHA_{MCL} of different compositions were produced in *Pseudomonas* sp. strain DR2, depending on various carbon sources. Overall, 37.34% and 23.52% PHA were produced in *Pseudomonas* sp. strain DR2 using corn oil and waste vegetable oil, respectively. 3-HDD was not synthesized, but 45.67% 3-HO was accumulated from waste vegetable oil. MCL-3HA-CoA monomer is supplied from sugar, glycerol, and long carbon number fatty acids *via* several metabolic pathways; thioesterase gene *tesA* [*via* β-oxidation pathway (*fadR*, *fadB*)], transacetylase gene *phaG* [25], 3-ketoacyl-ACP synthase III gene *fabH*, and 3-ketoacyl-acyl carrier protein reductase gene *fabG* [20]. Specifically, PHA_{MCL} might be produced from fatty acids and vegetable oil *via* a β-oxidation pathway of fatty acids as the sole PHA biosynthetic route [31]. *Pseudomonas* sp. strain DR2 showed the fastest growth rate among the vegetable-oil-degrading isolates; however, model strain *Pseudomonas putida* KT2440 did not grow (Fig. 1i and Fig. 3).

The present results of this study indicate that *Pseudomonas* sp. strain DR2 produces a gluey, sticky bioplastic from waste vegetable oil and vegetable oil. An inexpensive substrate and waste treatment technology lead to a moderate price for the PHA products for use in industrial application [9]. Waste vegetable oil is a very useful substrate for the production of PHA from bacteria, since it has a similar composition [glycerol, palmitic acid (16:0), oleic acid (18:1), and linoleic acid (C18:2)] to corn oil. Refined corn oil has been examined as the substrate for quality comparison and it has been reported that various substrates (sugar, alcohol, whey, styrene, phenylacetic acid, and palm oil) can be used to produce PHAs [34]. However, corn oil and waste vegetable oil are less expensive than most other vegetable oils, and can be stably supplied for the industrial PHA production. *Pseudomonas* sp. DR2 uses varieties of substrates such as oil, fatty acid, and sugar. In the present study, fatty acids in corn oil and waste vegetable oil were utilized at a similar rate for cell growth and PHA production (Fig. 4). *Pseudomonas* sp. DR2 was isolated using Nile blue A staining and GC/MS analysis. PHA granules were not directly stained by Nile blue or pink in bacteria grown on vegetable-oil-amended media, as Nile blue- or pink-stained lipids remaining in the media. Microscopic photographs of cells stained by Nile blue or pink showed clear granules accumulated in *Pseudomonas* sp. DR2 using different substrates (Fig. 3).

Pseudomonas sp. DR2 synthesized 3-HD and 3-HDD from acetate and butyrate, but they were not esterified as an aliphatic polymer in strain DR2. 3-Hydroxy-fatty acids are present in the membranes of bacteria [5, 33] or in polyhydroxyalkanoic acids [30]. 3-HD and 3-HDD are components in the lipopolysaccharides of membranes [8], whereas 3-HD is a component of amnolipids, a biosurfactant [17]. When grown on acetate and butyrate, *Pseudomonas* sp. strain DR2 might produce 3-HD and 3-HDD as components of the membrane and surfactant, but are not accumulated as polymers. 3-HB was detected in the cell lysates when *Pseudomonas* sp. strain DR2 was grown on glucose and corn oil. The 3-HB remaining in the cells might temporarily be produced through beta-oxidation, but then not polymerized, or the PHA_{MCL} might have been degraded by depolymerase and then 3-HB might be reserved within the cytoplasm or membrane [22]. In this context, the interest on the PHA in this study lies in its potential use of degradable plastics based on waste treatment technology, with a moderate price of bioplastics products. *Pseudomonas* sp. strain DR2 is capable of degrading waste vegetable oil and producing PHA. Raw materials, such as inexpensive vegetable oil and waste vegetable oil, are alternative substrates that can be used to decrease the production cost of PHA.

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