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ORIGINAL PAPER

Polyhydroxyalkanoate production from anaerobically treated palm oil mill effluent by new bacterial strain *Comamonas* sp. EB172

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Abstract A new isolate designated as strain EB172 was isolated from a digester treating palm oil mill effluent and was investigated by polyphasic taxonomic approach. The cells were rod-shaped, Gram-negative, non-pigmented, non-spore-forming and non-fermentative. Phylogenetic analysis using the 16S rRNA gene sequence showed that the strain clustered with the genus *Comamonas*. Its closest neighbours were the type strains *Comamonas terrigena* (96.8%), *Comamonas koreensis* (93.4%), *Comamonas composti* (92.9%), and *Comamonas kerstersii* (91.1%). The ability of the strain EB172 to produce polyhydroxyalkanoates (PHA) when supplied with organic acids made this bacterium unique among *Comamonas* species. The bacterial strain was clearly distinguished from all of the existing strains by phylogenetic analysis, fatty acid composition and a range of physiological and biochemical characteristics. The G+C content of the genomic DNA was 59.1 mol%. The strain showed good growth in acetic, propionic and *n*-butyric acids. *Comamonas* sp. EB172 produced 9.8 g/l of cell dry weight and accumulated 59 (wt%) of PHAs when supplemented with mixed organic acids from anaerobically treated palm oil mill effluent. It is evident from the genotypic, phenotypic data and ability to produce PHAs that strain EB172 represents a new strain in the genus *Comamonas* (GeneBank accession no. EU847238).

Keywords *Comamonas* sp. · Palm oil mill effluent · Polyhydroxyalkanoates · Phylogenetic · Taxonomy

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Introduction

Palm oil mill effluent (POME) is one of the major sources of pollutant produced during oil palm processing. POME is currently treated using different approaches such as lagoon, open and closed digesters as part of the wastewater management system in Malaysia. In recent years, it has gained great attention by the research institutions and industrial sectors, due to its potential as sources of carbon and nitrogen for microbial growth. The presence of unique microflora inside the lagoon, open and closed digesters coupled with rich cellulosic and lipid waste materials in POME led to new findings in recent years. Production of organic acids (Hassan et al. 1997; Tabassum et al. 2008), methane (CH_4) (Yacob et al. 2006) and biohydrogen (Chong et al. 2009) from POME were evidences of the presence of potential carbon and nitrogen sources al well as biological biodiversity in the POME sludge. Production of mixed organic acids from anaerobicallay treated palm oil mill effluent has introduced it as a shown renewable and cheaper carbon sources for PHAs production (Hassan et al. 1997, 2002; Zakaria et al. 2008).

During the characterization of the microflora from an open digester treated-POME, we isolated a bacterium

which was able to grow and accumulate polyhydroxyalkanoate (PHA) from organic acids, *Comamonas* sp. EB172 (Zakaria et al. 2008). Owing to the nature of organic acids produced from the anaerobic treatment of POME, the strain was capable of producing poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3-HV)] copolymer in shake flasks and fed-batch cultivation process. P(3HB-*co*-3-HV) copolymer had better thermal and physical properties in comparison to P(3HB) homopolymer. The thermal and physical properties of the P(3HB-*co*-3-HV) copolymer was influenced by the incorporation of P(3HV) unit monomers in the P(3HB-*co*-3-HV) copolymers (Doi 1990).

At the time of writing this paper, the genus *Comamonas* encompasses 10 species with validly published names: *C. aquatica, C. badia, C. composti, C. denitrificans, C. kerstersii, C. koreensis, C. nitrativorans, C. odontotermitis, C. terrigena, and C. testosteroni*. Those strains were isolated from various environmental origins with different functions and roles. However, only a few of the strains in the genus showed a capability to accumulate PHAs. The present study aimed to characterize a new isolate *Comamonas* sp. EB172 by polyphasic taxonomic approaches. Phenotypic and genotypic properties of the strain were reported and distinguished with other existing strains in the genus of *Comamonas*. Furthermore, the ability of the isolate to grow and accumulates PHAs from mixed organic acids derived from anaerobically treated palm oil mill effluent made this strain a promising candidate for industrial PHA production.

Materials and methods

Bacterial strain and cultivation

During the characterization of strain *Comamonas* sp. EB172, the culture was grown on nutrient agar (NA) plate and slant, at 30°C for 16–24 h. Subcultivation was performed on NA and cultures were kept at −80°C in 20% glycerol for preservation purposes. The methods for growing and PHA accumulation of strain EB172 was described elsewhere (Zakaria et al. 2008). Fed-batch cultivation was conducted in 2 l fermenter (Sartorius, Germany) with 900 ml of production medium. The medium composition used as follows: (in grams per liter of distilled water) KH_2PO_4, 5.0 g; $(NH_4)_2SO_4$, 1.5 g; $MgSO_4$, 0.2 g; Na_2HPO_4, 1.5; mixed organic acids from anaerobically treated POME (5 g/l) and 3.0 ml microelements. The initial medium pH was adjusted at 6.8 with 2 M H_2SO_4 and 2 M NaOH. The cultures were operated in batch mode for 12 h and switch to pH- stat fed- batch mode by adding stock solution of mixed organic acids (50 g/l) derived from anaerobically treated POME. The pH was controlled at pH 7.0 through out the study period and dissolve oxygen (DO) was set at 40% air saturation using cascade mode. The fermentation period was monitored for 60–70 h.

Morphology and taxonomical studies

Morphological features were studied using a phase-contrast microscope (Olympus BH-2, New York, USA). 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 described previously (Cole and Popkin 1981) and observed with a CM-20 Philips transmission electron microscope at a voltage of 100 kV. The Gram reaction was determined by Ryu non-staining KOH method (Powers 1995). Staining of polysaccharides and inclusion bodies was carried out using Sudan black and Nile blue A, respectively (Ostle and Holt 1982). Oxidase and catalase activities were analyzed according to Cole and Popkin (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 nutrient broth (NB) medium with the addition of NaCl (Merck, Germany) at a desired concentration. Biochemical characteristics of tested strain were determined using the API 20NE, API 50 CH and API ZYM (BioMérieux) following the manufacturer's instructions. 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 in the range of pH 5–9 at 30°C for 24 h. Growth was checked by monitoring optical density (OD) at 600 nm. All the tests were carried out in triplicates.

Identification and 16S rRNA gene sequencing

The analysis of quinones, cellular fatty acids, G+C contents and DNA–DNA hybridizations were performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig (DSMZ) Germany. *C. terrigena* (DSM 7099^T) was used as reference strain through out the study. The extraction of genomic DNA of strain EB172 was performed using genomic DNA extraction kit (Vivantis Technologies Sdn. Bhd., Malaysia) according to manufacturer's instruction. Bacterial universal primers used for PCR were: forward primer, pAF 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer, pAR 5′-AAGGAGGTGATCCAGCCGCA-3′.

Each PCR mixture (25 µl) contained 2.5 µl PCR buffer (Fermentas, Maryland, USA), 0.5 µl of 10 mM dNTPs, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of each bacterial primer (10 µM) (1st Base, Malaysia), and 0.2 µl of *Ampli*Taq DNA polymerase (Fermentas, Maryland, USA). This mixture was completed to 25 µl with 18.3 µl of sterile distilled water

containing 100 ng of extrcated genomic DNA. The PCR amplification was performed in a T Gradient Thermoblock, (Biometra, Germany) and the reaction parameters were an initial denaturation 94°C for 3 min followed by 35 cycles of 94°C for 40 s, 54°C for 40 s, and 72°C for 1 min and finally 72°C for 10 min. An expected PCR product of ~1500 bp was purified using a gel extraction kit (Qiagen, Germany) and the PCR products were sent to Medigene Sdn. Bhd. (Malaysia) for sequencing.

Phylogenetic analysis of 16S rRNA gene sequence

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. 1999). Sequence alignment was performed with the multiple sequence alignment using CLUSTAL_W on the website http://www/ebi.ac.uk/embl/. Phylogenetic tree construction and 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 substitution model (Kimura 1980). Phylogenetic trees were generated from the distance matrixes using a neighbor joining tree-building algorithm (Saitou and Nei 1987). Statistical support for the branching nodes was obtained by bootstrap (1000 replicates).

Analytical procedures

Optical density (OD) of the culture broth was measured at 600 nm using a spectrophotometer (Hitachi U-2900, Japan) and cell dry weight (CDW) was measured by filtering the broth culture using pre-weighted membrane cellulose nitrate filters of 0.45 µm (Sartorius, Germany). Organic acids concentration from the broth media was determined by high performance liquid chromatography (HPLC) (Shimadzu, LC-10 AS) (Hassan et al. 1997). PHA content and composition of the lyophilized cell were determined using gas chromatography (Agilent, model 7890A) using an ID-BP1 capillary column, 30 × 0.32 × 0.25 µm (SGE). A total of 25 mg of lyophilized cells were subjected to methanolysis in the presence of methanol and sulfuric acid [85%: 15% (v/v)]. The resulting hydroxyacyl methyl esters were then analyzed according to standard method (Braunegg et al. 1978).

Results and discussion

Morphological and taxonomical identification

The wild type strain *Comamonas* sp. EB172 was observed actively motile, aerobic, non-fermentative, non-spore forming,

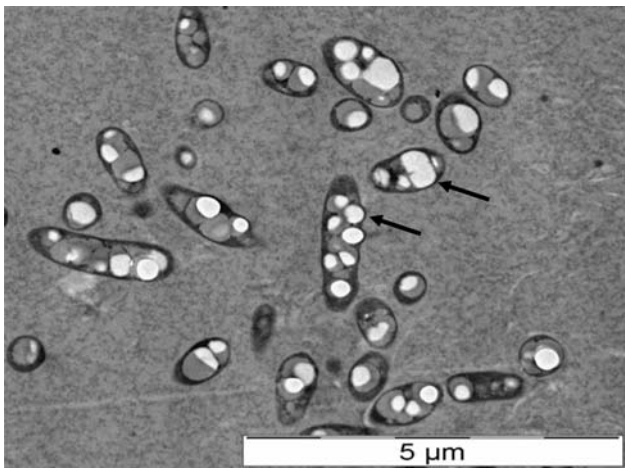

Fig. 1 Transmission electron micrograph of strain EB172 containing P(3HB-*co*-3HV) granules in fed-batch cultivation using mixed organic acids derived from anaerobically treated palm oil mill effluent as substrates. The presence of P(3HB-*co*-3HV) granules in the cell bodies was observed after 60 h of cultivation period. *Bar* represents 5 µm

and Gram- negative rod, with 0.71 × 2.64 µm in size. Colonies on NA developed after 24 h of incubation period were circular (1–2 mm in diameter) with transparent cream colour. The strain EB172 was oxidase and catalase positive. This bacterium grew well at temperatures in the range of 25–37°C and pH ranging from 6 to 8. Optimum growth was observed at 30°C and pH 7. In nutrient broth containing NaCl, the strain EB172 was able to grow in the range of 0–1.5% NaCl. The electron micrograph of negative staining showed the presence of flagella in multipolar tufts. All validly reported *Comamonas* strains were having flagella and motile except *C. koreensis* (Chang et al. 2002). Thin sections electron micrograph of strain EB172 revealed typical PHA granules which clearly showed the existence of intracellular PHA in the cell during unfavourable growth condition normally under nitrogen limitation (Fig. 1). Several *Comamonas* strains have been reported to be capable of PHA accumulations such as *C. denitrificans* (Gumaelius et al. 2001), *C. testosteroni* (Thakor et al. 2003), and *C. composti* (Young et al. 2008).

In API 20NE tests, strain EB172 showed positive results for oxidase, and catalase test. While negative results were observed for nitrate reduction, indole production, fermentation of glucose, arginine dihydrolase, urease, hydrolysis of esculin, gelatin, β-galactosidase, assimilation of glucose, arabinose, mannose, mannitol, maltose, N-acetylglucosamine, gluconate, caprate, adipate, malate, citrate and phenyl acetate. In API ZYM tests, positive results were recorded for alkaline phosphatase, C4 esterase, C8 lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-galactosidase, while negative results were obtained for C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β-galactosidase, β-glucuronidase, α-glucosidase,

Table 1 Genotypic, phenotypic and nutritional characteristics that distinguish strain EB172 from other *Comamonas* species

Characteristics	1	2	3	4	5	6	7	8
Source	POME sludge	Food waste	Wetland	Soil	Hay-infusion	Activated sludge	Fresh water	Human
Motility	+	+	−	+	+	+	+	+
Nitrite reduction to N_2	−	−	−	−	−	+	−	−
Alkaline phosphatase	+	+	+	+	+	−	−	−
Acid phosphatase	+	+	+	+	+	+	+	+
C8 lipase	+	+	+	+	+	−	+	+
C14 lipase	−	+	+	−	−	−	−	−
Valine arylamidase	−	+	+	+	+	+	−	+
Cysteine arylamidase	−	−	−	+	−	−	+	−
Assimilation of								
D-Gluconate	−	+	+	+	+	−	+	−
Caprate	−	−	−	+	−	−	−	−
Adipate	−	+	+	+	+	−	+	+
Malate	−	+	+	+	−	−	+	+
Citrate	−	−	+	+	−	−	−	−
Phenylacetate	−	−	−	−	−	−	−	−
Oxidation of								
Acetate	+	+	+	+	−	−	+	+
cis-Aconitae	−	+	−	−	+	+	−	−
Propionate	+	+	+	+	+	−	+	−
Hydroxybutyrate	+	+	+	−	−	−	+	+
Itaconate	−	+	−	−	−	−	+	+
D-Serine	−	−	+	−	−	−	−	−
L-Serine	−	−	+	−	−	−	−	−
L-Threonine	+	−	+	+	−	+	−	+
L-phenylalanine	−	−	+	−	−	−	−	+
Hydroxy-L-proline	−	+	+	−	−	−	−	−
L-Ornithine	−	−	−	−	−	−	−	−
Glycyl-L-aspartate	−	−	−	+	−	+	−	−
Glycyl-L-glutamate	−	−	+	+	−	−	−	−
N-Acetyl-D-galactyosamine	−	−	−	−	−	−	−	−
Tween 40	+	+	+	+	−	−	−	+
Tween 80	+	+	+	+	−	−	+	+
Susceptibility to								
Ampicillin	I	S	R	R	S	S	S	R
Gentamicin	S	R	S	S	S	S	S	S
Penicillin G	R	S	R	R	S	S	S	S
Streptomycin	I	R	S	R	S	S	S	S
G+C (mol%)	59.1	62.8	66	62.5–64.5	64	60.8	64	61
PHA accumulation	+	+	−	+	nd	+	nd	nd

Taxa: (1) Strain EB172; (2) *C. composti*; (3) *C. koreensis* DSM 18232^T; (4) *C. testosteroni* DSM 50244^T; (5) *C. terrigena* LMG 1253^T; (6) *C. denitrificans* DSM 17887^T; (7) *C. aquatica* LMG 2370^T; (8) *C. kestersii* LMG 3475^T. All the reference data was obtained from Young et al. (2008). +, Positive; −, negative; *R*, resistant; *S*, sensitive; *I*, intermediate; *ND*, no data available

β-glucosidase, α-mannosidase, N-acetyl-β-glucosaminidase and α-fucosidase reactions. Details of biochemical test results are shown in Table 1.

The 16S rRNA sequence of strain EB172, consisting of 1,446 nucleotides (GeneBank accession no. EU847238) was obtained in this study. The resultant sequence was checked for similarity against deposited data in GenBank and the top 50 hits, including non-cultivated organisms were retrieved and merged into the alignment. Phylogenetic analyses, in which this sequence was compared with

the corresponding sequences of representatives of the family *Comamonadaceae,* indicated that the strain belonged to the genus *Comamonas* in subclass of *β-Proteobacteria* (Fig. 2). The 16S rRNA sequence similarities between strain EB172 and its nearest neighbors, *C. koreensis* (93.4%), *C. composti* (92.9%), *C. kestersii* (91.1%), *C. testosteronii* (90.2%), and the type strains of other *Comamonas* species were low enough to categorize strain EB172 as species from the previously described *Comamonas* species. Only *C. terrigena* (96.8%) showed to be genetically rather close and related to strain EB172. However, none of the validly described species showed more than 97% 16S rDNA similarity (Stackebrandt and Goebel 1994). Bootstrap resamplings (100%) showed that the strain possessed a statistically significant association with *C. kerstersii*. Therefore, on the basis of this finding, the strain EB172 was classified as a new strain of the genus *Comamonas*.

The DNA–DNA hybridization was performed to investigate the DNA relatedness between strain EB172 and the reference strain *C. terrigena* (DSM 7099^T). The DNA–DNA relatedness between those strains was high (90%) and was above the cut-off point recommended for assignment of the strains to the genomic species (Wayne et al. 1987). From the results obtained, it was clear that that strain EB172 belonged to *C. terrigena* species. However, other properties like biochemical and phenotypic features should also be taken into consideration in describing new species. Studies previously conducted 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 (Wauters et al. 2003). These three bacteria were previously named as *C. terrigena* and later amended to new name after those analyses were carried out. The other tests conducted confirmed that the strain EB172 was distinct from the other existing *Comamonas* species. *Comamonas* sp. EB172 contained ubiquinone Q-8 (98%) as the major component of the quinone system while menaquinone and rhodoquinone were 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 strain EB172 were 16: 0, 18: 1/18: 1w 7c and summed feature 3. The fatty acid pattern of the strain EB172 was shown in Table 2 in comparison with other representative of *Comamonas* species. The fatty acid profile of strain EB172 was in good agreement with data obtained for other members of the genus *Comamonas* (Chang et al. 2002; Wauters et al. 2003). For G+C content calculations, the DNA sample was prepared in duplicate and degraded enzymically into nucleosides (Mesbah et al. 1989). The obtained nucleoside mixture was then separated with a HPLC system. The G+C content of strain EB172 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 showed

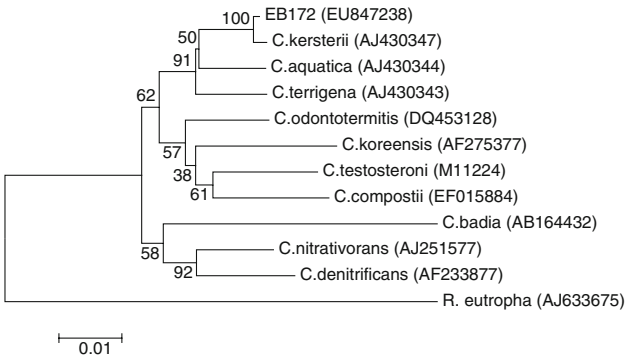

Fig. 2 Rooted neighbor joining tree based on 1,446 nucleotide positions of 16S rRNA gene sequences showing relationships between strain EB172 and closely related taxa (accession numbers in parentheses). *R. eutropha* was used as an out group. *Percentages at the nodes* indicate levels of bootstrap support based on neighbor-joining analyses of 1000 resampling datasets. Bar 0.01 substitutions per nucleotide position

Table 2 Comparison of the fatty acid compositions of strain EB172 and other *Comamonas* species

Fatty acid	1	2	3	4	5	6	7	8
10: 03-OH	4.3	5.6	3.5	4.8	5.3	4.2	5	4.5
12: 0	3	3.2	2.3	2.4	2.8	3	3	2.6
14: 0	4.5	1.4	1	1	3.3	3.2	3.9	2.9
15: 0	0.5	–	9.4	1	3.7	–	–	–
15: 02-OH	–	–	0.6	–	–	–	–	–
16: 0	31.9	33.3	29.9	30.4	27.5	17.8	25.2	23.4
16: 02-OH	–	–	2.2	2	–	–	–	–
16: 12-OH	–	–	–	0.6	–	–	–	–
17: 0	–	1	2.6	0.8	1.5	–	–	0.6
17: 0 cyclo	4.7	1.4	12.3	3.8	2.4	–	–	0.7
17: 1	–	–	0.7	–	–	–	–	–
18: 1/18: 1w 7c	14.8	12.9	9.6	17.9	14.9	22.4	19	36.1
19: 0 cyclo	–	–	–	0.9	–	–	–	–
20: 0 iso	–	–	–	1.1	–	–	–	–
Summed feature 3	32.5	40.8	26.1	33.1	38.4	48.6	42.4	28.2

Taxa: (1) strain EB172; (2) *C. composti*; (3) *C. koreensis* DSM 18232^T; (4) *C. testosteroni* DSM 50244^T; (5) *C. terrigena* LMG 1253^T; (6) *C. denitrificans* DSM 17887^T; (7) *C. aquatica* LMG 2370^T; (8) *C. kestersii* LMG 3475^T. All the reference data was obtained from Young et al. (2008). Values are percentages of total fatty acids; –, fatty acids representing less 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

another feature that distinguished this microorganism from other existing *Comamonas* species.

Growth and polyhydroxyalkanoates production by *Comamonas* sp. EB172

Based on the biochemical test results obtained, *Comamonas* sp. EB172 utilized most of the organic acids such as acetic, propionic, butyric, valeric, $_{D,L}$-lactic acid, and hydroxybutyric acids. While the tests showed negative results for all sugars. Acetic, propionic and *n*-butyric acids were used in the screening test for suitable substrates in batch experiments and the concentration of acids was in the range of 10–80 mM as shown in Fig. 3. The results obtained showed that *Comamonas* sp. EB172 was acid–tolerant bacterium which could stand higher concentration of *n*-butyric, acetic and propionic acids. The strain achieved its optimal growth at 60 mM when grown on acetic (~3.6 g/l) and *n*-butyric (~5.3 g/l) acids, and at 20 mM (~1.5 g/l), when propionic was used. Higher concentrations of acids applied into the medium inhibited the bacterial growth. Effect of propionic acid concentration on *Comamonas* sp. EB172 was obvious in which the concentration greater than 20 mM dramatically inhibited the bacterial growth. Several researchers have shown the effect of propionic acid as substrates on bacterial growth and P(3HB-*co*-3HV) copolymer production (Kim et al. 1992; Lee et al. 1994). Thus it was necessary to make sure that the concentration of the acids in the cultivation medium was lower than the inhibitory level during PHA production process. To overcome this problems, pH-stat continuous feeding is a suitable methods used in fed-batch cultivation process (Kobayashi et al. 2000; Sugimoto et al. 1999).

Effect of C/N ratio on the cell growth and PHA accumulation

The effect of carbon to nitrogen (C/N) ratio on *Comamonas* sp. EB172 growth and accumulation of PHA was studied. The pre-grown cultures were transferred into mineral salts medium containing different C/N ratio ranging from 10 to 50. *n*-butyric was used as the carbon sources while ammonium sulfate was used as the nitrogen sources. Specific growth rate and PHA accumulation in *Comamonas* sp. EB172 was depicted in Fig. 4. Various C/N ratios showed significant effect on both growth and PHA accumulations. Based on the results obtained, the C/N ratio of 30 was the best ratio for *Comamonas* sp. EB172 in which specific growth rate of (0.1 h^{-1}) and PHA accumulation of 46 (wt%) were recorded. However, the C/N ratio could be variables based on the types of carbon source, nitrogen source as well as bacterial strain used (Grothe et al. 1999; Rahayu et al. 2008). Therefore, the bacterial growth and polyester accumulations are influenced by the selection of suitable carbon and nitrogen sources.

Fed-batch cultivation using pH-stat continuous feeding of mixed organic acids derived from POME

Fed-batch cultivation of *Comamonas* sp. EB 172 using mixed organic acids derived from anaerobically treated POME was performed in 2 l fermenter. Pre-grown inoculums (1 g/l) were transferred into 900 ml of production medium containing 10 g/l of sodium acetate. Cell dry weight, PHA content and organic acids profiles are presented in Fig. 5a and b. pH was controlled at 7.0 during fermentation as pH increased upon acids consumption (data

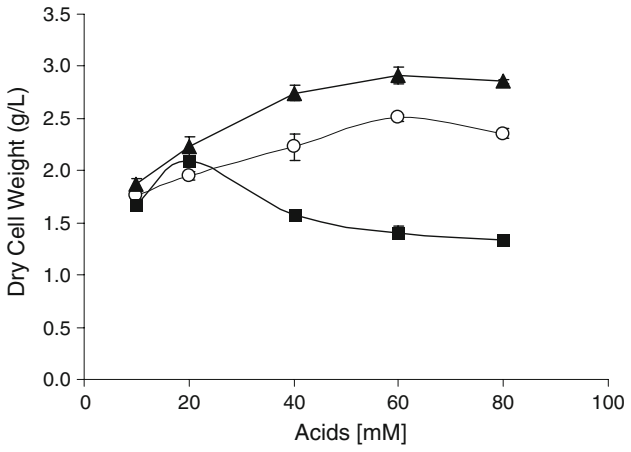

Fig. 3 The effect of organic acids concentration on growth of *Comamonas* sp. EB172 in the shake flask cultivation. *Close triangle* *n*-butyric, *close circle* propionic, *open circle* acetic acids

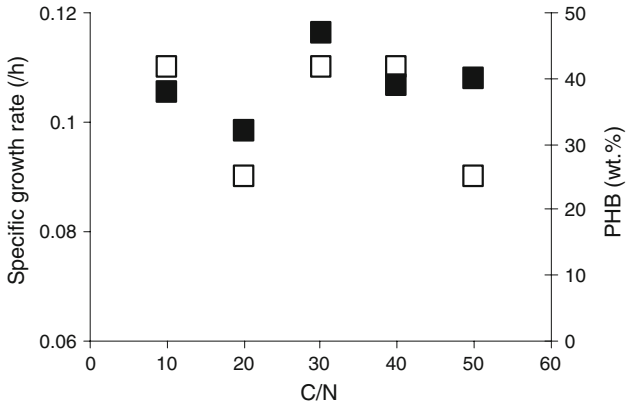

Fig. 4 The effect of specific growth rate and PHB accumulation by *Comamonas* sp. EB172 when supplied with 5 g/l of *n*-butyric acids. The cells were cultivated at 30°C for 30 h. *Open square* specific growth rate, *close square* PHA (wt%)

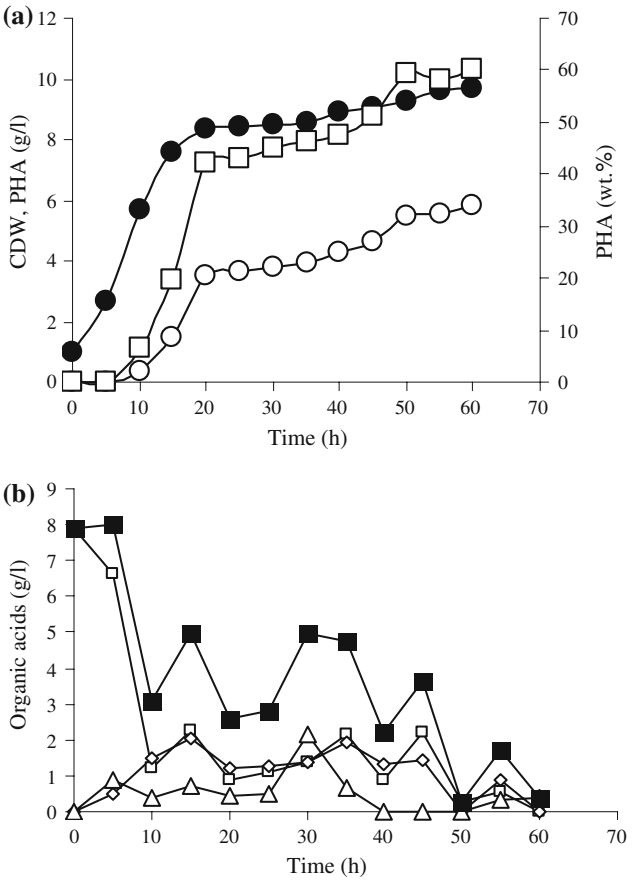

Fig. 5 **a** Time course of cell dry weight, PHA (g/l) and PHA content (wt%) of *Comamonas* sp. EB172 over time. P(3HB-*co*-3HV) copolymers containing of P(3HV) monomer unit (21 mol%) was obtained in the end of cultivation period (60 h). *Close circle* CDW (g/l), *open square* PHA (wt%), *open circle* PHA (g/l). **b** Organic acids profiles during fed-batch cultivation process of *Comamonas* sp. EB172 using pH-stat continuous feeding mode. *Close square* total acids, *open square* acetic, *open diamond* propionic, *open triangle* *n*-butyric acids

not shown). Development of cell biomass and PHA accumulation during this process was monitored and reached 8.2 g/l with 45 (wt%) of PHA after 20 h of incubation. However, the development of cell biomass was observed dramatically slow towards the end of 60 h of incubation period. This phenomenon could be explained by rapid cell biomass development during first 20 h which was due to the favorable condition expressed as carbon to nitrogen sources in the medium suitable for bacterial growth and cell division. Once, the nitrogen source was limited or depleted in the medium, unfavorable growth was imposed on the bacteria in which the entry of acetyl-coA into the Krebs cycle was restricted, and surplus of acetyl-coA was channeled to PHA biosynthesis (Doi 1990). During this stage, active PHA granules formation took place with minimal increment of cell biomass. Other factors also might be due to large volume of organic acids fed into bioreactor has diluted the broth medium. Furthermore, mixed organic acids from anaerobically treated POME used were suitable substrates for PHA production by *Comamonas* sp. EB172. Thus more concentrated feedstock solution could be applied during fed-batch cultivation to avoid the dilution of the broth medium. At the end of the fermentation period (60 h), CDW and PHA accumulation were recorded at 9.8 g/l and 59 (wt%), respectively. The incorporation of P(3HV) monomer unit (21 mol%) in the P(3HB-*co*-3HV) copolymers was obtained in this study due to the presence of propionic acid in the mixture of organic acids from anaerobically treated POME. Several reports have shown successful fed-batch cultivation using pH-stat feeding methods (Sugimoto et al. 1999; Kobayashi et al. 2000; Hassan et al. 2002). The idea was to supply enough carbon and nitrogen sources at suitable concentration to achieve maximum cell biomass production, proceed by PHA accumulation in nutrient limited environment. The development of high cell density cultivation of *Comamonas* sp. EB172 for PHA production using mixed organic acids derived from anaerobically treated POME is ongoing to meet the market potentials and reduction of PHA production cost.

Conclusion

Comamonas sp. EB172 isolated from open digester tank treating POME was characterized by polyphasic taxonomic approaches. Biochemical test, microscopic observation, cellular fatty acids analysis and 16S rRNA gene sequence analyses revealed that this strain was placed in the cluster of genus *Comamonas* and differed from the existing *Comamonas* species. Furthermore, the strain was capable of accumulating PHA up to 59% of CDW in fed-batch cultivation process with pH-stat continuous feeding of mixed organic acids derived from anaerobically treated POME. Further studies are required to improve the cell biomass formation as well as P(3HB-*co*-3HV) copolymer formation with higher incorporation of P(3HV) monomer unit using organic acids derived from POME as cheaper and renewable carbon sources.

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