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Microbiology of the Enhanced Biological Phosphate Removal process

[1]Raja Sonal Anand, [1]Dharmender Yadav, [1]Pramod Kumar and [2]Vikas Pruthi

[1]Department of Civil Engineering, Indian Institute of Technology Roorkee, Roorkee-247667, Uttarakhand, India
[2]Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee-247667, Uttarakhand, India

***Abstract :*Enhanced biological phosphorus removal (EBPR) is a well-established technology for removing phosphorus from wastewater. The process has been implemented in many wastewater treatment plants worldwide. The process remains operationally unstable in many systems, primarily because there is a lack of understanding regarding the microbiology of EBPR. Recent studies in this field have addressed this problem utilizing a wide range of approaches from studying the microorganisms that are primarily responsible for or detrimental to this process to determining their biochemical pathways and developing mathematical models that facilitate better prediction of process performance. A literature review of the recent developments in the process is presented in this paper which indicate that there are two types of microorganisms: Phosphorus Accumulating Organisms (PAOs) such as *Acinetobacter spp., Pseudomonas fluorescens, Bacillus cereus, Pseudomonas mendocina, Moraxella phenylpyruvica*etc. that have been observed as major groups of organisms responsible for EBPR; and phosphorus solubilizing microorganisms like *Pseudomonas chlororaphis, Acinetobacterbaumannii, Bacillus spp., Pseudomonas pickettii* etc. that enhance phosphorus uptake by other microorganism via making it available to them in the soluble form. Common metabolic pathways of EBPRsystems and metabolic models have also been discussed in this paper.**

Keywords: Enhanced Biological Phosphorus Removal, Phosphorus Accumulating Organisms, Wastewater Treatment, Microbiology

1. INTRODUCTION

It is well known that excess nutrients such as phosphorus (P) in water bodies result in serious eutrophication, which is currently a global problem. Eutrophication may affect the general aspects of water bodies, decreasing their aesthetic appeal and making necessary treatment for drinking water difficult and expensive. Aquatic life is also adversely affected by this excess vegetable matter due to its depletion of oxygen, slowing down the currents and sometimes producing toxic matters.

One strategy to decrease phosphorus in wastewater effluent is retrofitting the existing plants for enhanced biological phosphorus removal (EBPR), which is dependent on the capability of select microorganisms to remove phosphate from the liquid phase and convert it to the sludge phase in the form of intracellular polyphosphate. These microorganisms, known as polyphosphate-accumulating organisms (PAOs), are capable of luxury uptake-the accumulation of phosphorus beyond what is required for growth. While organisms in conventional activated sludge typically accumulate approximately 2% of dry biomass as phosphorus, EBPR organisms typically accumulate 4 to 8% in full-scale treatment plants and over 10% in laboratory systems using synthetic wastewater . The EBPR process typically can remove over 85% of phosphorus from domestic wastewater (Gebremariam et al., 2011).

When operated successfully, the EBPR process is a relatively inexpensive and environmentally sustainable option for P removal; however, the stability and reliability of EBPR can be a problem.Microbial competition between PAOs and another group of organisms, known as the glycogen (non-polyphosphate) accumulating organisms (GAOs), has been hypothesized to be the cause of the degradation in P removal. Like PAOs, GAOs are able to proliferate under alternating anaerobic and aerobic conditions without performing anaerobic P release or aerobic P uptake, and thus do not contribute to P removal from EBPR systems. Since GAOs consume VFAs without contributing to P removal, they are highly undesirable organisms in EBPR systems (Oehmen et al., 2007).

A deeper understanding of the microbiology of the EBPR process is required for improved performance and reliability of P removal from wastewater, better prediction and management of P removal failures andrefined design of EBPR plants leading to savings in construction and operating costs(Blackall et al., 2002).

While EBPR is indeed capable of efficient phosphorus (P) removal, disturbances and prolonged periods of insufficient P removal have been observed at full-scale plants on numerous occasions even under conditions that are seemingly favorable for EBPR. Recent studies in this field have utilized a wide range of approaches to address this problem, from studying the microorganisms that are

primarily responsible for or detrimental to this process, to determining their biochemical pathways and developing mathematical models that facilitate better prediction of process performance.

The objective of this paper is to understand the types of microorganisms involved in the EBPR process and to study their biochemical pathways.

2. MICROBES INVOLVED IN EBPR

The bacteria that are involved directly or indirectly in the EBPR process are the fermentative (acid-producing) bacteria, the PAOs; phosphorus solubilizing microbes and the GAOs.

2.1. Fermentative (Acid-Producing) Bacteria

Short-chain volatile fatty acids (VFAs) are absorbed by PAOs and stored as polyhydroxybutyrate, or PHBs, within the cells of these microorganisms. These VFAs are generated from complex particulate and soluble organic molecules during the fermentation process, which consists of three steps– hydrolysis, acidogenisis, and acetogenesis. Recent studies show that hydrolysis seems to be carried out by relatively few and specialized species. Several filamentous bacteria are involved, for example, *Microthrix* producing lipase and consuming long chain fatty acids, and *Chloroflexi* and the epiphytic bacteria *CandidatusEpiflobacter* producing proteases and consuming amino acids (Nielsen et al., 2010). Nielsen et al. (2012) found that fermenting bacteria constitute a large fraction (20%) of the microbial community in EBPR, and are dominated by *Firmicutes* and *Actinobacteria*. They however observed that bacteria involved in hydrolysis and fermentation are not well investigated in full-scale EBPR plants.

2.2. Phosphorus Accumulating Organisms

According to Seviour and McIlroy (2008) any population which accumulates more P than it requires for growth and which stains positively for polyPshould be considered as a putativePAO, regardless of whether it synthesizes PHA anaerobically or not. Several studies have indicated that *Accumulibacter* are the major PAO populations in both denitrifying and conventional EBPR processes, emphasizing the metabolic versatility of these bacteria. However, differences in physiologies among *Accumulibacter* strains may exist (Sidat et al., 1999).

Traditionally, based on cultivation experiments, *Gammaproteo* bacteria of the genus *Acinetobacter* were believed to be the only PAOs. However, today it has become clear that *Acinetobacter* can accumulate polyphosphate but does not possess the above described PAO metabolism. Furthermore, cultivation-independent methods and quantitative fluorescence in situ hybridization (FISH) have demonstrated that the relative abundance of *Acinetobacter* in EBPR systems was dramatically overestimated due to cultivation biases, further confirming that *Acinetobacter*is not of importance for EBPR (Hesselmann et al., 1999).

2.3. Phosphorus solubilizing organisms

These organisms enhance phosphorus uptake by other microorganism via making it available to them in the soluble form. Examples include *Pseudomonas chlororaphis, Bacillus spp., Xanthomonasmaltophilia, Acinetobacterbaumannii* etc.

2.4. Glycogen Accumulating Organisms

The GAOs have the ability under anaerobic conditions to assimilate substrates like acetate and use these to synthesize intracellular Polyhydroxyalkanates (PHA) under aerobic conditions. The GAOs, like the PAOs, are thought to metabolize this stored PHA, but they synthesize intracellular glycogen instead of polyP that the PAOs do. GAOs are viewed as potential competitors of the PAOs for anaerobic substrate uptake and thus a likely cause of EBPR failure (Seviour et al., 2003; Oehmen et al., 2007). Therefore it is recognized that maintaining conditions favoring the proliferation of PAOs over GAOs is critical for the stability of EBPR process. Since GAOs seem always to be present, but are suppressed as a minority in well-functioning EBPR processes, they may be scavengers for soluble COD, and may become the dominating population in deteriorated EBPR processes.The precise identity of these GAOs is still largely unknown. This is a major reason for the difficulty in fully comprehending the role of different GAOs in EBPR system failure.

3. MODELS RELATED TO EBPR PROCESS

3.1 The Comeau-Wentzel model

The Comeau-Wentzel model was initially developed in 1985. The salient points of this model are that (1) the model accepts the genus *Acinetobacter* as typical of the PAO group, and the carbon and phosphorus biochemical pathways specific to *Acinetobacter spp.* are recognized in this model; (2) the ATP/ADP and the NADH/NAD ratios are identified as the key parameters that may regulate these pathways (Liu et al., 2010).

- **Under Anaerobic Conditions**

The high extracellular acetate concentration allows passive diffusion of acetate into the cell. In the Comeau-Wentzel model, the intracellular acetate is activated to acetyl-CoA by coupled ATP hydrolysis, while the ATP hydrolysis releases cations (e.g., K^+ or Mg^{2+}) and the anion $H_2PO_4^-$.

Two acetyl-CoA molecules condense to form acetoacetyl-CoA, which is further reduced by $NAD(P)H_2$ to form hydroxybutyryl-CoA, which then is polymerized to form poly-β- hydroxybutyrate (PHB). Conversion of intracellular

acetate to PHB maintains a favorable concentration gradient for further diffusion of acetate into the cell. Organisms with stored PHB are able to use these as carbon and energy sources to grow and to assimilate phosphate to synthesize polyP under aerobic conditions (Liu et al., 2010).

To supply the reducing power $NAD(P)H_2$ needs to convert acetoacetyl-CoA to hydroxybutyryl-CoA and part of acetate is metabolized via the tricarboxylic acid (TCA) cycle. As a result, partial acetate is oxidized to carbon dioxide by TCA cycle for providing reducing power; meanwhile partial acetate is used for formation of PHB. The ATP required in the process is regenerated from ADP by transfer of an energy-rich phosphoryl group from polyphosphate (Poly P) to the ADP. Originally, this transfer was proposed to be direct, catalyzed by the enzyme ATP, e.g., polyphosphate phosphotransferase according to the following reaction:

$$(PolyP)n + ADP \leftrightarrow (PolyP)n-1 + ATP \quad (3.1)$$

However, evidence shows that there is an intermediate step in the ATP generation mediated by the combined action of the enzymes and AMP, i.e., polyphosphate phosphor transferase and adenylate kinase according to the following reactions:

$$(PolyP)n + AMP \leftrightarrow (PolyP)n-1 + ATP \quad (3.2)$$

$$ADP + ADP \leftrightarrow ATP + AMP \quad (3.3)$$

Whichever pathway is operative, the net result is a decrease in the stored Poly P concentration and a generation of ATP. Conversion of acetate (Ac) to PHB $(C_4H_6O_2)_n$ can be summarized as follows:

$$2nAc + 2nATP + nNADH_2 + CoASH \rightarrow (C_4H_6O_2)nCoA + nNAD + 2nADP + 2nPi \quad (3.4)$$

Metabolism of acetate via the TCA cycle for production of reducing power can be written as:

$$nAc + nATP + 4nNAD \rightarrow 4nNADH_2 + nADP + nPi + 2nCO_2 \quad (3.5)$$

The net result of these processes can be expressed as:

$$9nAc + 9nATP + CoASH \rightarrow (C_4H_6O_2)4nCoA + 9nADP + 9nPi + 2nCO_2 \quad (3.6)$$

It appears from Eq(3.6) that for every acetate utilized, one ATP is required, and one ADP and one Pi are generated. This gives a theoretical molar ratio of acetate uptake to P release of 1:1 (Liu et al., 2010).

3.2. The Mino Model

The Mino model was developed to explain observations on a laboratory-scale anaerobic/aerobic system receiving an artificial substrate of acetate, propionate, glucose, and peptone and observations on batch tests conducted using sludge from the laboratory-scale system.

In the laboratory-scale anaerobic/aerobic system measured the changes in soluble P, polyP, PHB, acetate, and intracellular carbohydrate. They observed a decrease of intracellular carbohydrates in the anaerobic phase and an increase in the subsequent aerobic phase. Evaluation of these results is hampered by uncertainty as to whether the analytical methodology used to determine carbohydrate adequately differentiated between extracellular and intracellular carbohydrates, while in some methods, extracellular carbohydrates are not separated from intracellular carbohydrates. For this reason, in describing the Mino model, no differentiation is made between extracellular and intracellular carbohydrates. Another point that requires clarification is whether the changes in PHB and carbohydrates are mediated by the same organism type, or by different organism types that may present in the mixed culture systems. To explain their results assumed that a single organism type would cause the observed changes in both carbohydrates and PHB. Obviously, this point requires further experimental clarification (Liu et a., 2010).

- **Under Anaerobic Conditions**

Acetate is first taken up by the organism, and intracellular acetate is activated to acetyl-CoA by coupled hydrolysis of ATP (P released to the bulk solution). The ATP required in Eq. (3.2) is supplied by the accumulated polyP. PHB is synthesized from acetyl-CoA (AcCoA) according to the following reaction:

$$2nAcCoA + nNADH_2 \rightarrow (C_4H_6O_2)n + nNAD + 2nCoASH \quad (3.7)$$

Up to this stage the Mino model is in agreement with the Comeau-Wentzel model. The main difference between the Comeau-Wentzel and the Mino model is the production of reducing equivalents required for the conversion of acetyl-CoA to PHB. The Mino model suggests thatreducing equivalents is produced by the conversion of glycogen to acetyl-CoA via pyruvate, and not by oxidation for acetyl-CoA via TCA cycle. Under anaerobic conditions, intracellularly stored glycogen $(C_6H_{10}O_5)_n$ is converted to pyruvic acid via the Embden-Meyerhof-Panas (EMP) pathway with the production of reducing equivalents ($NADH_2$). The pyruvic acid is further converted to acetyl-CoA with the production of carbon dioxide. The overall reaction for the breakdown of carbohydrate to acetyl-CoA can be expressed as follows:

$$(C_6H_{10}O_5)_n + 3ADP + 3nPi + 4nNAD + 2nCoASH \rightarrow 2nAcCoA + 4nNADH + 3nATP + 2nCO_2 \quad (3.8)$$

Thus, the reducing equivalents ($NADH_2$) required in the reduction of acetate to PHB under the anaerobic conditions are supplied by the consumption of carbohydrate via the

EMP pathway. By combining the reaction for the consumption of glycogen with that of the activation and conversion of acetate to PHB, the following net reaction for changes in intracellular carbon is obtained:

$(C_6H_{10}O_5)_n + 6nAc + 3nATP \rightarrow (C_4H_6O_2)_n + 3nADP + 3nPi + 2nCO_2$ (3.9)

For the bioenergetics of anaerobic substrate assimilation and PHA synthesis by PAOs, glycogen catabolism is thought to provide ATP for PHA production besides ATP from polyp degradation, and the amount of energy produced by glycogen depends on the pathway for glycogen catabolism (Liu et al., 2010.)

3.3. The Adapted Mino Model

- **Under Anaerobic Conditions**

Compared to the Mino model, the reducing equivalents in the adapted Mino model that convert acetate to PHB are supplied by consuming carbohydrates through the Entner–Doudoroff (ED) pathway. In fact, this has a significant influence on the stoichiometry of P release and acetate uptake because consumption of carbohydrates through the ED pathway produces markedly less energy than that produced through the EMP pathway, thus more energy production via polyP breakdown will be necessary to convert acetate to acetyl-CoA.

Consumption of carbohydrates via the ED pathway can be written as follows:

$(C_6H_{10}O_5)_n + 3nNAD + nNADP + 2nADP + 2nCoASH + 2nPi \rightarrow 2nAcCoA + 3nNADH + nNADPH_2 + 2nATP + 2nCO_2$ (3.10)

Eq. (3.10) shows that only 2 ATPs are produced, while Eq.(3.8) indicates that in the EMP pathway, 3 ATPs are generated per carbohydrate consumed. Acetyl-CoA produced by the consumption of carbohydrates is further converted to PHB according to Eq. (3.7). Combining Eq.. (3.10) and (3.7) gives the overall equation for the consumption of carbohydrates:

$(C_6H_{10}O_5)_n + 2nNAD + nNADP + 2nADP + 2nPi \rightarrow (C_4H_6O_2)_n + 2nNADH_2 + nNADPH_2 + 2nATP + 2nCO_2$ (3.11)

Assuming that Eq. (3.4) is acceptable for the production of PHB from acetate; the overall process can be summarized as:

$(C_6H_{10}O_5)_n + 6nAc + 4nATP \rightarrow (C_4H_6O_2)_n + 4nADP + 4nPi + 2nCO_2$

(3.12)

Here NAD and NADP have been used interchangeably, i.e., either form can be used in PHB synthesis. Comparison of Eq. (3.12) with Eq. (3.9) for the EMP pathway shows that in the ED pathway 4Ps are released for every 6Ac taken up, i.e., molar ratio of Ac taken up to P released is about 1.5:1; however, in the EMP pathway 6 moles of Ac are taken up for every 3 moles of P released, i.e., molar ratio of Ac taken up to P released is 2:1 (Liu et al., 2010).

- **Under Aerobic Conditions**

In the Comeau-Wentzel model, PHB is broken down and used for either anabolic or catabolic metabolism. In anabolism, carbon skeletons generated from PHB are incorporated into cell mass. In catabolism, the PHB is broken down to acetyl-CoA, which enters the TCA and associated glyoxylate cycles. Reducing equivalents ($NADH_2$) generated in these cycles are subsequently oxidized via the electron transfer pathway, and simultaneous oxidative phosphorylation generates ATP. The ATP generated is further used for cell energy requirements (e.g., biosynthesis) and synthesis of polyP. Phosphate uptake for polyP synthesis occurs via the hydroxyl mediated antiport, and cation uptake via the proton mediated antiport. However, it should be pointed out that the model does not explain the increase in intracellular carbohydrate and increase in extracellular carbohydrate.

4. CONCLUSIONS

- Phosphorus is an essential metabolic nutrients required for microbial growth and concentration as low as 0.1 ppm may lead to eutrophication of surface waters.
- The microbial community of the EBPR process seems to be diverse and consists of several major groups of microorganisms.
- Reducing power needed for PHA formation is produced mainly through degradation of internally stored glycogen, and not through the TCA cycle. The possibility of partial contribution of the TCA cycle to generation of reducing power, however, cannot be excluded. With the increase of evidence favoring a key role of glycogen in EBPR, the Mino model is now widely accepted.
- Metabolic models can serve as a bridge between research and practice, since they are ideal to serve as a representation of the state-of-the-art of our biochemical knowledge, and minimise the calibration effort required in full-scale applications.

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