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Full Length Research Paper

Utilization of *Enterobacter aerogenes* 12Bi Strain for Polyhydroxybutyrate (PHB) Biosynthesis from Domestic Wastewater

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A strain of poly- -hydroxybutyrate (PHB)-accumulating bacterium was isolated and identified as *Enterobacter aerogenes* (designated *E. aerogenes* 12Bi) by using biochemical and phylogenetic characterization. The accumulation of a large amount of granules in its cells cultured in the domestic wastewater medium (DWWM) were showed by transmission electron microscopy (TEM). When PHB production by our strain was determined by Hypochlorite method, it was found that PHB production ranged from 16.66 to 96.25% (w/w). The highest PHB yield by our microorganism was up to 96.25% within 18 h in DWWM 5 (supplemented with 100% DWW). This is the first report of the use of DWW for production of PHB by *E. aerogenes*. The results obtained in the study demonstrated that PHB could be efficiently produced to a high concentration with high productivity by using DWWM as an inexpensive substrate. Thus, it can contribute to the reduction of high production cost of PHB.

Key words: Poly- -hydroxybutyrate (PHB), bioplastics, domestic wastewater, *Enterobacter aerogenes,* biochemical identification, phylogenetic identification.

INTRODUCTION

The most widely produced microbial plastics are polyhydroxyalkanoates (PHAs) and their derivatives (Suriyamongkol et al., 2007; afak et al., 2002; Pozo et al., 2002; Wiltholt and Kessler, 1999). Poly- - hydroxybutyrate (poly-3-hydroxybutyrate=PHB=P3HB) containing only 3-hydroxybutyrate (HB) as its constituent, is one of the most extensively studied PHAs (Khanna and Srivastava, 2005).

PHB is also known as bioplastic (biobased polymer) that is synthesized and catabolized by numerous microorganisms (Akar et al., 2006; Verlinden et al., 2007).

This polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by microorganisms as a form of energy storage material accumulated intracellularly to be metabolized when other common energy sources are not available (Steinbuchel and Doi, 2002; Lugg et al., 2008). Conditions for optimal production of PHB usually include an excess of carbon source and exhaustion of a single nutrient such as nitrogen, sulphur, phosphate, iron, magnesium, potassium or oxygen (Anderson and Dawes, 1990). With the production of PHB is produced a bioplastic. A generic process for PHB production by bacterial fermentation consists of three basic steps: fermentation, isolation and purification, and blending and palletising. After fermentation, these polymers can be obtained by solvent extraction (PRO-BIP, 2009). The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics (Steinbuchel and Doi, 2002; Apostolis et al., 2006; Suriyamongkol et al., 2007; Akaraonye et al., 2010). The advantages of bioplastics over petroleum-based polymers were listed in Table 1.

The total maximum technical substitution potential of bioplastics replacing their petrochemical counterparts is estimated at 270 Mt (million metric tons/year), or 90% of the total polymers that were consumed in 2007 worldwide. Bioplastics, with consumption of only just

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Table 1. The advantages of bioplastics over petroleum-based polymers.

List of of advantages of bioplastics over petroleum-based
polymers PHB and other bioplastics are natural polymers
Thermoplastic or elastic properties with melting-points ranging from 40° to 180°C
Degradation in microbe active environments in 5-6 weeks
Degradation in process ultimately leave behind carbon dioxide and water, which are environmentally friendly byproducts
The released carbon dioxide and water are absorbed during photosynthesis in nature
The synthesis and biodegradation of bioplymers are totally compatible to the carbon-cycle
Bioplastics can be produced from renewable carbon resources. As long as there is fuel shortage & rise in crude oil prices traditional energy sources are safe
Conservation of finite fossil resources like mineral oil and coal and their neutrality with regard to the emission of CO_2
The wider use of bioplastics in daily life will solve the increasing problem of organic waste
The use of biodegradable plastics will decrease the country's dependence on other countries for fossil fuels
Biocompatible and hence is suitable for medical applications. Therefore can be implanted in the body without causing inflammations
Some possible bioplastics applications include: biodegradable carriers that demonstrate the ability to deliver drugs for a given time within the individual's body, surgical needles, suture materials, bone tissue replacement, etc. The advantage of using biodegradable plastics is that it does not require surgical removal
Their biocompatibility and a low oxygen permeability which allow further applications for the production of films and coatings and special biomedicals (patch materials, stents, bone implantats, drug delivery systems, scaffolds for tissue engineering)

Table 2. Manufacturing capacities of termoplastic biopolymers.

Capacity (1000 tons/year)	2007	2010
Petrochemical raw material base	44	110
Petrochemical additives/Blend components	81	386
Renewable raw material base	189	901
Total	314	1,397

above 225,000 tons in 2008 will reach a level of about 900,000 tons by 2013 (PRO-BIP, 2009) . Manufacturing capacities of thermoplastic biopolymers summarised in Table 2. From 2003 to the end of 2007, the global average annual growth rate was 38%. The global capacity of emerging bioplastic at 0,36 Mt by the end of 2007. In EU, annual growth rate was as high as 48% in the same period (PRO-BIP, 2009). In EU, potentials for bioplastics were presented in Table 3.

The factors affecting PHB production are included as carbon and nitrogen sources in culture medium, concentration of microbial cell, oxygen limitation, pH and temperature (Kim, 2000; U ur et al., 2002). Several factors affect the production cost of PHB, such as PHB productivity, content and yield, the cost of the carbon substrate. One of the limiting factors in the commercial success of PHB and other PHAs production schemes is the cost of the sugar substrate used for PHB formation (Choi and Lee, 1999) . According to Choi and Sang (1997), 40 to 48% of the total production costs are ascribed to the raw materials where the carbon source could account for 70 to 80% of the total expense.

Until recently, the PHB and its copolymers had been produced from glucose and propionate on a semi-commercial scale first by Zeneca BioProducts (Billingham, UK), and later by Monsanto (St. Louis, MO, USA). Although, the cost of polyolelins is less than US \$1 kg^{-1}, the price of PHB is much higher than that of synthetic plastic (Tsuge, 2002). The price of PHAs such as PHB will be further reduced by the use of more inexpensive carbon sources. It has been also calculated that 3 tones of glucose as carbon substrate must be used for each tone of polymer produced (Collins, 1987).

In recent years, there has been an increasin trend

Table 3. Potentials for bioplastics*.

Bioplastic applications	Potential (tons/year)
Catering	450,000
Bags to gather biological-organic waste	100,000
Biodegradable mulch films	130,000
Nappies made completely of biodegradable polymers	80,000
Light packages, trays, tubs	400,000
Fruit and vegetable packages	400,000
Components for vehicle tires	200,000
Total	1,760,000

*Estimation was done by COPA (Committee of Professional Agricultural Organisations in EU) and COGECA (General Committee for Agricultural Cooperation in EU) in 2001, Europe.

towards more efficient utilization of relatively cheaper substrates for PHB biosynthesis via fermentation. To date, used various inexpensive substrates for PHB (and its copolymers) biosynthesis were represented in Table 4. Finding a less expensive substrate is, therefore, a major need for a wide commercialisation of PHB (Pozo et al., 2002). Low cost and PHB production is also dependent on the restructuring of traditional fermentation and recovery technologies (Xu et al., 2010). A production process based on waste carbon sources is the requirement of the day, instead of noble ones (Wolf et al., 2005).

The main anthropogenic waste, domestic wastewater (DWW) is mainly regarded as organic pollutant due to its high biological oxygen demand (BOD) and its priority organic and inorganic constituents. These are carbohydrates, lignin, fats, soaps, synthetic detergents, proteins and their decomposition products, oxygen-demanding substances, soluble inorganic material (ammonia, road-salt, sea-salt, cyanide, hydrogen sulfide, thiocyanates, thiosulfates, etc.), toxic chemical compounds (heavy metals, pesticides, herbicides, arsenic, cadmium, chromium, copper, lead, mercury, zinc, etc.) (Vigneswaran and Sundaravadivel, 2004). Although, small differences, major constituents of typical DWW as chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), nitrogen (as N) and phosphorus (as P) are 100 to 500, 100 to 300, 20 to 85 and 6 to 20 mg/L, respectively (UN Department of Technical Cooperation for Development, 1985).

DWW is now rapidly becoming a waste product with an associated disposal cost (DWW Facilities Manual, 1997). Supposing the consumption of each individual requires a BOD of 40 g/day, it is estimated that the wastewater BOD weights 924 tons every day, 349 tons of which are drained. That is, 575 tonnes are discharged into rivers. DWW accounts for 60% of pollutants that cause river pollution, and ranks as the major source of river pollution. Construction of public underground sewerage systems is the key to minimizing domestic water pollution. Generally, 48.25% of DWW is treated. (http://www.epa.gov.tw/en/epashow.aspx?list=102&path=135&guid=c4b6ad0f-13e5-4259-be98-8356037dc862&lang=en-us). The remaining portion of DWW may be assessed as inexpensive substrate in PHB production process. The use of this substrate will also reduce environmental pollution caused by this material.

Therefore, we studied the possibility to use DWW as feedstock for *Enterobacter aerogenes* to produce PHB high-efficiency. This is the first report to our knowledge the use of DWW as carbon source in the production of PHB. We thought that the use of a cheaper feedstock/without cost sustrate will be provided to reduce the high production cost of PHB. Also, this study is different from other studies in another aspect. A strain of *E. aerogenes* was firstly presented amoung PHB-synthesing bacteria.

MATERIALS AND METHODS

Microorganism

The microorganism used in all experiments were strain 12B wasi (we signated it as strain 12Bi), originally newly isolated from cooling towers of a petrochemical industry plant, in Turkey in this study. The strain was identified by the biochemical methods and the 16S rRNA gene sequencing. Stock cultures were grown and maintained at 37 and 4°C, respectively by periodic transfer on nutrient agar (Difco) slants.

Biochemical characterization of microorganism

On the MacConkey agar growth of the strain 12Bi was investigated, then the biochemical characterization of it was determined using API 20E idenification kit (Biomérieux). This set for enterics allowed the determination of the carbohydrate metabolism of the strain within 48 h at 30°C. The biochemical profile determined was used to identify an isolate by comparison with known strains using a computer software containing an APILAB Plus Program. This allows identification of our strain using results obtained with API strips.

DNA purification

The genomic DNA was extracted from 1 ml of bacterial culture using the ZR fungal/bacterial DNA Extraction kit (ZymoResearch). Purified DNA was amplified by FastStart Taq DNA Polymerase

Table 4. PHB (and its copolymers) biosynthesis from inexpensive substrates. The table includes substrate, microorganism and reference.

Substrate	Microorganism	Reference
Sugar cane liquor	*Ralstonia pickettii* 61A6 *Pseudomonas fluorescens* A2a5	Bonatto et al. (2004) Jiang et al. (2008)
Sugar cane and beet molasses	*Bacillus cereus* M5	Yilmaz and Beyatli (2005)
Starch-based materials	*Azotobacter chroococcum* and Recombinant *Escherichia coli* strain	Kim (2000)
Molaesses	Recombinant *Escherichia coli*	Liu et al. (1998)
Cellulose hydrolysates, xylose etc.	*Burkholderia cepacia* IPT 048 and *B. sacchari* IPT 101	Silva et al. (2004)
Dairy whey	Recombinant *Escherichia coli* *Pseudomonas hydrogenovora* *Methylobacterium sp.* ZP24	Ahn et al. (2001) Koller et al. (2008) Nath et al. (2008)
Corn steep liquor	*Bacillus* sp. CFR 256	Vijayendra et al. (2007)
Corn steep liquor and molasses	*Bacillus megaterium* ATCC 6748	Chaijamrus and Udpuay (2008)
Corn syrup	*Cupriavidus necator**	Daneshi et al. (2008), Daneshi et al. (2010)
Plant oils from biotechnological rhamnose production	*Alcaligenes eutrophus*	Fukui and Doi (1998)
Crude palm kernel oil	*Cupriavidus* sp. USMAA2-4	Kek et al. (2010)
Fatty acids and waste glycerol	*Ralstonia eutropha* *Pseudomonas aeruginosa* NCIB 40045 *Cupriavidus necator*	Kahar et al. (2004) Fernández et al. (2005) Cavalheiro et al. (2009)
Paper mill wastewater	Activated sludge	Bengtsson and Werker (2008)
Swine waste liquor	*Azotobacter vinelandii* UWD.	Cho et al. (1997)
Petrochemical plastic waste	*Pseudomonas putida* CA-3	Goff et al. (2007)
Sunflower cake, soy bran and olive mill	*Pseudomonas hydrogenovora*	Koller et al. (2005)
Soy and malt wastes	Mixed bacteria	Wang et al. (2007)
Waste activated sludge	*Bacillus sp.*	Thirumala et al. (2010)
Dairy waste and sea water	*Bacillus megaterium*	Ram Kumar Pandian et al. (2010)
Waste water from olive oil mills (called alpechín)	*Pseudomonas putida* KT2442 *Azotobacter chroococcum* H23	Ribera et al. (2001) Pozo et al. (2002)
Acid-hydrolysed malt waste	Recombinant *Bacillus subtilis* strains	Law et al. (2003)

** Cupriavidus necator=Ralstonia eutropha=Alcaligenes eutrophus= Wautersia eutropha.*

dNTPack kit (Roche).

16S rRNA gene sequence analysis

PCR amplification was performed with the 16S rRNA- specific universal primer pairs. The primer pairs were 27F and 1522R (Thermo Scientific) (Lee et al., 2003). The PCR mixture consisted of PCR reaction-polymerase buffer (with 20 mM $MgCl_2$), 10 l of GC-rıch solution, 1 l of PCR Grade Nucleotid Mix (dNTP Mix), 5 l of each of the two primers, and 0.4 l of FastStart Taq DNA polymerase (5 U/ l), template DNA (200 to 500 ng) brought to 50 l of with deionized water PCR was performed with a thermal cycler (GeneAmp PCR system 9700).

Primer	Nucleotid sequence
27F	5'-AGAGTTTGATCMTGGCTCAG-3'
1522R	5'- AAGGAGGTTATCCANCCRCA-3'

The thermal cycling program used was as follows: initial denaturation at 95°C for 5 min; 35 cycles consisting of 95°C for 55 s, 50°C for 40 s, and 72°C for 1.5 min; and a final extension step consisting of 72°C for 7 min. Amplified PCR products and DNA marker (1000 bp, Fermentas DNA ladder) were separated on a 1% agarose gel by agarose gel electrophoresis containing 15 l of ethidium bromide (10 mg/ml) for visualization on an ultraviolet light box (254 nm) and were sequenced at RefGen (Gen Researches and Biotechnolgy Centre, Turkey). The sequences of partial 16S rRNA were compared with the 16S rRNA sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using their World Wide Web site (http://www.ncbi.nlm.nih.gov), and the BLAST (Basic Local Alignment Search Tool) algorithm (Lee et al., 2003; Adiguzel et el., 2009)

The 16S rRNA gene sequences of the species most closely related to our strain was retrieved from the database. Retrieved sequences were aligned by using the ClustalX program (Thompson et al., 1997) and manually edited. Phylogenetic tree was constructed by the neighbor- joining method using software package MEGA 4.0 (The Molecular Evolutionary Genetics Analysis 4.0) (Tamura et al., 2007).

DWW as nutrient source in culture medium for PHB production

DWW used as nutrient source in the present study was taken from ten households. The collected wastes were mixed thoroughly. The main composition of this mixture was remains of various vegetables and fruits 25% (w/w), remains of juicy meaty food and cooked rice 22% (w/w), remains of cakes and sweets 18% (w/w), water from household cleaning 10% (w/w), water from shower 10% (w/w). This mixture was pre-treated for removal of solids prior to its use for PHB accumulation. For this objective, wastewater was filtered through a porcelain filter three times in a row and then it was sterilized by Millipore filter with a por size of 0.45 mm (Khardenavis et al., 2007).

This filtrate with COD (185 mg/L) and the BOD_5 (the biochemical oxygen demand at 20°C over 5 days) (110 mg/L) was used as DWW in culture medium for PHB synthesis in this paper. In filtrate, the concentrations of nitrogen (as N) and phosphorus (as P) were 30 and 8 mg/L respectively. These contents were analyzed as described in American Public Health Association (APHA) Standard Method for the Examination of Water and Wastewater (1995).

Bacterial growth conditions for PHB production

PHB production of strain 12Bi was performed as described Ram Kumar Pandian et al. (2009) with some modifications. A loop of strain 12Bi was inoculated into 20 ml of yeast nitrogen base broth (YNB) medium (Difco) (pH 7.0) in a 100 ml erlenmeyer flask, which was subsequently aerobically cultured at 37°C for 24 h. Then, 2 ml of the cultured broth was inoculated to a 1 L shake-flask containing 200 ml of YNB medium with adding different concentrations of domestic wastewater (10, 30, 50, 80, 100% (v/v)) (DWWM). Table 5 shows different DWW contents of medium and symbols of them in this study. The flasks were maintained at 37°C for 48 h. Cell growth was determined by measuring the dry wight (g/L) of harvested biomass.

Observation of PHB granules

PHB granules have been recognized by their affinity for the dye Sudan black B, which is a presumptive test for the presence of PHB (Smibert and Krieg, 1981). Therefore, the colonies grown on DWWM (with 1.5% agar) were stained with Sudan black B and safranin using thin smears (Weibuli, 1953). The stained preparations were examined under the microscope to determine cellular PHB accumulation in this study. After the detection of PHB-production capability of our strain by Sudan black staining method, it was confirmed using transmission electron microscopy (TEM).

The sample preparation for TEM was done as described by Chien et al. (2007). Bacterial cells in broth DWWM at stationary phase were centrifuged (at 8,000 g for 13 min), washed and fixed with in potassium phosphate buffer (pH 7.0) and then prefixed with 4% paraformaldehyde and 2.5% glutaraldehyde. Strain 12Bi cells were then fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide in phosphate buffer (pH 7.0), stained with 1% uranyl acetate and examined under the TEM (Jeol 100 SX).

Determination of biomass

The biomass was harvested by centrifuging 100 ml of culture(10,000 rpm, 15 min, 4°C) and the cell pellet was washed in distilled water and dried to constant weight (105°C, 24 h) (Ram Kumar Pandian et al., 2009).

Determination of PHB using analytically procedures

The extraction of PHB was modified according to the previously reported Hypochlorite method (Rawte and Mavinkurve, 2002; Sayyed et al., 2009). For this, isolate 12Bi was grown in 250 ml erlenmeyer flasks containing DWWM (with different concentrations of DWW). These flasks were incubated at 37°C for 48 h in a shaker at 150 rpm. Diverse incubation temperatures were also tested as 25, 28, 30, 37 and 40°C (data not given). The best result of PHB extraction was obtained at 37°C and so we selected this temperature for incubation. Cell suspension (10 ml) was centrifuged at 6,000 rpm for 10 min. The cell pellet was washed once with 10 ml saline and was recentrifuged to get the pellet. The cell pellet was then suspended in 5 ml sodium hypochlorite (4% active chlorine) and incubated at 37°C for 10 min with stirring. This extract was centrifuged at 8,000 rpm for 20 min and the pellet of PHB was washed with 10 ml cold diethyl ether. The pellet was again centrifuged at 8,000 rpm to get purified PHB and was dried to constant weight (105°C, 24 h).

Calculation of % PHB

The following formula was used for the detection of percentage of PHB:

Table 5. Symbols and domestic wastewater contents of mediums in this study.

Symbol of medium (DWWM*)	Wastewater content of medium (%), (v/v)
DWWM1	10
DWWM2	30
DWWM3	50
DWWM4	80
DWWM5	100

* DWWM is YNB plus different concentrations of DWW.

% PHB = (Amount of PHB / Amount of biomass) x 100

RESULTS

Identification of microorganism

Strain 12Bi was short rod- shaped under the microscope, gram-negative, and motile. We inoculated the isolate onto MacConkey medium to understand that it was enteric or not. Enterics that can ferment lactose (lactose positive) are called coliforms and usually are considered to non-pathogenic in the intestinal tract (example, *E. aerogenes*). MacConkey agar is both selective and differential for *E. aerogenes*. It contains bile salts and the dye crystal violet, which inhibit the growth of gram-positive bacteria and select for gram-negative bacteria. It also contains the carbohydrate lactose, which allows differentiation of gram-negative bacteria based on their ability to ferment lactose. Because of typical pink colored growth of our strain on MacConkey plate, we characterized biochemically using API 20E identification kit.

Biochemical characterization was based on the utilization of 9 carbohydrates and 13 additional tests as summarised in Table 6. The biochemical profiles seemed to ferment all of carbohydrates in API 20E identification kit by our strain. The APILAB PLUS program revealed our strain could also be related closely to *E. aerogenes* (96% identity to carbohydrate utilization pattern of *E. aerogenes*).

Partial 16S rRNA sequence from our strain was compared against the sequences available in the GenBank, included numerous public databases, by using a BLAST search (blastn). The 16S rRNA nucleotide sequence of this strain determined in this study has been deposited in the GenBank database under accession#FJ799902.

Binary sequence comparisons showed that the sequence of strain 12Bi was similar to those of the strains *E. aerogenes* (100 or 99% homology). Our isolate was thus identified as *E. aerogenes*, and called *E. aerogenes* strain 12Bi.

In Figure 1, the amplified 16S rRNA of *E. aerogenes* 12Bi was compared to other eight selected gene sequences from the same group of *Enterobacter* sp. and other related-groups. Their relationships were then determined. The results were analysed using MEGA 4.0 software to understand its evolutionary distance by reconstructing a phylogenetic tree of these bacteria. The strains were *E. aerogenes* NCTC10006T (AJ251468), *Escherichia coli* ATCC 11775T (X80725), *Proteus vulgaris* (X07652), *P. vulgaris* DSM 30118 (AJ233425), *Pseudomonas stutzeri* DSM 50238 (U26416), *Pseudomonas aeruginosa* (AU0416), *P. aeruginosa* (NGKCTS), *Staphylococcus aureus* (185060).

The partial 16S rRNA sequence of *E. aerogenes* 12Bi (1381 bp) was closely related to that of *E. aerogenes* NCTC10006T (100% homology). The results suggested that our strain was closely related to the *E. aerogenes* NCTC10006T (AJ251468) with a genetic distance 0.040 base substitutions per site. Strain 12Bi and NCTC10006T are sisters group which had minimum genetic distance.

Screening of PHB granules using Sudan black staining method and TEM

Our strain was tested positive for presence of lipophilic PHB granules (positive Sudan black staining). PHB granules were observed dark gray-black in the pink-sole red vegetative cells by using Sudan black staining method. The PHB granules in *E. aerogenes* 12Bi cells was photographed with a TEM (Figure 2). TEM photograph of the cells grown in the DWWM illustrates the formation of these granules which change the cell shape from rod to oval and spherical to accommodate the size of the growing granules.

Analytical determination of PHB

Biomass of *E. aerogenes* 12Bi in DWWMs containing different concentrations of DWW as substrate is shown in Figure 3. Results showed that the growth of our strain increased when the concentration of DWW in culture was increased except 100% of DWW concentration. For example, in DWWM4 bacterial biomass (as dry cell

Table 6. Biochemical characterization results of strain 12Bi based on API 20E identification kit.

Test	Substrate	Reaction/ Enzymes	Strain 12Bi
ONPG	Ortho-nitro-fenil-β-D-galactopyranoside	β-galactocidase	+
ADH	Arginine	Arginine dihydrolase	+
LDC	Lisin	Lysine decarboxylase	+
ODC	Ornitin	Ornitine decarboxylase	+
CIT	Sodium citrate	Use of citrate	+
H_2S	Sodium thiosulfate	Production of H_2S	-
URE	Urea	Urease	-
TDA	Tryptophane	Triptofan deaminase	-
IND	Tryptophane	Production of indole	-
	Creatine sodium pyruvate	Production of acetoin	+
	Gelatine of Kohn	Gelatinase	-
GLU	Glucose	Fermentation/Oxidation	+
MAN	Mannitole	Fermentation/Oxidation	+
INO	nositole	Fermentation/Oxidation	+
SOR	Sorbitole	Fermentation/Oxidation	+
RHA	Rhamnose	Fermentation/Oxidation	+
SAC	Saccharose	Fermentation/Oxidation	+
MEL	Mellibiose	Fermentation/Oxidation	+
AMY	Amigdalin	Fermentation/Oxidation	+
ARA	Arabinose	Fermentation/Oxidation	+
OX	On filtration paper	Cytochrome-oxidase	+
NO^{3-}	NO^{2-}	Reduction	+
	N_2	Reduction	+

+:Positive, -: Negative.

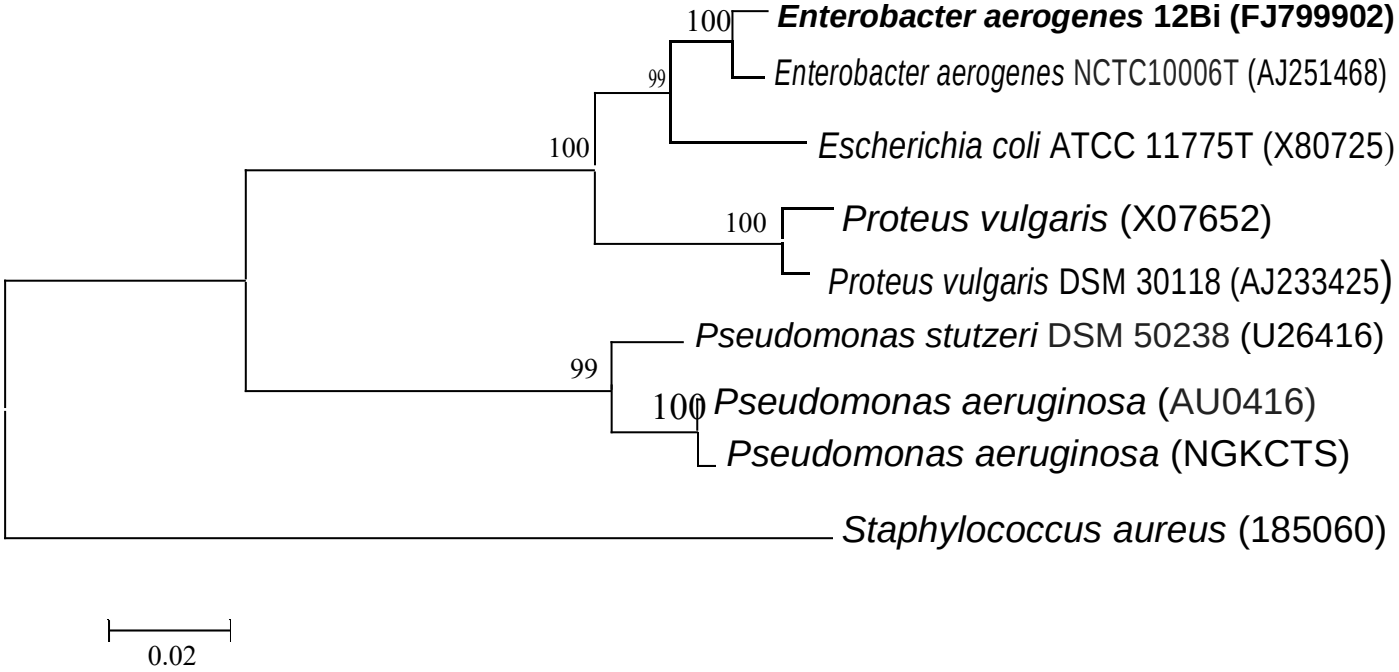

Figure 1. Phylogenetic tree of *E. aerogenes* 12Bi (FJ799902) among related species from reconstructed of BLAST results. The tree was constructed with the similarity and neighbor by MEGA 4.0. Accession numbers are given in parenthesis after the taxonomic assignment. Bar represents 0.02 (2%) sequence divergence.

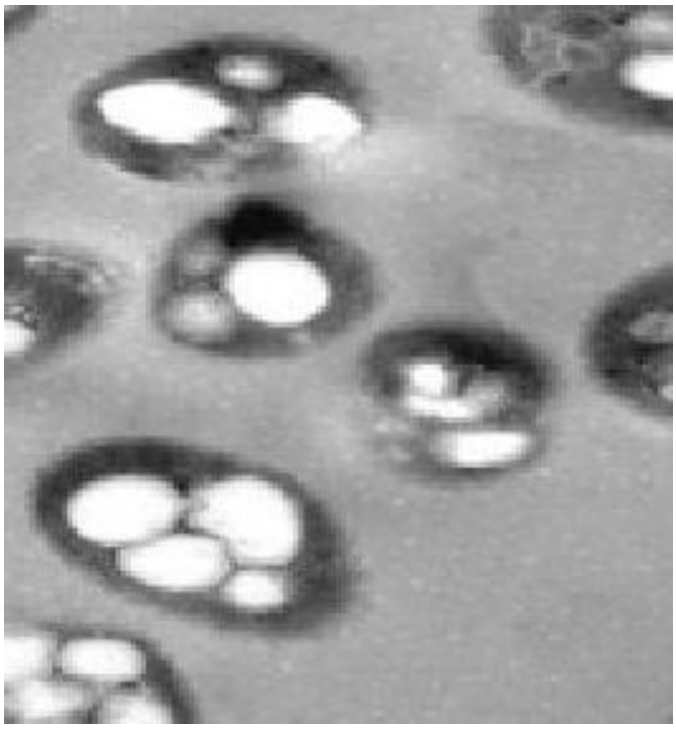

Figure 2. Transmission electron microphotograph of thin section of *E. aerogenes* 12Bi (magnification ~7000). Cells were grown in DWWM and were collected in stationary phase (about 18-28h). PHB granules was shown as light-bright regions.

weight) were 5.8, 6.2 and 5.3 g/L at 18, 24 and 30 h, respectively, whereas in DWWM5 bacterial biomass were 4.0, 4.9 and 3.9 g/L at 18, 24 and 30 h, respectively. The highest growths in all mediums were obtained at 24 h (Figure 3).

In Figure 4, accumulation of PHB in *E. aerogenes* 12Bi cells. These bacterial cells were grown in DWWMs. According to the results, the yields of PHB obtained from *E. aerogenes* 12Bi in medium added DWW were changed from 16.66 to 96.25%.

Several studies have stressed the high potential of other feedstocks to enhance microbial PHB production. For example, Quillaguaman et al. (2008) reported the utilisation of starch hydrolysates as carbon source in fermentations carried out with *Halomonas boliviensis* to produce a PHB content of 35%. Huang et al. (2006) used extruded rice bran as carbon source in a fed-batch fermentation carried out by *Haloferax mediterranei* to produce a total dry weight of 140 g/L with a PHA content of 55.6%. Commercial protein hydrolysates (that is, yeast extract, casein hydrolysates) and by-products of agro-industrial origin (that is, corn steep liquor) have been employed as sources of nitrogen. Bormann et al. (1998) reported the production of 65 g/L total dry weight with a PHB content of 60 to 80% when 20 to 30 g/L casein peptone or casamino acids were used as sole sources of nitrogen.

In our work, the accumulation of PHB granule in cells of *E. aerogenes* 12Bi significantly depended on the DWW in the medium culture. The highest PHB production was observed after 18 h of growth (96.25 w/w, dry weight) when 100% DWW was used, whereas the highest biomass (5.8 g/L) was obtained at 80% DWW.

This may be result from more carbon content in DWWM with 100% DWW than in DWWM with 80% DWW. Even if less amount of biomass, PHB production primarily depends on the abundance of carbon source in medium (Kim, 2000).

Finally, our results suggest that *E. aerogenes* 12Bi is a good candidate for the production of this biopolymer by fermentation, since the medium contains sources of carbon as the main substrate, a very cheap or free and abundant resource for PHB accumulation in shake-flask culture.

DISCUSSION

Many studies have therefore being focused on the efficient production of PHB- based polymers with desirable material properties by wild-type bacteria from different habitats or recombinants as summarised in Table 4. However, no research has been found on PHB productivity of *E. aerogenes*, used in this work and originated from the industry,.

Earlier studies on PHB production by several bacteria suggested the need for developing a new culture medium and cultivation strategy in order to achieve increased volumetric productivity (Wang and Lee, 1997; Quillaguamán et al., 2007).

The use of DWW as a complex carbon and nitrogen source makes it difficult to control the supply of nutrients for achieving high cell density as well as optimal PHB productivity. In this work, a defined medium was formulated and optimized for PHB production through cultures of *E. aerogenes* 12Bi.

Also, DWW filtrate used in this work was characterized for their pollution characteristics. The ratio of COD to BOD_5, nitrogen (as N), phosphorus (as P) were detected as 1.68:1, 30 and 8 mg/L respectively. These values are high for direct discharge safely into the environment and this wastewater has pollution potentials.

This shows that it is important to evaluate this DWW as a resource of carbon and energy for microorganisms rather than discharged to the environment.

PHAs such as PHB have been considered to be good candidates for biodegradable thermoplastics and are mainly produced by microbial fermentation processes. They also have the further advantage of being produced from renewable resources (Flieger et al., 2003; Nath et al., 2008). Due to the large impact of the carbon and nitrogen source price on production costs, one of the most important approaches to reduce costs is to use wastes and by-products as raw material for the fermentation process. However, the use of wastes has

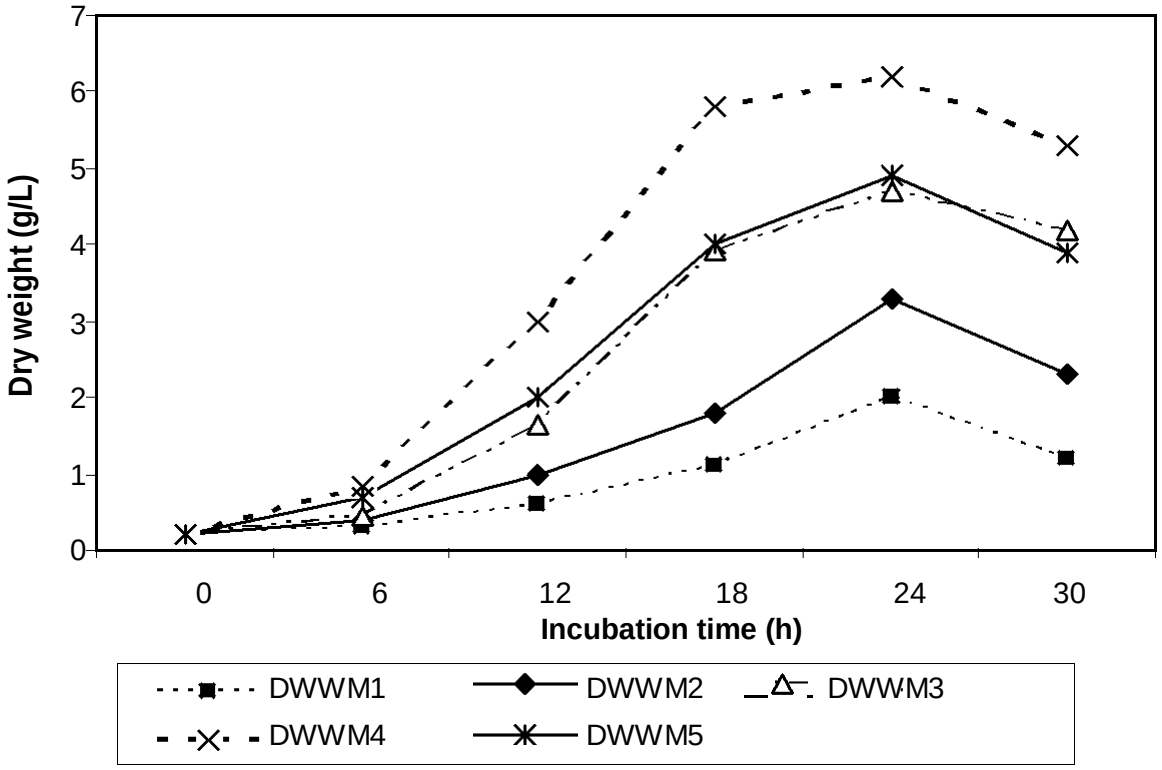

Figure 3. Biomass of *E. aerogene* 12Bi grown in DWWM containing different concentrations of DWW (10, 30, 50, 80, 100%, v/v). According to dry cell weight (biomass/medium).

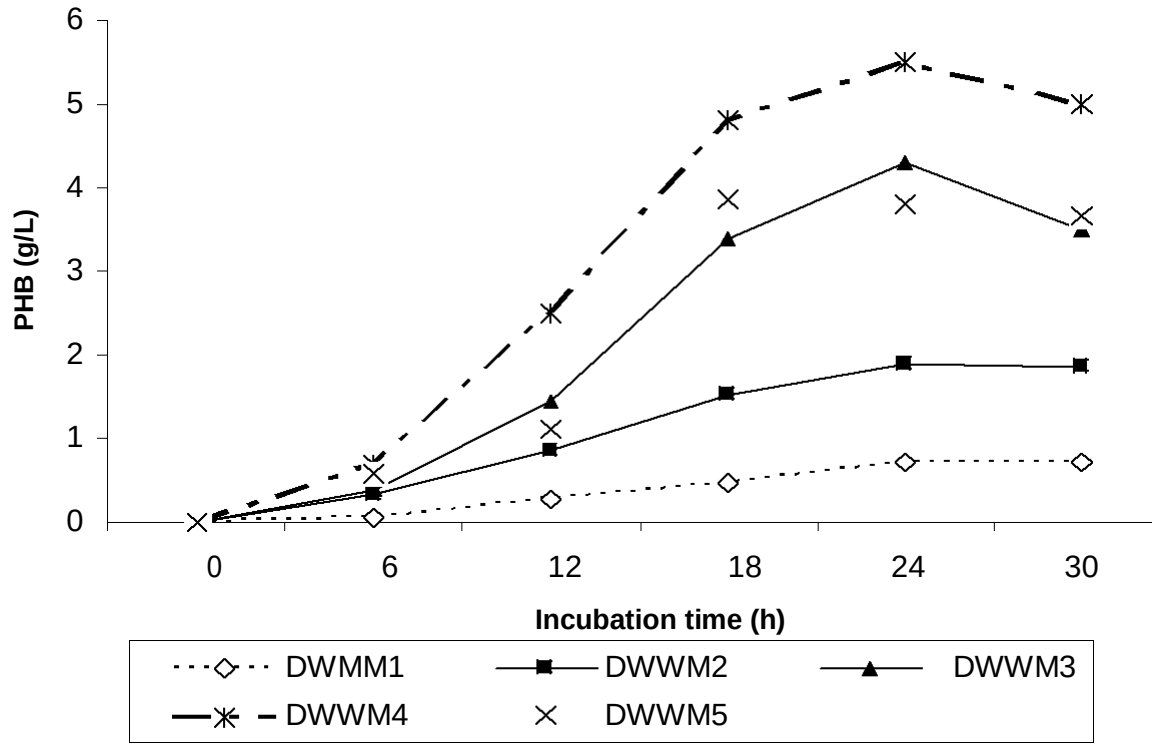

Figure 4. Accumulation of PHB in *E.aerogenes* 12Bi cells. Cells were grown in DWWM containing different concentrations of DWW (10, 30, 50, 80, 100%, v/v). According to dry cell weight (PHB/medium).

hardly been explored at all, which may well be due to the complexity of their composition (Castilho et al., 2009).

On the other hand, for PHB production, use of inexpensive renewable carbon sources such as waste materials is essential to reduce the production cost further.

Cheaper raw material and its efficient use have direct impact on the final price of the product in market. In the near future, all of these efforts will overcome the current problems concerning the production and application of PHB. In our study, DWW was successfully used as a cheap-substrate to produce PHB by *E. aerogenes*. Cavalheiro et al. (2009) exploited the use of waste glycerol, a by-product from the biodiesel industry, as primary carbon source for cell growth and polymer synthesis in their study. *Ralstonia eutropha* DSM 545 was used to accumulate PHB from GRP as substrate. In other study, PHA production of *P. aeruginosa* 42A2 with agro-industrial oily wastes, such as waste frying oils or with sub-products from the vegetable oil refining process was reported by Fernández et al. (2005). PHA accumulation ranged between 66.1% when waste-free fatty acids from soybean oil were used as carbon substrate, 29.4% when waste frying oil was used and 16.8% when glucose was used in bacterial culture. *Pseudomonas resinovorans* accumulated 15% of the cellular dry weight of PHAmcl from tallow (Cromwick et al., 1996). In our paper, PHB yield by *E. aerogenes* was detected 35.5% when DWW was 10% in DWWM and 57.27% when DWW was 30% in DWWM in 24 h.

In Ram Kumar Pandian et al. (2010), dairy waste was directly used for the synthesis of PHB as a sole carbon source, and similarly, we used directly that DWW from household wastes as a substrate instead of expensive sources. This may also reduce the final production cost of the biopolymers such as PHB as about 40% of total production cost for PHB production depends upon the substrate.

Corn steep liquor was chosen as a cheap nitrogen source for PHB production by *Bacillus megaterium* in order to reduce the cost of PHB production by Gouda et al. (2001) . The best growth was obtained with 3% molasses, while maximum yield of PHB (46.2% per mg cell dry matter) was obtained with 2% molasses. The different molasses by *Azotobacter* strains (Purushothaman et al., 2001), two types of fermented organic waste (trade and industry waste and fruit and vegetable waste) by *R. eutropha* were converted into PHB (Ganzeveld et al., 1999). Ganzeveld et al. (1999) obtained that the yield of PHB in medium with the fermented organic waste from fruit and vegetable industry used as growth substrate was 40 w% of dry cell weight of *R. eutropha*. Whereas, we detected that the PHB yield of *E. aerogenes* 12Bi in DWW medium (DWWM5) including wastewater by 100% was 96.25 w% of cell dry weight (Figure 3).

We also proved the increase in PHB volumetric productivity in high biomass cultures. Such that, in DWWM 3 maximum PHB production (91.48%) was obtained with maximum dry cell weight 4.7 g/L. In other work, the same provision was also detected with another PHB producing microorganism. According to mentioned study, the maximum cell concentration of *Cupriavidus necator* was 68.8 gDW/L at 30.5 h with a maximum PHB accumulation of 38% (Cavalheiro et al., 2009) by using waste glycerol as nutrient source.

In our study, it was observed that the trends of both biomass production and PHB production by time increased except 30 h of incubation period. Our microorganism has accumulated more PHB during the stationary phase of growth in which was the maximum dry weight of cells.

Depending on PHB accumulation kinetics, bacteria can be divided in two groups. The first group is formed by bacteria that require the limitation of some nutrients. To this group belong *R. eutropha, Pseudomonas oleovorans* or *B. megaterium*. In these bacteria, PHB is produced apparently in response to conditions of physiological stress (Fernández et al., 2005). So, in the work of Gouda et al. (2001) it was found that the synthesis of PHB by a *B. megaterium* strain is favored by environmental stresses such as nitrogen limitation, so PHB formation was studied under nitrogen limiting conditions. The production medium with 2% sugarcane molasses was supplemented with different levels of corn steep liquor from 0.5 to 7%. Maximum yield of PHB (49.12% per cell dry matter) was obtained with 3% corn steep liquor (CSL) in the medium after 48 h incubation. The use of 7% CSL decreased the amount of PHB by about 62% in comparison with 3%. On the other hand, maximum growth was obtained with 1% CSL and then began to decrease. Lee and Chang (1994) studied the effect of CSL concentration on PHB production by recombinant *E. coli* from a concentration of 0.1 to 1% and reported that the best concentration was 1% which yielded 44.9% PHB. The effect of nitrogen limitation was also studied by Ramsay et al. (1992).

The highest PHB production was observed after 45 h of growth (43% w/w, dry matter) when 4% substrate was used, whereas the highest biomass (7.2 g/L) was obtained at 4 to 6% substrate by other researches (Fabiane et al., 2007). This indicated that bacterial growth increased as substrate concentration increased, whereas the PHB accumulation of *B. megaterium* ATCC 6748 decreased. In our work differently, the PHB yield of *E. arogenes* 12Bi was maximum at 18 h and when bacterial biomass decreased to 4.2 g/L in DWWM 3, PHB yield was calculated 83.33%. As the results of this paper, PHB production by *E. aerogenes* 12Bi cells was both at high levels and quickly using DWWM as fermentation medium than many other previously studied bacteria and mediums.

Bacteria of the second group do not depend on nutritional limitation as they accumulate PHB during cell

growth. PHB was produced by these kind of microorganisms under the environmental stresses such as nutrient limitation, that is, nitrogen, phosphorus or oxygen limitation (Thakor et al., 2003). The microorganism and the strategy of production were affected on duration of fermentation, growth rate, carbon source concentration, etc. Some examples are *Alcaligenes latus*, *Azobacter vinelandii*, Pseudomona *putida* , *P. aeruginosa* 47T2 and r-*E. coli* (Thakor et al., 2003; Fernández et al., 2005). Also detected ın our study, was high PHB productivity in both log-phase and stationary-phase of *E. aerogenes* (Figure 4). Such that, yields of PHB were determined as 60.00% at 30 h, 57.27% at 24 h, 91.48% at 24 h, 88.70% at 24 h, 96.25% at 18 h (in DWWM 1, DWWM 2, DWWM 3, DWWM 4 and DWWM 5 respectively). In other study, when incubated in aerobic submerged mineral medium with waste cooking oil as carbon source, PHB productivity by *P. aeruginosa* 47T2 was 32% of 15.0 g/L cell dry weight at 20 h (Haba et al., 2007).

The accumulation of PHB granule in cells of *E. aerogenes* 12Bi was significantly depended on the DWW in the medium culture. The highest PHB production was observed after 18 h of growth (96.25 w/w, dry weight) when 100% DWW was used, whereas the highest biomass (5.8 g/L) was obtained at 80% DWW. This indicated that bacterial growth increased as DWW concentration increased, also the PHB accumulation was very similar. So, the formation rate of PHB up to 0.95/h and specific growth rate up to 1.8/h were observed during growth. In a similar work, typical culture characteristics of *Alcaligenes latus* ATCC 29713 for intracellular PHB production were 0.075/h maximum specific growth rate, 0.38 g/L h^{-1} maximum specific sucrose consumption rate, and 0.15 g/L h^{-1} maximum specific PHB production rate (Grothe et al., 1999).

Previous studies showed that enterobacteria did not accumulate PHAs naturally, requiring the cloned genes introduction. However, we studied *E. aerogenes* from enterobacteria of natural occurrence was able to accumulate PHB in cells. This is a novelty for PHB-production researches, except *Serratia* sp another strain from enterobacteriaceae (Lugg et al., 2008).

Bioplastics, and especially PHB, will certainly play an important role in the plastics market in the future due to their biodegradability and to the use of renewable resources for their production. Although, their manufacturing costs today are still too high to compete with conventional, petroleum-based polymers, advances in fermentation processes using inexpensive substrates, will certainly improve PHB competitiveness and make a broad use of these biopolymers possible in the future.

The focus of our study was on the development of an economic process for the production of PHB by *E. aerogenes* 12Bi using water of household wastes (DWW).

Our present data indicate that DWW could be alternatively used for high amounts of PHB production by this bacterium from a low cost process. Additionally, the use of DWW may contain pollutants in production of useful products such as PHB is very important in terms of environmental pollution and controlling of waste. With the use of this cheaper (even without cost) raw materials as carbon source in our study, we provided that about 40 to 48% of the total cost of PHB-production reduced. The adaptation assays to industrial scale should be performed by the next works.

Conclusıon

The results shown above demonstrated that the bacterium was identified as *E. aerogenes* 12Bi (accession # FJ799902). It was able to produce high amounts of PHB (up to 90% of dry cell weight) in the presence of excess domestic wastewater (DWW) as carbon source in the domestic wastewater medium (DWWM) . This is the first time using of DWW as substrate are described in PHB production by novel strain. Use of wastes as inexpensive substrates such as DWW could contribute to reducing the nearly half of PHB production cost.

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