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Contributed Paper

Culturable Bacterial Community in a High Temperature EBPR Reactor

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ABSTRACT

An enhanced biological phosphorus removal (EBPR) reactor operated at high temperature (28°C) exhibited good phosphorus removal efficiencies of 99% throughout a 2-year operation. To investigate the culturable bacterial community of this system, isolation of bacteria was carried out on agar media containing nutrients similar to that in the EBPR reactor. Analyses of 16S rRNA gene sequences of selected bacterial isolates were found to belong to α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Flavobacteria, Bacilli, Sphingobacteria and Actinobacteria. The dominant community was β-Proteobacteria which included *Acidovorax* spp., *Achromobacter* spp., and *Hydrogenophaga* spp. Out of the 24 species identified, some of the bacterial strains were not commonly found in previously reported EBPR processes. Thus, this has contributed to the uniqueness of microbial population in EBPR process operated at high temperature.

Keywords: enhanced biological phosphorus removal, temperature, isolation, bacterial community, polyphosphate accumulating organisms, *Candidatus* 'Accumulibacter phosphatis'

1. INTRODUCTION

Removal of phosphorus from wastewater is a key step in preventing eutrophication of downstream water bodies. To achieve this, enhanced biological phosphorus removal (EBPR) process is one of the popular mechanisms applied in municipal wastewater treatment plants. This biological treatment process relies on the ability of some microorganisms in activated sludge to accumulate phosphorus in excess of their growth requirement and store in the form of polyphosphate. The alternating anaerobic-aerobic condition of the EBPR process enables the selection and proliferation of these polyphosphate accumulating organisms (PAOs). PAOs release phosphorus along with carbon uptake under anaerobic condition and remove phosphorus which is

more than the released amount under aerobic condition. Thus, the excess phosphorus from wastewater will be removed via biomass settling and wastage. *Canditatus* 'Accumulibacter phosphatis' ('Accumulibacter') a β-proteobacterium affiliated with the *Rhodocyclus* group, is currently thought to be the PAOs primarily responsible for phosphorus accumulation in EBPR process.

The EBPR process is a relatively inexpensive and environmentally sustainable option for phosphorus removal if it is operated successfully. However, maintaining the stability and reliability of this biological process could be a great challenge sometimes. It is widely known that EBPR processes may experience deterioration in performance and even failures, causing violations to discharge regulations [1, 2]. In some cases, temperature plays a decisive role in EBPR performance. Poor phosphorus removal efficiencies have often been observed in the EBPR process operated at temperature higher than 20°C [3, 4, 5, 6]. Rabinowitz *et al.* [7] and Gu *et al.* [8] also reported deterioration of EBPR during summer seasons. It was claimed that the deterioration of EBPR process at higher temperature was associated with the predominance of glycogen accumulating organisms (GAOs), undesirable microorganisms which take up carbon source faster than PAOs but without performing phosphorus uptake. All these imply that the operation of EBPR process could be more problematic in a tropical climate.

Contrary to the previous observations, high phosphorus removal efficiency was achieved in a lab-scale EBPR reactor operated in our laboratory at 28°C, the average temperature of municipal wastewater in our tropical region. During the two-year operation, the reactor had been stably and reliably producing effluent with very low phosphorus, less than 1.0 mg/L. Thus, its microbial ecology is of great interest. To gain preliminary insights into the culturable microbial community of this high temperature EBPR reactor, bacteria was isolated from the reactor and later subjected to sequencing of the 16S rRNA genes analysis. Since microbial cultivability depends on suitable nutrients and familiar environment, agar media with nutrients similar to that in the reactor were used in order to reveal greater culturable microbial diversity. An attempt was also made to isolate the 'Accumulibacter' which till date remain uncultured.

2. MATERIALS AND METHODS

2.1 Reactor Operation

A 2-L anaerobic/aerobic sequencing batch reactor (SBR) was set up for EBPR process. The reactor was operated at room temperature of 28 °C ± 1 and in 6 cycles per day with 4 hours every cycle. The operational pH was controlled at 7.00 ± 0.05 by adding either 0.5 M HCl or 0.5 M NaOH. Each SBR cycle consists of five phases, 11 minutes of filling, 1 hour of anaerobic and 2 hours of aerobic conditions, followed by 40 minutes of settling and 9 minutes of decanting. Anaerobic period was achieved by nitrogen purging during the first 10 minutes of anaerobic period while aerobic condition was maintained by delivering air from air compressor to the mixed liquor. Solids retention time (SRT) and hydraulic retention time (HRT) applied to this process was 10 days and 10 hours respectively.

Synthetic wastewater was used in the SBR operation. The SBR was operated at 50 mg C/L feed, with acetate, yeast extract and peptone as carbon sources. The nutrients concentrations in the feed were previously reported in Ong *et al.* [9].

The performance of EBPR process were monitored by measuring mixed liquor volatile suspended solids (MLVSS), total organic carbon (TOC), orthophosphate (PO_4^{3-}) and intracellular polyhydroxybutyrate (PHB) content, as detailed in Ong *et al.* [9].

2.2 Fluorescent *in situ* Hybridization (FISH)

FISH was applied to the sludge samples to detect the presence of *Candidatus* 'Accumulibacter phosphatis' ('Accumulibacter'). The FISH procedure was performed according to Amann [10]. The oligonucleotide probes EUBmix (equimolar concentrations of EUB 338, EUB 338-II and EUB 338-III) were 5' labelled with FITC; PAOmix (equimolar concentrations of PAO 462, PAO 651, PAO 846) were 5' labelled with Cy3. All the probes used were commercially synthesized by Proligo Singapore, Singapore. Samples after FISH procedure were observed under a fluorescence microscope (Model DM 2500, Leica, German) and images were captured with a cooled charged-coupled device CCD) camera (Model DFC 310 FX, Leica, German). A minimum of 20 microscopic fields was captured randomly for each sample. The areas of each image that were positive for the PAOmix and EUBmix probes were measured using image analysis software, VideoTesT- Morphology 5.1. Cells hybridized to PAOmix probe in each field were statistically expressed as a percentage of the total area of bacteria hybridizing to the EUBmix.

2.3 Isolation

Before cultivation, mixed liquor was collected from the EBPR reactor and then centrifuged for 5 minutes at 3500 rpm (Sigma 3-16 P, United Kingdom). Supernatant of the samples were removed and the samples were resuspended in a solution of 0.9 % (w/v) NaCl. Subsequently, 0.1 mL of sludge sample (10^{-3} dilution with 0.9% (w/v) NaCl solution) was spread onto each culture medium containing agar. Two types of agar culture media were used, namely Medium A and Medium B. Medium A was prepared by using the synthetic wastewater recipe listed in Ong *et al.* [9] with addition of 1.5 % (w/v) of Bacto™ agar (Difco, USA). Medium B was similar to Medium A except the water used in the preparation of Medium A was replaced by the effluent from the EBPR reactor. All plates were incubated for 3 - 7 days at room temperature of 28°C. Individual colonies showing different morphology on the agar plate of each medium were isolated and purified. Isolates were grouped based on phenotypic similarities. One representative isolate was chosen from each group for subsequent 16S rRNA gene sequence analysis.

2.4 DNA Extraction

For genomic DNA extraction of the isolates, a loopful of bacterial cells was transferred into a 1.5 mL microcentrifuge tube and suspended in 1 mL of sterile ultra pure water. The DNA extraction was carried out by using NucleoSpin® kit (Macherey-Nagel, Germany) based on manufacturer's instructions.

2.5 PCR Amplification

PCR was performed in a total volume of 50 µL, prepared in parallel using universal bacteria primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r

(5'-TACGGYTACCTTGTTACGACT-3') [11]. The PCR mixture contained 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (0.2 mM), 0.25 µL *Taq*-polymerase (Promega, USA), 1 µL of each primer (10 µM),

32.75 μL of sterile ultra pure water, and 1 μL DNA extract. PCR amplification was carried out in a thermocycler (Swift™ Maxi, Esco, Singapore) with an initial denaturation step at 95°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 30 seconds, and elongation at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. Amplicons were purified by the NucleoSpin® Extract II PCR Clean-up Kit (Macherey-Nagel, Germany).

2.6 DNA Sequencing and Phylogenetic Analysis

Sequencing of the 16S rRNA genes of selected isolates was carried out using ABI 3730xl DNA analyzer. Similarity searches against the NCBI database were carried out using BLAST search (http://www.ncbi.nlm.nih.gov/) and EzTaxon-e Server [12]. A phylogenetic tree was constructed using the neighbour-joining method with MEGA 5 [13].

3. RESULT AND DISCUSSION

3.1 EBPR Reactor Performance

Two weeks after the reactor start-up, the conventional activated sludge used as the inoculums acclimated to the EBPR conditions by exhibiting carbon and phosphorus transformations like those found in established EBPR process. Figure 1(A) illustrates typical concentration profile of mixed liquor phosphorus and TOC and intracellular PHB of a monitoring SBR cycle on day 500 of operation. Figure 1(A) showed that the carbon sources were readily sequestered during the anaerobic stage, accompanying the release of phosphate. In the subsequent aeration stage, phosphate decrease rapidly from 50 mg P/L to 10 mg P/L in 40 minutes, and continuously decrease to below 1.0 mg P/L in one hours. As for PHB, it increased from 3 mg C /L to 50 mg C /L in the anaerobic stage and then decreased to 4 mg C /L at the end of aeration stage.

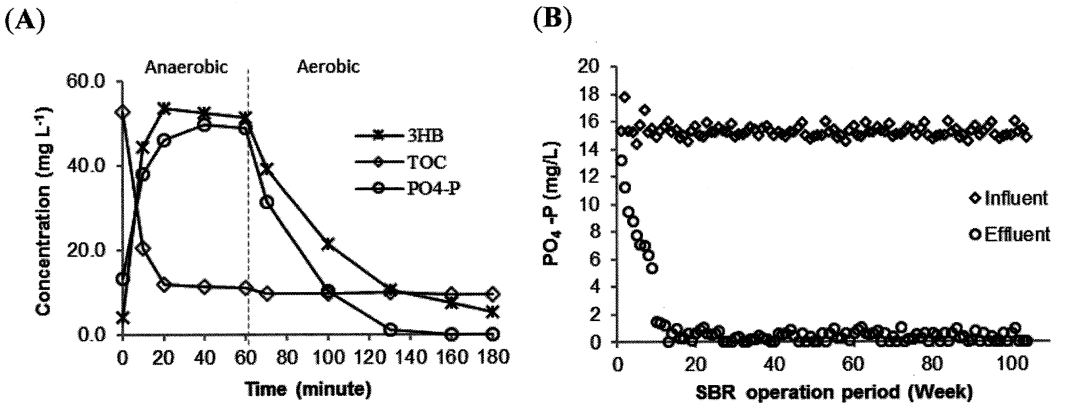

Figure 1. (a) Typical concentration profiles of TOC, PO_4-P and 3HB (mg C/L) in one SBR cycle on day 500 of SBR operation; (b) Concentration profiles of PO_4-P in the monitored cycles throughout the 2-year operation of EBPR reactor.

The continuous operation of EBPR process at 28°C for a period of 2 years showed stable MLVSS concentration of 4100 - 4500 mg/L. EBPR capacity continued to increase with phosphorus concentration in effluent stably and reliably maintained below 1.0 mg/L after 15 weeks of reactor operation and resulted a phosphorus removal efficiency of 99%, as shown in Figure 1(B).

From the FISH image showed in Figure 2, the numbers of 'Accumulibacter' detected by FISH was accounted for around 36% of the total eubacterial cells in the sludge sample collected during day 500 of reactor operation. The 'Accumulibacter' observed under the microscope were in the coccobacilli shape which is similar to that reported by Chua *et al.* [14].

Figure 2. Overlay FISH images of sludge sample from day 500 of reactor operation showing the PAOs (orange cell clusters) hybridized with both FITC-labelled EUBmix probe (green) and Cy3-labelled PAOmix probe (red). (Scale bar = 10mm).

3.2 Culturable Bacterial Strains

In the past studies that applied culture dependent approaches to examine the bacterial community present in activated sludge, culture media such as R2A agar, TS agar, Luria broth agar, were commonly used [15, 16, 17]. Realize that conventional nutrient medium may limit the number and diversity of microbes recovered from environmental samples due to the different of nutrient condition present, two agar media that better mimic the nutrient environment in the aforementioned EBPR reactor were formulated in this study.

With the two media used, isolates obtained were later screened and grouped based on morphology, size and color. These isolates were phylogenetically clustered into seven major classes and twenty four lineages or species, resulting 33.2% of the culturable bacterial community was b-Proteobacteria. The remaining 66.8% was distributed among a-Proteobacteria (n = 130; 29.5%), g-Proteobacteria (n = 49; 11.1%), Flavobacteria (n = 31; 7.0%), Bacilli (n = 64; 14.5%), Sphingobacteria (n = 10; 2.3%) and Actinobacteria (n = 10; 2.3%). This result is similar with the results reported by Lu *et al.* [16] which showed □-Proteobacteria was the most dominant group in sludge samples, in contrast to the observation by K□mpfer *et al.* [15], which showed that members of

g-Proteobacteria were the most dominant group. Table 1 lists the identification of isolates based on 16S rRNA gene sequences analysis. Besides, a phylogenetic tree constructed together with their closest relatives was shown in Figure 3.

Table 1. Identification of isolates based on 16S rRNA gene sequences analysis.

Affiliation	Identification (the nearest genus)	Similarity (%)	Number of isolates	OTU
α-Proteobacteria	Bosea	98-99	24	1
	Paracoccus	100	35	1
	Rhodobacter	97	17	1
	Shinella	98-100	16	2
	Sphingobium	99	19	1
	Sphingomonas	98-99	8	1
	Sphingopyxis	99-100	11	1
β-Proteobacteria	Acidovorax	99	37	1
	Achromobacter	98-100	69	4
	Hydrogenophaga	98-100	40	1
γ-Proteobacteria	Escherichia	99-100	12	1
	Pseudomonas	100	16	1
	Pseudoxanthomonas	100	8	1
	Stenotrophomonas	100	13	1
Flavobacteria	Chryseobacterium	99	31	1
Bacilli	Lysinibacillus	99	23	1
	Bacillus	100	41	1
Sphingobacteria	Sphingobacterium	99-100	10	1
Actinobacteridae	Streptomyces	99-100	7	1
	Micrococcus	99-100	3	1

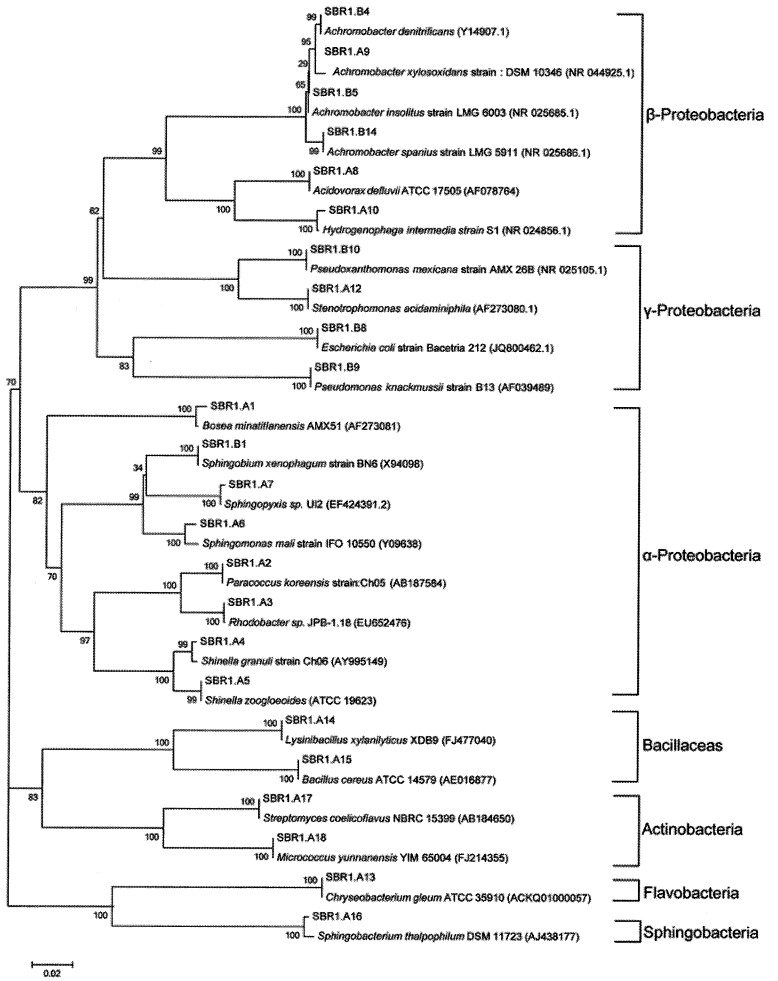

Figure 3. Neighbor- joining trees deduced from partial sequences of 16S rRNA genes of culturable bacterial from EBPR sludge sample. Bootstrap confidence values obtained with 1,000 replicates are given at the branch.

Those isolates assigned to the class of α-Proteobacteria, were closely related to seven different known genera which are *Bosea*, *Shinella*, *Paracoccus*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis* and *Rhodobacter*. The member of genus *Bosea*, a Gram-negative and straight to curved rod bacteria, obtained from this study, is closely related to a strain AMX51 (Accession number AF273081) isolated from anaerobic digester sludge [18]. As for the Gram-negative coccus shaped *Paracoccus* sp. found, it possess 100% pairwise similarity with a *Paracoccus koreensis* strain Ch05(T) (Accession number AB187584) isolated from granules of an upflow anaerobic sludge blanket (UASB) reactor [19]. Moreover, members in genus *Sphingomonas* have been reported and suggested as glycogen accumulating organisms (GAOs) as they were abundant in EBPR communities with poor P removal which the biomass had high glycogen content [20]. The presence of *Sphingomonas* sp., a potential GAO in this high temperature EBPR reactor without overruled the phosphorus removal further indicates the uniqueness of its microbial community in

sustaining good EBPR performance.

β-Proteobacteria was the most dominant group in this high temperature EBPR sludge. The isolates assigned to the class of β-Proteobacteria, were closely related to 3 different genera. Among these β-Proteobacteria members found, *Acidovorax* sp. was commonly reported in activated sludge processes, including EBPR process [21, 22, 23]. The species was well known for the utilization of a wide spectrum of carbon sources. With capability of reducing nitrate, *Acidovorax defluvii* could also produce polyhydroxybutyrate (PHB), which might be denitrifying polyphosphate accumulating organisms (DPAO) [24]. *Achromobacter* sp. and *Hydrogenophaga* sp. have rarely been reported previously in any EBPR communities. However, the *Hydrogenophaga* genus was observed in an activated sludge process [21] and found as the predominant type of bacteria in a fail EBPR process [23].

Four genera found related to γ-Proteobacteria, were *Escherichia, Pseudomonas, Pseudoxanthomonas,* and *Stenotrophomonas. Stenotrophomonas acidaminiphila* a Gram-negative and straight to curved rod bacteria obtained, is closely related to a strain AMX19(T) (Accesssion number AF273080) which was isolated from a lab scale UASB reactor treating petrochemical wastewater. Although the role of *Pseudomonas knackmussii* in the EBPR process is unclear, *Pseudomonas* sp. was reported to be capable of accumulating phosphorus.

Members of the class Actinobacteria which have been widely shown to participate in both laboratory and full scale EBPR plants [25, 26] were also found in the EBPR reactor in this study. *Streptomyces coelicoflavus* highly similar with strain NBRC 15399(T) and *Micrococcus yunnanensis* has 99% pairwise similarity with strain YIM 65004(T). The genus *Micrococcus* was also reported to present in an EBPR process treating municipal wastewater by Melasniemi *et al.* [22].

The only Flavobacteria affiliated species isolated is *Chryseobacterium gleum* which closely related to strain ATCC 35910(T). Flavobacteria was found to be an important group in mediating the liberation of inorganic orthophosphate from detrital organic phosphate of an aerobic activated sludge wastewater treatment process [27]. Since Bacilli and Sphingobacteria affiliated species have rarely been reported in EBPR communities, the ecological roles of these species obtained are unclear. However, most of *Baclilus* sp. can produce endospores that confer their survival in extreme environments; this genus is ubiquitous in nature with ability to degrade a wide range of substrates.

Despite the enrichment of 'Accumulibacter' in the high temperature EBPR reactor, its recovery from the agar culture media used was still without success. In the studies carried out by Kampfer *et al.* [15] and Lu *et al.* [16] on the analysis of microbial communities in EBPR process using culture dependent approaches via TS agar and R2A agar, the 'Accumulibacter' was also uncultured.

Differences between the growth environment applied in the EBPR reactor and culturing plates may have transformed the 'Accumulibacter' that were enriched in the reactor developed into dormant forms during culturing process. The differences include, changing from liquid growth environment to solid growth environment, negligence of feast and famine feeding condition, lack of pH control in the culturing plates and lack of proper control on the sequential anaerobic aerobic condition which provides a unique ecological niche for PAOs. Thus, to enhance the culturability of 'Accumulibacter', these differences need to be overcome or

minimized during the isolation procedure.

Most of the strains found in this study were not commonly reported in EBPR communities operated at temperate climate [28, 29]. It could have suggested a distinctive microbial community in the EBPR process operated at high temperature. At the same time, the culture media used would also exert a selective pressure towards the culturable bacterial composition. Nevertheless, this study has provided some useful preliminary microbial data for the EBPR process operated under tropical climate which have been very limited till date.

4. CONCLUSIONS

Isolation of bacteria from a high temperature EBPR reactor was performed using agar media that mimic the nutrients condition in the reactor. Phylogenetic analysis of partial 16S rRNA gene sequences of the isolates obtained showed that β-proteobacteria were the dominant microorganisms in the EBPR sludge sample, followed by α-Proteobacteria, γ-Proteobacteria, Flavobacteria, Bacillaceas, Sphingobacteria and Actinobacteria. Some of the culturable bacterial strains obtained in this study, have not been reported in EBPR process operated at milder temperature ($< 25°C$). Although the presence of 'Accumulibacter' PAOs was detected by FISH, it was not isolated through cultivation method. This observation reinforced that different approaches should be performed to better reveal the microbial diversity in this high temperature EBPR sludge. This study though is preliminary but it has provided useful information to the microbial data of high temperature EBPR process which is scarce till date.

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Conflict of Interests

All authors of this manuscript would like to declare that there is no any direct financial relation with the commercial identities mentioned in the paper that might lead to a conflict of interests.

REFERENCES

[1] Blackall L.L., Crocetti G., Saunders A.M. and Bond P.L., A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants, *Antonie van Leeuwenhoek,* 2002; **81**: 681-691.

[2] Thomas M., Wright P., Blackall L., Urbain V. and Keller J. Optimisation of Noosa BNR plant to improve performance and reduce operating costs, *Water Sci. Technol.,* 2003; **47**: 141-148.

[3] Panswad T., Doungchai A. and Anotai J. Temperature effect on microbial community of enhanced biological phosphorus removal system, *Water Res.,* 2003; **37**: 409415.

[4] Erdal U.G., Erdal Z.K. and Randall C.W. The competition between PAOs (phosphorus accumulating organisms) and GAOs (glycogen accumulating organisms) in EBPR (enhanced biological phosphorus removal) systems at different temperatures and the effects on systems performance, *Water Sci. Technol.,* 2003; **47**: 1-8.

[5] Whang L.M. and Park J.K. Competition between polyphosphate-and glycogen accumulating organisms in enhanced biological phosphorus removal systems:

Effect of temperature and sludge age, *Water Environ. Res.*, 2006; **78**: 4-11.

[6] Cao Y.S. 2011. *Biological Phosphorus Removal Activated Sludge Process In Warm Climates*. IWA Publishing, London.

[7] Rabinowitz B., Daigger G.T., Jenkins D. and Neethling J.B., *The Effect of High Temperatures on BNR Process Performance*, WEFTEC, 77th Annual Technical Exhibition and Conference, Oct. 2-6, 2004, New Orleans, USA.

[8] Gu A.Z., Saunders A.M., Neethling J.B., Stensel H.D. and Blackall L., *Investigation of PAOs and GAOs and Their Effects on EBPR Performance at Full-scale Wastewater Treatment Plants in US.* WEFTEC 05, Oct. 29- Nov. 2, 2005, Washington, DC, USA.

[9] Ong Y.H., Chua A.S.M., Lee B.P., Ngoh G.C. and Hashim M.A., An observation on sludge granulation in an enhanced biological phosphorus removal process, *Water Environ. Res.*, 2012; **84**: 3-8.

[10] Amann R., Ludwig W. and Schleifer K.H. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation, *FEMS Microbiol. Rev.*, 1995; **59**: 143-169.

[11] Lane D.J., 1991. 16S/23S sequencing, in: Stackebrandt E. and Goodfellow M., eds., *Nucleic Acid Techniques in Bacterial Systematics*, John Wiley, New York, 115-175.

[12] Kim O.S., Cho Y.J., Lee K., Yoon S.H., Kim M., Na H., Park S.C., Jeon Y.S., Lee J.H., Yi H., Won S., and Chun J. Introducing EzTaxon-e: A prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species, *Int. J. Syst. Evol. Microbiol.*, 2012; **62**: 716-721.

[13] Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.*, 2011; **28**: 2731-2739.

[14] Chua A.S.M., Eales K.., Mino T., Seviour R. The large PAO cells in full-scale EBPR biomass samples are not yeast spores but possibly novel member of *β-Proteobacteria*, *Water Sci. Technol.*, 2004; **50**: 123-130.

[15] Kämpfer P., Erhart R., Beimfohr C., Böhringer J., Wagner M. and Amann R., Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification verse *in situ* identification using group- and genus-specific rRNA-targeted oligonucleotide probes, *Microbial Ecol.*, 1996; **32**: 101-121.

[16] Lu S.P., Park M.J., Ro H.S., Lee D.S., Park W.J. and Jeon C.O., Analysis of microbial communities using culture dependent and culture independent approaches in an anaerobic/ aerobic SBR reactor, *J. Microbiol.*, 2006; **44**: 155-161.

[17] Jin D.C., Wang P., Bai Z.H., Wang X.X., Peng H., Qi R., Yu Z.S. and Zhuang G. Q. Analysis of bacterial community in bulking sludge using culture dependent and independent approaches, *J. Environ. Sci.*, 2011; **23**: 1880-1887.

[18] Ouattara A.S., Assih E.A., Thierry S., Cayol J-L., Labat M., Monroy O., Macarie H. *Bosea minatitlanensis* sp. nov., a strictly aerobic bacterium isolated from an anaerobic digester, *Int. J. Syst. Evol. Micr.*, 2003; **53**: 1247-1251.

[19] La H.J., Im W.T., Ten L.N., Kang M.S., Shin D.Y., Sung-Taik Lee, S.T. *Paracoccus koreensis* sp. nov., isolated

from anaerobic granules in an upflow anaerobic sludge blanket (UASB) reactor, *Int. J. Syst. Evol. Micr.*, 2005; **55**: 1657-1660.

[20] Beer, M., Kong, Y.H. and Seviour, R.J. Are some putative glycogen accumulating organisms (GAO) in anaerobic: Aerobic activated sludge systems members of the *alphaproteobacteria*? *Microbiol.*, 2004; **150**: 2267-2275.

[21] Snaidr J., Amann R., Huber I., Ludwig W., Schleifer K.-H. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge, *Appl. Environ. Microb.*, 1997; **63**: 2884-2896.

[22] Melasniemi H., Hernesmaa A., Pauli A. S.L., Rantanen P. and Salkinoja-Salonen M., Comparative analysis of biological phosphate removal (BPR) and non-BPR activated sludge bacterial communities with particular reference to *Acinetobacter*, *J. Ind. Microbiol. Biotechnol.*, 1998; **21**: 300-306.

[23] You S.J, Hsu C.L, Ouyang C.F. Identification of the microbial diversity of wastewater nutrient removal processes using molecular biotechnology, *Biotechnol. Lett.*, 2002; **24**: 1361-1366.

[24] Willems A., Falsen E., Pot B., Jantaen E., Hoste B., Vandamme P., Gillis M., Kersters K., Deley J., *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delajieldii*, E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delajieldii* comb. nov., and *Acidovorax temperans* sp. nov., *Int. J. Syst. Bacteriol.*, 1990; **40**: 107-119.

[25] Crocetti G.R., Hugenholtz P., Bond P., Schuler A., Keller J., Jenkins D. and Blackall L.L., Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantification, *Appl. Environ. Microbiol.*, 2000; **66**: 1175-1182.

[26] Kong Y.H., Nielsen J.L, Nielsen P.H. Identity and ecophysiology of uncultured actinobacterial poly P-accumulating organisms in full-scale enhanced biological phosphorus removal plants, *Appl. Environ. Microbiol.*, 2005; **71**: 4076-4085.

[27] Van Ommen Kloeke F. and Geesey G.G., Localization and identification of populations of phosphatase-active bacterial cells associated with activated sludge flocs, *Microbial Ecol.*, 1999; **38**: 201-214.

[28] Oehmen A., Lemos P.C., Carvalho G., Yuan Z., Keller J., Blackall L.L. and Reis M.A.M., Advances in enhanced biological phosphorus removal: From micro to macro scale, *Water Res.*, 2007; **41**: 2271-2300.

[29] Gebremariam S.Y., Beutel M.W., Christian D. and Hess T.F., Research advances and challenges in the microbiology of enhanced biological phosphorus removal - a critical review, *Water Environ. Res.*, 2011; **83**: 195-219.