Poll Res. 40 (2) : 526-534 (2021)
Copyright © EM International
ISSN 0257–8050

SCREENING MICROORGANISMS FOR PHOSPHORUS REMOVAL IN SALINE WASTEWATER

RAFITAH HASANAH[1,2], TSUYOSHI IMAI[1,*], ARIYO KANNO[1], TAKAYA HIGUCHI[1], MASAHIKO SEKINE[1] AND KOICHI YAMAMOTO[1]

[1]*Division of Environmental Engineering, Graduate School of Sciences and Technology for Innovation, Yamaguchi University, Yamaguchi 755-8611, Japan*
[2]*Faculty of Fisheries and Marine Sciences, Mulawarman University, Samarinda, Kalimantan Timur 75123, Indonesia*

(Received 18 October, 2020; accepted 1 December, 2020)

ABSTRACT

This study aimed at screening salt-tolerant phosphorus (P) accumulating organisms (PAOs) and investigating the P release and uptake of the organisms in saline wastewater. Batch experiments were conducted over 98 days in solutions with a salinity of 3.5% and P concentrations of 1, 5, 10, and 20 mg-P/L. The P-uptake ability of microorganisms increased by increasing P concentration from 1 to 20 mg-P/L. A high P removal percentage with an average of 85% was obtained at 10 mg-P/L after day 56. The uptake and release of P were observed in saline wastewater, signifying that salt-tolerant PAOs could grow in the saline solution. Bacterial screening by isolation and sequence analysis using 16S rRNA demonstrated that two cultivated strains, TR1 and MA3, had high similarity with *Bacillus* sp. and *Thioclava* sp. EIOx9, respectively.The colony morphology analysis showed that the colonies of TR1 were rod-shaped, milky-colored, round, shiny-viscous, smooth with a defined margin, while colonies of MA3 were cream-colored with smooth surfaces and raised aspect. The TR1 was gram-stain-positive with approximately 6-10 µm long and 1.2 µm wide cells, and MA3 was gram-stain-negative withabout 0.9 µm long and 0.5 µm widecells. The results demonstrated the involvement of *Bacillus* sp. and *Thioclava* sp. in the release and uptake of P, owing to their ability to grow in saline wastewater.

KEY WORDS: Phosphorus removal, Saline-wastewater, Bacterial screening, batch test, 16S rRNA

INTRODUCTION

Numerous industrial sectors, such as seafood processing, preservative industries, and cheese processing, likely generate highly saline wastewater. This wastewater contains large amounts of dissolved inorganic salts with elevated salinity levels up to the level of seawater (i.e., 3.5% w/w of sodium chloride, NaCl). Wastewater with salinity range 1-10 g/L is defined as saline wastewater. It features significant concentrations of salt and is high in nitrogen (N) and phosphorus (P) (Intrasungkha *et al*., 1999; Pernetti and Di Palma, 2005). Untreated saline wastewater may cause serious environmental pollution, where the excessive P discharge in seawater results in eutrophication. An important factor in improving the quality of aquatic ecosystems is to reduce P contamination in wastewater before discharge into water bodies (Hanrahan *et al*., 2005; Aydin *et al*., 2009).

Enhanced biological P-removal (EBPR) systems are simple and widely adopted for P removal, which is environmentally friendly and inexpensive. These systems include communities of microorganisms, some of which are phosphorus accumulating organisms (PAOs). They help remove P from wastewater by accumulating it inside their cells as polyphosphate (Wentzel *et al*., 1989). They use P for cell maintenance, nucleic acid synthesis, construction of cell membranes (as phospholipids),

and chemical reactions in cell energy transfer (such as adenosine triphosphate, ATP), while some are stored for future use (Krishnaswamy *et al*., 2009).

In accordance with previous studies, the removal of biological P in wastewater is highly dependent on its release under anaerobic conditions and the process of excess uptake under aerobic conditions. Controlling both conditions is critical to biological P removal (Li *et al*., 2003). The first stage of the EBPR process occurs under anaerobic conditions, in which an organic substrate in the form of volatile fatty acids (VFA) is taken in by PAOs and stored as polyhydroxyalkanoates (PHA), an internal polymer (Zou and Wang, 2016; Acevedo *et al*., 2017; Jiang *et al*., 2018). In aerobic conditions, PAOs use PHA as a source of carbon and energy for metabolism and cell growth and also restore stocks of glycogen and polyphosphate in the aerobic zone. To restore polyphosphate, PAOs take in excess P from solution (Bao *et al*., 2007; Jiang *et al*., 2018).

EBPR systems are sensitive to water quality fluctuations and environmental conditions, such as pH, temperature, and salinity. The biological treatment of saline wastewater makes the survival of salt-intolerant microorganisms impossible. Salt in wastewater triggers an outflow of intracellular-water and cell dehydration, leading to the loss of cell activity (Welles *et al*., 2015). Studies demonstrate that ammonia-oxidizing bacteria can tolerate NaCl concentrations of up to 33 g/l, while nitrite-oxidizing bacteria are intolerant to high-saline conditions. Only a few studies demonstrate the effect of salinity on biological P removal (Bassin *et al*., 2011; Wang *et al*., 2017; Welles *et al*., 2015). Here, we investigate the P removal ability of phosphorus accumulating organisms (PAOs) by isolating and identifying them under salinity conditions similar to those of seawater.

MATERIALS AND METHODS

Sample collection

Seawater and sediment samples were taken 1 m below the water surface during tidal flat conditions in Yamaguchi Bay (Yamaguchi, Japan). The samples were transferred into 500 ml plastic non-sterile bottles for analysis.

Artificial saline wastewater containing phosphorus

The aerobic medium was prepared from distilled water with the addition of 3.8 mg/l of NH_4Cl and artificial seawater with a salinity of 35.00‰. The anaerobic medium was synthesized so as to have the same composition as an aerobic medium with the addition of 680 mg/l of CH_3COONa and 540 mg/l of C_2H_5COONa. Various concentrations of P (1, 5, 10, and 20 mg/l of PO_4-P from KH_2PO_4) were added to both the anaerobic and aerobic mediums. To adapt of seawater pH the medium was adjusted to 7.9 using 1 M NaOH and 1 M HCl solutions.

Culture enrichment

The sediments (100 g) were added to 150 mL of artificial saline wastewater as an anaerobic medium. The cultures were given by feed medium every three days at 25 °C and shaken at 140 rpm. The hydraulic retention time of the cultivation was 16 h and 8 h under anaerobic and aerobic conditions, respectively. A 10 sponges, made of polyurethane with dimensions of 2 cm^3 were put in Erlenmeyer flasks, and was used as a bio-carrier surface for microorganisms to adhere to. Water was passed over the sponge surface to acclimatize the microorganisms growing outside the sponge as well as within its pores, ensuring sufficient growth surface. The cultivation duration was 112 days.

P-release and P-uptake measurements

A batch test was performed under both anaerobic and aerobic conditions, the shaker in mode off for anaerobic condition and when aerobic the shaker in mode on. To investigate the release and uptake of P by the microorganisms in saline wastewater sampling was done every two weeks for analyzing. The schematic diagram of the batch experiment setup is shown in Figure 1. The artificial feed medium contained indispensable nutrients for the growth of bacteria enabling P removal. However, to understand the effect of P concentration on its release and uptake by microorganisms, the experiments were conducted using different initial P concentrations. All tests were conducted in 300 ml Erlenmeyer flasks with 10 sponges, and 150 ml of artificial saline wastewater was used. The experiment was conducted in triplicates. The batch tests were operated in a horizontal shaker (EYELA-multi shaker IMS-1000; Rikakikai Co., Ltd. Japan) at 140 rpm and incubated at 25 °C under two alternating phases: anaerobic conditions (4 h) for P release and aerobic conditions (8 h) for P uptake.

PO_4-P was measured to evaluate P concentrations using the molybdenum blue method (Japanese Industrial Standards-JIS K102.46.3-2000) and a UV-

visible spectrophotometer analyzer (Spectrophotometer-800; Shimadzu Co., Ltd. Japan) at a wavelength of 880 nm. P concentrations were measured after filtration through a 0.45 µm syringe filter before and after the anaerobic and aerobic periods.

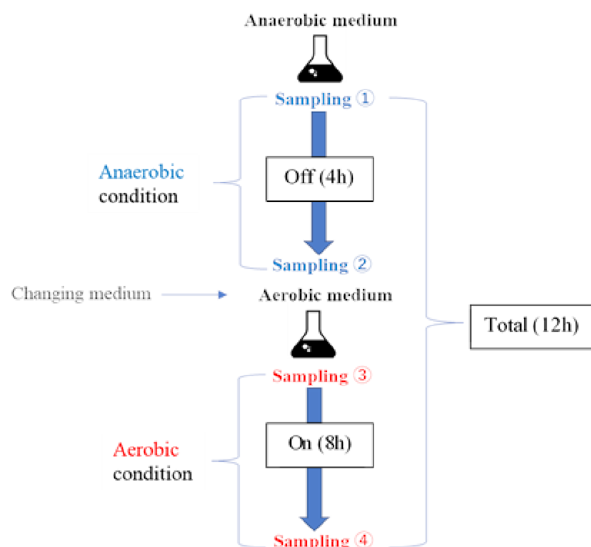

Fig. 1. Schematic diagram of the experimental setup.

Isolation and purification of bacteria strain

The bacteria were isolated from sponges that enriched in the Erlenmeyer flask. Microbial isolation was carried out by the pouring method by making a 10^{-3}-10^{-5} dilution series. R2A agar (Nihon Seiyaku, Japan) and Marine 2216 agar (Difco, Detroit, MI, USA) were used to grow and isolate. The plates were incubated at a temperature of 25-30 ° C for 3-5 days for isolation under aerobic and anaerobic conditions, for anaerobic condition the plate was given AnaeroPack (Mitsubishi Gas Chemical, Japan) to avoid the entry of oxygen during incubation. After being isolated, the next step is to carry out purification in order to obtain the desired pure culture without any contaminants from other microbes. Well-isolated colonies were purified by re-streaking on the same medium. Selection of purified microbial colonies based on differences in the appearance of colony morphology, observing the morphology of bacterial colonies with the naked eye to determine the general shape, margins, elevation, texture, and pigmentation. The Quebec colony counter was used because it had a magnifying glass and a backstage light for better viewing. After reaching pure culture, further analysis was carried out on it. The pH of Marine agar 2216 and R2A is adjusted to 7.6 and 7.2, using 1 M NaOH and 1 M HCl.

Microscope examination

Bacteria cells were Gram-stained according to (Smith and Hussey, 2005), andtheir cell morphology was examined by an optical microscope (IX70, Olympus, Tokyo) with 40x (UPlanFL N, Olympus) and 100x objectives (UPlanFL N, Olympus). Images were taken by a CMOS camera (DCC1645C-HQ, Thorlabs). For scanning electron microscopy (SEM), sample preparation was carried out according to the method suggested by (Kouzui, Tokikawa *et al.*, 2016). Bacteria cells were fixed on the surface of the coverslips coated with 0.01% Poly-L-Lysine (SIGMA) using a fixative of 2.5% Glutaraldehyde (Electron Microscopy Sciences), 2 mg/ml tannic acid in 20 mM MOPS pH 7.0. After 20 min of incubation, the samples were washed with distilled water (DW) and dehydrated by a series of ascending t-butyl alcohol to replace water inside cells. Samples were frozen at -20 °C and placed overnight in a plastic desiccator which was kept at low pressure by a rotary pump. The dried samples were coated with a thin layer of platinum with a sputter coater (MSP-1S, Vacuum Device, Japan) and immediately observed with a scanning electron microscope (S-4300Y, Hitachi) operated at a 2 kV accelerating voltage.

Determination of 16S rRNA gene sequence and phylogenetic analysis

The isolates were identified by 16S rRNA sequencing analysis using two universal bacterial primers: forward primer 27f and reverse primer 1492r. The polymerase chain reaction (PCR) amplification was performed in a 25 µl (total volume) reaction with 12.5 µl of PCR master mix (Emerald, Takara Bio Inc. Japan), 0.5 µl for each forward and reverse, 1.5 µl of template DNA and 10 µl of ddH_2O (double-distilled H_2O). It was performed by initial denaturation at 94 °C for 10 min, followed by 30 cycles of thermal cycling (denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and an extension at 72 °C for 1 min), followed by one final extension at 72 °C for 10 min. PCR products were run on 1.5% agarose gel stained with Ethidium Bromide (EtBr) (100 V for 30 min), visualized under blue light and photographed with Bio-Rad Universal Hood. The primers amplified 1,400–1,500 base pairs (bp). Amplicons of 16S rRNA were purified using a PCR purification kit

(Macherey-Nagel, Germany). The sequences in public databases at the National Center for Biotechnology Information (NCBI) were contrasted with the 16S rRNA gene sequences. To find similarities between colonies, a search was undertaken on the basic local alignment search tool (BLAST) of NCBI. Then, 1,000 replications were used as the basis for bootstrap values. Phylogenetic analysis was performed using MEGA version 6.0 (Tamura *et al*., 2013), with distance options according to the Kimura two-parameter model and clustering using neighbor joining (Saitou and Nei, 1987) and maximum likelihood (Felsenstein, 1985) methods. In each case, bootstrap values were calculated based on 1,000 replications.

RESULTS AND DISCUSSION

Phosphorus release and uptake

The ability of cultivated organisms in taking P from saline water was studied varying the P concentration. During a long-term operation, the alternating aerobic and anaerobic condition was provided by periodic switching off shaking process. Figure 2 illustrates the P-release and P-uptake ability, during batch-type experiments under anaerobic and aerobic conditions at various PO_4-P concentrations in saline wastewater for 98 days. The figure shows that P tended to be released and taken in during anaerobic and aerobic conditions, respectively. P release in the anaerobic phase is related to the uptake of the synthetic substrate by using energy generated via hydrolysis of stored polyphosphate and degradation of glycogen from intracellular stock (Wu *et al*., 2013). Poly-p hydrolysis releases orthophosphate into the solution. The accumulated substrate is then transported and stored as polyhydroxyalkanoate (PHA) (Bunce *et al*., 2018). In the subsequent aerobic conditions, the microorganisms are exposed to oxidizing conditions, leading to oxidation of PHA. The energy generated by oxidation is used for the uptake of P and synthesis of glycogen for cell-growth and regeneration of polyphosphates (Marais *et al*., 1983).

The visible P-release and P-uptake during cultivation and long-term batch testing implied the involvement of PAOs. In biological P removal by PAOs, the P release under anaerobic conditions is critical to P uptake in the subsequent aerobic phase, which ultimately affects the total efficiency of P

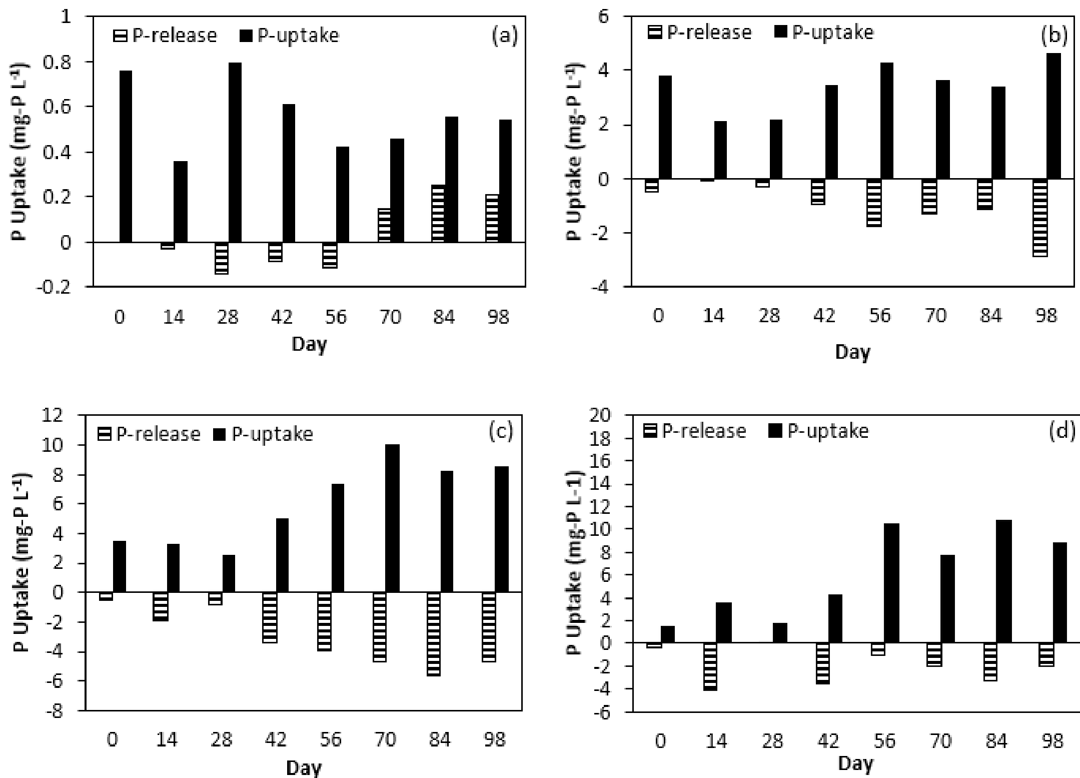

Fig. 2. P uptake and P release during 98 days in various P concentrations: (a) 1 mg-P/l, (b) 5 mg-P/l (c) 10 mg-P/l, and (d) 20 mg-P/l.

transfer (Li *et al*., 2003). The ability of PAOs to remove P decreases with increasing salinity (>2 %) owing to the reduction of the microbial community (He *et al*., 2020). However, in this study, P release and uptake were still observed at high P levels and salinity of 3.5%, denoting the growth of salt-tolerant PAOs. The P release and uptake demonstrated the existence of PAOs sufficiently feeding on P (Zhou *et al*., 2016; Wu *et al*., 2012).

Figure 2(a-d) shows that the P-uptake ability of PAOs increased with an increasing concentration of P from 1 to 20 mg-P/l. This clearly indicates that the organisms require P in the solution as an energy source for their growth. The higher P concentration provided, the higher P uptake obtained from each P concentration solution, which signifies that the higher amount of PAOs in higher concentration of P. Figure 2(b-d) demonstrates that PAOs in P concentration of 5, 10, and 20 mg-P/l needed 56-70 days to achieve the optimum P uptake and then fluctuated, even though a decrease was observed in 5 mg-P/l in the first 14 days.

P-uptake of PAOs in concentration of 1 mg-P/l reached maximum at day 28 (0.80 mg-P/l), after that decrease until day 56, and then fluctuated until day 98 (Figure 2(a)). Moreover, P-release was not observed with anaerobic conditions on some days, especially from day 70 to 98, whereas P uptake was detected. This decrease signified a lack of available P for microbial growth in aerobic conditions, and the bacteria involved in P removal were not yet enriched. Additionally, the competition with other organisms such as glycogen accumulating organisms (GAOs) could cause this ability decline. The limited P condition is limiting for PAOs growth while GAOs are favored under this limiting condition. The GAOs take in the synthetic substrate for their metabolism without releasing P (Mulkerrins, Dobson *et al*., 2004). This could explain the absence of P-release from day 70 in P concentration of 1 mg-P/l.

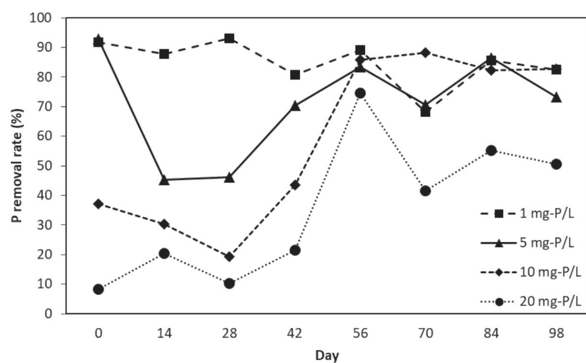

Fig. 3. Net P removal during 98 days in various P concentrations.

In order to investigate the effectivity of PAOs (that was cultivated from 100 grams of wet sediment) in removing P from solution, the P removal rate was defined. The P removal rate is P-uptake percentage that corresponds to its influent and effluent P concentration. The P removal rate during 98 days in various P concentrations is shown in Figure 3. Even though the P-uptake ability increased when the P concentration increased to 20 mg-P/l, however, the increase of PO_4-P concentration to 20 mg-P/l did not cause an increase of the uptake removal rate. After day 56, PAOs took in only around half of the P provided at 20 mg-P/l of PO_4-P concentration; however, they took in around 80% P at lower PO_4-P concentrations. This deteriorated removal rate indicted the saturation of the biomass provided on the sponge for P uptake. The capacity of PAOs to accumulate phosphate as poly-P depends on the biomass in previous condition. The maximum amount of poly-P accumulation is limited for existing PAOs in the reactor, they are not able to uptake any more P when they replenished all their possible poly-P. These results signify that 10 mg-P/l is the maximum effective P uptake by the cultivated salt-tolerant PAOs.

Colony and cell morphology

We therefore investigated the differences of colony morphology between strain TR1 and MA3 over specific agar mediums. Figure 4 shows two different types of colonies from each isolate which were obtained by using R2A for strain TR1 and Marine agar 2216 for strain MA3. From the appearance of the colonies, the strain TR1 appeared to be roundish, milky in color, and possessed a shiny and smooth surface with flat aspect in elevation.

To directly observe the cell morphological differences between strain TR1 and MA3, a SEM study was carried out. As shown in Figure 5, strain TR1 that was tested positive for Gram staining exhibited a rod-shaped form with 6-10 µm long and 1.2 µm wide in average. Meanwhile, strain MA3 that was tested negative for Gram staining showed short-rod-shaped cells with 0.9 µm long and 0.5 µm wide in average. The results showed that there were substantial differences between strain TR1 and MA3 morfologies.

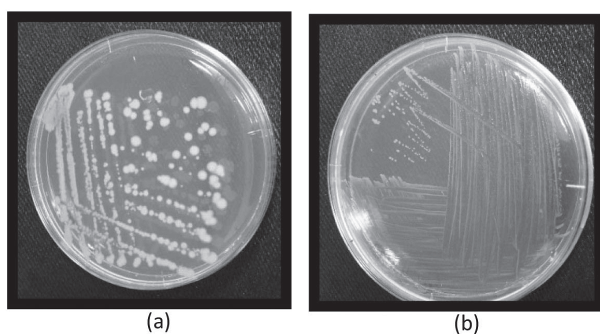

Fig. 4. Morphology of (a) strain TR1 colonies on R2A agar and (b) strain MA3 colonies on Marine agar 2216.

Molecular analysis

To obtain the detailed information of two cultivated strains, MA3 and TR1, the 16S rRNA sequencing analysis was conducted. The analysis using 16S rRNA gene sequencing showed that *Bacillus* bacterium was more abundant compared to *Thioclava*. By using NCBI's BLAST, the strain TR1 showed high similarity with *Bacillus* sp., *Bacillus megaterium*, and *Bacillus aryabhattai*, while the MA3 strain showed the highest similarity with *Thioclava* sp. EIOx9, *T. nitratireducences* 25B10-4, and *T. dalianensis* D2. The BLAST search of Genbank for all strains provided the percentage similarity between the cultivated microorganisms andthose detected in Genebank for three most similar representatives as shown in Table 1.

Moreover, the neighbor-joining tree based on 16S rRNA gene sequences was created to investigate the relationship between cultivated strains and their representative species (Figure 6). Thus, the molecular analysis results showed that the cultivated strains, TR1 and MA3, belonged to *Bacillus* and *Thioclava* species.

In order to confirm the ability of *Bacillus* sp. and *Thioclava* sp. in eliminating phosphorus from saline water, reviewing of some literatures was conducted.The *Bacillus* sp. tend to be tolerant to extreme physical and chemical conditions and can persist under various conditions owing to their ability to form endospores (Fajardo-cavazos and Nicholson, 2006). Further, they are able to sporulate under unfavorable conditions with low nutrition and oxygen concentration, which are essential for their growth (Choi *et al*., 2002). Two bacterial strains, *Bacillus aryabhattai* and *Bacillus* sp., has been found in the water of intensive catfish-pond sludge, and they showed the ability to take in polyphosphate (Khoi and Diep, 2013). Particularly, *Bacillus* is a group of proteolytic bacteria, which are cellulolytic, lipolytic, aerobic, or facultative anaerobes widely used in treating wastewater (Groudev, 1987). *Bacillus* sp. showed a reasonably high phosphate-accumulating ability (Brodisch and Joyner, 1983), whereas wastewater treatment system in Korea is operated with dominant bacilli strains which has capability of efficiently removing P(Choi *et al*., 2002).

While *Thioclava* sp. has been found to be salt-tolerant and they can grow both at 0 and 20 g/l salinity, even this bacterium can still be observed at salinities >20 g/l (Chen *et al*., 2018). *Thioclava* sp. can found every where in the marine environment and are distributed in various ecological niches, which

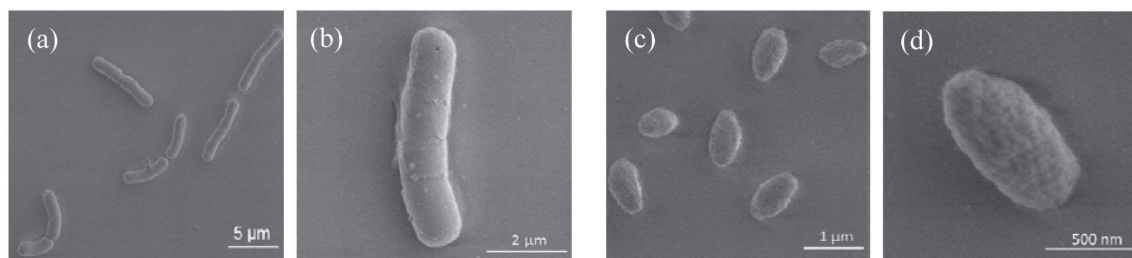

Fig. 5. Scanning electron microscopic micrographs of (a-b) strain TR 1 and(c-d) strain MA3.

Table 1. Similarity percentage of cultivated strains toward representative species in BLAST search

Strains	Representative species	Similarity percentage (BLAST)
TR1	*Bacillus* sp. (LC484742)	99.93%
	Bacillus megaterium (MK307786)	99.93%
	Bacillus aryabhattai (MN555367)	99.93%
MA3	*Thioclava* sp. EIOx9 (MG208121)	96.72%
	T. nitratireducences 25B10-4 (NR157648)	96.41%
	T. dalianensis D2 (MG547693)	95.08%

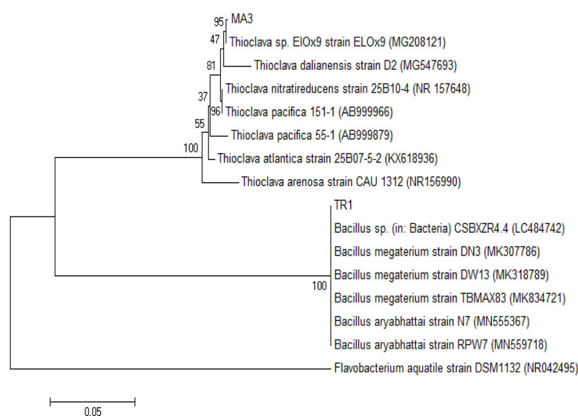

Fig. 6. Neighbor-joining tree showing the phylogenetic position of strain MA3, TR1, and related species, based on 16s rRNA gene sequence. *Flavobacterium aquatile* DSM1132 was used as an outgroup.

confirms their ability to live in saline environments (Liu *et al*., 2017). The consortium was found to include dominant genera that share significant sequence similarity to the sulfur-oxidizing genus, *Thioclava* sp., which has demonstrated chemoautotrophic growth on intermediate sulfur compounds including thiosulphate, and heterotrophic growth on simple organic matter including glucose (Sorokin *et al*., 2005). It is suggested that *Thioclava* sp. can improve the removal of N and P from saline wastewater. The study of *Thioclava* sp. to remove P in saline wastewater requires further research.

CONCLUSION

We demonstrated the growth of salt-tolerant PAOs through experiments using batch tests in saline media. The ability of PAOs to release and take in P was strongly affected by the concentration of PO_4-P. With very low PO_4-P concentrations 1 mg-P/l, the P-removal-efficiency was high on day one and tended to decrease with time due to the competition of getting nutrients with GAOs. The P-uptake ability of microorganisms increased by increasing P concentration from 1 to 20 mg-P/l. A high P removal percentage with an average of 85% was obtained at 10 mg-P/l after day 56. Bacterial screening by isolation and sequence analysis using 16S rRNA showed that *Bacillus* sp. (TR1) was Gram-stain-positive with rod-shaped and approximately 6-10 µm long and 1.2 µm wide cells. On the other hand, *Thioclava* sp. (MA3) was identified as Gram-stain-negative with short-rod-shaped and 0.9 µm long and 0.5 µm wide cells. The TR1 colonies were milky-colored, round, shiny-viscous, smooth with an entire margin, while MA3 colonies were cream-colored, with smooth and raised surfaces. Both isolates were identified in the saline wastewater, implying that they may be representative of PAOs and could live in environments with a salinity of 3.5%. Further, the two bacteria recorded in the study could be used for improving P removal in saline wastewater.

ACKNOWLEDGEMENTS

This work was supported by the Indonesian Ministry of Research and Higher Education Technology and funded by the Islamic Development Bank (IDB) Scholarship. We are very grateful to Prof. Katsuya Shimabukuro and Mr. Tatsuya from the National Institute of Technology, Ube College for helping us to prepare and analyze the samples for Scanning Electron Microscopy.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Acevedo, B., Murgui, M., Borras, L. and Barat, R. 2017. New insights in the metabolic behaviour of PAO under negligible poly-P reserves. *Chemical Engineering Journal.* 311 : 82-90.

Aydin, I., Aydin, F., Saydut, A. and Hamamci, C. 2009. A sequential extraction to determine the distribution of phosphorus in the seawater and marine surface sediment. *Journal of Hazardous Materials.* 168: 664-669.

Bao, L, Li, D., Li, X., Huang, R., Zang, J., LV, Y. and Xia, G. 2007. Phosphorus accumulation by bacteria isolated from a continuous-flow two-sludge system. *Journal of Environmental Sciences.* 19 : 391-395.

Bassin, J. P., Pronk, M., Muyzer, G., Kleerebezem, R., Dezotti, M. and van Loosdrecht, M. C. M. 2011. Effect of elevated salt concentrations on the aerobic granular sludge process: Linking microbial activity with microbial community structure. *Applied and Environmental Microbiology.* 77: 7942-7953.

Brodisch, K. E. U. and Joyner, S. J. 1983. The role of micro-organisms other than Acinetobacter in biological phosphate removal in activated sludge processes. *Water Science and Technology.* 15:

117-125.

Bunce, J. T., Ndam, E., Ofiteru, I. D., Moore, A. and Graham, D. W. 2018. A review of phosphorus removal technologies and their applicability to small-scale domestic wastewater treatment systems. *Frontiers in Environmental Science.* 6 : 1-15.

Chen, Y., He, H., Liu, H., Li, H., Zeng, G., Xia, X., and Yang, C. 2018. Effect of salinity on removal performance and activated sludge characteristics in sequencing batch reactors. *Bioresource Technology.* 249 : 890-899.

Choi, Y. S., Hong, S. W., Kim, S. J. and Chung, I. H. 2002. Development of a biological process for livestock wastewater treatment using a technique for predominant outgrowth of Bacillus species. *Water Science and Technology.* 45 : 71-78.

Fajardo-cavazos, P. and Nicholson, W. 2006. Endospores Isolated from Granite: Close Molecular Relationships to Globally. *Distributed.* 72 : 2856-2863.

Felsenstein, J. 1985. Confidence Limits on Phylogenies/ : An Approach Using the Bootstrap Author (s): Joseph Felsenstein Published by/: Society for the Study of Evolution Stable Society. 39 : 783-791.

Groudev, S. N. 1987. Use of heterotrophic microorganisms in mineral biotechnology. *Acta Biotechnologica.* 7 : 299-306.

Hanrahan, G., Salmassi, T. M., Khachikian, C. S. and Foster, K. L. 2005. Reduced inorganic phosphorus in the natural environment: Significance, speciation and determination. *Talanta.* 66 : 435-444.

He, Q., Wang, H., Chen, L., Gao, S., Zhang, W., Song, J. and Yu, J. 2020. Elevated salinity deteriorated enhanced biological phosphorus removal in an aerobic granular sludge sequencing batch reactor performing simultaneous nitrification , denitrification and phosphorus removal. *Journal of Hazardous Materials.* 390 : 121782.

Intrasungkha, N., Keller, J. and Blackall, L. L. 1999. Biological nutrient removal efficiency in treatment of saline wastewater. *Water Science and Technology.* 39(6) : 183-190.

Jiang, L., Wang, M., Wang, Y., Liu, F., Qin, M., Zhang, Y. and Zhou, W. 2018. The condition optimization and mechanism of aerobic phosphorus removal by marine bacterium *Shewanella* sp. *Chemical Engineering Journal.* 345 : 611-620.

Khoi, L. Q. and Diep, C. N. 2013. Isolation and phylogenetic analysis of polyphosphate accumulating organisms in water and sludge of intensive catfish ponds in the Mekong Delta, Vietnam. *American Journal of Life Sciences*. 1 : 61-71.

Kouzui, H., Tokikawa, K., Satomi, M., Negoro, T., Shimabukuro, K. and Fujii, K. 2016. *Gilvimarinus japonicus* sp. Nov., a cellulolytic and agarolytic marine bacterium isolated from coastal debris. *International Journal of Systematic and Evolutionary Microbiology.* 66 : 5417-5423.

Krishnaswamy, U., Muthusamy, M. and Perumalsamy, L. 2009. Studies on the Efficiency of the Removal of Phosphate Using Bacterial Consortium for the Biotreatment of Phosphate Wastewater. *European Journal of Applied Sciences.* **1** : 6-15.

Li, J., Xing, X. H. and Wang, B. Z. 2003. Characteristics of phosphorus removal from wastewater by biofilm sequencing batch reactor (SBR). *Biochemical Engineering Journal.* 16: 279-285.

Liu, Y., Lai, Q. and Shao, Z. 2017. A multilocus sequence analysis scheme for phylogeny of Thioclava bacteria and proposal of two novel species. *Frontiers in Microbiology.* 8 : 1-11.

Marais G. v. R., Loewenthal, R. E. and Siebritz, I. P. 1983. Observations supporting phosphate removal by biological excess uptake. A review. *Water Science and Technology.* 15(3-4) : 15-41.

Mulkerrins, D., Dobson, A. D. W. and Colleran, E. 2004. Parameters affecting biological phosphate removal from wastewaters. *Environment International.* **30**(2): 249-259.

Pernetti, M. and Di Palma, L. 2005. Experimental evaluation of inhibition effects of saline wastewater on activated sludge. *Environmental Technology.* 26: 695-704.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 4 : 406-425.

Smith, A. C. and Hussey, M. A. 2005. Gram stain protocols. *American Society for Microbiology.* 1: 14.

Sorokin, D. Y., Tourova, T. P., Spiridonova, E. M., Rainey, F. A. and Muyzer, G. 2005. Thioclava pacifica gen. nov., sp. nov., a novel facultatively autotrophic, marine, sulfur-oxidizing bacterium from a near-shore sulfidic hydrothermal area. International *Journal of Systematic and Evolutionary Microbiology.* 55 : 1069-1075.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution.* 30 : 2725-2729.

Wang, Y., Sun, C., Wang, Z., Zhao, H., Zhang, Y. and Zhou, W. 2017. Phosphorus removal by marine bacterium enhanced with iron in the presence of silicon. *J. Chem Technol Biotechnol.* 92: 1884-1890.

Welles, L., Lopez-Vazquez, C. M., Hooijmans, C. M., van Loosdrecht, M. C. M. and Brdjanovic, D. 2015. Impact of salinity on the aerobic metabolism of phosphate-accumulating organisms. *Applied Microbiology and Biotechnology.* 99 : 3659-3672.

Wentzel, M. C., Ekama, G. A., Loewenthal, R. E., Dold, P. L. and Marais, G. 1989. Enhanced polyphosphate

organism cultures in activated sludge systems. Part II: Experimental behaviour. *Water S.A.* 15(2): 71-88.

Wu, D., Hao, T., Lu, H., Chui, H. K., Van Loosdrecht, M. C. M. and Chen, G. H. 2012. Phosphorus release and uptake during start-up of a covered and non-aerated sequencing batch reactor with separate feeding of VFA and sulfate. *Water Science and Technology.* 65(5) : 840-844.

Wu, X., Du, Y. G., Qu, Y. and Du, D. Y. 2013. Ternary cycle treatment of high saline wastewater from pesticide production using a salt-tolerant microorganism. *Water Science and Technology.* 67(9): 1960-1966.

Zhou, W., Huang, Z., Sun, C., Zhao, H. and Zhang, Y. 2016. Bioresource Technology Enhanced phosphorus removal from wastewater by growing deep-sea bacterium combined with basic oxygen furnace slag. *Bioresource Technology.* 214 : 534-540.

Zou, H. and Wang, Y. 2016. Phosphorus removal and recovery from domestic wastewater in a novel process of enhanced biological phosphorus removal coupled with crystallization. *Bioresource Technology.* 211 : 87-92.