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# **Production of polyhydroxybutyrate by activated sludge performing enhanced biological phosphorus removal**

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## **Abstract**

Recovery of biodegradable plastics from activated sludge is a promising sustainable technology. In this study, polyhydroxybutyrate (PHB) – a biodegradable plastics material - was produced by activated sludge performing enhanced biological phosphorus removal (EBPR) in batch experiments under anaerobic, aerobic and anaerobic/aerobic conditions. Under anaerobic conditions, the PHB content of 28.8% of dry biomass weight was obtained, while under aerobic or anaerobic/aerobic conditions, the maximum PHB content of 49-50% was achieved. The PHB production rate with respect to the volatile suspended solids (VSS) was 70 mg/g VSS·h under aerobic conditions, and this increased to 156 mg/g VSS·h under anaerobic condition and to 200 mg/g VSS·h under aerobic conditions with energy also supplied from polyphosphate. A side stream, with initially anaerobic conditions for PHB accumulation and phosphorus release, and then aerobic conditions for PHB accumulation, was proposed. In this side stream, biomass with a high PHB content and a high PHB production rate could be both achieved.

**Keywords:** Activated sludge; biodegradable plastics; enhanced biological phosphorus removal; polyhydroxybutyrate

## **1. Introduction**

In wastewater treatment processes, storage of organic carbon can be considered as one type survival mechanism for microorganisms experiencing dynamic “feast” and “famine” conditions (van Loosdrecht et al., 1997). In these processes, polymer-accumulating organisms can be enriched, and two important carbon polymers, polyhydroxyalkanoate (PHA) and glycogen, are usually stored (Dircks et al., 2001). When acetate and propionate/glucose are the carbon substrates, polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) are the main PHAs, respectively (Hood and Randall, 2001).

PHA plays an important role in wastewater treatment processes, and it can be sustainably recovered for the production of biodegradable plastics (Satoh et al., 1998); simultaneously, sludge reduction can be achieved. As a consequence, the costs for PHA production and sludge disposal will be decreased. Different wastewater treatment processes (aerobic dynamic substrate feeding processes and

anaerobic/aerobic processes) have been investigated for the accumulation of PHA (Satoh et al., 1998; Takabatake et al., 2000; Serafim et al., 2004). An aerobic dynamic substrate feeding process can be optimized for PHA accumulation and is suitable for treating industrial wastewater with excess organic carbon but with limited nitrogen and phosphorus. Anaerobic and aerobic alternating processes, such as enhanced biological phosphorus removal (EBPR), on the other hand, would be a better choice for treating wastewater with high carbon and phosphorus concentrations. In the EBPR process, polyphosphate-accumulating organisms (PAOs) can be acclimated to simultaneously accumulate PHA and polyphosphate for potential recovery, e.g., PAOs can store polyphosphate up to 15% for possible use as a fertilizer. At the same time, if PAOs in EBPR are able to accumulate a high proportion of PHA, EBPR processes could be used for both phosphorus and PHA recovery. Many studies have been carried out to examine the function and composition of PHA by EBPR microbial communities (Lemos et al., 1998; Pijuan et al., 2009). However, only limited studies have been carried out on the PHA accumulation in EBPR processes for the purpose of recovery (Satoh et al., 1998; Takabatake et al., 2000; Perez-Feito and Noguera, 2006; Kasemsap and Wantawin, 2007). Among these studies on the PHA recovery from EBPR, some only focused on PHA accumulation inside the treatment reactors (Perez-Feito and Noguera, 2006), while others focused on PHA accumulation potential (Takabatake et al., 2000). It is important to examine the metabolic processes of PAOs by including the dynamics of various polymers so as to optimize the system for both wastewater treatment and resource recovery.

PAOs can accumulate PHA under anaerobic conditions with the energy supplied from polyphosphate degradation or under aerobic conditions with excess external organic carbon being available. In this study, PHB production potential by activated sludge performing EBPR under anaerobic, aerobic and anaerobic/aerobic (initially anaerobic and then aerobic) conditions was investigated in batch experiments. Only PHB was focused on because acetate was used as the main carbon substrate and acetate is mainly stored as PHB (Hood and Randall, 2001). Furthermore, a strategy was proposed for both resource recovery and wastewater treatment.

## **2. Materials and methods**

### **2.1. EBPR acclimation**

A sequencing batch reactor (SBR) was operated at 20 °C and the reactor had a working volume of 5.4 litres. The SBR had four cycles per day and each cycle comprised the following phases: fill (15 min), anaerobic (105 min), aerobic (180 min), settle (40 min) and draw/idle (20 min). In each cycle, 1.8 litres of treated wastewater were exchanged with a new batch of synthetic wastewater. The reactor was constantly stirred with a magnetic stirrer at 500 rpm during the fill, anaerobic and aerobic phases; during the aerobic phase, air was supplied with an air diffuser located at the bottom of the reactor. Once a day, mixed liquor of 750 ml was withdrawn from the reactor just before the end of the aerobic phase, resulting in a solids retention time (SRT) of 7.2 days assuming no solids loss occurred during the settle phase.

The components of the synthetic wastewater were: 750 mg/l sodium acetate, 18 mg/l yeast extract, 120 mg/l NH<sub>4</sub>Cl, 200 mg/l K<sub>2</sub>HPO<sub>4</sub>, 130 mg/l MgSO<sub>4</sub>·7H<sub>2</sub>O and 18 mg/l CaCl<sub>2</sub>·6H<sub>2</sub>O. Trace elements (1 ml) were added following Barat et al. (2008).

Concentrated sulfuric acid (1.4 drops in 1 litre) was added to the synthetic wastewater to adjust pH to approximately 6.8. The reactor was seeded with activated sludge taken from Tuam Wastewater Treatment Plant, Co. Galway, Ireland.

## 2.2. Batch experiments

Batch experiments were carried out to examine PHB accumulation potential under (i) anaerobic, (ii) aerobic and (iii) anaerobic/aerobic conditions. 750 ml of activated sludge mixed liquor was withdrawn from the SBR and used for each condition.

For the anaerobic PHB accumulation, the 750 ml mixed liquor was settled in a glass flask for 20 minutes. Then 250 ml of the supernatant was removed. The remaining mixed liquor was purged with argon gas to remove oxygen, and 250 ml of synthetic wastewater was added to again provide a total of 750 ml mixed liquor. The 250 ml of synthetic wastewater had the same composition as that fed into the parent SBR, except that the sodium acetate concentration was increased to around 2865 mg/l. In addition, the synthetic wastewater was also purged with argon gas to remove oxygen before adding to the glass flask. The glass flask with 750 ml mixed liquor was sealed with a rubber stopper and stirred at 300 rpm in an incubator at 20 °C. Samples were taken at intervals to test soluble and particulate components including: orthophosphate (PO<sub>4</sub>-P), sodium acetate, carbohydrate and PHB. The top of the glass flask was supplied with argon gas during sampling so as to maintain anaerobic conditions. pH ranged from 7.3 to 7.5 during this anaerobic batch experiment.

For the aerobic PHB accumulation, the 750 ml mixed liquor was withdrawn from the SBR and then transferred to a 1000 ml glass flask. The glass flask was placed in an incubator at 20 °C. Air was continuously supplied at the rate of 2 l/min and the dissolved oxygen (DO) concentration was above 4 mg/l during the whole experimental period. Only sodium acetate was added at 0 (2539 mg/l after addition) and 120 minutes (2296 mg/l after addition) from the start of aeration. With the addition of only acetate, nitrogen limitation was adopted and this benefited PHB accumulation (Ntaikou et al., 2009). Samples were taken at intervals to test soluble and particulate components including: PO<sub>4</sub>-P, sodium acetate, carbohydrate and PHB. The initial pH was 7.6 and this increased to 9.4 by the end of the aerobic batch experiment.

For the anaerobic/aerobic PHB accumulation at 20 °C, the procedure combining the anaerobic PHB accumulation and the aerobic PHB accumulation was used. Firstly, the anaerobic PHB accumulation was carried out as described previously. After the anaerobic PHB accumulation, the mixed liquor was settled for 20 minutes, and the supernatant liquid (approximately 1/3 of the total volume) was exchanged with the effluent from the SBR to remove the released phosphorus and other nutrients. These settle and exchange processes (washing) were carried out twice. After dilution, nitrogen limitation occurred in the mixed liquor for benefiting PHB accumulation. Then the mixed liquor (around 700 ml) was aerated for PHB accumulation with only excess sodium acetate (2160 mg/l after addition) added at the start of the aeration. Samples were taken at intervals to test soluble and particulate components. The pH ranged from 7.4 to 7.5 during the initial anaerobic batch experiment; then increased to 7.77 after washing; and finally increased to 9.5 by the end of the aerobic batch experiment.

### 2.3. Analytical methods

NH<sub>4</sub>-N, nitrite (NO<sub>2</sub>-N), nitrate (NO<sub>3</sub>-N) and PO<sub>4</sub>-P was analyzed using a Konelab 20 analyzer (Thermo Clinical Labsystems, Vantaa, Finland). Sodium acetate was measured with high performance liquid chromatography (HPLC, Agilent 1200, Agilent Technology, USA) using a UV index detector and an Aminex HPX-87H column (Bio-Rad, USA). Separation during HPLC tests was achieved using a mobile phase of 1% (vol/vol) H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.6 ml/min, a column temperature of 65 °C, and a detector temperature of 40 °C. Suspended solids (SS), volatile suspended solids (VSS) and total phosphorus (TP) were determined according to standard methods (APHA, 1995). pH was measured using the WTW pH probe.

Total carbohydrate was measured after Maurer et al. (1997) by digesting activated sludge with a final HCl concentration of 0.6 M at 100 °C for 2 hours, and the samples were mixed at intervals during digestion. After digestion and centrifugation, carbohydrate concentrations were measured by means of the sulfuric-phenol method (Dubois et al., 1956).

PHB concentration was detected by the HPLC according to Karr et al. (1983) using the modified procedure as follows: (i) 2 ml of mixed liquor was centrifuged at 14,000 rpm for 5 minutes; (ii) the centrifuged biomass was washed with 50%, 75% and 96% ethanol (each 3 minutes) to dehydrate the biomass; (iii) the biomass was then transferred to a screwed glass tube by washing with 0.5 ml concentrated sulfuric acid twice; (iv) the tube containing the dehydrated biomass and the 1 ml concentrated sulfuric acid was heated at 100 °C for 30 minutes; (v) sodium 3-hydroxybutyrate (Sigma-Aldrich, Ireland) was digested at the same condition as those samples and used for calibration; and (vi) the HPLC conditions used for PHB detection were the same as those used in the acetate detection. The PHB content was presented as the ratio of PHB/SS.

## 3. Results and discussion

### 3.1. System performance

In this study, EBPR was achieved after 11 days acclimation, with the effluent PO<sub>4</sub>-P concentrations below 1 mg/l thereafter. The following batch experiments were carried out after more than 4 SRTs acclimation. With the influent PO<sub>4</sub>-P concentration of 36.3 mg/l, the effluent PO<sub>4</sub>-P concentration was below 0.9 mg/l, resulting in a removal percentage above 97.5%. The phosphorus content in the biomass was 12.8±0.4%. Nitrification occurred in the reactor, with only NO<sub>3</sub>-N detected in the effluent and its concentration below 4 mg/l.

Soluble (acetate and PO<sub>4</sub>-P) and particulate (PHB and carbohydrate) parameters dynamics in a typical cycle are shown in Fig. 1. Under the anaerobic phase, acetate uptake, phosphorus release, carbohydrate degradation and PHB production occurred. The pH decreased from 7.44 after fill to 7.30 by the end of the anaerobic phase. The ratio between phosphorus release and acetate uptake was 0.61 mol-P/mol-C (R<sup>2</sup>=0.98, n=4), which is in the theoretical range of 0.25-0.75 mol-P/mol-C as suggested by Smolders et al. (1994). The ratio of PHB production to acetate uptake was 1.31 mol-

C/mol-C ( $R^2=0.98$ ,  $n=5$ ), which is close to the theoretical value of 1.33 mol-C/mol-C in the PAO model of [Smolders et al. \(1995\)](#). Under the aerobic phase, PHB was utilized,  $PO_4$ -P was taken up, and carbohydrate was replenished. The ratio between carbohydrate production to PHB utilization was 0.33 mol-C/mol-C ( $R^2=0.80$ ,  $n=9$ ). The net  $NH_4$ -N utilized was 5.57 mg, corresponding to the produced biomass ( $C_5H_7NO_2$ ) of 44.9 mg or carbon of 23.8 mg (1.99 mmol-C). With the net PHB utilization of 7.55 mmol-C, the proportion used for biomass production was 26%.

### 3.2. PHB production in the anaerobic batch experiment

Under anaerobic conditions with excess acetate addition ([Fig. 2](#)), acetate was taken up with degradations of polyphosphate and carbohydrate, and simultaneously PHB was produced. Acetate uptake and PHB production continued even when there was no phosphorus release, and carbohydrate degradation was still observed. This indicates that carbohydrate could be used as the energy source by PAOs under excess organic carbon conditions, which has also been observed in other studies ([Erdal et al., 2008](#); [Zhou et al., 2008](#)). In this study, with the high phosphorus content in the biomass (12.8%) and a high influent P/C ratio (phosphorus/carbon, wt/wt) of 0.16, glycogen-accumulating organisms (GAOs) – competitors of PAOs in anaerobic and aerobic alternating processes – could have been washed out ([Liu et al., 1996](#)). Under the excess acetate condition, phosphorus of 276 mg/l was released. The TP concentration in the biomass was 349 mg/l, showing that 79% of the TP was released for energy supply. The ratio between phosphorus release and acetate uptake was 0.58 mol-P/mol-C ( $R^2=0.99$ ,  $n=3$ ). The ratio of PHB production to acetate uptake was 1.28 mol-C/mol-C ( $R^2=0.97$ ,  $n=8$ ). After 3 hours of anaerobic conditions, no significant increase in PHB was observed. The PHB content inside the dry mass increased from an initial of 4.7% to a final of 28.8% by the end of the anaerobic condition. During the initial 90 minutes of phosphorus release, the PHB production rate was 228 mg/l-h, or 156 mg/g VSS-h with respect to the initial VSS; thereafter, with the carbohydrate as the main energy source, the PHB production rate was reduced to 96 mg/l-h, or 66 mg/g VSS-h with respect to the initial VSS.

### 3.3. PHB production in the aerobic batch experiment

PHB production, acetate utilization, phosphorus release and carbohydrate utilization under aerobic conditions are presented in [Fig. 3](#). Under aerobic conditions, acetate uptake was accompanied by PHB production. The ratio between PHB production and acetate utilization was 0.96 mol-C/mol-C ( $R^2=1$ ,  $n=5$ ) during the first 2 hours and 0.62 mol-C/mol-C ( $R^2=0.99$ ,  $n=11$ ) thereafter. Although aeration was continued all the time and DO concentrations were above 4 mg/l, phosphorus release and carbohydrate utilization were observed. PAOs can release phosphorus under aerobic or anoxic conditions with excess external organic carbon being available ([Kuba et al., 1994](#); [Ahn et al., 2007](#)). Under aerobic conditions, with an initial TP of 319 mg/l in the biomass, the final released phosphorus was 196 mg/l, which showed that 62% of TP was released and used as an energy source. Carbohydrate degradation under aerobic conditions contributed to PHB accumulation, which was confirmed from the high ratio of PHB production to acetate utilization of 0.96 mol-C/mol-C during the first 2 hours. The PHB content increased from 1.5% to 45.9% after the 10 hours aerobic period, and this only slightly increased to 50% during a further 14 hours. During the first 2 aerobic hours, the PHB production rate was 309 mg/l-h, or 200 mg/g VSS-h



with respect to the initial VSS. In the next 8 aerobic hours, the PHB production rate was 109 mg/l·h, or 70 mg/g VSS·h with respect to the initial VSS. Considering the total aerobic period, the ratio between PHB production and acetate uptake was 0.74 mol-C/mol-C.

### 3.4. PHB production in the anaerobic/aerobic batch experiment

The dynamics of acetate, phosphorus, carbohydrate and PHB under aerobic conditions after an initial anaerobic phase are presented in Fig. 4. At the end of the anaerobic phase, the PHB content increased from 2% to 26%, which is similar to that obtained in the anaerobic experiment above. After the anaerobic phase and subsequent washing, there was a loss of biomass from 1.98 g SS/l to 1.64 g SS/l and the soluble PO<sub>4</sub>-P concentration was reduced from 260 mg/l to less than 13 mg/l. A slight increase in the PHB content from 26% to 30% was observed after washing, and could be because the biomass with high PHB content had a higher density than other biomass without PHB accumulation, and as a result, could settle more efficiently. This density difference between microorganisms with and without polymer accumulation has been used to isolate PHB accumulating organisms (Oshuki et al., 2008). Under aerobic conditions, acetate uptake was accompanied by PHB production. The ratio between PHB production and acetate utilization was 0.59 mol-C/mol-C ( $R^2=0.99$ ,  $n=13$ ). The PHB content was increased from 30% to 49% after 6 aerobic hours, and there was no significant increase thereafter. The PHB production rate during the aerobic condition was 84 mg/l·h, or 72 mg/g VSS·h with respect to the initial VSS. Combining the total anaerobic and aerobic periods, the ratio between PHB production and acetate uptake was 0.78 mol-C/mol-C.

### 3.5. PHB production rate and its content, and potential production strategy

Under aerobic conditions, the PHA production rate of 70 mg/g VSS·h or 42 mg/g SS·h is similar to that of 93 mg/g VSS·h by GAOs (Bengtsson et al., 2008) or 3.8-48.9 mg/g SS·h by activated sludge taken from various wastewater treatment plants (Takabatake et al., 2002). Under anaerobic conditions, the PHB production rate was increased to 156 mg/g VSS·h; furthermore, this could be improved to 200 mg/g VSS·h under aerobic conditions with energy also supplied from polyphosphate degradation. The higher PHB rate observed with polyphosphate degradation could have been due to (i) less energy supply limitation by using intracellular polyphosphate rather than oxygen; and (ii) the lower initial PHB content since the PHB production rate may decrease with increasing intracellular PHB contents (de Kreuk et al., 2007). These results showed that a high PHB production rate could be obtained with intracellular polymers as energy sources. A high PHA production rate up to 1090 mg/g SS·h was obtained by Kasemsap and Wantawin (2007) where intracellular polymers such as polyphosphate and carbohydrate were used as energy sources.

The maximum PHB content obtained in this study was 28% under anaerobic conditions and 50% under aerobic conditions. Kasemsap and Wantawin (2007) obtained a PHA content of 51% with a polyphosphate content of 8% in the biomass, and Takabatake et al. (2000) obtained PHA contents of 17-57% with anaerobic/aerobic sludge. These values are much higher than those of 8-25% obtained directly from EBPR reactors (Perez-Feito and Noguera, 2006). In the anaerobic and aerobic alternating processes, other possible microorganisms such as GAOs could also

be enriched, while a high PHB proportion could be still obtained. For example, the PHA content in GAOs was shown to be up to 42% during treatment of a paper mill wastewater (Bengtsson et al., 2008).

For PHB recovery from EBPR, using only anaerobic conditions, the PHB content inside the biomass was too low to be sustainably recovered; by using only aerobic conditions, the efficiency of carbon transfer from acetate to PHB was decreased due to the energy requirement for other purposes, and the polyphosphate content inside the biomass may decrease the content of PHB and further increase the recovery cost. Therefore, based on the above results, PHB accumulation, initially under anaerobic conditions, and then under aerobic conditions, could be a better choice and a proposed process for PHB production is given in Fig. 5. By this method, PHB accumulating organisms are enriched in the main stream, while PHB accumulation is achieved in the side stream as was shown also in other studies (i.e., Takabatake et al., 2000). In the present study, anaerobic and aerobic conditions are adopted in the side stream. In the side stream, phosphorus released under anaerobic conditions can be also recovered as fertilizer, such as through calcium phosphate or struvite crystal precipitation in fluidized bed reactors (i.e., de-Brshnan and Bashan, 2004; Le Corre et al., 2009; and references there); the sludge will be settled and separated for further PHB production under aerobic conditions. Under aerobic conditions, phosphorus will not be taken up if there is excess organic carbon available, and its recovery may be carried out after PHB production. By this means, phosphorus recovery from the liquid and PHB recovery from the solid could be processed together at the end of the aerobic phase and no separation will be required between the anaerobic and aerobic conditions. Nutrient removal and resource recovery (phosphorus and PHB) could be simultaneously achieved in the proposed system. Further studies on PHB recovery from EBPR should be carried out, such as in the area of (i) extending the aerobic phase in the main stream to enrich PHB accumulating microorganisms; (ii) producing short chain organic carbon by acidifying or alkaline hydrolysis of activated sludge for PHB production; and (iii) separating PHB accumulating microorganisms based on the different density between PHB accumulating microorganisms and other microorganisms without polymer accumulation.

## 5. Conclusions

PHB production using an acetate substrate by activated sludge performing EBPR was investigated under anaerobic, aerobic and anaerobic/aerobic conditions. The following can be concluded: (i) with the phosphorus content of 12.8% in the biomass, PHB accumulation under anaerobic conditions reached 28.8% of dry biomass weight; (ii) a PHB content of 50% was achieved under aerobic conditions, or anaerobic conditions followed by aerobic conditions; (iii) a high PHB production rate occurred when polyphosphate was used as the energy source alone or combined with aeration; and (iv) a system combining initially anaerobic conditions and then aerobic conditions would be better for both PHB production and phosphorus recovery.

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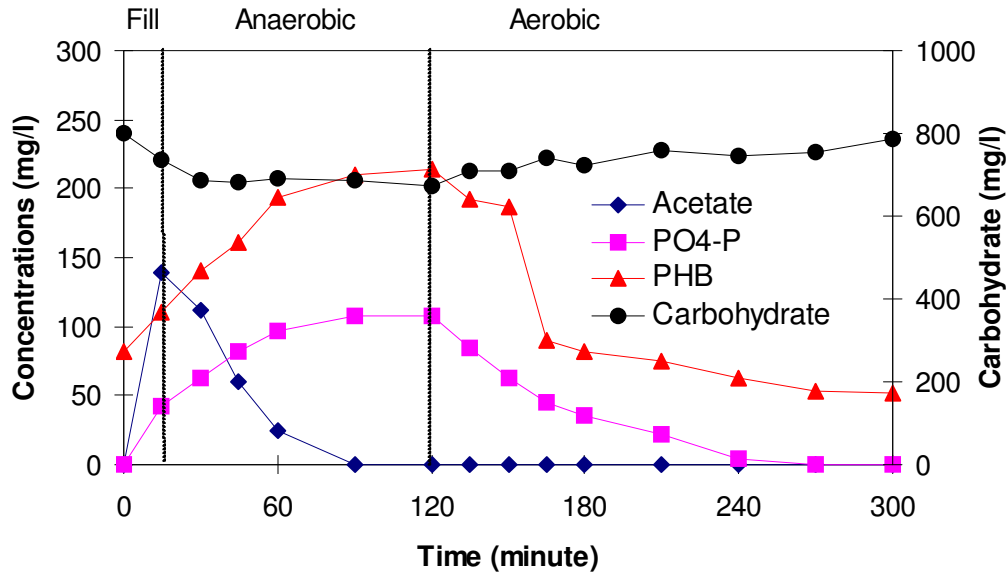


Figure 1. Dynamics of soluble acetate and PO<sub>4</sub>-P in the liquid, and particulate PHB and carbohydrate in the biomass in a typical reaction cycle.

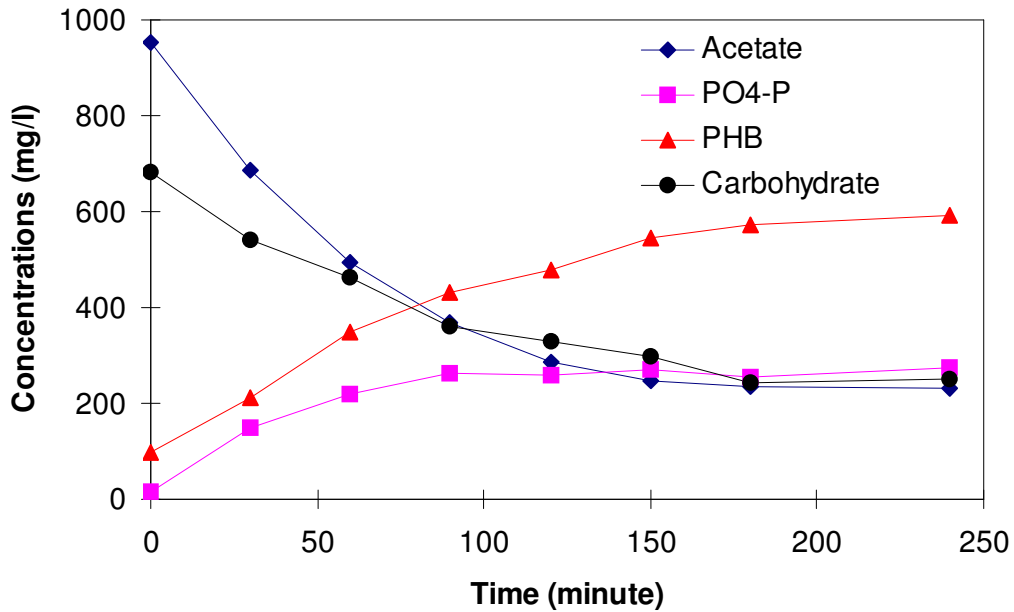


Figure 2. PHB accumulation under anaerobic conditions with excess sodium acetate (955 mg/l) addition. The initial volatile suspended solids concentration was 1.46 g/l.

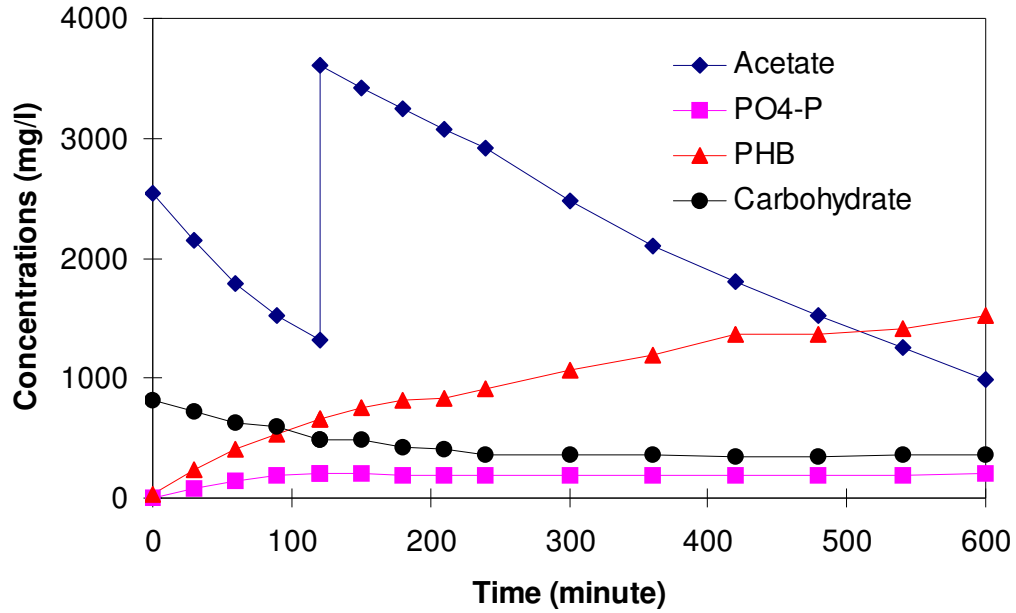


Figure 3. PHB accumulation under aerobic conditions with excess sodium acetate addition twice at Minutes 0 (2539 mg/l) and 120 (2296 mg/l). The initial volatile suspended solids concentration was 1.55 g/l.

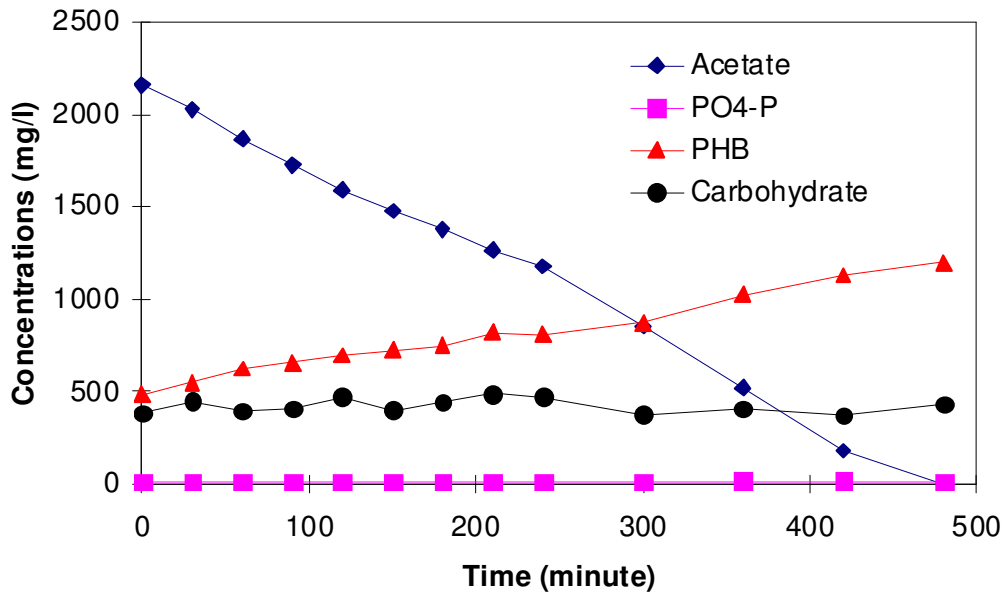


Figure 4. Accumulation of PHB under aerobic conditions with excess sodium acetate addition (2160 mg/l) after an initial anaerobic accumulation. The initial volatile suspended solids concentration was 1.42 g/l.

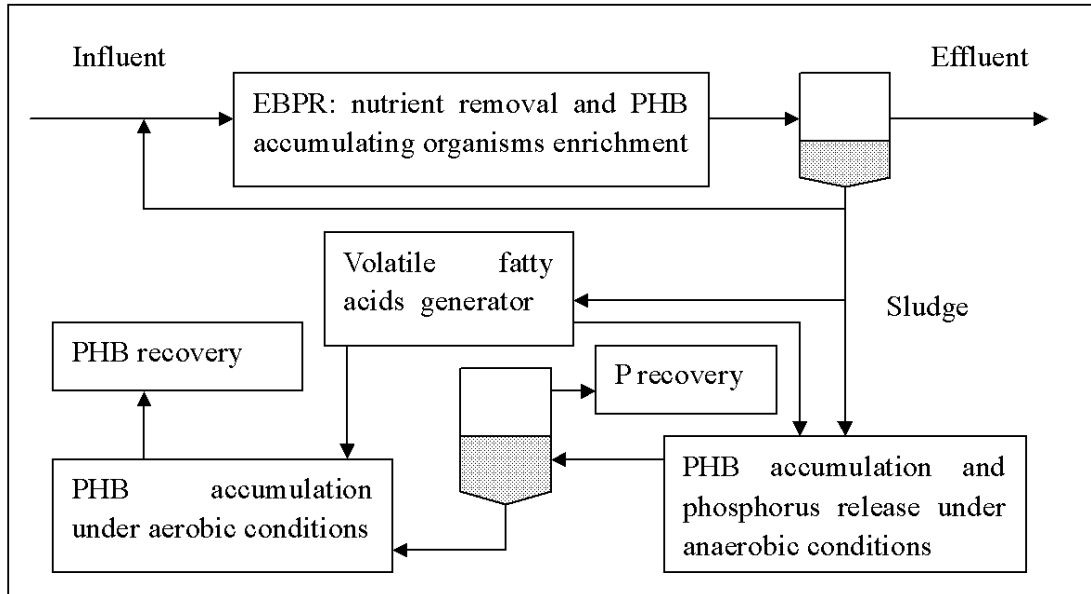


Figure 5. The proposed PHB production system by activated sludge performing EBPR.