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Performance of Short Chain Length Polyhydroxyalkanoate Production by *Comamonas* sp EB172 in Large Scale Bioreactors Based on Constant Impeller Tip Speed

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Authors' contributions

This work was carried out in collaboration between all authors. Author TM develop the protocol, carried out the study and drafted the manuscript. Authors MM and LNY collaborated in large scale fermentations and managed the analyses of the study. Authors SAA, PLY, HA, YS and MAH participated in interpreting the experimental results and critically revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In this study, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or PHBV, a short chain length polyhydroxyalkanoates (PHAs) production by a local isolate *Comamonas* sp. EB172 using 500 g/L mixed organic acids were investigated in 10 L and 150 L stirred tank bioreactors based on constant impeller tip speed (minimum 0.56 m/s and maximum 2.22 m/s).

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Results: The ratio of the three acids i.e., acetic, butyric, and propionic, were 2:1:1, mimicking clarified mixed acids obtained from anaerobically treated palm oil mill effluent. In pH controlled fed-batch cultivation, in 10 L bioreactor, the strain could accumulate 84% PHBV with biomass concentration of about 4.08 g/L under dual nutrient limitation strategy i.e., dissolved oxygen and nitrogen limited conditions. By maintaining similar tip speed in 150 L bioreactor, a final biomass concentration of 5.35 g/L with a PHBV content of 72% and volumetric productivity of 0.083 g/L/h were obtained. The calculated yield was 0.259 g PHBV/g mixed acids.
Conclusion: Both the yield and PHA content in 150 L bioreactor were comparable to that obtained in 10 L scale where PHBV content ranged from 70- 90% (w/w) of the cell with yield of 0.27- 0.4 g PHBV/g mixed acid, respectively. This is the first report of utilizing mixed organic acids for PHBV biosynthesis in large scale.

Keywords: Comamonas sp. EB172; mixed organic acids; PHBV; impeller tip speed.

1. INTRODUCTION

Because of their biodegradability and biocompatibility, polyhydroxyalkanoates (PHAs), have been extensively researched for use in medicine, drug-delivery, agriculture and horticulture, the fiber industry, and consumer products [1]. As a potentially attractive environmentally benign substitute for petroleum-derived polymers, PHAs have gone through many years of efforts towards commercialization, with limited successes. Among two groups of PHAs, short chain- length (SCL) PHAs, consisting of 3–5 carbon atoms, has been commercially produced by several companies whereas medium-chain-length (MCL) PHAs, consisting of 6–14 carbon atoms, has yet to be produced in large scale owing to relatively lower yield compared to SCL-PHA [2]. To name a few scientific breakthroughs which led to the successful large scale production of SCL-PHAs include poly-(R)-3-hydroxybutyrate (PHB) by Chemie Linz AG, Austria; copolymer PHBV by ICI, UK and TianAn, China; and copolymer PHBHHx (3-hydroxybutyrate-co-3-hydroxyhexanoate), by the joint efforts of Tsinghua University, KAIST and P&G. In view of the estimated production capacity of 50,000 and 10,000 tonnes/year in 2009 by Metabolix (USA) and Tianjin Green Bioscience (China), respectively, a new wave of PHA development focusing on new applications is expected [3].

Even though, production of PHAs from renewable, bio-based materials, wastewater and solid wastes have been gaining momentum, the utilization of waste substrate as carbon sources has not yet been developed in commercial scale. Similar to any other microbial fermentation, PHA production also differs by an order-of-magnitude when carried out in shake flasks under batch mode or in controlled bioreactors under fed-batch mode. While shake-flask experiments are the necessary first stages in developing a new product using inexpensive substrates; the potential of a novel system cannot be truly assessed unless it has been operated in larger volume where culture conditions at least approach those of industrial fermentations [4]. To optimize the chance of a successful fermentation at a pilot plant scale, the process parameters need to be set as the lab scale and translated into large scale fermentation.

In our previous study, we have reported the synthesis, characterization and high accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) PHBV granules in *Comamonas* sp EB172 – a local isolate from the palm oil mill effluent (POME)-treating anaerobic digester in 2 L scale [5,6]. Under dual nutrient limitation in pH-controlled fed-batch cultivation, PHBV productivity of 0.1318 g/L/h was achieved using mixed organic acids [7]. Therefore, clarified mixed organic acids from POME can be an industrially relevant carbon source for the production of PHBV by *Comamonas* sp. EB172. However, larger scale experiments are required before commercial implementation as the productivities reported above were obtained in 2 L laboratory scale fermenter.

The main aim of this study is the scale-up production of PHBV in pH-stat fed-batch cultivation of *Comamonas* sp. EB172 (GeneBank accession no. EU847238) on technical grade organic acid mixtures in 10 L and 150 L bioreactors based on constant impeller tip speed. This can be considered as an important first step towards the development of an industrial large-scale (>50 m^3) cultivation protocol.

2. MATERIALS AND METHODS

2.1 Pilot Scale Fermenters

The geometrical dimensions of 10 L and 150 L stirred tank fermenters, manufactured by B. Braun Biotech International, Germany, used in this study, are given in Table 1. The 10 L fermenter (Biostat C, B. Braun, Germany) with a 8 L total working volume was made up of a jacketed stainless steel culture vessel with a borosilicate glass side viewing window. Mixing was facilitated by means of three adjustable Rushton impellers. The fermenter was equipped with pH and temperature control systems. The top-plate consisted of openings for the introduction of alkali, inoculums and feed medium. The measurement and control systems for the fermenter were integrated in the control units of the BIOSTAT® C.

The 150- L process controlled automatic fermenter with SCADA (MFCS D/A) (Biostat D 100, B. Braun International), was made up of a jacketed stainless steel culture vessel. The 100-L fermenter had a bottom drive system and the stirrer shaft was sealed by mechanical seals. The vessel was equipped with three adjustable Rushton turbine impellers and pH, DOT, foam, mixing and temperature control systems. The thermostat system was an open, pressurized water system with pumps controlled by pulse-modulated valves. pH was controlled by automatic addition of sulfuric acid from a built-in peristaltic pump. Samples were taken by means of the outlet pipe located at the bottom of the culture vessel. The top-plate, consisted of openings for the introduction of alkali, acid, inoculums and feed solution. The measurements and control systems for the fermenter were investigated in the control units of the BIOSTAT® D. The pH of the medium after sterilization was around 6.8 and was adjusted to 7.0 before inoculation. The dissolved oxygen tension (DOT) was set at 100% air saturation at the beginning of the experiment and was kept above 30% of air saturation by adjusting the stirrer speed and air flow into the bioreactor.

2.2 Inoculation Procedure and Cultural Conditions

In order to produce a sufficient amount of viable biomass to inoculate the 10 L and 150 L bioreactors, the following procedure was applied.

Step 1. The starter inoculum of *Comamonas* sp. EB172 was prepared by transferring a loopful of culture from nutrient agar streak plate in the shake flasks containing 10 mL of growth medium (8 g/L nutrient broth and 1 g/L yeast extract, pH 7.0) and allowed to grow for 24 h at 30℃ and 200 rpm.

Step 2. Cultures were transferred to 250 mL conical flasks containing 50 mL of growth media and were incubated for 24 h at 30℃ at 200 rpm.

Step 3. In case of 10 L bioreactor, 100 mL inoculum from step 2 was used to inoculate 2 L bioreactor with 1 L of working volume. For 150 L bioreactor, 500 mL of culture from shake-flask in step 2 were transferred to 10 L bioreactors with the initial volume of 4.5 L of growth medium and were grown for 12-24 h a pH 7.5 and 30℃.

Table 1. Geometric dimension of the 10 L and 150 L bioreactors used in this study

Design parameters	Scale of fermenter		
	2 L	**10 L**	**150 L**
Total working volume	1.5 L	10 L	100 L
Liquid volume used	1.0 L	8 L	50 L
Diameter of inner tank(D_t)	13.0 cm	21.3 cm	45.72 cm
Height of tank(H_t)	24.0 cm	56 cm	101.6 cm
Number of baffles	4	4	4
Type of impellers	6-bladedisk	Rushton turbine	Rushton turbine
Number of impellers	2	3	3
Distance between impellers	8.8 cm	8.5 cm	10 cm
Impeller diameter of disc(D_i)	5.3 cm	8.5 cm	16.51 cm
Impeller blade width (W_i)	1.0	1.0 cm	3.2 cm
Impeller blade length (L_i)	1.25 cm	1.7 cm	4.9 cm
H_t/D_t	1.85:1	2.62:1	2.22:1

Step 4. The final pilot-plant cultivation in 10 L and 150 L bioreactor was carried out with an initial volume of 6 L and 40 L of growth medium, respectively. Batch operation was continued till late log phase. The inoculum size in all cultivations was 10% (v/v). A semi-defined PHA production medium containing (in g L^{-1}): 1.0 $NaHCO_3$, 0.01 $CaCl_2.2H_2O$; 0.123 $MgSO_4.7H_2O$; 0.024 $Fe(NH_4)_2(SO_4)_2.6H_2O$; 5.0 CH_3COONa and 1.0 ml trace elements solution [8] was then fed to the fermenters. After the complete addition of substrate, pH-controlled, fed batch operation was started by feeding substrate stream containing mixed technical grade organic acids. The fermentation was continued until no further increase in product concentration observed.

2.3 Operating Condition in the 10 L and 150 L Bioreactors

In this study, fermentation strategy previously developed in the 2 L scale [5] had been employed in 10 L and 150 L scale. A simple, pH-stat, fed-batch fermentation strategy for the biosynthesis of PHBV in 2 L consisted of a growth phase followed by a production phase in the same vessel consecutively by introducing nitrogen-free mineral media in the 12 hour culture (pre-grown in nutrient rich medium) and initiating feeding with mixed acids at pH 7.0.

However, three procedural differences existed between lab-scale and pilot scale fermentations were as follows:

First, the concentration of acid and base solution was increased from 1 M to 3 M and 1 M to 5 M for H_2SO_4 and NaOH, respectively, in pilot-scale studies.

Second, the feeding solution consisted of technical grade organic acids e.g., acetic, propionic and butyric acids in 2:1:1 ratio which mimics the actual clarified organic acids from fermented palm oil mill wastewater. Concentrated substrate stream (500 g/L) was used to avoid the dilution of culture broth during pH-stat feeding.

Third, in 150 L stirred tank fermenter, working volume was 50 L, to minimize the washing out of cells during foaming.

2.4 Scale-up Criteria

In this study, scale-up fermentation was carried out on the basis of impeller tip speed. The impeller tip speed for 10 L and 150 L bioreactor was kept constant during fermentation. The tip speed was calculated according to equation (1).

$$\text{Impeller tip speed (m/s)} = \frac{\pi N_i D_i}{60} \quad (1)$$

Where

π = 3.142
N_i = impeller speed (rotation/min)
D_i = impeller diameter (m)

Agitator was initially set at 145 rpm in 10 L which corresponded to the initial impeller tip speed value in 2 L bioreactor operation (0.56 m/s). Similarly, in 150-L bioreactor, agitation speed up to 258 rpm was used based on the eq (1) corresponding to the minimum (0.56 m/s) and the maximum impeller tip speed (2.22 m/s) value obtained in 2 L bioreactor.

2.5 Assays

Measurement of biomass as cell dry weight (CDW), PHA, organic acid and ammonium nitrogen concentration were carried out as previously described [9]. The pH, DOT and rotational speed were monitored throughout the fermentation. The mean value was calculated from two independent experiments, using Microsoft Excel and was used to plot the graph.

3. RESULTS

3.1 Scaling up Studies in 10 L Bioreactor

Fig. 1a shows the time course of biomass concentration, residual organic acid concentration, optical density and PHA content during fermentation in 10 L. Generally, the two main medium parameters that are known to affect the PHA yield are the pH of the medium and the dissolved oxygen tension (DOT) [10]. In our previous study, % DOT was shown to affect the growth of *Comamonas* sp EB172 and failure to keep DOT at 30% during growth phase led to poor growth and lower the PHA yield.

In order to provide similar process conditions as in 2 L fermenter, the upper limit for pH was set to 7.3 and lower limit to be 7.0 during pH-stat feeding. Both agitation speed and air flow rate was varied to the maximum to provide sufficient oxygen for biomass formation and PHA accumulation. The cell mass increased steadily and reached 4.14 g/L at 52 h of cultivation. The

cell mass increased nearly 35-folds during the fermentation (Fig. 1a). Little or no accumulation was observed during cultivation in nutrient rich medium and the copolyester concentration in the cells increased from 0.1 g/L at 16 h to a maximum of 3.65 g/L at 48 h (Fig. 1a).

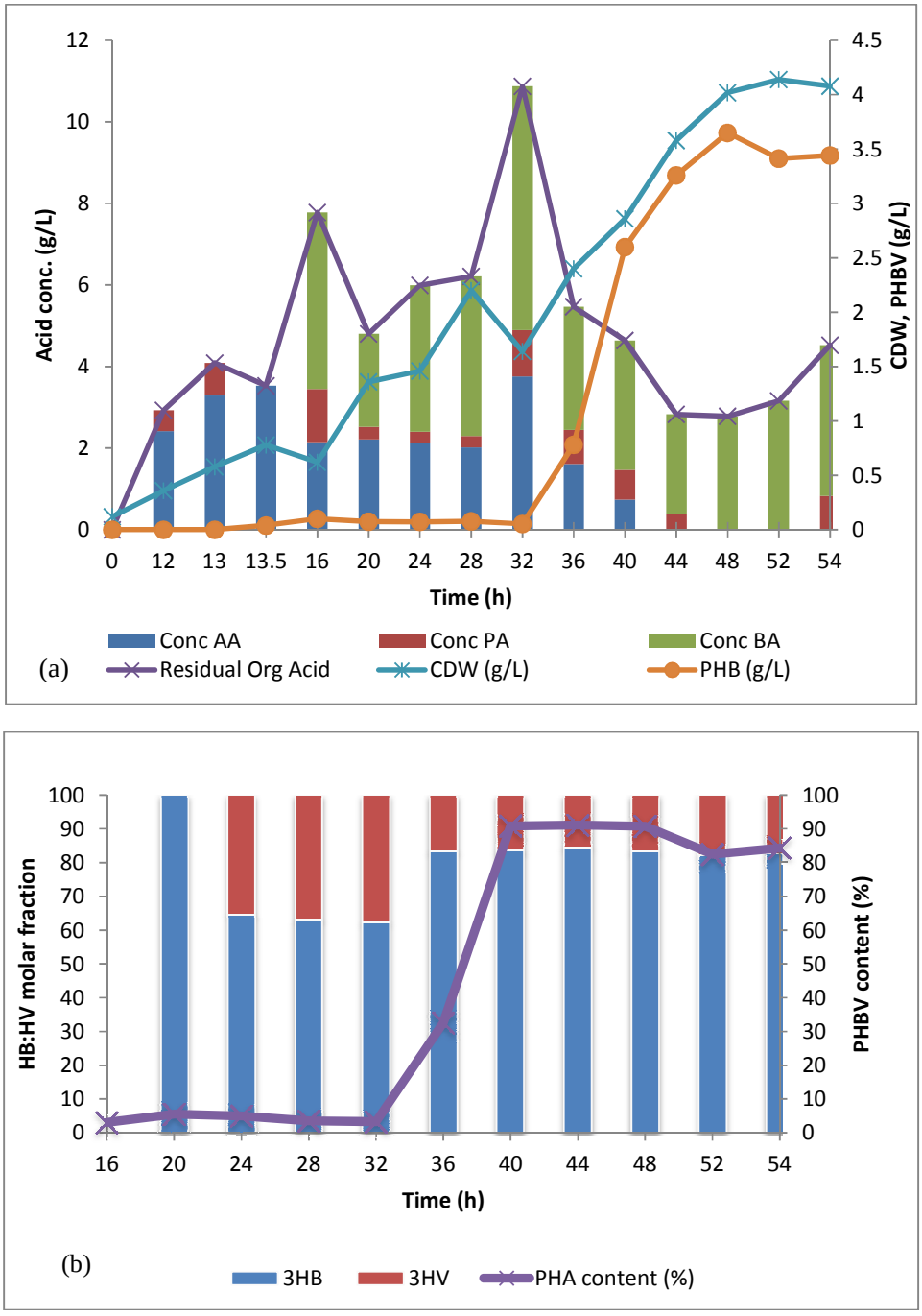


Fig. 1. Fermentation profile of *Comamonas* sp EB172 in 10 L bioreactor with controlled DOT strategy (a) time course of CDW, AA-Acetic acid, BA-butyric acid and PA-Propionic acid concentration, residual acid concentration and PHBV concentration and (b) changes of PHBV monomer composition and PHBV content during the fermentation

The residual acid concentration was varied from a minimum of 2.93 g/L to maximum of 10.87 g/L throughout the fermentation. It is to be mentioned here that mixed organic acids were automatically fed to the bioreactor depending on the fluctuation of pH in the culture broth which resulted in the variation in the residual acids in the broth. As can be seen in Fig. 1a, the growth was slightly reduced at 32 hour probably because both propionic acid and butyric acid concentration exceeded the tolerance level. Concentration above 1.5 g/L and 5.3 g/L for propionic acid and n-butyric acid, respectively, are reported to be inhibitory to *Comamonas* sp. EB172 [11]. During 44 – 52 h of fermentation, out of three acids, only butyric acid was prevalent in the broth in the concentration of 2.3 -3.1 g/L. Small amount of propionic acid was detected in 44 hour old culture and was not detected in the broth until 52 h. In this study, acetic acid (CH_3COOH) was consumed faster than propionic acid (CH_3CH_2COOH) followed by n-butyric acid ($CH_3CH_2CH_2COOH$). Therefore, consumption of acids was followed by the number of carbon atoms or molecular size of each organic acid- the shorter being utilized first. Thus more rapid decrease of the residual acetic acid concentration in the broth was observed compared to the other two acids.

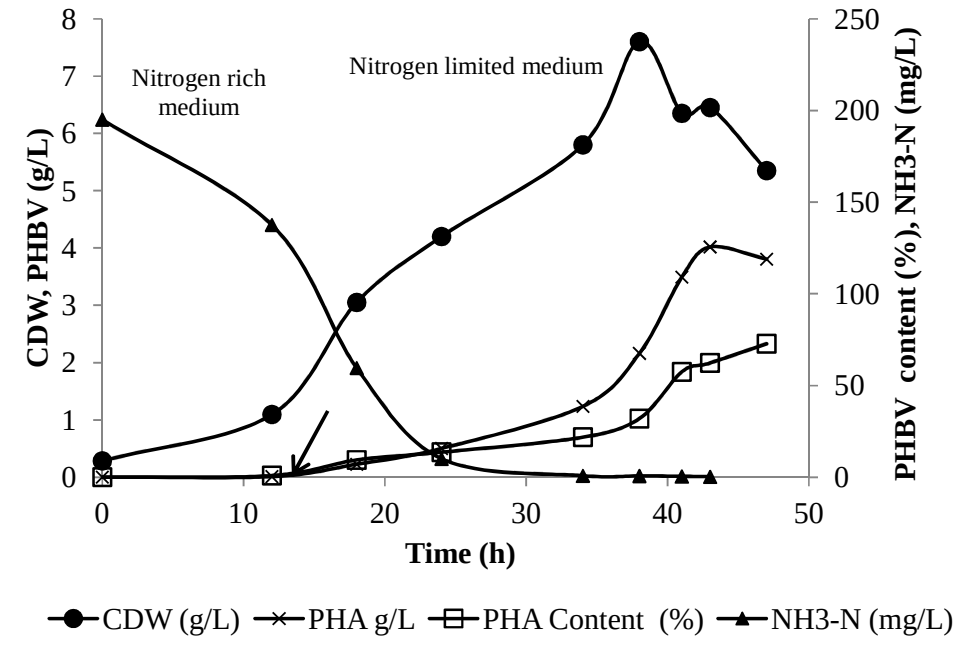
The PHA accumulation detected at 16 h was below 5% and was not considerable until 32 h. PHBV content in the cells reached a maximum, 91% of CDW and was maintained from 44-48 h of cultivation. Gas chromatogram (GC) of PHBV revealed variation in molar composition throughout the fermentation. During accumulation of PHBV, molar composition of the HB and HV units in the synthesized copolymer varied on the concentration of mixed organic acids; particularly on the availability of HV precursor-propionic acid in the medium. At initial stage, GC analysis of dried cells revealed PHB homopolymers and as time progresses, HB- the principal monomer unit shifted to HV and the mole fraction of HV was 35.36%. 3HV fraction increased to the maximum value of 37.81% during fermentation (Fig. 1b). However, after 32 hour, more HB was incorporated and HV fraction reached 17.38% at the end of the study. Similar observation in the fluctuation of HV molar fraction during fed-batch fermentation was reported in *E. coli* feeding with glucose and propionate [12] and also during PHBV fermentation by *Rhodococcus aetherivorans* IAR1 grown on toluene [13].

Moreover, it was observed that towards the end of fermentation from 36 h until 54 h, the HV content in the copolymer was maintained between 16-18%. Rhee et al. [14] showed that the molar fraction of HV in P(HB-co-HV) can be controlled by regulating the concentration of ammonium in the feed. In this study, the feeding solution contained mixture of three acids and were devoid of any nitrogen source. In this study, the residual ammonium nitrogen in the broth ranged between 65-100 mg/L during the accumulation period so that a slight but constant growth of the cells could be maintained (profile not shown). This may have played a role in the stability of HV content in the copolymer and also the reason for the longer time span at which the accumulation remained constant.

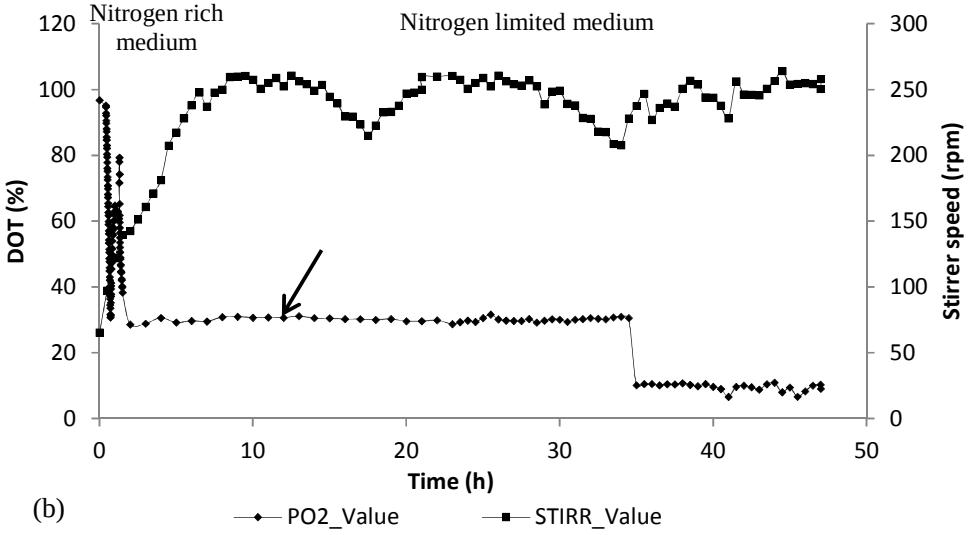
3.2 Scale up Studies in 150 L Bioreactor

The time profile of PHBV production in 150 L bioreactor is shown in Fig. 2. The initial biomass concentration was around 0.30 g/L and was increased to 1 g/L after 12 h. Initially, the stirrer speed was set at 50 rpm and it shoot up to around 250 rpm during exponential growth phase. After adding production media, the cell concentration increased steadily and five-fold increase in cell titre was noticed within 12 to 34 h of cultivation. The dissolved oxygen concentration was maintained at 30% during this period (Fig. 2b). On the other hand, PHBV continuously accumulated after its production was detected at 18 h of cultivation. The PHBV concentration increased from 0.5 g/L at 24 h to 4.0 g/L at 38 h and 3.8 g/L at 47 h of fermentation. The biomass reached maximum of 7.6 g/L at 38 h of cultivation.

A very sharp increase in the PHBV content resulted from the limited oxygen environment as shown in Fig. 2b. After 38 h, dissolved oxygen concentration value (pO_2) dropped to 10% because of the high oxygen tension. The stirrer was operated at its maximum speed limit (258±4 rpm) and could not be increased further to keep impeller tip speed similar to that in smaller scale bioreactor. PHBV content in the cells reached its maximum, 72.80% of CDW, at 47 h of cultivation. However, as shown in Fig. 2a, from 34 h onwards, the ammonium nitrogen in the broth was not detected and complete nitrogen deficiency in the broth led to cell inhibition. As a result, dry cell mass reduced to 5.35 g/L at the end of fermentation. Since the PHBV content in the cells was highest (72.80% of CDW), the fermentation was terminated at 47 h as prolonged cultivation to achieve higher PHBV concentration would be counterproductive.



(a)



(b)

Fig. 2. Fermentation profile of *Comamonas* sp EB172 in 150 L bioreactor with controlled DOT strategy. (a) Time course of CDW (g/L), PHBV concentration and PHBV content and (b) Changes of dissolved oxygen and stirrer speed during fermentation. Arrow indicates the onset of feeding - transition from nitrogen rich phase to nitrogen deficient phase

It has been reported that the presence of small amount of ammonium in the fermentation media is crucial to maintain the growth capacity of the organism, because only viable residual biomass has the biochemical capability to synthesize and store the PHA. Thus, even though nitrogen source limitation stimulated PHA synthesis, total elimination of nitrogen is detrimental to culture

physiology resulting in biomass death and biodegradation of stored PHA. In contrast, excessive feeding of nitrogen during PHB accumulation phase has been reported to degrade the accumulated PHB and to reduce PHB synthesis [15]. Therefore, a balance between cell growth and PHA accumulation must be achieved to avoid incomplete accumulation of PHA or premature termination of PHA synthesis at low cell concentration.

4. DISCUSSION

Scale-up for the growth of microorganisms is usually based on maintaining a constant oxygen transfer rate or k_La (oxygen mass transfer coefficient) which is suitable for cultures which require high levels of oxygen and also grow fast enough to cause oxygen depletion. Another way to a scale-up is to have the speed of the end (tip) of the impeller equal the velocity in both the laboratory bioreactor and the pilot scale bioreactor. Even though, a number of research papers have described PHA production in pilot and/or industrial scale, the details on the parameters used for scaling-up was ambiguous [16-21]. Recently, Elbahloul and Steinbuchel [22] reported the production of PHO, poly(3-hydroxyoctanoic acid) -a medium chain length PHA at 400 litre volume using Biostat D 650 stainless steel reactor,. However, the scale up was not based on any criteria but the feeding strategy was modified in order to improve the fermentation performance.

In our continuous effort to utilize POME-derived mixed organic acids for PHA production, the accumulation capability of single culture of *Rhodobacter spheroides*, and *Alcaligenes eutrophus* in shake-flask and laboratory-scale fermenters were studied and have been reported [8,23]. Using acetic acid from POME, 45% PHA content with a yield of 0.32 and volumetric productivity of 0.09 g PHA $l^{-1}h^{-1}$ were obtained previously in the 2 L fed-batch production of PHA by *Alcaligenes eutrophus* [8]. Employing same strain, Ganzeveld et al. [24], obtained a yield of 0.16 g/g and productivity of 0.025 g/L/h from the bioconversion of fermented organic waste containing volatile organic acids into PHBV under oxygen limitation. Using thermophillic UASB (Upflow Anaerobic Sludge Blanket) reactor to produce organic acids from starchy wastewater, Yu [25] has been able to show 34.1% PHA content in *C. necator* with a concentration of 1.2 g/L within 48 h resulting in a similar productivity of 0.025 g/L/h.

Table 2 shows the results of our scale-up study compared with previous studies in small scale bioreactor. It is to be mentioned that the substrate used in scale-up studies were a mixture of three, technical grade organic acids whereas in 2L scale this mixed acid has been obtained by clarification and extraction of acidified POME. However, the fermentation performance was found similar regardless of the origin of mixed acids. From Table 2, it can be seen that the fed-batch study with variable feeding has the lowest volumetric productivity than others. The longer fermentation time and lower biomass resulted in low productivity even though the product yield was comparable to others. When the novel fermentation strategy was applied, all three kinetic parameters increased and biomass concentration also increased three fold. Scaling up of fermentation to 10 L and 150 L bioreactor did not affect much on the product yield however, the biomass yield coefficient as well as the overall productivity was reduced to half.

Table 2. Summary of kinetic data from pH controlled fed-batch fermentation in lab-scale to pilot-scale

Fermentation conditions	Biomass yield coefficient Yx/s (g/g)	Product yield coefficient Yp/s (g/g)	Volumetric productivity Φ (g/L/h)	PHBV content (%)	References
Variable C/N feeding in 2 L	0.365	0.31	0.040	85.81	[7]
Novel fermentation strategy with C feeding under dual limitation strategy in 2 L	0.555	0.35	0.1318	70.10	[5]
Scale up in 10 L	0.381	0.3238	0.070	84.37	This study
Scale up to 150 L	0.356	0.259	0.083	72.80	This study

In this study, technical grade acetic, propionic and n-butyric acids were mixed in 2:1:1 ratio simulating the clarified organic acid from POME. The feeding solution had a concentration of 500 g/L which was fed to a 150 L bioreactor from 13 to 47 h using pH-stat method. In this method, the mixed acid feeding rate was controlled by the fluctuation of pH due to substrate utilization and was automatically controlled at pH 7.0. Since the feeding of mixed acids was operated in response to control the pH, the amount of acids in the broth was not constant. The feed rate also varied from 36 mL/h to 69 mL/h and reduced to 8 mL/h at the end of fermentation. Since the concentration of acid used in lab-scale and pilot scale were different, it is better to express feed rate in terms of g/h. The range of feeding rate in lab-scale fermentation was around 0.275 to 0.584 g/h whereas in pilot scale fermenter the value obtained was in the range of 0.36-0.69 g/h throughout the fermentation. The feeding rate of mixed acid was highest during 13-16 h of fermentation and at the onset of both ammonium nitrogen and dissolved oxygen limitation or so called dual-nutrient-limitation zone.

In the present study, the results obtained in fed-batch cultivations of *Comamonas* sp. EB172 on technical grade mixed organic acid at pilot-plant and lab scale were highly similar. This represents an important step forward towards commercial-scale production of PHA-rich biomass via this cultivation mode. Scaling up in 150 L bioreactor resulted in biomass concentration of 5.35 g/L with final PHA content of 72.80% by applying dual nutrient limitation strategy. The calculated yield was 0.259 g PHBV/g mixed acids, close to that obtained in 2 L. Sangkarak and Prasertsan [26] pointed out that acetate is 10% less expensive than the other carbon sources (including the industrial standard of glucose) and therefore could be commercially beneficial in large scale PHA production. Compared to other pure reagents for fermentation processes- acetic, butyric and propionic acids derived from POME will provide increased PHA yields at a lower cost.

5. CONCLUSION

In this study, scaling up fermentation based on constant tip speed in 150 L bioreactor resulted in biomass concentration of 5.35 g/L with final PHBV content of 72.80% and volumetric productivity of 0.083 g/L/h. The calculated yield was 0.259 g PHBV/g mixed acids. Both yield and PHBV content were comparable to that obtained in 2 L scale in which, the maximum biomass was 10.2 g/L with residual acid around 2-6 g/L and volumetric productivity of 0.1318 g/L/ h [5]. PHBV content in 2 L scale ranged from 70-90% (w/w) of the cell with yield of 0.27-0.4 g PHB/g mixed acid, respectively. Furthermore, it can be extrapolated that, if this organic acid mixtures can be produced in bulk quantities in palm oil mills and integrated with PHBV production-the whole process will be more economic and contribute towards the zero discharge of palm oil mill wastewater.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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