Clean Techn Environ Policy (2017) 19:935–947
DOI 10.1007/s10098-016-1286-9

ORIGINAL PAPER

Polyhydroxyalkanoates production from fermented paperboard mill wastewater using acetate-enriched bacteria

A. Farghaly[1] · A. M. Enitan[2] · S. Kumari[2] · F. Bux[2] · A. Tawfik[1]

Received: 24 May 2016 / Accepted: 19 September 2016 / Published online: 6 October 2016

Abstract The aim of the study was to investigate the use of dark fermented paperboard mill wastewater (PMW) containing volatile fatty acids for polyhydroxyalkanoates (PHA) production. Six sequencing batch reactors (SBRs) were initially fed with synthetic feed containing acetate and operated at different organic loading rates (OLRs) of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 gCOD l^{-1} day^{-1} for PHA-storing bacteria enrichment. The maximum PHA content and yield ($Y_{PHA/S}$) were 67.44 ± 5.88 % and 0.45 ± 0.39 C-mol C-mol^{-1} at OLR of 1.5 gCOD l^{-1} day^{-1}, respectively. The synthetic feed was replaced by dark fermented PMW effluent rich in acetate, butyrate, propionate, and lactate at the accumulation stage resulting in a PHA content and yield of 58.57 ± 4.02 % and 0.46 ± 0.09 C-mol C-mol^{-1}, respectively. The maximum specific PHA production rate (q^{max}_{PHA}) amounted to 0.29 ± 0.1 C-mol C-mol^{-1} X^{-1} h^{-1}. Illumina MiSeq sequencing of bacterial 16S rRNA gene showed that *Proteobacteria* and *Bacteroidetes* increased from 37.4 to 77.6 % and from 2.49 to 17.66 % at enrichment and accumulation stages, respectively. *Actinobacteria* (15.44 %), *Chloroflexi* (8.15 %), *Planctomycetes* (7.46 %), and *Acidobacteria* (6.0 %) were detected at the enrichment SBRs.

✉ A. Farghaly
Ahmed.abdelwahhab@ejust.edu.eg

A. Tawfik
Ahmed.Tawfik@ejust.edu.eg

[1] Environmental Engineering Department, Egypt - Japan University of Science and Technology (E-JUST), New Borg El Arab City, Alexandria 21934, Egypt

[2] Institute for Water and Wastewater Technology, Durban University of Technology, Durban 4000, South Africa

Graphical Abstract

Keywords Paperboard mill wastewater · Polyhydroxyalkanoates · Organic loading rate · Acetate-enriched bacteria · Illumina MiSeq sequencing

Introduction

The paperboard industry is considered not only from the largest polluting sources in developing countries, but also produces a huge amount of severe wastewater. The composition of paperboard mill wastewater (PMW) is largely fluctuated and characterized by high chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), and total suspended solids (TSS), which cannot be discharged into the sewerage network and/or water streams (Farghaly et al. 2015). PMW could be a promising substrate for energy production in the form of hydrogen and/or methane via dark fermentation processes (Foglia et al. 2011). The produced biofuels represent a clean energy source and a better alternative to the depleted fossil fuels (Al-Shorgani et al. 2014). Nevertheless, considerable concentrations of

organic matter, soluble metabolic products, and nitrogenous compounds remain in the dark fermented effluents causing serious problems for the environment. Fortunately, the volatile fatty acids (VFAs) contained in these effluents could be easily utilized for value-added products such as polyhydroxyalkanoates (PHA) (Shahzad et al. 2013). Furthermore, PHA production from such wastewaters is accompanied by considerable reductions in organics and nitrogenous compounds (Bengtsson et al. 2008).

The PHA content and yield depends mainly on the imposed organic loading rate (OLR) and the type of substrate (Reddy and Mohan 2012a, 2012b; Khandpur et al., 2012). Carvalho et al. (2014) found that the maximum specific PHA production rate decreased from 0.11 to 0.05 C-mol C-mol^{-1} X^{-1} h^{-1} by increasing the OLR from 90 to 120 C-mmol l^{-1} day^{-1}, respectively. The excessive available substrate deteriorated the PHA production resulting in the death of the storing bacterial cultures (Khandpur et al. 2012). On the other hand, using a synthetic feed for PHA-storing bacteria enrichment was found to provide a high PHA yield as compared to real dark fermented effluents. For instance, Johnson et al. (2009) achieved 89 % of polyhydroxybutyrate (PHB) using acetate at a retention time of 7.6 h. Using a mixture of acetate and lactate (1:1) for enriching the storing bacteria was followed by 84 % of PHB accumulation after 8 h. In addition, Chua et al. (2003) found that the supplementation of municipal wastewater with acetate increased the PHA content by 1.5-fold. However, only 48 % of PHA was produced using dark fermented PMW effluent (Bengtsson et al. (2008). This is related to the presence of non-VFAs in the dark fermented effluents which affect negatively the PHA-storing bacteria enrichment performance. In this case, the cell growth is prioritized rather than PHA accumulation and the growth of non-PHA-storing bacteria might take place (Bengtsson et al. 2008; Tamis et al. 2014).

Therefore, acetate has been selected in this study to achieve a high PHA production from the dark fermented PMW effluent, where acetate is considered the most assimilated carbon source for enriching the PHA-storing bacteria as reported earlier by Chua et al. (2003). The specific objectives of the present study were to 1. Investigate the efficiency of the dark fermentation process by using an anaerobic baffled reactor for VFAs production from PMW at different OLRs. 2. Assess the capability of sequencing batch reactors (SBRs) fed with a synthetic feed containing acetate for PHA-storing bacteria enrichment. 3. Study the effect of replacing the synthetic feed with dark fermented PMW effluent on PHA accumulation, and 4. Identify the microbial community at the enrichment and accumulation stages by using the next generation sequencing (NGS) analysis (Illumina MiSeq).

Materials and methods

Paperboard mill wastewater (PMW)

The end-of-pipe effluent of the El-Dar Bidaa paperboard manufacturing factory was daily collected and immediately analyzed and fed to the anaerobic baffled reactor. The factory is situated in new Borg Al-Arab city and generates approximately 700 m^3 day^{-1} of PMW, which is discharged into sewerage network without treatment. Recycled papers and paperboard wastes are the main raw materials for manufacturing processes. The factory produces approximately 60 ton day^{-1} of paperboard. The physicochemical characteristics of the PMW in g l^{-1} are as follows: total COD (1.19 ± 0.16), soluble COD (0.92 ± 0.12), TSS (0.96 ± 0.2), volatile suspended solids (VSS: 0.46 ± 0.07), total VFAs (0.16 ± 0.11), ammonium nitrogen (NH_4-N: 0.004 ± 0.003), total nitrogen (TN: 0.023 ± 0.004), and pH (7.21 ± 0.04). The BOD_5/COD ratio was 0.57 which is suitable for the dark fermentation process.

Dark fermentation of PMW at different organic loading rates

An anaerobic baffled reactor (ABR) of 30 L was operated for VFAs production from PMW via dark fermentation as described earlier by Farghaly and Tawfik (2016) (Fig. 1). The OLR was maintained at 0.75, 1.1, 1.5, and 2.2 gCOD l^{-1} day^{-1} by controlling the pump flow rate. The reactor was continuously fed with PMW and operated at an ambient temperature of (22–30 °C). The fermented PMW effluents were collected and used as influents for the PHA accumulation stage. The physicochemical characteristics of the fermented PMW effluents at different OLRs are listed in Table 1.

Sequencing batch reactors (SBRs) for the production of polyhydroxyalkanoates

The use of synthetic feed containing acetate for PHA-storing bacteria enrichment

The enrichment of PHA-storing bacteria was carried out by using six identical sequencing batch reactors (SBRs) fabricated from Perspex glass with a working volume of 4 l (Fig. 1). The SBRs were equally inoculated with 2 l of aerobic sludge harvested from a full-scale municipal wastewater treatment plant (Alexandria, Egypt). The reactors were fed with synthetic feed containing acetate and operated at different OLRs of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 gCOD l^{-1} day^{-1}. NH_4Cl and KH_2PO_4 and were supplemented to the feed resulting in C:N:P ratio of 100:8:1 (Carvalho et al. 2014). 1 M

HCl and NaOH were occasionally supplied to the SBRs in order to maintain the pH value at a level of 7.0 ± 0.2. The SBRs were operated under the aerobiosis process (feast and famine) at a hydraulic retention time of 24 h, two cycles per day. Each cycle consisted of influent feeding (5 min), aerobiosis (11 h), settling period (40 min), and supernatant decanting (15 min). The SBRs were aerated by a fine bubble diffuser at a flow rate of 0.9 ml min^{-1}. The sludge residence time was kept constant at 10 days by intentionally withdrawing the excess sludge (Albuquerque et al. 2010a, 2010b). The steady state was achieved after about 1 month (62 cycles) of operation when the TSS at the end of each cycle and the length of the feast phase became stable.

PHA accumulation stage using acetate-enriched bacteria supplemented with dark fermented PMW effluents

The steady-state enriched PHA-storing bacteria were fed with the fermented PMW effluents at the end of the famine phase as described by Albuquerque et al. (2010a, b). Prior to starting the experiments, the suspended solids were removed from the fermented PMW effluents by centrifugation (6000×*g*, 10 min). The SBRs were aerated through a fine bubble diffuser with a constant airflow rate of 0.9 ml min^{-1} for 12 h, and the biomass was periodically harvested and concentrated by using centrifuge (6000×*g*, 10 min). The pH value of the feed was adjusted at 7.0 ± 0.2 using 1 M HCl or 1 M NaOH, and the temperature was maintained at 30 °C.

Calculations

$$\text{PHA content } \% = \frac{\text{gPHA}}{\text{gVSS}} \times 100 \quad (1)$$

$$\text{Active biomass } (X) = \text{gVSS - gPHA} \quad (2)$$

$$Y_{\text{PHA/S}} = \frac{\text{PHA}}{\text{COD}_{\text{consumed}}} \frac{\text{C - mol}}{\text{C - mol}} \quad (3)$$

$$Y_{X/S} = \frac{X}{\text{COD}_{\text{consumed}}} \frac{\text{C - mol}}{\text{C - mol}} \quad (4)$$

where VSS is the volatile suspended solids, $Y_{\text{PHA/S}}$ is the PHA yield, and $Y_{X/S}$ is the active biomass yield. Maximum specific substrate uptake rate ($q_s^{\max}$ in C-mol C-mol^{-1} - X^{-1} h^{-1}) and maximum specific PHA production rate ($q_{\text{PHA}}^{\max}$ in C-mol C-mol^{-1} X^{-1} h^{-1}) were calculated by linear regression of the concentrations, divided by the average active biomass concentration in the intervals. The specific active biomass growth rate (μ) was determined from the linear regression of the trend in ln*X* versus time over the batch experiments. The active biomass concentration was converted from g l^{-1} into carbon moles per liter (C-mol l^{-1}), assuming a composition of $CH_{1.8}O_{0.5}N_{0.2}$ and a molecular weight including ash of 25.1 g C-mol^{-1} (Katja et al. 2009). PHA concentration (in C-mol l^{-1}) corresponds to the sum of PHB and PHV monomer concentrations (in C-mol l^{-1}) (Bengtsson et al. 2008; Tamis et al. 2014). The results of each experiment were assumed to be independent with a different variance. The single-factor analysis of variance (ANOVA) using Microsoft Excel 2010 was performed, and the statistical significance was considered at a p value <0.05.

Analytical methods

The VFAs, acetate (HAc), butyrate (HBu), propionate (HPr), and lactate (HLa) were analyzed using a high-performance liquid chromatography (HPLC) (LC-10AD,

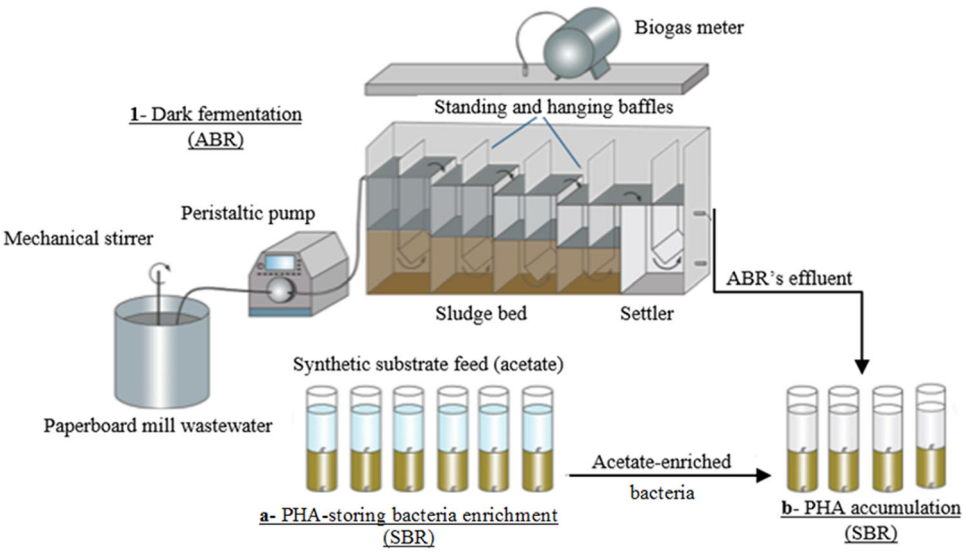


Fig. 1 A schematic diagram of PHA production system using acetate-enriched bacteria and dark fermented PMW effluents at different OLRs

Table 1 Average physicochemical characteristics of the fermented effluents resulted at different OLRs

Parameters (mg l^{-1})	OLRs (gCOD l^{-1} day^{-1}) (average ± STD)			
	0.75	1.1	1.5	2.2
Total COD	746 ± 25.1	589 ± 29.3	477 ± 12.8	247 ± 4.7
Soluble COD	482 ± 42.4	318 ± 31	253 ± 18.1	112 ± 9.5
Carbohydrates	142.7 ± 10.2	121.5 ± 10.2	83.2 ± 10.2	62.4 ± 10.2
Total VFAs	225 ± 14.58	241 ± 19.6	259 ± 17.11	340 ± 21.1
NH_4-N	5.8 ± 0.41	7.7 ± 0.39	13.6 ± 0.57	16.1 ± 1.17
TN	31.3 ± 2.36	35.7 ± 0.76	39.6 ± 0.95	43.6 ± 1.28

Shimadzu, Japan). It is provided with an ultraviolet detector using a Shim-pack HPLC column (4.6 × 250 mm, VP-ODS, vertical). The temperature of the column oven was 40 °C. Four mM H_2 SO_4 was used as a mobile phase (flow rate 0.5 ml min^{-1}) for 22 min followed by 0.4 ml min^{-1} for 8 min. Sludge analysis, total COD, soluble COD, BOD_5, MLSS, MLVSS, NH_4-N, TN, and carbohydrates were determined according to APHA (2005). Dissolved oxygen (DO), temperature, and pH were measured by using Thermo Scientific Orion StarTM A111 meters.

Extraction and analysis of PHA

Samples harvested for PHA analysis were added to 15-ml falcon tubes with five drops of formaldehyde to stop the biological activity (Katja et al. 2009). Extraction and estimation of PHA were performed based on the procedure reported by Reddy and Mohan (2012a). The collected biomass was separated from the substrate by centrifugation (6000×*g* for 30 min), and the resulting pellets were continuously washed with acetone and ethanol individually for the removal of unwanted materials. The pellets were subsequently suspended in an equal volume of sodium hypochlorite (4 %) and incubated at a room temperature for 3 h. The resulting mixture was centrifuged (6000×*g* for 30 min), and the supernatant was discarded. After washing simultaneously with acetone and ethanol, the pellets with lysed cells were dissolved in hot chloroform and passed through a glass fiber filter (0.45 µm pore size) to separate the polymer from the cells debris. The chloroform filtrate was used to estimate the PHA colorimetrically (Double-beam V-630 UV–Vis Spectrophotometer, Japan). PHA polymer extracted in chloroform was subjected to evaporation followed by the addition of 10 ml sulfuric acid (36 N) and heated at a temperature of 100 °C for 10 min. The addition of sulfuric acid converts the polymer to crotonic acid. The cooled solution was measured at an absorbance of 235 and 285 nm for PHB and PHV concentration, respectively. The sulfuric acid was used as a blank sample. The standard curve was prepared using pure poly-3 (hydroxybutyrate-co-hydroxyvalerate) (copolymer; natural origin, Aldrich).

Microbial community analysis

Sample collection and extraction of genomic DNA

Samples at the best storage performance during the aerobiosis and accumulation stages were collected from the reactors for microbial analysis. Total genomic DNA was extracted using fast DNA Spin Kit (MP Biomedicals, LLC, Solon, OH) following the manufacturer's instructions. Two milliliters of the samples was centrifuged at 9600×*g* for 5 min, and the resulted pellets were washed twice using 1 × PBS. The latter step was repeated twice to remove all the debris from the samples, after which the extraction was performed. The quality and quantity of the extracted DNA were assessed using a Nanodrop Spectrophotometer (ND-1000), Qubit fluorimeter analysis and confirmed by agarose (1 %) gel electrophoresis. The samples were subsequently stored at −20 °C for further analysis.

Illumina MiSeq sequencing and bioinformatics analysis

The extracted DNA was analyzed using an Illumina MiSeq sequencing platform, using the universal bacterial fusion primer sets derived from the V3–V4 region of the 16S rRNA with adapter primers attached to its 3′-end (Klindworth et al. 2013). The products of PCR were sequenced using the Illumina (MiSeq) next generation sequencing platform. The obtained sequence was analyzed using CLC Main Bench software. The raw sequence dataset was cleaned by removing all sequences containing ambiguous nucleotides and low-quality reads using a based pipeline for NGS data. The generated metagenomics dataset, raw paired-end reads were trimmed by their quality with a minimum quality score of 30 ($Q > 30$) and a minimum read length of 50 bp (Meyer et al. 2008) with the remaining clean reads used for further analysis. The obtained itags were aligned and subjected to BLASTN against the known 16–18S rRNA gene tag database. The maximum *E*-value

cutoff of 0.005 based on the entire available source databases was used for the analysis through genomic CLC software. The dissimilarity cutoff of 0.03 was used to cluster the cleaned reads into operational taxonomic units (OTUs) to yield more biologically meaningful OTUs (Chen et al. 2013; IRTEL et al. 2015; Nguyen et al. 2016). A group of taxa was manually searched in the National Center for Biotechnology Information taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/). Taxonomic classification into domain, phylum, order, class, families, and genus was performed with a set confidence threshold based on OTU diversity and reads (OTU abundance). The fastQ file has been uploaded to the NCBI Sequence Read Archive.

Results and discussion

Organic loading rate controls volatile fatty acids production from paperboard mill wastewater

Figure 2 shows the effect of organic loading rate (OLR) on volatile fatty acids (VFAs) production from paperboard mill wastewater (PMW). The tested OLRs were 0.75, 1.1, 1.5, and 2.2 gCOD l^{-1} day^{-1}. The VFAs concentration increased from 0.225 ± 0.015 to 0.340 ± 0.021 g l^{-1} by increasing the OLR from 0.75 to 2.2 gCOD l^{-1} day^{-1}, respectively. The yield of VFA (Y_{VFA}) transformed from 0.21 ± 0.09 to −0.21 ± 0.16 gVFA $gCOD^{-1}$ by decreasing the OLR from 2.2 to 0.75 gCOD l^{-1} day^{-1}, respectively. This was concomitant with the pH decrease from 7.21 ± 0.06 (influent) to 5.33 ± 0.08 (effluent) by increasing the OLR from 0.75 to 2.2 gCOD l^{-1} day^{-1}, respectively. Increasing the OLR increased the VFA production, and subsequently, the pH values dropped due to the excessive activity of hydrolytic and acidogenic bacteria (Basri et al. 2009; Kim and Kang 2015).

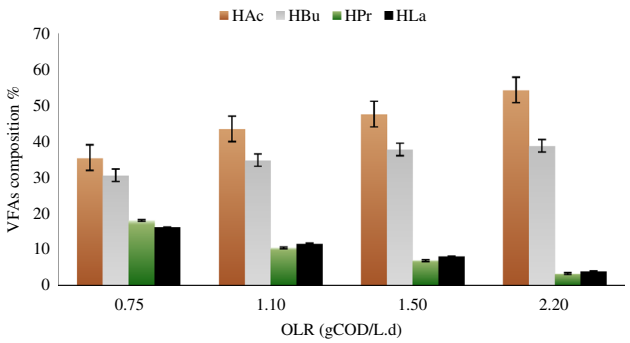


Fig. 2 Volatile fatty acids composition in the fermented PMW effluents at different OLRs. The *error bars* represent the standard deviations from triplicates

The acetate (HAc), butyrate (HBu), propionate (HPr), and lactate (HLa) concentrations varied at different OLRs as shown in Fig. 2. The HAc and HBu increased from 35.3 ± 3.0 to 54.1 ± 6.3 % and from 30.5 ± 2.1 to 38.7 ± 4.6 % by increasing the OLR from 0.75 to 2.2 gCOD l^{-1} day^{-1}, respectively. This was not the case for HPr and HLa as shown in Fig. 2. HPr and HLa in treated effluent amounted to 3.3 ± 1.9 and 3.8 ± 2.7 % at OLR of 2.2 gCOD l^{-1} day^{-1}, which increased up to 18.1 ± 1.8 and 16.1 ± 1.1 % at OLR of 0.75 gCOD l^{-1} day^{-1}, respectively. The metabolic pathways of HAc and HBu production appeared to be favored at the pH value of 5, while higher or neutral pH is believed to promote HPr production (Farghaly and Tawfik 2015). Thanwised et al. (2012) found that HAc increased from 1390.75 to 1726.85 mg l^{-1} by increasing the OLR by 1.5-fold.

The results in Fig. 3 show the hydrogen production rate (HPR), methane production rate (MPR), and soluble COD (SCOD) concentration in effluent at different OLRs. It was found that HPR decreased from 0.37 ± 0.02 to 0.14 ± 0.01 l day^{-1} by reducing the OLR from 2.2 to 0.75 gCOD l^{-1} day^{-1}, respectively. Low OLR exhibits poor metabolic activity for the acidogens which is responsible for H_2 production. Moreover, the bioconversion of SCOD decreased from 0.72 ± 0.51 to 0.58 ± 0.23 g l^{-1} by increasing the OLR from 0.75 to 2.2 gCOD l^{-1} day^{-1}, respectively (Fig. 3). The increasing trend of HPR with decreasing the SCOD indicates the main contribution of soluble organic carbon to H_2 production, which was confirmed earlier by Tawfik and El-Qelish (2014). However, the MPR were 0.10 ± 0.01 and 0.25 ± 0.13 l day^{-1} at OLR of 2.2 and 0.75 gCOD l^{-1} day^{-1}, respectively. This indicates that the OLR is a crucial design parameter for energy production from PMW.

Polyhydroxyalkanoates (PHA) production

PHA-storing bacteria enrichment by using synthetic feed containing acetate at different organic loading rates

Figure 4 shows the effect of OLR on the PHA production. The produced PHA was the homopolymer polyhydroxybutyrate (PHB) at all imposed OLRs. This can be due to the conversion of acetate into acetyl-CoA, which represents a direct precursor for PHB synthesis. The highest PHA content of 67.44 ± 5.88 % was registered at an OLR of 1.5 gCOD l^{-1} day^{-1}. PHA content was relatively low (26.61 ± 3.11 %) at an OLR of 0.5 gCOD l^{-1} day^{-1}, which was due to the insufficient substrate availability for the bacterial consortium; therefore, it reduces the ability of the PHA-storing bacteria to compete against other cultures in storing PHA. On the other hand, the low PHA content of 37.01 ± 3.14 % was recorded at OLR of 2.0

gCOD l^{-1} day^{-1} and the process stability was negatively affected at OLRs of 2.5 and 3.0 gCOD l^{-1} day^{-1} (Fig. 4). High OLR > 2.0 gCOD l^{-1} day^{-1} exhibited no significant successive feast and famine phases during the enrichment period. The microorganisms which are only able to grow on the high OLRs (either storing or non-storing cultures) could survive and dominate in the system. Jia et al. (2013) reported that high carbon source concentrations (OLRs) resulted in substrate inhibition during PHA production.

The highest PHA yield ($Y_{PHA/S}$) of 0.50 ± 0.39 C-mol C-mol^{-1} was registered at an OLR of 1.5 gCOD l^{-1} day^{-1}, which dropped to 0.39 ± 0.22 C-mol C-mol^{-1} by raising the OLR up to 2.0 gCOD l^{-1} day^{-1}. This was consistent with the PHA production and dissolved oxygen (DO) profiles in the SBRs (Fig. 4). The selective pressure to enrich the bacterial consortium to produce PHA reduced at OLRs exceeding 1.5 gCOD l^{-1} day^{-1}. Similar results were achieved by Karlsson (2010) who found that $Y_{PHA/S}$ significantly decreased from 0.42 to 0.19 C-mol C-mol^{-1} by doubling the OLR from 6 to 12 gCOD l^{-1} day^{-1}, respectively. However, the $Y_{X/S}$ showed a slight increase from 0.21 ± 0.08 to 0.31 ± 0.14 C-mol C-mol^{-1} by increasing the OLR from 0.5 to 3.0 gCOD l^{-1} day^{-1}, respectively. This implies that the bacterial consortium assimilates the most of carbon source to store PHA in combination with the nutrients uptake for biomass growth.

The maximum specific substrate uptake rate (q_S^{max}) increased in a polynomial relationship ($R^2 = 0.8620$) by increasing the OLR during the feast phase, which was comparable to Wang et al. (2010) findings. The highest q_s^{max} of 7.43 ± 5.51 C-mol C-mol^{-} X^{-1} h^{-1} was obtained at an OLR of 3.0 gCOD l^{-1} day^{-1}. This corresponds to the specific growth rate (μ) trends, which increased from 0.18 ± 0.08 to 0.29 ± 0.09 h^{-1} by raising the OLR from 1.5 to 3.0 gCOD l^{-1} day^{-1}, respectively. However, this was not accorded to the maximum specific PHA production rate (q_{PHA}^{max}) of 0.46 ± 0.18 Cmol $Cmol^{-1}$ X^{-1} h^{-1} that was achieved at an OLR of 1.5 gCOD l^{-1} day^{-1}. The $q_{PHA}{}^{max}$ significantly ($p < 0.005$) dropped by a value of

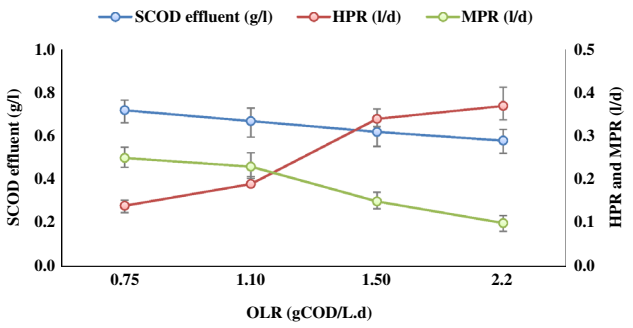


Fig. 3 Effect of OLR on SCOD bioconversion, hydrogen production rate (HPR), and methane production rate (MPR). The *error bars* represent the standard deviations from triplicates

77.48 % at an OLR of 3.0 gCOD l^{-1} day^{-1}. This indicated that the substrate was mainly consumed for cell growth not for PHA storage at an OLR exceeding 1.5 gCOD l^{-1} - day^{-1}. In addition, the results show that there was no substrate inhibitory effect, which explained the increasing of μ with increasing OLR (polynomial relationship, $R^2 = 0.86$). The biomass growth inhibition occurred due to either insufficient carbon source concentrations or the lack of nutrients (Muller et al. 1999). Therefore, it can be concluded that increasing the OLR up to 1.5 gCOD l^{-1} - day^{-1} changed the biomass response from storage to growth.

Feast/famine (F/F) ratio is a key parameter for PHA-storing performance and was found to have a profound effect by changing the imposed OLR. The F/F ratio increased from 4.4 ± 1.2 to 14 ± 2.2 % by increasing the OLR, respectively, from 0.5 to 1.5 gCOD l^{-1} day^{-1}, which coincided with the PHA production trends (Fig. 4). The highest PHA contents were registered at the end of the feast phase as shown in Fig. 4. Long famine phases (at OLR of 1.5 gCOD l^{-1} day^{-1}) resulted in physiological adaptation of the bacterial consortium and thus enhanced the PHA storage performance. Lower OLRs (low substrate concentrations) exhibited the culture to utilize the stored PHA for synthesis and respiration (Jia et al. 2013). Subsequently, it ceased to accumulate PHA and stepped into the famine phase as the carbon source was depleted (Reddy and Mohan 2012a). Dionisi et al. (2007) and Albuquerque et al. (2010a, 2010b) found that the F/F ratio should be lower than 22 % to improve the PHA-storing process performance (Fig. 5).

PHA accumulation stage using acetate-enriched culture fed with PMW rich in VFAs

The acetate-enriched culture at an OLR of 1.5 gCOD l^{-1} - day^{-1} was used for PHA accumulation experiments. The maximum PHA content and yield of 58.57 ± 4.02 % and 0.46 ± 0.09 C-mol C-mol^{-1} were achieved using the fermented PMW at OLR of 1.5 gCOD l^{-1} day^{-1}. The maximum specific PHA production rate q_{PHA}^{max} amounted to 0.29 ± 0.10 C-mol C-mol^{-1} X^{-1} h^{-1} (Table 2). The obtained PHA content in the present study was higher than the percentage reported (48 %) by Bengtsson et al. (2008) using the fermented PMW effluent. This resulted from their use of the fermented PMW effluent that contained acetate, butyrate, propionate, and valerate as well as non-VFAs as substrate at both enrichment and accumulation stages. This showed that acetate-enriched conditions were more favorable and increased the ability of the PHA-storing bacteria in the reactor, since acetate is the easiest assimilated carbon substrate for culture enrichment; thus, a better performance would be obtained.

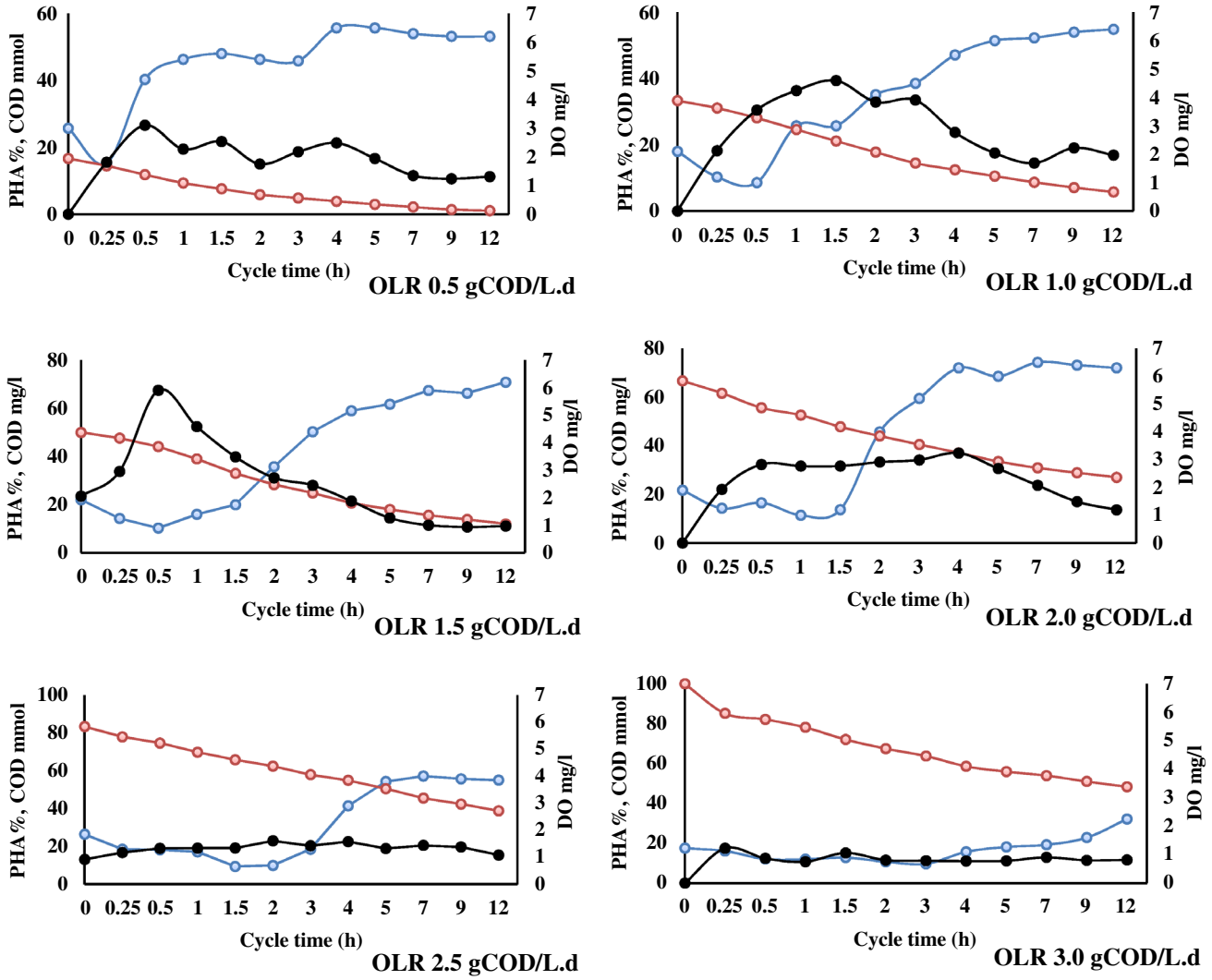


Fig. 4 Effect of OLR on the PHA-storing bacteria enrichment stage during 12-h cycle regarding: (*green line*) PHA %, (*red line*) COD (mmol), and (*blue line*) DO (mg/l)

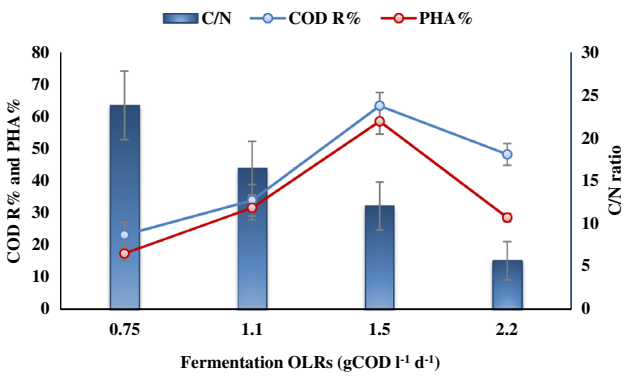


Fig. 5 Effect of C/N on PHA production and COD removal efficiency. The *error bars* represent the standard deviations from triplicates

Nevertheless, the PHA content dropped by a value of 8.87 %, due to shifting the feed substrate from acetate (synthetic) to fermented PMW effluent (real) during the enrichment and accumulation stages, respectively. The acetate-enriched culture stored the residual carbohydrates in the fermented PMW as glycogen rather than PHA production (Albuquerque et al. 2010a). Subsequently, the growth of a side population and the accumulation of non-PHA compounds might take place in the reactor.

The composition of the accumulated PHA of 92:8 and 84:16 % (PHB:PHV) % was obtained by using the fermented PMW effluent at OLR of 1.5 and 0.75 gCOD l^{-1} day^{-1}, respectively. This can be attributed to the concentration of the produced soluble metabolites which were 1.51-fold at OLR of 1.5 gCOD l^{-1} day^{-1} as compared to 0.75 gCOD l^{-1} day^{-1}. Furthermore, the low accumulated PHV content was related to the dominance of HAc and HBu with low HPr levels in the fermented PMW effluents. Taken into consideration the complexity of the metabolic pathway of HPr utilization by active biomass, which is the main precursor for PHV production (Carvalho

Table 2 PHA accumulation stage performance for PHA production and nitrogen removal

Fermentation OLR (gCOD l^{-1} day^{-1})	Enrichment OLR (gCOD l^{-1} day^{-1})	PHA accumulation		*R* %	
		$Y_{PHA/S}$ (C-mol C-mol$-^{1}$)	q^{max}_{PHA} (C-mol C-mol^{-1} X^{1} h^{-1})	NH_4-N	TN
0.75	1.5	0.28 ± 0.008	0.11 ± 0.007	50.66 ± 3.72	52.14 ± 6.09
1.1		0.39 ± 0.01	0.18 ± 0.11	44.29 ± 4.92	46.05 ± 4.86
1.5		0.46 ± 0.09	0.29 ± 0.1	42.98 ± 4.14	44.5 ± 3.33
2.2		0.38 ± 0.012	0.17 ± 0.08	30.14 ± 2.11	34.76 ± 2.83

et al. 2014). In addition, using acetate for storing bacteria enrichment might promote the growth of specific bacteria favors PHB production rather than PHV. These results are comparable to those obtained by Albuquerque et al. (2010a) using fermented molasses effluent containing HAc, HBu, and HPr, where (PHB:PHV) % amounted to 87:13 % at the end of accumulation stage. Indeed, when mixtures of HAc, HBu, and HPr are used for PHA production, acetyl-CoA and propionyl-CoA are formed as precursors for PHA production, resulting in a copolymer of P(HB-co-HV) (Katja et al. 2009).

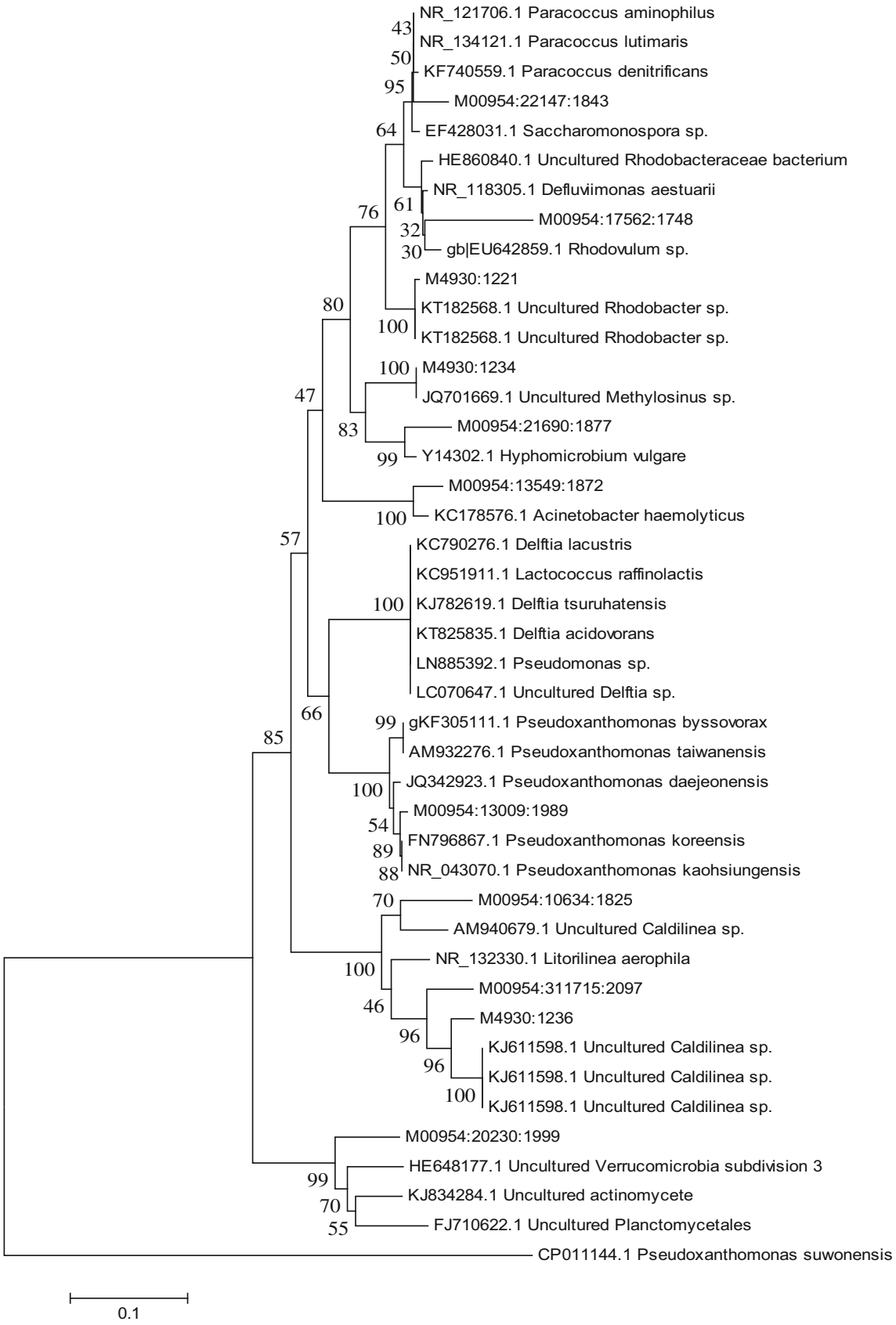
COD removal of 63.4 ± 4.2 % was achieved using the fermented PMW effluent at an OLR of 1.5 gCOD l^{-1} - day^{-1}. Nonetheless, this was not the case for nitrogen compounds removal (Table 2), where the NH_4-N and TN removal peaked at 50.66 ± 3.72 and 52.14 ± 6.09 %, respectively, using the fermented PMW at OLR of 0.75 gCOD l^{-1} day^{-1}. This was due to the decrease in the C/N ratio from 23.83 ± 4.02 to 5.67 ± 2.22 by increasing the OLR from 0.75 to 2.2 gCOD l^{-1} day^{-1}, respectively. As reported by Johnson et al. (2010), the high carbon uptake rates were found at an average C/N ratio of 9.6, whereas C/N ratio (≈19.5) was characterized by high nitrogen uptake rates.

Microbial community

Diverse microbial communities of storing and non-storing bacteria were detected in the present study at the general level. However, only the dominant sequences with the highest hits were selected for further analysis, due to the huge number of data generated at the genus level. The most dominant species detected at the two different stages (accumulation and aerobiosis) are represented in Fig. 6a, b, respectively. The most dominant phyla were found to belong to *Proteobacteria* (37.4 %) including (*Alpha-*, *Beta-*, *Delta-*, and *Gamma-Proteobacteria*), *Armatimonadetes* (21.78 %), *Actinobacteria* (15.44 %), *Chloroflexi* (8.15 %), *Planctomycetes* (7.46 %), *Acidobacteria* (6.0 %), and *Bacteroidetes* (2.49 %) at an OLR of 1.5 gCOD l^{-1} day^{-1}. However, *Proteobacteria* (77.6 %), *Bacteroidetes* (17.66 %), *Nitrospira* (1.75 %), and *Armatimonadetes* (1.30 %) were detected and dominated at the accumulation stage. Minor phyla belonged to *Actinobacteria, Acidobacteria, Verrucomicrobia,* OD1, *Planctomycetes, Chloroflexi,* and an unknown culture was identified and detected in the SBRs (Fig. 7). Increasing the relative abundance of *Proteobacteria* at the accumulation stage might be due to the use of fermented PMW effluent containing VFAs. Wang and Liu (2013) found the dominance of *Proteobacteria* (*Beta-*, *Gamma-*), *Arcobacter* spp, *Bacteroidetes* sp., and *Bacillus* sp. during PHA production. Other researchers have also demonstrated the association of these species and other diverse PHA-storing bacterial communities such as *Acidobacteria, Actinobacteria, and Alpha*-Proteobacteria in the reactors (Reddy and Mohan 2012b; Dai et al. 2015). Moreover, different species of genus *Paracoccus,* order *Rhodobacterales* that are known as PHA-storing bacteria were detected in the sequence reads generated from a sample taken during the aerobiosis stage. These organisms are known to utilize a broad range of substrates for PHA production.

Different genera were detected during the accumulation stage as shown in Fig. 6a. Particularly, fifteen species of *Pseudomonas* (*P. putida, P. taiwanensis,* etc.), *Thauera aromatica, Azoarcus denitrificans, Methylocaldium szegediense,* and others were detected and identified, and this was mainly due to phosphorus and nitrogen limitation. *Pseudomonas putida* KT2442 and *P. oleovorans* ATCC 29347 have been found to accumulate PHA (Lee et al. 2000; Lageveen et al. 1988). Other taxa were detected in this study such as *Plasticicumulans acidivorans* and *Plasticicumulans lactativorans* of class *Gamma-Proteobacteria* (Jiang et al. 2011 2014).

Fig. 6 **a** Taxonomic classification of core OTUs in the 16S rRNA ▶ gene sequences from bacterial communities at the accumulation stage. The evolutionary history was inferred using the neighbor-joining method, and the analyses were conducted in MEGA6 (Saitou and Nei 1987). **b** Phylogenetic tree based on nucleotide sequences of 16S rRNA gene generated during aerobiosis stage at the highest PHA production (OLR 1.5 gCOD $^{-1}$ day^{-1}). The evolutionary history was inferred using the neighbor-joining method, and the analyses were conducted in MEGA6 (Saitou and Nei 1987)


NR_121706.1 Paracoccus aminophilus
NR_134121.1 Paracoccus lutimaris
KF740559.1 Paracoccus denitrificans
M00954:22147:1843
EF428031.1 Saccharomonospora sp.
HE860840.1 Uncultured Rhodobacteraceae bacterium
NR_118305.1 Defluviimonas aestuarii
M00954:17562:1748
gb|EU642859.1 Rhodovulum sp.
M4930:1221
KT182568.1 Uncultured Rhodobacter sp.
KT182568.1 Uncultured Rhodobacter sp.
M4930:1234
JQ701669.1 Uncultured Methylosinus sp.
M00954:21690:1877
Y14302.1 Hyphomicrobium vulgare
M00954:13549:1872
KC178576.1 Acinetobacter haemolyticus
KC790276.1 Delftia lacustris
KC951911.1 Lactococcus raffinolactis
KJ782619.1 Delftia tsuruhatensis
KT825835.1 Delftia acidovorans
LN885392.1 Pseudomonas sp.
LC070647.1 Uncultured Delftia sp.
gKF305111.1 Pseudoxanthomonas byssovorax
AM932276.1 Pseudoxanthomonas taiwanensis
JQ342923.1 Pseudoxanthomonas daejeonensis
M00954:13009:1989
FN796867.1 Pseudoxanthomonas koreensis
NR_043070.1 Pseudoxanthomonas kaohsiungensis
M00954:10634:1825
AM940679.1 Uncultured Caldilinea sp.
NR_132330.1 Litorilinea aerophila
M00954:311715:2097
M4930:1236
KJ611598.1 Uncultured Caldilinea sp.
KJ611598.1 Uncultured Caldilinea sp.
KJ611598.1 Uncultured Caldilinea sp.
M00954:20230:1999
HE648177.1 Uncultured Verrucomicrobia subdivision 3
KJ834284.1 Uncultured actinomycete
FJ710622.1 Uncultured Planctomycetales
CP011144.1 Pseudoxanthomonas suwonensis
0.1

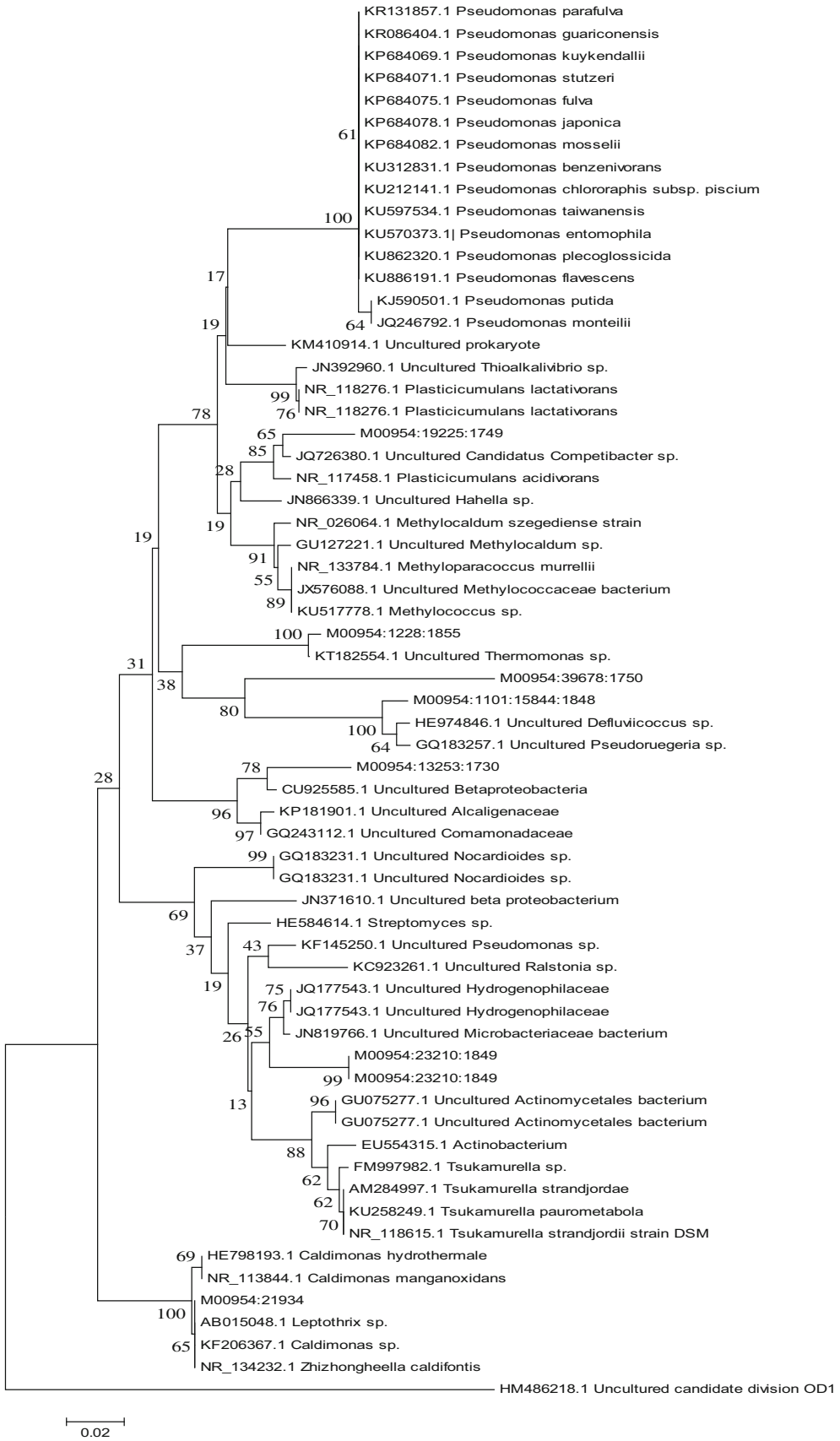


Fig. 6 continued

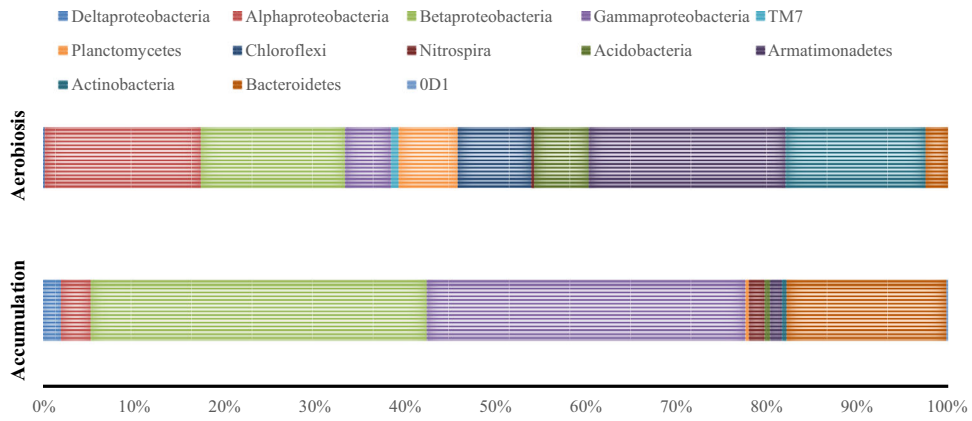


Fig. 7 Structure of bacterial community in PHA production system using Illumina MiSeq sequencing

The taxonomic distribution showed that there was a significant shift in the composition of bacterial communities between the aerobiosis and accumulation stages. *Burkholderiales*, family *Comamonadaceae* of the genus *Delftia, Ralstonia, Caldimonas, Zhizhongheela, Paracoccus,* and others were detected at the aerobiosis stage (Fig. 6b). Interestingly, *Deftia acidivorans* are known as a wild PHA-storing bacterium containing high fractions of 4-hydroxybutyrate (4-HB) and potential strains for the synthesis of PHA used in medical applications (Loo and Sudesh 2007). Likewise, *Ralstonia sp.,* especially *R. eutropha,* are known to capture and utilize CO_2 for the production of chemicals that are of commercial value, thus contributing to the organic carbon removal. Moreover, they store PHA in the form of short chains of copolymer (PHB-co-PHV) within the cytoplasm. As expected, most of the dominant bacteria belonged to HAc- and HBu-utilizing groups were identified at the accumulation stage. This could be explained by the presence of high concentrations of accumulated PHB as compared to PHV in this stage.

Although TM7 phylum and *Bacteroidetes* as protein-hydrolyzing organisms contributed to the organic carbon removal improvements (Ibarbalz et al. 2013), their ability in storing PHA has yet to be explored. Other dominant bacterial phyla were detected among the other communities including *Nitrospira* and *Actinobacteria*. Several studies have shown the dominance of these bacterial phyla in biological industrial wastewater treatment systems of nitrogen removal, but no report on the accumulation of PHA has been found in the literature (Ibarbalz et al. 2013).

Conclusions

The present study showed that an acetate-enriched culture plays a key role for PHA production from fermented PMW effluents; however, the PHA content and yield were OLR dependents. The highest PHA yield ($Y_{PHA/S}$) of 0.50 ± 0.39 C-mol C-mol^{-1} was registered at OLR of 1.5 gCOD l^{-1} day^{-1}, which significantly dropped to 0.39 ± 0.22 C-mol C-mol^{-1} by raising the OLR to 2.0 gCOD l^{-1} day^{-1}. Nevertheless, the PHA content in the SBRs fed with fermented PMW effluent dropped by a value of 8.87 %, due to shifting the feed substrate from acetate (synthetic) to fermented PMW effluent (real) during the enrichment and accumulation stages, respectively. The most dominant phyla belonged to *Proteobacteria* (37.4 %), *Armatimonadetes* (21.78 %), *Actinobacteria* (15.44 %), *Chloroflexi* (8.15 %), *Planctomycetes* (7.46 %), *Acidobacteria* (6.0 %), and *Bacteroidetes* (2.49 %) at an OLR of 1.5 gCOD l^{-1} day^{-1}. However, *Proteobacteria* (77.6 %), *Bacteroidetes* (17.66 %), *Nitrospira* (1.75 %), and *Armatimonadetes* (1.30 %) were detected and dominant at the accumulation stage.

Acknowledgments The first author is very grateful to the Ministry of Higher Education (MOHE), Egypt, and Egypt–Japan University of Science and Technology (E-JUST) for providing him with financial and technical support (Ph.D. scholarship) for this research.

References

Albuquerque MGE, Concas S, Bengtsson S, Reis MAM (2010a) Mixed culture polyhydroxyalkanoates production from sugar molasses: the use of a 2-stage CSTR system for culture selection. Bioresour Technol 101:7112–7122. doi:10.1016/j.biortech.2010.04.019

Albuquerque MGE, Torres CV, Reis MM (2010b) Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: effect of the influent substrate concentration on culture selection. Water Res 44:3419–3433. doi:10.1016/j.watres.2010.03.021

Al-Shorgani NKN, Tibin EM, Ali E et al (2014) Biohydrogen production from agroindustrial wastes via Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564). Clean Technol Environ Policy 16:11–21. doi:10.1007/s10098-013-0586-6

APHA (2005) Standard methods for the examination of water and wastewater, 25th edn. APHA, Washington, DC

Basri MF, Yacob S, Hassan M et al (2009) Improved biogas production from palm oil mill effluent by a scaled-down anaerobic treatment process. World J Microbiol Biotechnol 26:505–514. doi:10.1007/s11274-009-0197-x

Bengtsson S, Werker A, Christensson M, Welander T (2008) Production of polyhydroxyalkanoates by activated sludge

treating a paper mill wastewater. Bioresour Technol 99:509–516. doi:10.1016/j.biortech.2007.01.020

Carvalho G, Oehmen A, Albuquerque MGE, Reis MM (2014) The relationship between mixed microbial culture composition and PHA production performance from fermented molasses. N Biotechnol 31:257–263. doi:10.1016/j.nbt.2013.08.010

Chen W, Zhang CK, Cheng Y et al (2013) Methods for Clustering 16S rRNA Sequences into OTUs. PLoS ONE 8:e70837

Chua ASM, Takabatake H, Satoh H, Mino T (2003) Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent. Water Res 37:3602–3611. doi:10.1016/S0043-1354(03)00252-5

Dai J, Gliniewicz K, Settles ML et al (2015) Influence of organic loading rate and solid retention time on polyhydroxybutyrate production from hybrid poplar hydrolysates using mixed microbial cultures. Bioresour Technol 175:23–33. doi:10.1016/j.biortech.2014.10.049

Dionisi D, Majone M, Vallini G et al (2007) Effect of the length of the cycle on biodegradable polymer production and microbial community selection in a sequencing batch reactor. Biotechnol Prog 23:1064–1073. doi:10.1021/bp060370c

Farghaly A, Tawfik A (2015) Inoculation of paperboard sludge versus mixed culture bacteria for hydrogen production from paperboard mill wastewater. Environ Sci Pollut Res 23:3834–3846

Farghaly A, Tawfik A (2016) Simultaneous hydrogen and methane production through multi-phase anaerobic digestion of paperboard mill wastewater under different operating conditions. Appl Biochem Biotech. doi:10.1007/s12010-016-2204-7

Farghaly A, Tawfik A, Mona G (2015) Surfactant-supplemented mixed bacterial cultures to produce hydrogen from paperboard mill wastewater. Eng Life Sci 15:525–532. doi:10.1002/elsc.201400099

Foglia D, Wukovits W, Friedl A et al (2011) Effects of feedstocks on the process integration of biohydrogen production. Clean Technol Environ Policy 13:547–558. doi:10.1007/s10098-011-0351-7

Ibarbalz FM, Figuerola ELM, Erijman L (2013) Industrial activated sludge exhibit unique bacterial community composition at high taxonomic ranks. Water Res 47:3854–3864. doi:10.1016/j.watres.2013.04.010

Irtel J, Walser JC, Pichon S et al (2015) Estimating bacterial diversity for ecological studies: methods, metrics, and assumptions. PLoS ONE 10(4):e0125356. doi:10.1371/journal.pone.0125356

Jia Q, Wang H, Wang X (2013) Dynamic synthesis of polyhydroxyalkanoates by bacterial consortium from simulated excess sludge fermentation liquid. Bioresour Technol 140:328–336. doi:10.1016/j.biortech.2013.04.105

Jiang Y, Dimitry Y, Kleerebezem R et al (2011) *Plasticicumulans acidivorans* gen. nov., sp. nov., a polyhydroxyalkanoate-accumulating gammaproteobacterium from a sequencing-batch bioreactor. Int J Syst Evol Microbiol 61:2314–2319. doi:10.1099/ijs.0.021410-0

Jiang Y, Sorokin DY, Junicke H et al (2014) *Plasticicumulans lactativorans* sp. nov., a polyhydroxybutyrate-accumulating gammaproteobacterium from a sequencing-batch bioreactor fed with lactate. Int J Syst Evol Microbiol 64:33–38. doi:10.1099/ijs.0.051045-0

Johnson K, Jiang Y et al (2009) Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 10:670–676. doi:10.1021/bm8013796

Johnson K, Kleerebezem R, van Loosdrecht MCM (2010) Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res 44:2141–2152. doi:10.1016/j.watres.2009.12.031

Karlsson A (2010) Production of polyhydroxyalkanoates in an open mixed culture from fermented hydrolyzed sludge under high organic loading rates. Water Res 44:5196–5211

Khandpur P, Jabeen ET, Rohini KVL, Varaprasad Y (2012) Study on production, extraction and analysis of polyhydroxyalkanoate (PHA) from bacterial isolates. IOSR J Pharm Biol Sci 1:31–38

Kim J, Kang CM (2015) Increased anaerobic production of methane by co-digestion of sludge with microalgal biomass and food waste leachate. Bioresour Technol 189:409–412. doi:10.1016/j.biortech.2015.04.028

Klindworth A, Pruesse E, Schweer T et al (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41:1–11. doi:10.1093/nar/gks808

Lageveen R, Huisman GW, Preusting H, Ketelaar P, Eggink G, Witholt B (1988) Formation of polyester by Pseudomonas oleovorans: the effect of substrate on the formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl Env Microbiol 54:2924–2932

Lee S, Wong H, Choi J et al (2000) Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation of Pseudomonas putida under phosphorus limitation. Biotechnol Bioeng 68:466–470

Loo CY, Sudesh K (2007) Biosynthesis and native granule characteristics of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in Delftia acidovorans. Int J Biol Macromol 40:466–471. doi:10.1016/j.ijbiomac.2006.11.003

Meyer F, Paarmann D, D'Souza M et al (2008) The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinform 9:386. doi:10.1186/1471-2105-9-386

Muller S, Bley T, Babel W et al (1999) Adaptive responses of Ralstonia eutropha to feast and famine conditions analysed by flow cytometry. J Biotechnol 75:81–97

Nguyen NP, Warnow T, Pop M, White B (2016) A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. npj Biofilms Microbiomes 2:16004

Reddy MV, Mohan SV (2012a) Effect of substrate load and nutrients concentration on the polyhydroxyalkanoates (PHA) production using mixed consortia through wastewater treatment. Bioresour Technol 114:573–582. doi:10.1016/j.biortech.2012.02.127

Reddy MV, Mohan SV (2012b) Influence of aerobic and anoxic microenvironments on polyhydroxyalkanoates (PHA) production from food waste and acidogenic effluents using aerobic consortia. Bioresour Technol 103:313–321. doi:10.1016/j.biortech.2011.09.040

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Shahzad K, Kettl KH, Titz M et al (2013) Comparison of ecological footprint for biobased PHA production from animal residues utilizing different energy resources. Clean Technol Environ Policy 15:525–536. doi:10.1007/s10098-013-0608-4

Tamis J, Lužkov K, Jiang Y et al (2014) Enrichment of *Plasticicumulans acidivorans* at pilot-scale for PHA production on industrial wastewater. J Biotechnol 192:161–169. doi:10.1016/j.jbiotec.2014.10.022

Tawfik A, El-Qelish M (2014) Key factors affecting on bio-hydrogen production from co-digestion of organic fraction of municipal solid waste and kitchen wastewater. Bioresour Technol. doi:10.1016/j.biortech.2014.02.127

Thanwised P, Wirojanagud W, Reungsang A (2012) Effect of hydraulic retention time on hydrogen production and chemical oxygen demand removal from tapioca wastewater using anaerobic mixed cultures in anaerobic baffled reactor (ABR). Int J Hydrog Energy 37:15503–15510. doi:10.1016/j.ijhydene.2012.02.068

Wang Y, Liu S (2013) Polyhydroxyalkanoates (PHAs): biosynthesis, Production and Recovery. J Bioprocess Eng Biorefinery 2:61–72
Wang J, Yue ZB, Sheng GP, Yu HQ (2010) Kinetic analysis on the production of polyhydroxyalkanoates from volatile fatty acids by Cupriavidus necator with a consideration of substrate inhibition, cell growth, maintenance, and product formation. Biochem Eng J 49:422–428. doi:10.1016/j.bej.2010.02.005