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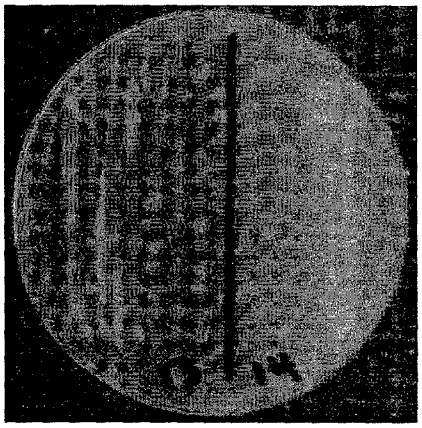
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(54) **Title**: A NOVEL BACTERIUM PRODUCING POLYHYDROXYALKANOATES FROM PALM OIL MILL EFFLUENT

Fig. 1



(57) **Abstract**: The bacterial strain EB 172, isolated from digester treating palm oil mill effluent, was investigated by polyphasic taxonomic approach. The cells were rod-shaped, Gram- negative, non-pigmented, non-spore-forming and non-fermentative. Phylogenetic analyses using the 16S rRNA gene sequence showed that the strain was placed in the cluster of genus *Comamonas*; its closest neighbours were the type strains C. *terrigena* (96.8 %), C. koreensis (93.4 %), C. *composti* (92.9 %), and C. *kerstersii* (91.1 %). The ability of C. *putranensis* to produce polyhydroxyalkanoates (PHA) when supplied with organic acids made this bacterium is unique in Comamonas species. The bacterial strain was clearly distinguished from all of the existing strains using phylogenetic analysis, fatty acid composition data and a range of physiological and biochemical characteristics. The DNA G+C content of the genomic DNA was 59.1 mol%. It is evident from the genotypic and phenotypic data that strain *Comamonas putranensis* represents a novel species in the genus *Comamonas*, for which the name *Comamonas putranensis* sp. nov. is proposed.

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A Novel Bacterium Producing Polyhydroxyalkanoates From Palm Oil Mill Effluent

FIELD OF INVENTION

The invention relates to isolation of a novel bacterium and a process for polyhydroxyalkanoates (PHA)s production from anaerobically treated palm oil mill effluent and other organic wastes.

BACKGROUND OF INVENTION

Various scientific and scholarly articles are referred to throughout the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains. Polymeric materials occurring naturally or produced from renewable resources were recognized as extremely useful many years ago. These materials have become increasingly more interesting and attractive in recent years as alternatives to petrochemical-based polymers and plastics. Meanwhile, consumers and the plastics industry have shown a growing concern about the disposal of plastics and their environmental impact. The industry is actively looking for ways to minimize the unnecessary use of plastics to complement recycling and reuse programs. Others are working on new materials or modifications to old ones to reduce the environmental impact of plastics. Palm oil mill effluent (POME) is the most polluted organic residue generated from palm oil processing and comprises of high nutrient content mainly oil and fatty acids. POME is able to support bacterial growth with the degradation of the waste to reduce its pollution strength. Most of the mills in Malaysia adopt open lagoons and open digester tanks for POME treatments. Under anaerobic conditions, biogas will be generated and will mainly consist of methane gas. Other potential by-products recovered from the anaerobic process of POME are volatile fatty acids, mainly acetic, propionic and butyric acids (Hassan *et al.*, 1997). These acids then will be used for polyhydroxyalkanoate (PHA) production (Hassan *et al.*, 2002). Several studies have reported on the isolation of PHA accumulating bacteria from the environments (Redzwan *et al.*, 1997; Alias and Tan, 2005; Berlanga *et al.*, 2006: Zakaria *et al.*, 2008) utilizing oils, glucose and organic acids as carbon and energy source. Since organic acids are abundantly available from the anaerobic process of POME, and due to existing intellectual property or licensing in commercialize it in future, an attempt to screen and isolate the wild type strains from the

anaerobic digester treating POME was carried out using conventional Nile Blue A staining method. The significance of this finding is that potential PHA producers were successfully isolated from oil palm wastewaters. The purified culture showed significant potential for PHBV accumulation utilizing those acids derived from anaerobically treated POME.

SUMMARY OF THE INVENTION

The invention pertains to a method of the isolation of bacteria from digester tanks treating POME. The invention pertains to a method used in describing newly isolated bacterium by polyphasic approach. The bacterial strain is in the family *Comamonadaceae* and novel for their combination of biochemical and genetic properties. These bacterial strains have ability for PHBV production from organic acids derived from POME treatment. Published work on POME treatment and organic acids recovery were performed by previous invention (Hassan, 2006). The invention pertains to propose a new name of newly isolated bacterium as *Comamonas putranensis* sp. nov. The invention pertains to a process for production of polyhydroxyalkanoates (PHAs) from synthetic / organic acids derived from palm oil mill effluent (POME) treatment which comprises the step of incubating of bacterial strains for a sufficient period of time and specified conditions to produce PHA in a culture medium. The invention pertains to a method to improve culture growth and production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [(P(3HB-*co*-3HV)] copolymers using single or combination of synthetic/ organic acids from POME. The invention pertains to a strategy to improve the incorporation of P(3HV) monomer units in the P(3HB-*co*-3HV) copolymer by controlling their culture conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the novel bacteria, *Comamonas putranensis* stained with Nile Blue A staining and were exposed under UV illumination. PHA containing cells exhibited pink-orange colour while colourless if no accumulation.

Fig. 2 shows the growth profiles of *C. putranensis* when supplemented with different organic acids.

Fig. 3 shows the electron micrograph with negative stained cells of strain EB172 under growth phase. Bar represents 1.0 µm.

Fig. 4 shows the electron micrograph of strain EB172 grown under nitrogen limitation for PHAs accumulation. Bar represents 5 µm.

Fig 5 shows rooted neighbor joining tree (Saitou and Nei, 1987) based on 1,446 nucleotide positions of 16S rDNA sequences showing relationships between strain EB172 (*C. putranensis*) and closely related taxa. *R. eutropha* was used as an out group. Percentages at the nodes indicate levels of bootstrap support based on neighbor-joining analyses of 1000 resampling datasets. Bar, 0.01 substitutions per nucleotide position.

Fig 6 shows 500 MHz 1H-NMR spectrum of PHBV produced by *C. putranensis* when supplied with 10 g/L organic acids derived from POME treatment.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Sampling

Sludge was obtained from an open digester tank (ODT) treating POME at the FELDA Serting Hilir mill located in the Negeri Sembilan, Malaysia. The ODT capacity was 3600 m^3 which was able to treat POME at HRT 20 days daily. The treated effluent in the ODT was withdrawn and collected in the 1L Schott bottles and kept on ice prior to use.

Screening and isolation process

Screening of PHA-producing bacteria from ODT was performed by direct plating and enrichment techniques. In direct plating technique, 50 mL of activated POME sludge from ODT was homogenized by vortex at moderate speed and a dilution series up to $10x^{-9}$ dilution was made to obtain a desired colony forming unit (c.f.u). Aliquots of 0.1 mL were plated onto nutrient agar (Merck, Germany) and the plates were incubated for 24 – 48 h at 30 or 37°C. Colonies developed on the agar were differentiated by color, elevation, form, and edge appearances (Dawes and Senior, 1973). For enrichment technique, the sludge was centrifuged at 3,500 rpm for 10 min at 4°C to remove residual oils. The supernatant was decanted and the pellet was transferred into enrich media containing 2 g/L of sodium acetate. The flask was agitated at 250 rpm for 2 weeks at 30 or 37°C. Concentration of the sodium acetate was maintained at 2 g/L by weekly addition of sodium acetate solutions into the enriched media. Loopfuls of the culture media was withdrawn and streak onto the nutrient agar plates using spread plating technique. Selected isolates were purified by dilution-streaking to obtain single colonies. The isolation for PHA producing bacteria was performed by randomly picking out and culturing the colonies onto solid mineral salt medium (MSM) containing components as proposed by Berlanga et al., (2006). All MSM compositions were same except the concentration of NaCl. MSM was supplemented with 2.5 g sodium acetate/L. 0.5 mg Nile blue A (Sigma, St. Louis, MO, USA) (DMSO)/mL was added into the MSM media. The cultures were incubated for 4 – 5 days at 30 - 37°C. The grown colonies from solid MSM were exposed under UV illuminator at the wavelength 280 - 360 nm. The orange fluorescence color observed from PHA positive colonies were different in brightness and it depends on the types of microorganisms able to store polymeric compounds (**Fig. 1**). The cultures from agar slants were then transferred in the 250 mL shake flask containing 50 mL NB. The cultures were

incubated at 30°C for 18 h. The cells were centrifuged at 6,000 rpm for 5 min at 4°C. Then the cultures were transferred into 250 ml shake flask containing 50 mL nitrogen limiting media with 2.5 g/L sodium acetate. The compositions of the nitrogen limiting media were as mentioned above excluding agar. The cultures were incubated for 4– 5 days at 30 - 37°C. As for PHBV productions, the MSM medium was supplied with 10 g/L of acids mixture from anaerobically treated POME with ratio (acetic 5: propionic 3: butyric 2).

Characterization of P(3HB-*co*-3HV) producer

Biochemical characterization, Quinones, 16S rDNA sequencing, DNA base composition, cellular fatty acids analysis and DNA–DNA hybridization were performed to determine the identity of the isolate, *C. putranensis*.

Morphological features were studied using a phase-contrast microscope (Olympus BH-2). For the determination of cell shape, size and the detection of flagella, cells were negatively stained with 1% (w/v) phosphotungstic acid according to the method of Cole & Popkin (1981) and observed with a CM-20 Philips transmission electron microscope at a voltage of 100 kV. Staining of polysaccharides was performed using Sudan black and staining of inclusion bodies with Nile blue A (Ostle and Holt, 1981). Oxidase and catalase activities were analyzed according to Gerhardt *et al.*, (1981) and antibiotic sensitivity tests were performed using antibiotic sensitivity discs (OXOID, England) on Muller-Hilton Agar (MHA) (Merck, Germany). Saline tolerance was investigated using NB medium and NA with the addition of NaCl (Merck) at required concentration.

Biochemical characteristics of test strain were determined using the API 20NE, API 50 CH and API ZYM (BioMérieux) following the manufacturer's protocols. Screening for carbon substrates utilized was performed with the BIOLOG GN2 microplate system release 4.0. The pH range for growth was checked using a 250 ml flask containing 50 ml NB with a pH of 5-9 at 30 °C. Growth was checked by monitoring optical density at 600 nm.

Quinones were characterized by HPLC using an EcoCart 125-3 (Lichrospher; RP- 18, 5 µm) column and acetonitrile/2-propanol (65:35, v/v) as mobile phase at a flow rate of 0.5 mL/min. The column was kept at 40 °C. The ubiquinone was detected by a UV detector at 254 nm

(Kroppenstedt, 1985). The analyses were performed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

For fatty acids analysis, fatty acid methyl esters were obtained by saponification, methylation and extraction using the method as described by Kuykendall et al. (1988). The fatty acid methyl esters mixtures were separated using Sherlock Microbial Identification System (MIS) (Microbial ID, Newark, DE 19711 USA.) which consisted of a Hewlett-Packard Model 5980 gas chromatograph fitted with a 5% phenyl–methyl silicone capillary column (0.2 mm · 25 m), a flame ionization detector, Hewlett-Packard model 7673A automatic sampler, and a Hewlett-Packard model KAYAK XA computer (Hewlett- Packard Co., Palo Alto, California, USA). Peaks were automatically integrated and fatty acid names and percentages calculated by the MIS Standard Software (Microbial ID). For the G+C content determination genomic DNA was prepared according to the procedure of Wilson (1987). The HPLC technique (Mesbah *et al.*, 1989) was used for the determination of the G+C content of strain *C. putranensis* and the control strain, *C. terrigena* (DSM 7099^T) was used. DNA–DNA hybridization by the initial renaturation rate was carried out under consideration of the modifications described by Huss et al. (1983) using spectrophotometer (Cary 100 Bio UV/VIS) equipped with a Peltierthermostatted 6X6 multicell changer and a temperature controller with in situ temperature probe (Varian). The hybridization temperature was 70℃ and the reaction was carried out in 2X standard saline citrate (SSC) and 10% formamide. Each value was the mean of at least two hybridization experiments.

16S rDNA sequence analysis and phylogenetic tree construction

The extraction of genomic DNA from strain *C. putranensis* was performed using genomic DNA extraction kit (Vivantis Tech., Malaysia) by following manufacturer's instruction. Bacterial universal primers used for PCR were: forward pAF 5`-AGAGTTTGATCCTGGCTCAG-3` and reverse pAR 5`-AAGGAGGTGATCCAGCCGCA -3`. PCR reaction mixture contained 25 µL: 10 X PCR buffer (Fermentas, USA), 0.5 µL: 10 mM dNTP mix (Fermentas, USA), 2.5 µL: 25 mM MgCl (Fermentas, USA), 0.5 µL: of each primer (10 µM) (1st Base, Malaysia), 0.2 µL: 5U/ µL Taq DNA polymerase (Fermentas, USA), 0.5 µL: total DNA sample and 17.8 µL: sterile de-ionized water to make total reaction mixture 25 µL. The amplification was performed by PCR machine; model T Gradient Thermoblock, (Biometra, Germany) with PCR condition as follows:

initial denaturation 94℃, 3 min: denaturation 94℃, 40 s: annealing 54℃, 40 s: elongation 72℃, 1 min 30 s and 72℃, 10 min: 35 cycles. An expected PCR product of approximately 1500 bp was purified using a gel extraction kit (Qiagen, Germany) and the PCR products were sent to Medigene Sdn. Bhd. (Malaysia) for sequencing. The sequences generated in this work were all searched for similarity against GenBank using the advanced gapped blast option (Altschul *et al.*, 1997) at the NCBI home page (http:// www.ncbi.edu) and Ribosomal Database Project II (RDP) (Maidak et al., 1997). Sequence alignment was performed with the multiple sequence alignment using CLUSTAL_W on the website http://www/ebi.ac.uk/embl/. Phylogenetic analyses were conducted by MEGA 3.1 software (Kumar et al., 2004). Phylogenetic distances between the cultures were calculated based on a Kimura 2-parameter (Kimura, 1980) substitution model. Phylogenetic trees were generated from the distance matrixes using a neighbour joining tree-building algorithm (Saitou and Nei, 1987). Statistical support for the branching nodes was obtained by bootstrap (1000 replicates).

Production of Polyhydroxyalkanoates (PHA)

Bacterial strain

C. putranensis used in this study was isolated from open digester treating POME collected in Serting Hilir palm oil mill located in the Negeri Sembilan, Malaysia. For preparation of inocula, the culture was grown at 30℃ under aerobic condition in nutrient broth (5 g of peptone, 3 g of meat extract, 1 g of yeast extract, 2.5 g of NaCl and 3 g of sodium acetate in 1 l distilled water) for 14 hours. The cultures were stored at -80 ℃ in 25 % (v/v) glycerol for maintenance purposes.

Growth and PHA biosynthesis by one- step cultivation

Precultured cells were transferred into 50 ml mineral salts medium (MSM) in 250 mL flasks containing (per liter of distilled water) KH_2PO_4, 6.7 g ; $(NH_4)SO_4$, 1 g ; $MgSO_4$, 0.2 g, 0.02 $CaCl_2$ and 0.1 mL microelements solution as reported by Hassan *et al.* (2002). The initial pH of the medium was adjusted to 7.0. Synthetic organic acids and their mixtures (total acids 5 g/L) were added into the medium and were extrapolated to the desired ratio. The cultures were incubated at 30℃ for 48 hours under aerobic condition with agitation speed 200 rpm. Cell growth was

monitored by measuring the optical density (OD) of the broth at 600 nm and correlation of OD versus cell dry weight (CDW) was established from this study.

Analytical procedures

PHA content and composition in the lyophilized cell material were determined using gas chromatography (Agilent, Series 2000) and nuclear magnetic resonance (^{1}H NMR) analyses. In GC analysis (Braunegg *et al.*, 1978), approximately 15 mg of lyophilized cell was subjected to methanolysis in the presence of methanol and sulfuric acid [85%:15% (v/v)]. The reaction mixture was incubated at 100°C for 2 h. The organic layer containing the reaction products was separated, dried over Na_2SO_4, and analyzed by GC.

In NMR analysis, the PHA was extracted from freeze-dried cells. 1.0 g freeze-dried cells were stirred in 100 mL of chloroform for 24 h at 30°C. The extract was filtered to remove cell debris, and the chloroform was concentrated to a volume of about 15 mL using a rotary evaporator. The concentrated solution was then added drop-wise to 150 mL of rapidly stirred methanol to precipitate the dissolved PHA. The precipitated PHA was recovered by filtration using a 0.45 µm PTFE membrane and dried overnight at room temperature. The purified PHA was dissolved in deuterated chloroform ($CDCl_3$) and subjected to the 400 MHz ^{1}H and 300 MHz ^{13}C NMR analyses.

Results:

Screening and Isolation of PHBV producer

Approximately 200 colonies were randomly screened for their ability to accumulate polyhydroxybutyrates (PHB). All colonies were transferred onto the selective medium containing the Nile Blue A dye with limiting nitrogen source. After several days of incubation, the viable colonies that showed a bright orange color under UV light were selected for further processed indicated the isolates were able to accumulate PHA. All the colonies that have native colors (orange or yellow) were discarded during the screening process because this color will influence the orange fluorescence exhibited and all the color strains tested previously did not promoted the PHB accumulation. Only the white colonies were selected for further

analysis. Eleven colonies were successfully isolated from the palm oil wastewaters using acetate as sole carbon and energy sources. The isolates namely as PHB 5 showed a good PHB accumulation and later designated as *C. putranensis*. Growth study of *C. putranensis* was performed using various organic acids intended to obtain suitable acids for PHB and PHBV production. As showed in **Fig. 2**, propionic acid is the best carbon source for growth of *C. putranensis* as determined by CDW, followed by acetic and butyric acids. The results obtained showed that this bacterium was able to consume propionic acids and possibly could produce higher hydroxyvalerate (HV) monomer units during PHA accumulation with POME acids mixture.

Morphological studies

C. putranensis is actively motile, aerobic, nonfermentative, Gram- negative rod, 0.71 by 2.64 µm size. Colonies on NA (nutrient agar) plates developed after 24 h incubation period were circular (1-2 mm diameter) and transparent cream- colour. Electron micrograph of Negative staining showed that presented of flagella in multipolar tufts during growth stage as showed in **Fig. 3**. All validly reported *Comamonas* strains were having flagella and motile except *C. koreensis* (Chang *et al.,* 2002). Thin sections electron micrograph of strain *C. putranensis* revealed typical PHA granules clearly show the existence of intracellular PHA granules (**Fig. 4**). There were several *Comamonas* strains showed capabilities of PHA accumulations: *C. denitrificans* (Gumaelius *et al.*, 2001), *C. testosteroni* (Thakor *et al.*, 2003), and *C. composti* (Young *et al.,* 2008). Other unique features that differed *C. putranensis* with other reported *Comamonas* species are described in later section.

DNA-DNA hybridization

The DNA–DNA hybridization was performed to investigate the DNA relatedness between *C. putranensis* with reference strain *C. terrigena* (DSM 7099). The DNA-DNA relatedness between those strains were high (90%) and this value is above the cut-off point recommended for assignment of the strains to the genomic species (Wayne et al., 1987). It was clear that from the results obtained that *C. putranensis* belongs to *C. terrigena* species. However, other properties like biochemical and phenotypic features are also should take into consideration in describing new species. Studies conducted by Wauters *et al.* (2003), showed that *C. aquatica* and *C. kerstersii* were the subgroups of the *C. terrigena* after performing additional analysis

like, SDS-PAGE, biochemical analysis, and immunodiffusion analysis. These three bacteria was previously named as *C. terrigena* and later amended to new name after those analyses was carried out.

G+C content

For G+C content calculations, the DNA sample was prepared in duplicate and degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated with a HPLC system. The G+C content of *C. putranensis* was 59.1±1.0 mol%, which was a little bit lower in comparison with other DNA G+C contents previously reported for *Comamonas* species (60.8–66.3 mol%) (Young *et al.,* 2008). This is another feature that differed this microorganism with other existing *Comamonas* species.

Analysis of quinone and cellular fatty acids

C. putranensis contained ubiquinone Q-8 (98%) as the predominant component of the quinone system while menaquinone or rhodoquinone was not detected. Fatty acid methyl esters were prepared, separated and identified according to the instructions of Microbial Identification System (MIDI; Microbial ID). Predominant fatty acids of *C. putranensis* were 16: 0, 18 : 1/ 18: 1w 7c and summed feature 3. The fatty acid pattern of the *C. putranensis* is shown in Table 1 in comparison with other representative *Comamonas* species. The fatty acid profile of *C. putranensis* was in good agreement with data obtained for other members of the genus *Comamonas* (Chang *et al.,* 2002; Wauters *et al.,* 2003; Tago &Yokota, 2004; Chou *et al.,* 2007) **(Table 1).**

Table 1 Comparison of the fatty acid compositions of strain EB172 and other *Comamonas* species.

Taxa: 1, strain EB172 (*C. putranensis* sp. nov.); 2, *C. composti*; 3, *C. odontotermitis* Dant3-8^T; 4, *C. koreensis* DSM 18232^T; 5, *C. testosteroni* DSM 50244^T; 6, *C. terrigena* LMG 1253^T; 7, *C. denitrificans* DSM 17887^T; 8, *C. nitrativorans* DSM 13191^T; 9, *C. aquatica* LMG 2370^T; 10, *C. kestersii* LMG 3475^T ; 11, *C. badia* DSM 17552^T. All the reference data was obtained from Young et al., (2008). Values are percentages of total fatty acids; -, fatty acids representing les

than 0.5%. The position of the double bond in the unsaturated fatty acids is obtained by counting from the methyl (ω) end of the molecule.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
10 : 0 3-OH	4.3	5.6	3.8	3.5	4.8	5.3	4.2	5	5	4.5	2.4
12 : 0	3	3.2	2.7	2.3	2.4	2.8	3	2.9	3	2.6	2.9
14 : 0	4.5	1.4	-	1	1	3.3	3.2	3.4	3.9	2.9	1.5
15 : 0	0.51	-	-	9.4	1	3.7	-	-		-	-
15 : 0 2-OH	-	-	-	0.6	-	-	-	-	-	-	-
16 : 0	31.9	33.3	33.6	29.9	30.4	27.5	17.8	21.2	25.2	23.4	33.3
16 : 0 2-OH	-	-	2.5	2.2	2	-	-	-	-	-	2.3
16 : 1 2-OH	-	-	-	-	0.6	-	-	-	-	-	-
17 : 0	-	1	-	2.6	0.8	1.5	-	-	-	0.6	-
17 : 0 cyclo	4.7	1.4	5.9	12.3	3.8	2.4	-	-	-	0.7	-
17 : 1	-	-	-	0.7	-	-	-	-	-	-	-
18 : 1/ 18: 1w 7c	14.8	12.9	16.2		17.9	14.9	22.4	23.8	19	36.1	13.1
19 : 0 cyclo	-						-	-	-	-	-
20 : 0 iso	-						-	-		-	-
Summed feature 3*	32.5						48.6	42.9		28.2	41.9

Phylogenetic analysis

Almost complete 16S rDNA sequence of *C. putranensis*, consisting 1,446 nucleotides have been determined. The resulting sequence was checked for similarity against deposited data in GenBank for the top 50 hits, including described and noncultivated organisms were retrieved and merged into the alignment. Phylogenetic analysis in which this sequence was compared with corresponding sequences of representatives of the family *Comamonadaceae* indicated this strain belongs to the genus *Comamonas* (**Fig. 5**). The 16S rDNA sequence similarities between *C. putranensis* and its nearest neighbors, *C. kestersii* (91.1%), *C. terrigena* (96.8%), *C. koreensis* (93.4%), *C. testosteronii* (90.2%), *C. composti* (92.9%), and the type strains of other *Comamonas* species are low enough to categorize *C. putranensis* as a species that is distinct from previous described *Comamonas* species. None of the validly described species showed more than 97% 16S rDNA similarity (Stackebrandt and Goebel, 1994). Bootstrap resampling showed that it possesses a statistically significant association with *C. kerstersii*. Therefore, on the basis of this data, strain *C. putranensis* should be placed in the genus *Comamonas* as a novel species, for which we propose the name *Comamonas putranensis* sp. nov.

Biosynthesis of PHA during one-step cultivation

The ability of *C. putranensiss* to accumulate PHA during one- step cultivation process was investigated using different types of carbon sources. Carbon sources such as acetic, propionic, butyric, lactic valeric acid, and glucose were used as solely or in combination with suitable ratios to observe *C. putranensis* growth and accumulation of polyesters. It was found that *C. putranensis* was able to produce homopolymer P(3HB) and copolymer P(3HB-*co*-3HV) when grown on mainly fatty acids as their carbon and energy sources. **Table 2** shows PHA content, CDW and monomer compositions were significantly different observed through out the study when supplied with various carbon sources. P(3HB) monomer was produced when acetic, lactic and butyric acid was used sole carbon sources.

Table 2 Cell dry weight, PHA content and PHA monomer compositions produced fro m various carbon sources by *Comamonas putranensis*[a].

Carbon sources	Cell dry weight (g/L)	PHA content (wt.%)[b]	PHA composition (mol%)[b]	
			3HB	3HV
Acetic acid	2.1 ± 0.15	16 ± 1.2	100	0
Butyric acid	3.8 ± 0.42	50 ± 17.0	100	0
Propionic acid	1.1 ± 0.32	15 ± 2.0	68 ± 9.3	29 ± 14.9
Lactic acid	3.0 ± 0.35	66 ± 7.1	100	0
Valeric acid	1.5 ± 0.45	10 ± 4.5	42 ± 9.6	58 ± 9.6
Acetic + Valeric	3.4 ± 0.70	27 ± 5.7	56 ± 11.8	44 ± 11.8
Glucose + Valeric	2.9 ± 0.90	19 ± 6.0	35 ± 4.2	65 ± 4.2
Glucose + Propionic	2.0 ± 0.85	34 ± 14.8	79 ± 0.6	21 ± 0.6
Acetic + Propionic	3.0 ± 0.10	20 ± 3.5	91 ± 5.0	9 ± 5.0

[a]Fermentation was conducted by single or in combination of carbon sources with total 5 g/L of substrates, 30℃, 200 rpm for 48 h.

[b]Determination by GC

On the other hand, P(3HB-*co*-3HV) was produced when propionic and valeric acids were used as carbon sources. Other carbon sources like glycerol and fructose also was carried out but there was no good results obtained (results not shown). The highest CDW (3.8 g/L) was produced when butyric acid was used as the carbon source and PHB accumulation 50 wt.%. Lactic acids, showed the highest PHB accumulation (66 wt.%) but with moderate CDW produced (3.0 g/L). An inhibition on cell growth was observed when propionic or valeric acids were fed into MSM media and resulted lower amount of CDW produced with 1.1 g/L and 1.5 g/L, respectively. However, combination of propionic and valeric acids with acetic, and glucose resulted in improvement of CDW, PHA content and mol % of HV units. Copolymers with a broad range of 3HV molar fractions (9-65 mol %) were obtained when the medium were co-fed with propionic and valeric acids.

P(3HB-co-3HV) copolymer production using acetic : propionic acid at different ratios

In order to obtain a P(3HB-*co*-3HV) copolymer containing varying mol % P(3HV) units, a study was conducted using acetic and propionic acids at different ratio. From the results obtained (Table 3), combination of substrates used resulted in better cell biomass formation and P(3HB-*co*-3HV) accumulations. The highest CDW and PHA content were obtained when acetic: propionic ratio (2:3) was supplied into the MSM medium resulted 2.8 g/L and 30 wt%, respectively. Thus suggesting the optimal acids mixture for acetic and propionic for P(3HB-*co*-3HV) formation. The results also showed that the mol% of HV units formed were proportional to propionic acids concentration in which P(3HV) monomers in the P(3HB-*co*-3HV) copolymers increasing when supplemented with higher propionic acids concentration. The highest P(3HV) monomer (17 mol%) was detected when the ratio of 1:4 (acetic: propionic) supplemented into the medium.

Table 3 Cell dry weight, PHA content and PHA monomer compositions produced from combination of acetic and propionic acids at different ratio[a].

Carbon sources Acetic: Propionic	Cell dry weight (g/L)	PHA content (wt.%)[b]	PHA composition (mol%)[b]	
			3HB	3HV
(5:0)	1.5 ± 0.2	15 ± 1.5	100	0
(4:1)	2.2 ± 0.3	18 ± 1.5	91 ± 2.1	9 ± 2.1
(3:2)	2.5 ± 0.1	23 ± 1.5	90 ± 1.0	10 ± 1.0
(2:3)	2.8 ± 0.3	30 ± 5.9	85 ± 2.5	15 ± 2.5
(1:4)	2.6 ± 0.1	21 ± 2.1	83 ± 2.5	17 ± 2.5

[a]Fermentation was conducted by combination of carbon sources with total 5 g/L of substrates, 30℃, 200 rpm for 48 h.

[b]Determination by GC.

Effect of pH on P(3HV) unit in the P(3HB-*co*-3HV) copolymers using sodium valerate as substrate precursor

The study using sodium valerate as precursor for P(3HB-*co*-3HV) copolymer production was carried out. Despite the ability of *C. putranensis* to consume and accumulates P(3HB-*co*-3HV) copolymer, the incorporation of P(3HV) unit monomer in the P(3HB-*co*-3HV) copolymers was improved by selecting initial medium pH. **Table 4** showed initial medium pH plays an important role in biomass production, PHA accumulation as well as incorporation of P(3HV) unit monomers. By selecting the initial medium pH (6.5 – 8.0), production of P(3HB-*co*-3HV) copolymers with varying P(3HV) monomer units in the range of 45- 86 mol % P(3HV) was obtained. pH 7.5 was observed the best pH for biomass production (2.1 g/L), meanwhile pH 7.0 is the best pH for PHA accumulation with 59 wt.%. Thus this result indicates the neutral pH is the best condition for biomass and PHA accumulations. The most interesting finding was

P(3HV) monomer units were observed in increasing trends towards the alkaline condition (86 mol % P(3HV)), however reduced in PHA accumulation. This finding was contradictory to the study reported by Loo *et al.* (2004), in which P(3HV) units formation increased towards lower pH. However, P(3HV) monomer units produced in this study were in the reported range and comparable to other studies (Loo *et al.*, 2004).

Table 4 Effect of pH on biosynthesis of P(3HB-*co*-3HV) copolymers production using sodium valerate by *C. putranensis*[a].

pH	Cell dry weight (g/L)	PHA content (wt.%)[b]	PHA composition (mol%)[b]	
			3HB	3HV
6.5	0.3 ± 0.2	15 ± 3.5	55 ± 1.5	45 ± 1.5
7.0	1.9 ± 0.4	59 ± 3.2	30 ± 1.5	70 ± 1.5
7.5	2.1 ± 0.2	26 ± 3.2	23 ± 2.1	77 ± 2.1
8.0	1.7 ± 0.2	30 ± 2.5	14 ± 1.5	86 ± 1.5

[a]Fermentation was conducted at 30°C, 200 rpm for 48 h.

[b]Determination by GC

^{1}H NMR

It was expected that the PHA produced from the cells is a P(3HB-*co*-3HV) copolymer due to the feedstock used during fermentation. In order to confirm this hypothesis, the extracted PHA sample was analyzed by ^{1}H NMR and the spectrum is shown in **Fig. 6**. The assignments of P(3HB-*co*-3HV) signals have been reported previously (Doi *et al.*, 1986; Kamiya *et al.*, 1990). Based on the reports and by the estimation of ^{1}H NMR chemical shifts using a ChemNMR program in a CS ChemDraw Ultra version 6.0, the peaks in Figure 4 were assigned. The assignments of the ^{1}H NMR signals revealed that the PHA produced by *C. putranensis* was a P(3HB-*co*-3HV) copolymers and hence, confirmed the speculation.

CLAIMS

1) A process for production of polyhydroxyalkanoates (PHAs) from synthetic acids / organic acids derived from palm oil mill effluent (POME) treatment which comprises the step of incubating of a biologically pure bacterial strain for a sufficient period of time and under specified conditions to produce said PHA in a culture medium comprising synthetic acids / organic acids derived from POME or other organic wastes.

2) A biologically pure bacterial strain as claimed in claim 1 which is isolated from digester-treated palm oil mill effluent (POME) identified by polyphasic approach.

3) A biologically pure bacterial strain as claimed in claim 1 which belongs to *Comamonadaceae* family in the genus of *Comamonas* in which proposed as *Comamonas putranensis* sp. nov.

4) The process as claimed in Claim 1 in which the synthetic acids are acetic, butyric, lactic, propionic, valeric acids and other organic acids.

5) The process as claimed in Claim 1 in which the polyhydroxyalkanoates are selected from the group consisting of polymer of a hydroxybutyric acid, hydrovaleric acid or other hydroxyl acids and copolymers thereof.

6) The copolymer as claimed in claim 5 which is a poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)].

7) The process according to Claim 1, wherein the PHA produced from the bacterial strain is capable of producing 10 – 66 % PHA by cell dry weight

8) The process according to Claim 5, wherein the said P(3HB-*co*-3HV) copolymer produced from the selected bacterial strain with P(3HV) monomer units is in the range of 0-86 mol %.

9) A biologically pure bacterial strain with 16S rDNA sequences as deposited in NCBI accession number of EU 847238.

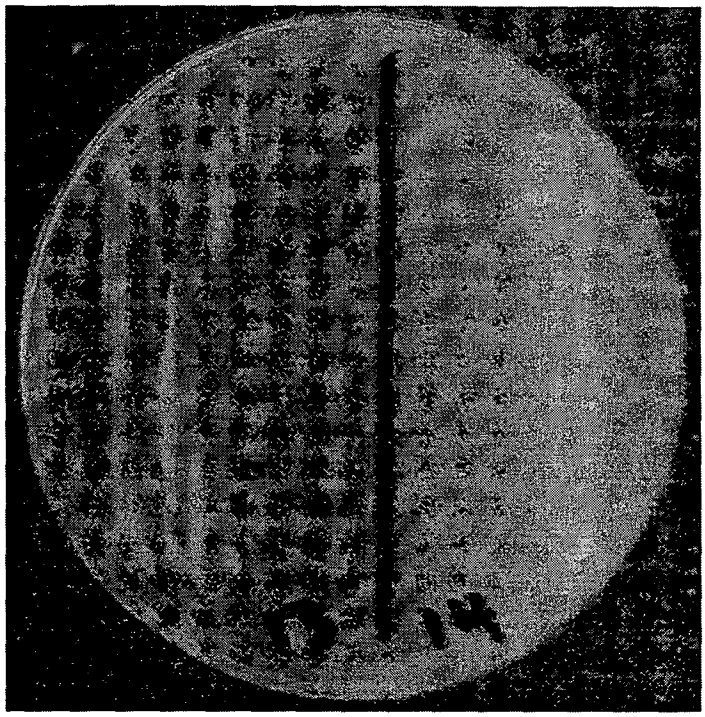


Fig. 1

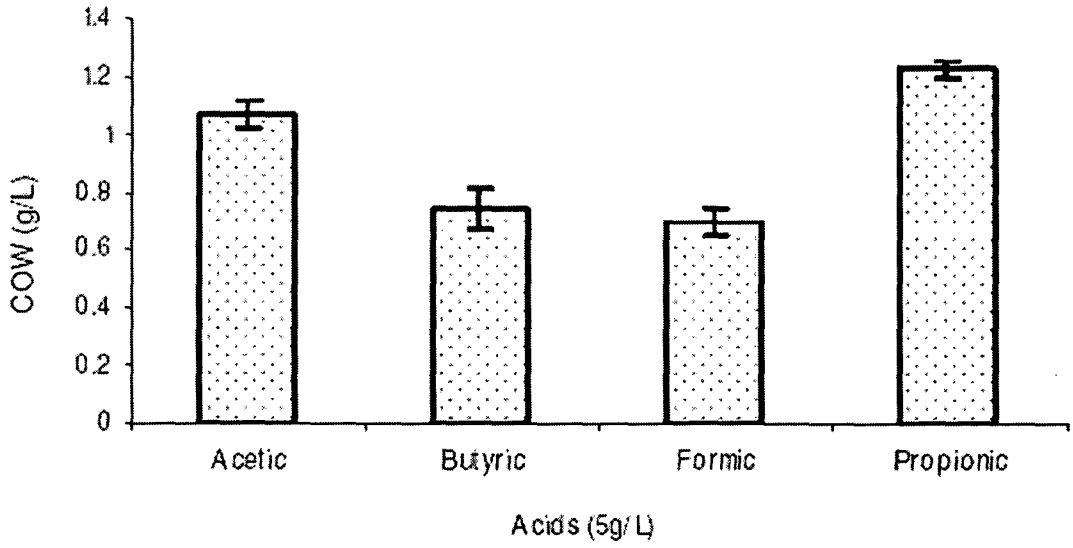

1.4
1.2
1
0.8
0.6
0.4
0.2
0
COW (g/L)
Acetic
Butyric
Formic
Propionic
Acids (5g/L)

Fig. 2

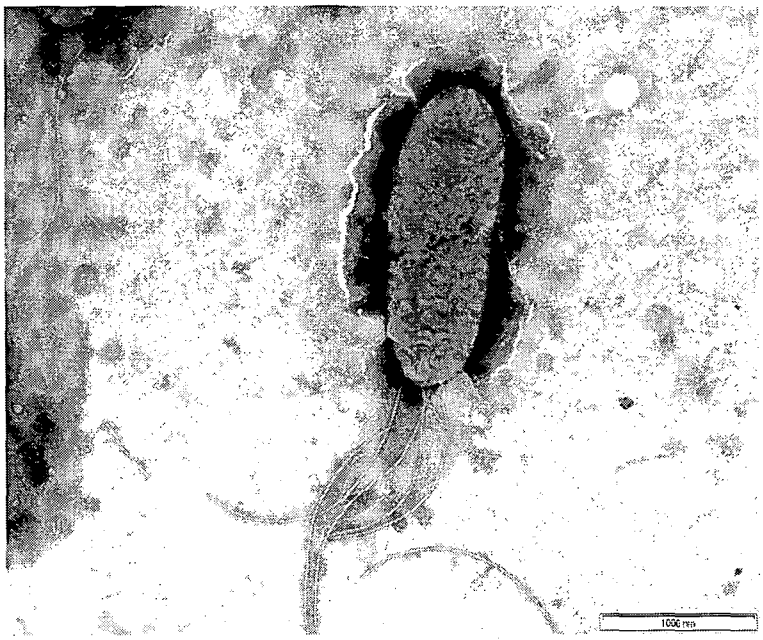


Fig. 3

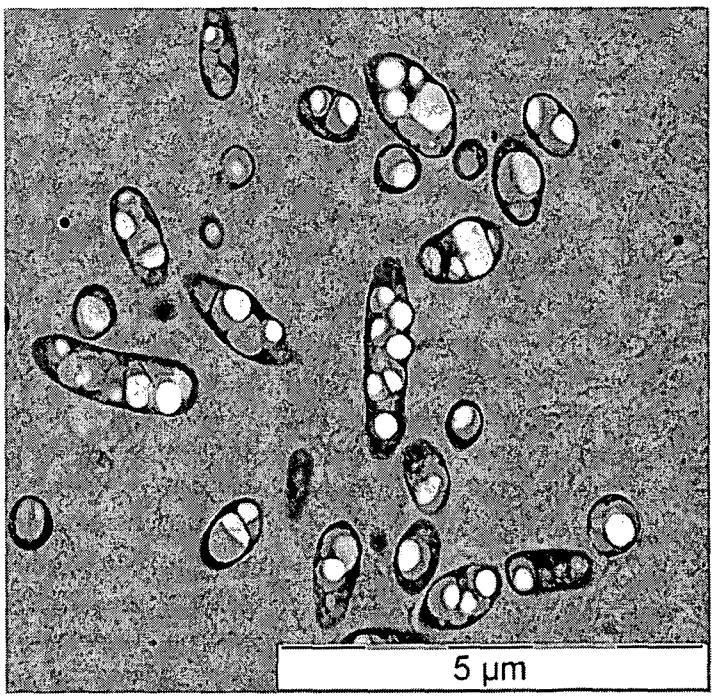


Fig. 4

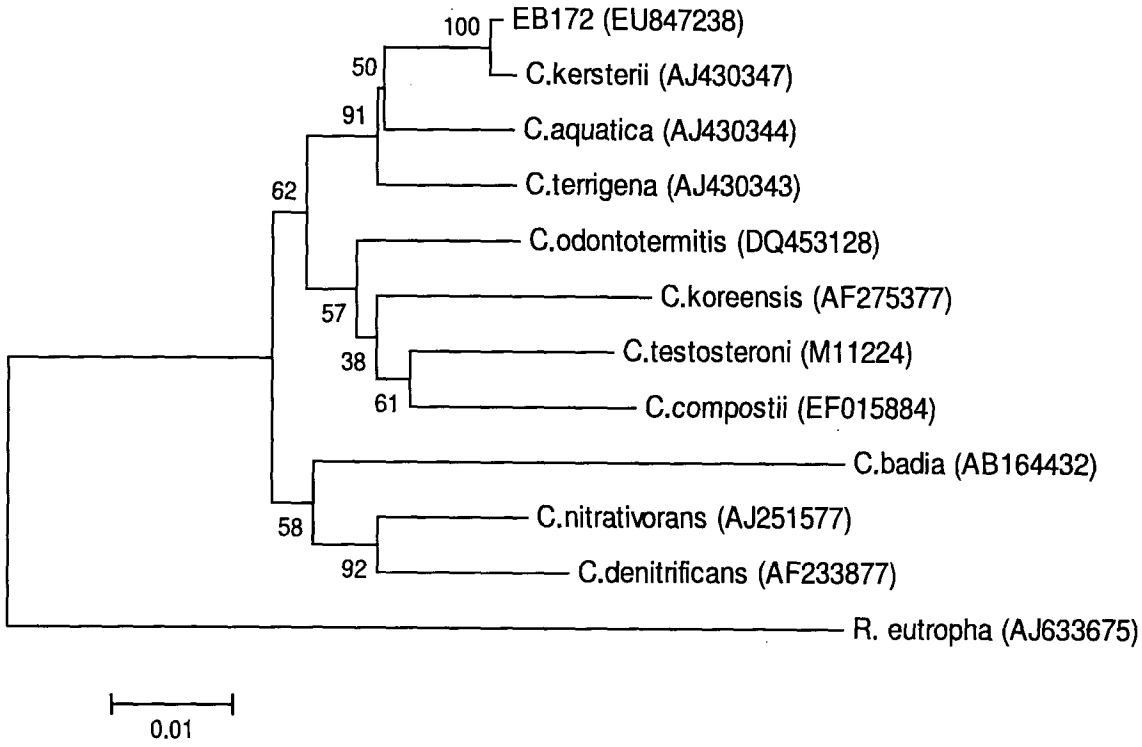

100
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C.aquatica (AJ430344)
62
C.terrigena (AJ430343)
C.odontotermitis (DQ453128)
57
C.koreensis (AF275377)
38
C.testosteroni (M11224)
61
C.compostii (EF015884)
C.badia (AB164432)
58
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C.denitrificans (AF233877)
R. eutropha (AJ633675)
0.01

Fig. 5

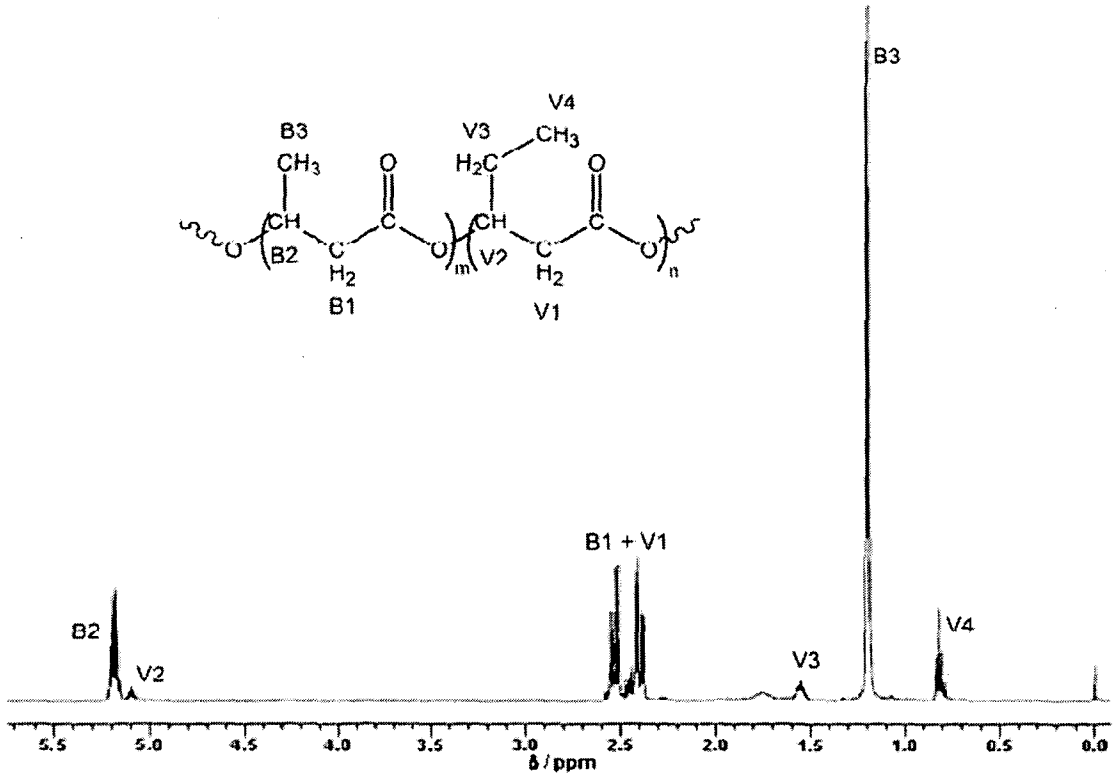

B3
V4
B3
V3
CH3
CH3
H2C
O
O
CH
CH
C
C
O
O
B2
m
V2
n
C
H2
C
H2
B1
V1
B3
B1 + V1
B2
V4
V2
V3
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
δ / ppm

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/MY2010/000004

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P1/04 C12P7/62 C12R1/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/57298 A1 (UFZ LEIPZIGHALLE GMBH [DE]; BABEL WOLFGANG [DE]; MASKOW THOMAS [DE]) 11 November 1999 (1999-11-11) page 4, line 7 - page 8, line 26	1-8
X	MR ZAKARIA ET AL.: "Comamonas sp. EB172 isolated from digester treating palm oil mill effluent as potential polyhydroxyalkanoate (PHA) producer." AFRICAN JOURNAL OF BIOTECHNOLOGY, vol. 7, no. 22, 19 November 2008 (2008-11-19), - 19 November 2008 (2008-11-19) pages 4118-4121, XP002588054 page 4119, left-hand column - page 4121, left-hand column	1-8

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 July 2010	27/07/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Stoyanov, Borislav

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INTERNATIONAL SEARCH REPORT

International application No
PCT/MY2010/000004

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	T. MUMTAZ ET AL.: "Fed-batch production of P(3HB-co-3HV) copolymer by Comamonas sp EB 172 using mixed organic acids under dual nutrient limitation" EUROPEAN JOURNAL OF SCIENTIFIC RESEARCH, vol. 33, no. 3, July 2009 (2009-07), - July 2009 (2009-07) pages 374-384, XP002588055 ISSN: 1450-216X page 375 - page 382	1-8

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/MY2010/000004

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DE 19820168 A1	04-11-1999
		JP 4416947 B2	17-02-2010
		JP 2002513584 T	14-05-2002
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