0273-2289/05/121–124/289–302/$30.00

Synthesis of Polyhydroxyalkanoate (PHA) from Excess Activated Sludge Under Various Oxidation–Reduction Potentials (ORP) by Using Acetate and Propionate as Carbon Sources

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Abstract

Accumulation of poly hydroxyalkanoate (PHA) from excess activated sludge (EAS) was monitored and controlled via the oxidation-reduction potential (ORP) adjusting process. The ORP was adjusted and controlled by only regulating the gas-flow rate pumped into the cultural broth in which sodium acetate (C2) and propionate (C3) were used as carbon sources. Productivity of PHA and the PHA compositions at various C2 to C3 ratios were also investigated. When ORP was maintained at +30 mV, 35% (w/w) of PHA of cell dry weight obtained when C2 was used as sole carbon source. The PHA copolymer, poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), accumulated by EAS with different 3-hydroxyvalarate (3HV) molar fractions ranged from 8% to 78.0% when C2 and C3 was used as sole carbon source, By using ORP to monitor and control the fermentation process instead DO meter, the ORP system provided more precise control to the PHA accumulation process from EAS under low dissolved oxygen (DO) concentrations. Adjusting the C2 to C3 ratios in the media could control the composition such as the 3HV/3HB ratios of the PHBV. Furthermore, it might be an effective way to adjust the 3HV molar fractions in PHBV by controlling the DO concentration via the ORP monitoring system. The 3HV molar fractions in the PHBV declined with increasing ORP from −30 mV to +100 mV by adjusting the gas-flow rate (i.e. the DO concentration). It is concluded that the DO plays a very important role in the synthesis of 3HV subunits in PHBV co-polymer from the EAS. Therefore, a hypothetic

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metabolic model for PHA synthesis from EAS was proposed to try to explain the results in this study.

Index Entries: Activated sludge; oxidation–reduction potential; ORP; polyhydroxyalkanoate; PHA.

Introduction

Polyhydroxyalkanoate (PHA), a biological polymer produced by microbial fermentation, has received much attention due to its biodegradability and as an alternative to the usual petroleum-derived plastics. A number of investigations have been attempted to lower production cost and put this kind of polymer production on wider applications and commercial scale. Those investigations have had good success by investigating the PHA production feasibilities from the co-culture of excess activated sludge in a sewage treatment plant (*1–8*).

This study examines the control of PHA contents under various oxidation-reduction potentials (ORP), in attempt to manipulate the PHA compositions and physical properties by using sodium acetate (C2) and sodium propionate (C3) as the carbon sources. ORP monitoring and controlling are the core technique to be investigated throughout the PHA accumulation process. ORP monitoring and controlling instead of direct dissolved oxygen (DO) measurement is adopted because accurate direct measurement of very low concentrations of a DO by a DO meter is difficult and unreliable. Fine adjustment of the DO is detectable by ORP meter but not a DO meter. ORP can provide real time, accurate, and flexible adjustments in the fermentation process, and it is highly sensitive to reflect the levels of dissolved oxygen, organic substrates, as well as organism activities.

Materials and Methods

Operation of Sequencing Batch Reactor and Biomass Harvesting

A laboratory-scale sequencing batch reactor (SBR) with 12-L effective volume was inoculated with activated sludge from a local sewage treatment plant in Hong Kong. A synthetic wastewater containing reconstituted milk at 1.92 g/L, which is equivalent to 1200 mg/L in terms of total organic carbon (TOC) was fed *(3).* The SBR was operated for a period of 60 d and the steady-state performance reached a 98.0% TOC removal. The suspended solid (SS) was analyzed as activated sludge concentration in accordance with the Standard Methods *(9)*; 7–8 g EAS was harvested at each batch, washed with distilled water to remove any residual nitrogenous matters, and then inoculated into a jar bioreactor with nitrogen-free medium for PHA synthesis fermentation.

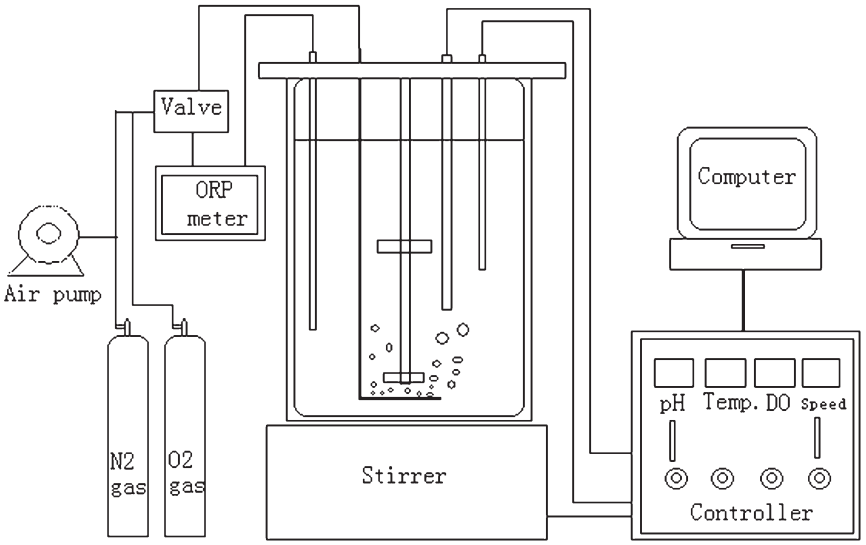

Fig. 1. Schematic diagram of cultivation set-up including ORP meter equipped with an ORP sensor, pH meter equipped with a pH sensor, magnetic stirrer, glass jar bioreactor, central controller, and computer.

Cultural media and ORP Monitoring and Controlling for the Fermentation Processes

An Automatic jar bioreactor of 3-L working volume (Bioengineering Model ALF, Ruti/Switzerland) equipped with an ORP meter (Cole-Parmer, pH/ORP Controller Model 5656-00) was operated for this study (Fig. 1). The operation conditions were the same as described by Chua et al. *(1,2)* and Hu et al. *(3)*. The pH was kept relatively constant at 6.8–7.0 by the addition of 2 *M* NaOH solution.

Two sets of cultural conditions are provided by varying the carbon sources and the ORP values.

Set I with Sodium Acetate (C2) as Sole Carbon Source

A nitrogen-free medium, named Medium-1, with 5.13 g/L (1500 mg/L of TOC) of C2 as sole carbon source, equivalent to TOC of 1500 mg/L, was employed for PHA-accumulation fermentation. An average of 2.5 g/L of EAS harvested from SBR was inoculated into the jar bioreactor, and then collected after 48 h fermentation at 30°C for further PHA analysis.

Other nutrients including supplementary trace minerals and growth factors with formulation were added as described by Chua et al. *(1)*. To avoid any growth inhibition caused by C2, the bioreactor was operated in a fed batch mode *(3)*.

In order to investigate the effect of ORP on the PHA accumulation, the ORPs of the cultural media were maintained at set levels of −30 mV, 0 mV, +30 mV, and +100 mV, respectively. Any variation of the ORP could

be adjusted back by controlling the gas-flow rate pumped into the jar bioreactor (i.e., the concentration of DO). The compressed air, pure oxygen, or nitrogen gas was added into the jar bioreactor if necessary (Fig. 1).

Set II with Sodium Acetate and Sodium Propionate as Carbon Sources

Another medium, Medium-2, C2 and sodium propionate (C3) were used as the complex carbon sources with the addition of supplementary trace minerals and growth factors. In separate batch cultures, the C2 to C3 mole ratios in the medium were 100:0, 80:20, 50:50, 20:80 and 0:100, respectively. The total TOC of the medium-2 was about 1500 mg/L contributed by C2 and C3. The fed batch mode was used as the same as in set I. Medium-2 with different C2/C3 ratios was introduced to prove that the composition of the poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV copolymer) with different 3-hydroxybutyrate (3HB) unit to 3-hydroxyvalerate (3HV) unit ratio synthesized in EAS could be controlled by regulating the carbon sources. In set II, the ORP was kept constant at +30 mV.

TOC measurement and PHA analysis

The initial and residual carbon concentration of the medium or cultural broth was measured as TOC by TOC-500 (Shimadzu).

A Gas Chromatography Analyzer (Hewlett Packard 5859 Series II) equipped with a 6 ft length of Supelco Packed Column (10% Carbowax 20M with 80/100 in mesh size Chromosorb WAW) was used to determine the PHA content and PHA composition. The methods for the pretreatment of EAS sample and analysis of PHA coincided with that of Braunegg et al. *(10)* reported.

Results and Discussion

Composition of PHA Synthesized at Different C2/C3 Ratios where ORP Maintained at +30 mV

Table 1 lists the TOC removal efficiency, polymer content, product yields, final 3HV molar fraction, and thermal property of the polymer after 48 h fermentation at five sets of carbon source ratios, respectively. The initial cell mass concentration in the bioreactor was about 3.0 g/L, final cell mass was maintained within the range of 3.0–3.2 g/L. Because the bacteria in EAS grow under aerobic conditions only, it could be considered that cell mass concentration remained approximately constant during the periods of the cultivation for PHA accumulation *(11)*.

As shown in Table 1, an increase in C3 in the media from 0 to 100 mol% resulted in a decline in polymer production yield, $Y_{p/s}$, from 0.48 to 0.38 g-polymer/g-TOC consumed. The PHA productivities were lowered by the inclusion of propionate in the carbon source of the medium *(7)*. The

Table 1
Productivity of PHA from EAS Under Various C2 to C3[a] Ratios

C_2 to C_3 (mol/mol)	C_2 and C_3 (g/L)		TOC removal (%)	Polymer content (wt%)	$Y_{p/s}$[b], (g/g)	3HV fraction (mol%)	T_m (°C)
	C_2	C_3					
100:0	5.13	0.00	98.2	35.0	0.48	8.0	168
80:20	3.72	1.09	98.8	33.0	0.45	22.0	140
50:50	2.05	2.40	95.6	28.0	0.39	55.0	108
20:80	0.73	3.42	96.3	26.1	0.36	70.0	98
0:100	0.00	4.00	90.4	25.8	0.38	78.0	95

[a]The total TOC (total organic carbon) of the cultural medium was 1500 mg/L added in a fed batch mode once every 12 h in order to avoid growth inhibition. ORP was kept at +30 mV.

[b]$Y_{p/s}$ = Polymer production yield (g polymer/g carbon), which was calculated as the polymer accumulated divided by the TOC consumed.

polymer content in the EAS also decreased from 35.0 to 25.8 wt%. It was close to the theoretical yield of 0.65 g-PHA/g-fatty acids by *Alcaligenes eutrophus (12),* which is a common genus in activated sludge. On the other hand, it was observed that the main bacterial genus in the activated sludge that accumulated the polymers was rod-shaped bacteria. *Alcaligenes* spp was the main group in the cultural broth after 48 h cultivation for the PHA accumulation. This result agreed well with that described by Dave et al. *(13)* and Hu et al. *(3).*

The infrared spectrums of the PHA produced by EAS were identical with those of the standard samples including PHB and PHBV. The maximal 3HV molar fraction of 78.0 mol% was achieved when C3 was used as sole carbon source. The molar fraction of 3HV in the PHBV copolymer increased proportionately with the C3 concentration in medium. This result agreed with the early observation *(3)* where butyric and valeric acids were used as carbon sources. Takabatake et al. *(7)* found similar results. It is demonstrated that the C3 could serve as the precursor for 3HV units in the PHBV copolymer as well as the pure culture described by Choi et al. *(14).* The PHBV copolymer accumulated in EAS with wide range of 3HV molar fractions (8 mol%–78 mol%) could be produced and adjusted by controlling the C2 to C3 ratios in the medium.

PHA Accumulation by Using C2 as Sole Carbon Source Under Various ORPs

It is illustrated in Fig. 2 that PHA content in the EAS increased with the increasing of ORP values by using C2 as sole carbon source. The minimum PHA content at ORP –30 mV was about 12% (w/w) of EAS dry weight, and the maximum PHA content of 35% (w/w) was achieved while

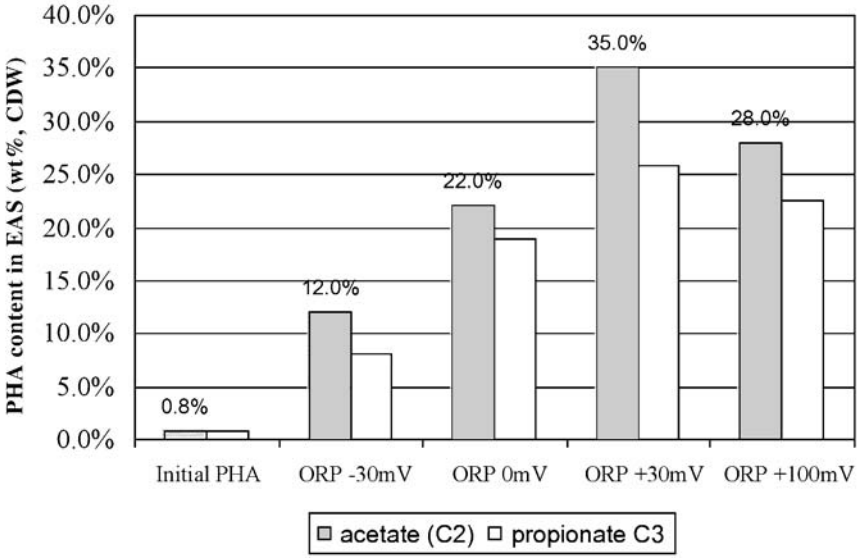

Fig. 2. PHA contents in EAS accumulated under various ORP by using acetate (C2) or propionate (C3) as sole carbon sources.

ORP being maintained at +30 mV. PHA content in the EAS declined to 28% of EAS dry weight when ORP was kept at +100 mV.

The accumulation of PHA in activated sludge is not yet understood very well, and there is no widespread accepted metabolic mechanism to describe the detail of PHA synthesis. Until now, only the metabolic pathway for PHA synthesis in enhanced biological phosphorus (EBPR) was studied intensively. In the EBPR process, PHA is known to play a very important role for the phosphorus uptake from wastewater. Under aerobic conditions, PHA previously accumulated in anaerobic stage is metabolized as the energy source for microorganism growth and the synthesis of glycogen and polyphosphate with phosphate uptake from the wastewater. Otherwise, under anaerobic condition, the activated sludge uptakes organic carbon substrates and accumulates it as PHA. Energy for this process comes from the breakdown of intracellular polyphosphate, glycogen consumption, and substrate degradation in the incomplete tricarboxylic acid cycle (TCA cycle) *(11).* But this metabolic mechanism cannot explain the phenomena found in this study.

The increase of PHA production with the increase of ORPs, equivalent the increasing of DO concentration, is probably due to the supply of energy by oxidation of the organic substrate acetate. Under the anaerobic conditions, where ORP is maintained at low level (for example, −30 mV), the energy source is limited by the amount of polymers such as polyphosphate and glycogen. Polyphosphate and glycogen are the limit factors for the PHA accumulation under anaerobic conditions. When energy sources are exhausted, the PHA accumulation will be stopped. Adjusting the ORP

to higher value of +30 mV, more oxygen is supplied, which results in an oxygen-limited condition. EAS takes up organic C2 to get energy by oxidative degradation of some part of it to meet the need for the PHA accumulation except for the degradation of polyphosphate and glycogen. When ORP was kept at +100 mV, close to an aerobic condition, the PHA stored in cells could be reused as carbon and energy sources for the accumulation of polyphosphate and glycogen, and therefore resulting in the declining of PHA content in EAS. It is concluded that just like the bacterial pure culture for PHA synthesis, DO plays a very important role in the PHA synthesis by EAS.

PHA Accumulation by Using C3 as Sole Carbon Source Under Various ORPs

As shown in Table 1, when propionate (C3) was used as sole carbon source (total of 4 g/L) and ORP was kept at +30 mV, the PHBV copolymer content, polymer production yield, and 3HV molar fraction were 25.8%, 0.38 (g/g), and 78.0 mol%, respectively. Moreover, the TOC removal efficiency was 90.4%. On the other hand, as illustrated in Fig. 2, the initial PHA was 0.8% CDW; when ORPs were preset at −30 mV, 0 mV, +30 mV and +100 mV, the PHA contents in the EAS were 8.0%, 18.9%, 25.8% and 22.5%, CDW, respectively. The PHA content in EAS was increased with the increasing of ORPs. Finally, when the ORP was +100 mV, the PHA content decreased in comparison with that of ORP +30 mV. As suggested in the previous report where C2 as sole carbon source, the presence of excess oxygen where +100 mV of ORP was maintained (i.e., pure oxygen was supplied), resulted in the balanced growth conditions, and acetyl-CoA was submitted to the TCA cycle for energy generation and biomass growth. In consequence, the concentration of free CoASH became higher. The key enzyme for PHA synthesis, 3-ketothiolase, was inhibited by high concentrations of free CoASH, and resulted in the repression of PHA synthesis *(15).*

All these observations are in accordance with that of C2 as carbon substrate. Furthermore, these results agreed well with the point that the increase of carbon skeleton length of fatty acid results in the declining of the performance of activated sludge.

3HV Molar Fraction in PHBV by Using C2 as Sole Carbon Source

When ORPs were maintained at −30 mV, 0 mV, and +30 mV, respectively, the PHAs accumulated in the EAS were PHBV co-polymer instead of PHB homopolymer even at +100 mV of ORP. This result agreed well with the other reports. Furthermore, it was also observed in this study that the 3HV molar fractions in the PHBV varied from 21 mol % to 8 mol% (Fig. 3).

Satoh et al. *(16)* stated that when acetate was used as sole carbon source, 87% of the PHA produced were 3HB, 11% were 3HV, and the remaining 2% were 3H2MB (3-hydroxy-2-methylbutyrate) and 1% 3H2MV

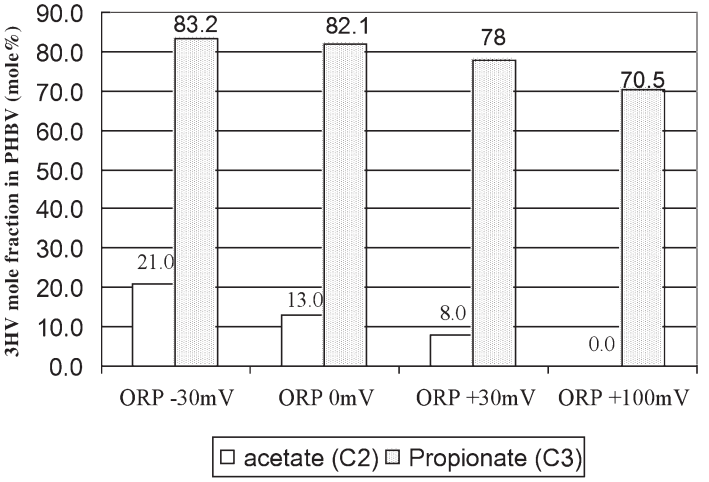

Fig. 3. 3HV molar fractions in the PHBV copolymer produced by the EAS under various set ORPs when C2 and C3 were used as sole carbon source respectively.

(3-hydroxy-2- methylvalerate). Liu et al. *(17)* found that when the GAO (glycogen-accumulating organisms) population from a EBPR process without biological phosphorus removal was conducted for the acetate uptake under anaerobic conditions, the PHA increase to 13.2% of total suspended solid (TSS) with 66.7 mol% of 3HB unit and 26.4 mol% of 3HV unit. Even under aerobic conditions, Randall et al. *(18*) found that PHBV copolymer with 3HB as dominant unit according with 3HV unit obtained when acetate as carbon source. Takabatake et al. *(7)* tested the activated sludge in the PHA-accumulating bacteria enrichment reactor, a modified SBR operated in the anaerobic–aerobic mode. The acclimated activated sludge then was submitted to PHA production under aerobic conditions. Consequently, PHBV copolymer with predominant 3HB and only 3% molar fraction of 3HV unit has been obtained when acetate was used as sole carbon source.

The ORP in this study was kept at set levels by only adjusting the DO concentration in the cultural medium. Therefore, the variation of 3HV molar fractions in the PHBV resulted from the changes of DO of the media. These observations have never been described earlier in the literature. In the pure culture of *Ralstonia eutropha* for PHA production from acetate as sole carbon substrate, only PHB homopolymer accumulated *(19)*. Serafim et al. *(8)* found that there was only PHB homopolymer produced by activated sludge co-culture when reactor operation was changed from anaerobic–aerobic conditions, to aerobic dynamic substrate feeding process where C2 was used as sole carbon source. Moreover, when the reactor operation was changed back to anaerobic-aerobic conditions, PHBV copolymer with predominant 3HB unit was accumulated *(8).* However, the authors did not find, mention, discuss this evidence that oxygen might play a very

important role to regulate the 3HV molar fraction in PHBV. This is a new challenge to that of currently existing biosynthetic pathways on the synthesis of PHA in activated sludge, because the existing mechanism cannot explain these observations very well.

It is believed that there are two possible explanations for these observations. Firstl, the changes of ORP (i.e., DO in this research) became a selecting force and resulted in the selected growth of bacteria in the EAS, and then led to a metabolic pathway for the accumulation of PHA with different 3HV mole fraction. Second, there must be some other metabolic mechanisms not found yet and the synthesis of 3HV unit in PHBV copolymer might be affected by the DO concentration. Thus, a hypothetic metabolic pathway for PHA production from EAS by using C2 as carbon source has been proposed and demonstrated in Fig. 4.

This hypothetic metabolic pathway is modified from Doi et al. *(15)*, and combined with recent research achievements such as succinate-propionate pathway, EBPR metabolic models *(11,16, 20–28).*

The most important view of this hypothetic pathway is that the TCA cycle and glyoxylate pathway are involved even under oxygen-limited and anaerobic conditions. Generally, it is believed and widely accepted that, only under aerobic conditions does the TCA cycle functions completely. However, recent research suggested that TCA cycle and glyoxylate pathway are fully or incompletely involved in the bacterial metabolisms in the activated sludge and PHA production even under anoxic and anaerobic conditions *(11, 27, 28).* Therefore, in this model, the TCA cycle and glyoxylate pathway are combined with the Doi's model proposed for the PHA production from *R. eutropha* pure culture *(15).*

It can be seen from the model, after being converted into acetyl-CoA from acetate, that part of acetyl-CoA is directly contributed to PHB production. Others enter TCA cycle and glyoxylate pathway for energy, reducing power generation and cell growth. As we know, a completely functioning TCA cycle must have excess oxygen or else only incomplete TCA cycle functions *(11, 20, 21, 27, 28).* In addition, in the absence of oxygen or oxygen-limited conditions, the step from succinate to fumarate in TCA cycle will be blocked (indicated by the black bar in Fig. 4). This is because the oxidation of succinate to fumarate in the TCA cycle requires a terminal electron acceptor with a redox potential ($E^{0\prime}$) more positive than that of fumarate/succinate couple (+32 mV). In this case, apparently only O_2 (O_2/H_2O, $E^{0\prime}$= +818 mV) meet this requirement *(29).* Therefore, under oxygen-limited (anoxic) and anaerobic conditions, succinate derived from the front part of TCA cycle or glyoxylate pathway cannot be oxidized to fumarate and consequently results in the accumulation of succinate. As illustrated in Fig. 4, the step from α-ketoglutarate to succinyl-CoA is irreversible reaction. Thus, accumulated succinate is forced to the pathway for formation of succinyl-CoA and then converted to propionyl-CoA as the precursor of 3HV for PHA synthesis. However,

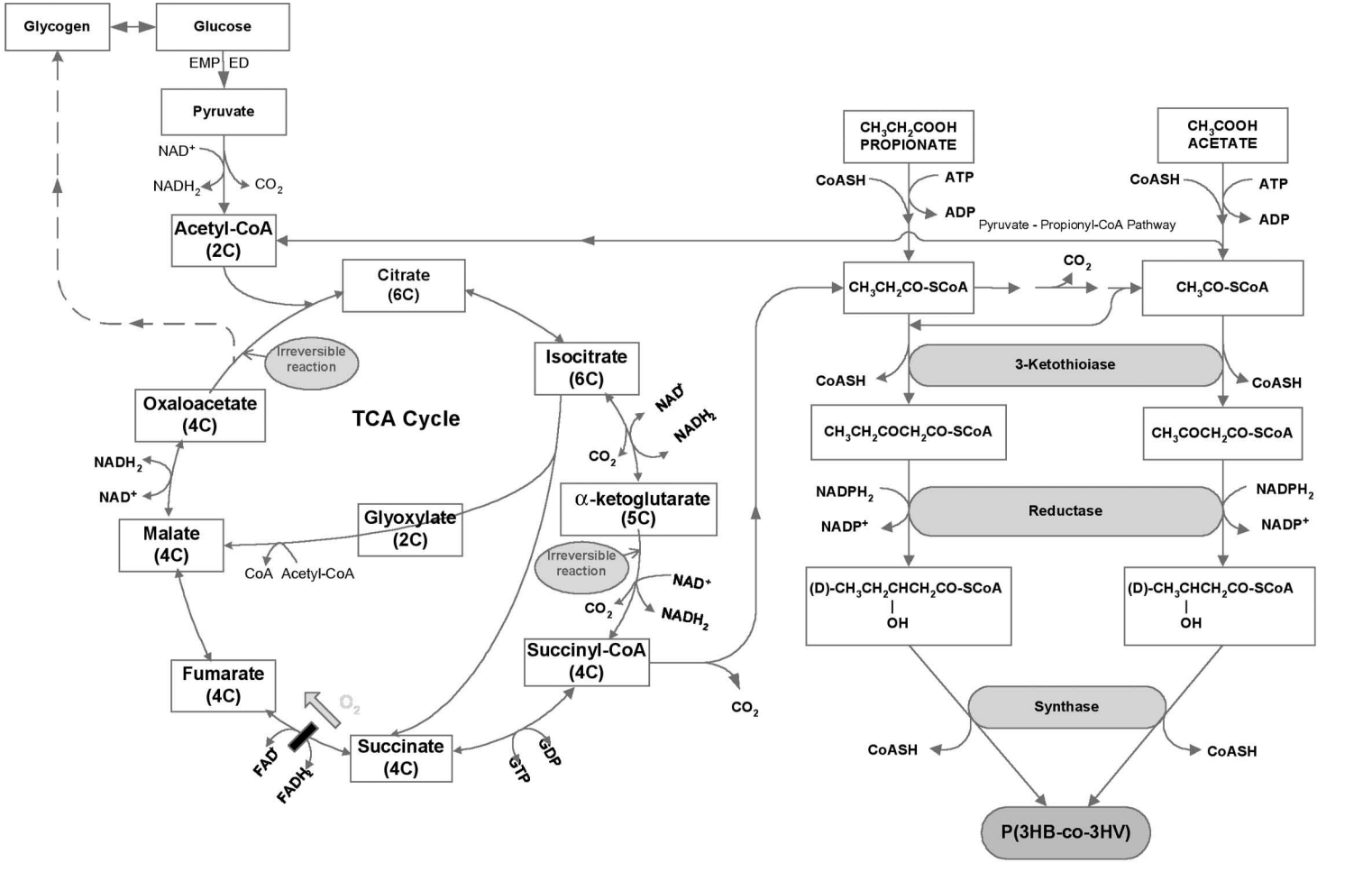

Fig. 4. Hypothetic metabolic pathway for PHA synthesis from EAS by using acetate and propionate as the sole or complex carbon sources. The TCA cycle, glyoxylate pathway, and succinate–propionate pathway are combined with Doi model *(15)*, and oxygen plays a very important role for adjusting 3HV molar fraction in PHBV co-polymer.

when ORP increased from −30 mV to +100 mV, i.e. alternating the anaerobic or oxygen-limited conditions to aerobic condition, the complete TCA cycle is functioning and there is no net succinate accumulated for the conversion of propionyl-CoA, and a few or no 3HV units will be synthesized, and hence PHB homopolymer is produced instead of PHBV copolymer.

On the other hand, the production of glycogen from fatty acids (C2 and C3, for example), and then the glycolysis of glycogen are also taken into account in the model. Comeau et al. *(21)* reported the accumulation of 3HV in addition to 3HB after the anaerobic uptake of various short chain organic acids. Based on the observation that glycogen in the activated sludge decreased in the anaerobic uptake of acetate, Mino et al. *(22)* suggested the significance of glycolysis as the supplier of reducing power in the anaerobic substrate uptake. Satoh et al. *(4, 16*) identified that the sink of carbon in the anaerobic uptake of acetate and propionate was PHA composed of 3HB, 3HV, 3H2MB, and 3H2MV. They measured the amount of substrate taken up, PHA in sludge increased, carbohydrates in sludge decreased, and phosphate released. The observed stoichiometric relations among the metabolic substances were in good agreement with their model, which assumes that glycolysis of glycogen supplies the reducing power necessary for the conversion of acetate or propionate into PHA. Thus the generation of glycogen from C2 and C3, and then glycolysis of glycogen, are involved in the hypothetic model (Fig. 4).

3HV Molar Fraction in PHBV by Using C3 as Carbon Source

It was demonstrated in Fig 3 that when C3 was submitted as sole carbon source to PHA production, the 3HV fractions in PHBV were decreased slightly from 83.2 mol% to 70.5 mol% with the increasing of ORPs from –30 mV to +100 mV, and in turn of the increasing of 3HB molar fraction from 16.8 mol% to 29.5 mol%. These results are in accordance with that of C2 as sole carbon source. However, the effect of DO concentration on the 3HV molar fraction in PHBV was not so efficient as that of C2 as sole carbon source. Obviously, these variations of 3HV molar fraction in the PHBA resulted from the changes of DO concentration in cultural broth via adjusting of ORP levels. Otherwise, notably no matter how much ORP was, the 3HV was the dominant unit in the PHBV copolymer while C3 as sole carbon source. The reasonable answer and explanations for these observed phenomena could be found from the hypothetic metabolic model in Fig. 4.

After entering the cells, part of C3 will be directly converted into propionyl-CoA as 3HV precursor; simultaneously, the other part of C3 will be decarboxylated and converted to acetyl-CoA for 3HV and 3HB synthesis, respectively. On the other hand, the acetyl-CoA derived from C3 will flow into the TCA cycle and glyoxylate pathway for energy and reducing power generation and biomass growth under aerobic condition. Noticeably, when C3 was used as carbon source the propionyl-CoA, the one of the 3HV

precursors, was converted directly and independently from C3 and was not through the TCA cycle and glyoxylate pathway in the absence of oxygen. Therefore, the effect of ORPs (i.e., DO concentration) on the 3HV fraction in copolymer is diminished even under anoxic and anaerobic conditions.

Conclusions

By using ORP to monitor and regulate the fermentation process instead a DO meter, the PHA accumulation process from EAS under low DO concentration could be controlled more precisely than the DO concentration does.

Adjusting the C2 to C3 ratios in the media could control the composition such as the 3HV/3HB ratios of the PHA from EAS. Furthermore, it might be an effective way to adjust the 3HV molar fractions in PHBV by controlling the DO concentration via ORP monitoring system.

When C2 and C3 were used as sole carbon sources, respectively, PHBV co-polymer accumulated in the EAS where the ORP was lower than +30 mV. The 3HV molar fractions in the PHBV co-polymer declined with the increasing of ORP from –30 mV to +100 mV by adjusting the gas-flow rate (i.e., the DO concentration). It is concluded that the DO plays a very important role in the synthesis of 3HV subunits in PHBV co-polymer from the EAS. Therefore, a hypothetic metabolic model was established.

This study implied the possibility to harvest the EAS from sewage treatment plant to produce PHA using co-culture of EAS instead of pure culture process. On one hand, it results in the decrease of the quantity of EAS for further treatment in the entire wastewater treatment process. On the other hand, it might make it possible to produce PHA in such a way that autoclaving and sterilization procedures become unnecessary compared with the conversional pure cultural process, and thereby result significant cost reduction of PHA production.

Acknowledgment

The authors gratefully acknowledge the support by a research grant from the Hong Kong Polytechnic University Research Committee.

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