Doctoral Dissertation

APPLICATION OF MARINE BACTERIA FOR PHOSPHORUS REMOVAL FROM SALINE WASTEWATER

(塩分含有廃水からのリン除去のための海洋バクテリアの利活用)

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ABSTRACT

Nutrient pollution is one of our most pervasive, expensive, and challenging environmental problems, according to the United States Environmental Protection Agency (EPA). Phosphorus is one of the nutrients that are essential for the growth of living organisms. However, excessive amounts of nutrients released into the environment by human activities can harm ecosystems and impact human health. In surface waters, phosphorus can contribute to an overgrowth of algae called algal "blooms" that can sicken or kill wildlife and endanger aquatic habitats. Algal blooms consume dissolved oxygen in the water, leaving little or no oxygen for fish and other aquatic organisms. Algal blooms can harm aquatic plants by blocking the sunlight they need to grow. Some algae produce toxins and encourage the growth of bacteria that can make people sick who are swimming or drinking water or eating contaminated fish or shellfish. Phosphorus is often a major limiting nutrient freshwater system. Consequently, many of the wastewater treatment plant discharged into freshwater systems such as lakes, ponds, and rivers have phosphorus discharge limits. In an attempt to prevent harmful environmental effects of excess phosphorus, several techniques have been designed to remove phosphorus from wastewater. These techniques range from adsorption and precipitation to enhanced biological phosphorus removal and constructed wetlands.

Biological phosphorus removal (BPR) was first used at a few water resource recovery facilities in the late 1960s. A common element in EBPR implementation is the presence of an anaerobic tank (no nitrate and oxygen) before the aeration tank. In the next aerobic phase, these bacteria can accumulate large amounts of polyphosphate in their cells and phosphorus removal is said to be increased. The group of microorganisms that are largely responsible for P removal are known as the phosphorus accumulating organisms (PAOs).

One of the options to remove phosphorus is to utilize bacteria from nature, besides being easy to obtain and inexpensive. The application of bacteria from sediment and seawater was able to reduce phosphorus in wastewater. In this study, for screening salt-tolerant phosphorus accumulating organisms (PAOs) and investigating the P release and uptake of the organisms in saline wastewater. The samples used were sediment and seawater from Yamaguchi Bay, Yamaguchi, Japan. Sediment and seawater added 150 mL of artificial saline wastewater with media (anaerobic media). The samples were then cultured and given feed media every three hours day 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. 10 sponges made of polyurethane with dimensions of 2 cm 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. 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 with about 0.9 µm long and 0.5 µm wide cells. 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.

Furthermore, *Bacillus* sp. (TR1) and *Thioclava* sp. (MA3) were assessed for their abiotic adaptability and phosphorus removal efficiency in saline wastewater. The effects of abiotic factors such as carbon source, pH, temperature, and salinity on bacterial growth were examined through a series of batch experiments. Both bacteria used carbon sources such as glucose, sucrose, and CH_3COONa for their growth. The pH study indicated that *Bacillus* sp. (TR1) preferred the pH range of 6–8 and *Thioclava* sp. (MA3) preferred the pH range of 6–9. *Bacillus* sp. favorably multiplied in the temperature range of 25- 40 °C, while 25–35 °C was preferred by *Thioclava* sp. Salinity range of 0%–10% was favorable for TR1, with optimum growth observed at 3.5%–5%, and *Thioclava* sp. (MA3) preferred the salinity range of 1%–10% with optimal growth at 4%, but was absent in non-saline water. *Bacillus* sp. and bacterial combination (TR1 and MA3) showed similar values for phosphorus removal efficiency (100%) at 1.0 mg-P/L total P compared to *Thioclava* sp. (38.2%). The initial phosphorus concentration of 2.5 mg-P / L showed a slightly higher 72.35% P removal efficiency compared to the individual strains. However, phosphorus removal did not increase, but showed a downward trend with increasing at initial phosphorus. The combination possibly built a synergistic activity between the individual strains to remove phosphorus. The results demonstrated that when used individually, *Bacillus* sp. showed a reasonably high phosphorus removal ability than *Thioclava* sp., and exhibited good synergy when used in combination to remove phosphorus from saline wastewater.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABRREVIATIONS

%	Percent
‰	Part per thousand
°C	degree Celsius
µm	Micrometer
µL	Microliter
ATP	Adenosine Triphosphate
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
C	Carbon
CH_3COONa	Sodium acetate
C_2H_5COONa	Sodium propionate
cm^3	Cubical centimeter
ddH_2O	double-distilled hydrogen monoxide
dNTP	deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic
HCl	Hydrochloric Acid
EtBr	Ethidium Bromide
EBPR	Enhanced Biological Phosphorus Removal
g	gram
g/L	gram per liter
GAOs	Glycogen Accumulating Organisms
h	hour
ml	milliliter
mg/L	milligrams per liter
N	Nitrogen
MEGA	Molecular evolutionary genetics analysis
nm	nanometer
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NCBI	National Center for Biotechnology Information
NH_4Cl	Ammonium chloride
NH_4NO_3	Ammonium Nitrate
KH_2PO_4	Potassium dihydrogen phosphate
OD	Optical density
P	Phosphorus
PAOs	Phosphorus-accumulating organisms
PHA	polyhydroxyalkanoates
PHB	poly-beta-hydroxy-butyrate
PCR	Polymerase Chain Reaction
PO_4-P	Phosphate as Phosphorus
Poly-P	polyphosphate
rRNA	ribosome-Ribonucleic Acid
rpm	Revolutions per minute
R2A	Reasoner`s 2A agar
SEM	scanning electron microscopy
VFA	Volatile Fatty Acid
w/v	weight per volume

CHAPTER I
INTRODUCTION

1.1 Background

Phosphorus (P) is an essential element for all forms of life. In natural environments, phosphorus is mainly present in particulate form as minerals with low solubility. The phosphorus content in natural waters is usually regulated by microorganisms, so there is a balance in the demand for phosphorus and the available ecosystem. However, if P input exceeds the level of consumption of the ecosystem, the problem of phosphorus increases concentration arises. it can also be considered a pollutant if the concentrations are high under specific environmental conditions (Smil, 2000).

Large quantities of phosphate-rich saline present in wastewater that negatively affects many natural water bodies, both fresh water and marine. Coastal marine eutrophication caused industrial activity such as agriculture, aquaculture industries, food processing industries, the drainage of saline farmlands or use of seawater for toilet flushing whereas, this wastewater contain phosphorus (Graaff et al., 2020; Lefebvre and Moletta, 2006; Sefelnasr and Sherif, 2014). Increased phosphorus concentration also results in higher water treatment costs, decreased recreational value, and livestock losses. There is also a high probability of the sub-lethal effect because of toxins from algae in the drinking water (Awual, 2019; Schmale et al., 2019; Wang et al., 2021).

Due to its ability to remove phosphorus, enhanced biological phosphorus removal (EBPR) has become a standard treatment procedure in recent years. This was accomplished by increasing the amount of group bacteria, which collect phosphate in excess of their metabolic requirements for development. The existence of an anaerobic tank before the aeration tank is a typical feature in EBPR implementation. Under these circumstances, a heterotrophic species of bacteria known as polyphosphate accumulating organisms (PAOs) are preferentially enriched in the bacterial population of activated sludge. During the following aerobic phase, these bacteria can amass huge quantities of polyphosphate in their cells, and phosphorus removal is stated to rise (Pasayeva et al., 2011; Pijuan et al., 2004; Welles, 2016; Yuan et al., 2012)

Biological treatment to remove phosphorus appears to be a good alternative in saline wastewater. To support phosphorus recovery, reliable and high-performance EBPR in saline wastewater is desirable to sequester as much phosphorus as possible to maximize the ability of bacteria. Despite years of EBPR research, questions remain regarding the microbial mechanism of phosphorus removal and the various problems that cause reliability and performance issues, particularly in saline wastewater under similar seawater conditions. However, those saline wastewaters contain nutrients (C, N, P) that need to be removed from the water before its discharge to the environment, to avoid severe environmental issues like

hypoxia and eutrophication. Several biological nutrient removal processes have been developed to remove C, N and P, but the microorganisms responsible for the biological nutrient removal processes may be severely affected by salinity. Although the salinity effects on the C and N removal have been studied, the salinity effects on P removal are unclear. Only few studies have been conducted on the EBPR process but the outcomes from these studies are inconclusive and inconsistent (Khannous et al., 2003; Uygur, 2006; Wang et al., 2018)

1.2 Dissertation objectives

The goal of this study phase was to learn more about the microbial communities and pure culture observed in EBPR process in saline wastewater. In the first part of this study, a mixed PAOs culture was enriched and its influence on the performance of the phosphorus release and uptake process in saline wastewater was investigated by a batch test. Whereas, in the second part of the study, the ability of pure culture PAOs to proliferate and remove phosphorus in saline wastewater under various initial phosphorus concentrations were observed. As a result, it is critical to discover and enrich PAOs that can thrive in variable phosphorus concentrations in saline wastewater, which was similar to that of seawater. The sample from seawater and sediment were taken 1 m below the water surface during tidal flat conditions in Yamaguchi Bay, Yamaguchi, Japan.

Four sub-objectives have been formulated to obtain the goal mentioned above:

1. To investigate P release and uptake ability of Phosphorus accumulating organisms (PAOs) in saline wastewater with various initial P-concentrations
2. To isolate and identification Phosphorus accumulating organisms (PAOs)
3. To examine the effect of environmental factors on the growth activity of *Bacillus* sp. and *Thioclava* sp.
4. To determine their individual and combination bacteria for P removal performance at different P concentrations from saline wastewater

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CHAPTER 2

LITERATURE REVIEWS

2.1. Phosphorus in saline wastewater

Seafood processing, pharmaceuticals, agricultural and textile industry, for example, produce saline effluent with high quantities of inorganic salts (Figure 2.1). These industries, produces significant amounts of saline wastewater with high levels of nutrients and organics, and turns 95 percent of the water it uses into sewage. Saline and hypersaline wastewater account for approximately 5% of total global influent to wastewater treatment plants (WWTP). Salts dissolved in saline wastewater will affect aquatic life in water bodies and inland lakes in this situation. (Lefebvre et al., 2004; Zhang et al., 2014; Zhao et al., 2020).

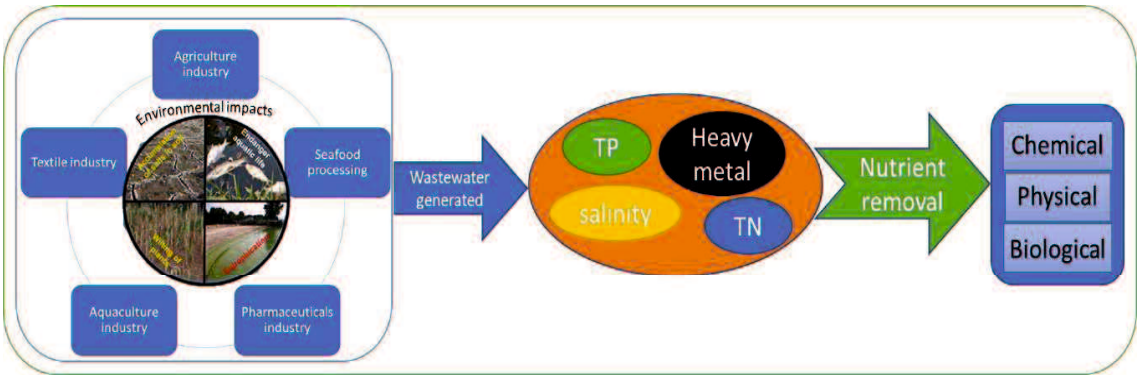

Figure 2.1 Saline Wastewater Generated (modified from Srivastava et al., 2021)

Wastewater is usually defined as saline wastewater when the salinity is below 10 g/ L, otherwise it is regarded as hypersaline wastewater or brines (Pernetti and Di Palma, 2005). Most of these saline wastewater's discharges are regarded as the primary source of the increasing P concentration in the water. The release of active phosphate, which is the most common type of phosphorus in phosphorus-rich saline wastewater, into aquatic ecosystems causes eutrophication, excessive algae reproduction, fish and other organism death, and ecological imbalance. Furthermore, high phosphorus levels in water may cause metabolic bone disease, which is hazardous to human health (Wang et al., 2021). The Australian and New Zealand guidelines for fresh and marine water quality state that to ensure the protection of aquaculture species, phosphate concentration from primary industries should be <0.1 mg/L in freshwater and <0.05 mg/L in salt water (NSW Health et al., 2000)

Salt is known to inhibit biological wastewater treatment processes in terms of chemical oxygen demand (COD) removal, nitrification, denitrification and phosphate removal (Uygur, 2006; Uygur and Kargi, 2004; Welles et al., 2015). To treat saline effluents, a

variety of technologies, such as biological and physics-chemical systems, can be used; however, physics-chemical procedures consume a lot of energy and have significant startup and operational costs. Alternative unit processes for saline wastewater treatment include anaerobic or aerobic biological treatments. As a result, saline wastewater is generally treated using biological procedures that include a significant number of microorganisms. Using bioreactors, phosphorus-accumulating organisms (PAOs) are routinely employed to extract phosphorus from sewage (Q. Liu et al., 2017; Pernetti and Di Palma, 2005; Ye et al., 2016).

However, using biological treatment strategies to treat saline wastewater may be problematic. A large number of research on the biological treatment of saline wastewater have revealed that salt has a considerable detrimental influence on the performance of biological systems. For example, it might lead to an increase in the concentration of suspended particles in effluent, a decrease in organic removal efficiency, and the inhibition of bacterial metabolism (Bassin et al., 2011; Chen et al., 2018; Qin et al., 2019).

The biological treatment of marine wastewater was unsuccessful in eliminating nitrogen and phosphorus because the capacity of microorganisms in activated sludge to extract these nutrients is reduced in saline settings. Another negative consequence of high salinity is decreased microbial adaptability, which reduces sludge settling in biological treatment (Bassin et al., 2011; Pronk et al., 2014). Because it is difficult for activated sludge to remove nutrients in the surrounding marine wastewater, microorganisms must be resistant or adaptable to high salinity in the marine wastewater in order for successful treatment to occur (Salmanikhas et al., 2016).

The presence of high salt in wastewater not only hinders the metabolic processes of heterotrophic bacteria involved in biological wastewater treatment, but it also affects the effectiveness of the remediation process. Various environmentalists have isolated and enriched salt-tolerant microorganisms to clean saline wastewaters. When treating saline phenolic wastewater, researchers used sea mud to cultivate marine activated sludge, and they discovered that this type of marine activated sludge performed exceptionally well in terms of COD, ammonium (NH4+-N), and phenol removal, with rates of 80 percent, 68 percent, and 99 percent, respectively (Tan et al., 2017).

2.2 Enhanced Biological Phosphorus Removal (EBPR)

Phosphorus is the most critical and limiting nutrient for aquatic plant populations, and the principal sources of P loading into water resources include municipal and industrial drainages (point sources), as well as agricultural runoff (non-point or diffuse sources) (Anand et al., 2014). Phosphorus removal is required for continued wastewater treatment to meet the reduced discharge limit at the effluent phosphorus concentrations. To overcome this condition, reducing the amount of phosphorus in wastewater effluent is essential.

Typically, influent wastewater has a total phosphorus concentration of 5-9 mg / l and is required in the waste stream for growth and biological treatment (Curtin et al., 2011; Pijuan et al., 2008). Adsorption, membrane separation, ion exchange, or electrodialysis are some of the chemical and physical technology treatment methods now employed in the disposal of salt wastewater (Fan et al., 2011; Takács et al., 2006). The removal of phosphorus through biological processes are more economical and environmentally friendly than the traditional phosphorus removal process (chemicals) for a number of reasons, including the cost of the chemical, the ability to release and recover phosphorus and phosphorus availability to plants in which the activated sludge (WAS) is used regularly. Phosphorus-removal techniques can take advantage of microorganisms to remove phosphorus from wastewater, this process is called enhanced biological phosphorus removal. EBPR relies on the selection and proliferation of a microbial population capable of up taking orthophosphate in greater amounts than their normal biological growth requirements (Ge et al., 2015; Mino, 2000; Shu et al., 2006). In figure 2.2 showed the A/O process in reactors.

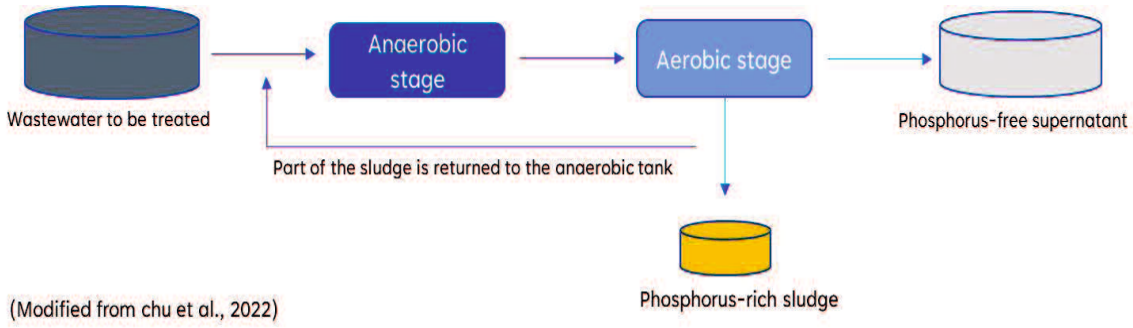

Figure 2.2 Typical biological removal configuration

Enhanced biological phosphorus removal is a process that uses alternating anaerobic and aerobic zones to provide an environment that encourages the growth of phosphorus accumulating organisms (PAOs). PAOs store excess polyphosphate in their cell mass and phosphorus is removed with the waste sludge (Ramasahayam et al., 2014; Seviour et al., 2003). Phosphorus accumulating organisms (PAOs) are a group of bacteria that, under certain conditions, facilitate the removal of large amounts of phosphorus from wastewater in a process, called enhanced biological phosphorus removal (EBPR). PAOs completes the removal of this phosphate by accumulating it in their cells as polyphosphate. PAOs is not the only bacteria that can accumulate polyphosphate in their cells and in fact, the production of polyphosphates is the ability widespread among bacteria. However, the PAOs has a lot of characteristics that are not owned by other organisms that accumulate polyphosphate, which allows it to be used in wastewater treatment. In particular, it is the ability to consume simple carbon compounds (sources of energy) in the absence of an

external electron acceptor (such as nitrate or oxygen) to generate energy from polyphosphate and glycogen stored internally (Seviour et al., 2003). Anaerobic phases can be considered as stress conditions for PAOs whereas they will use polyphosphate and glycogen stored in their cell as energy source to enable them to uptake volatile fatty acid (VFA). VFA are converted to polyhydroxyalkanoates (PHA) and stored in the cells of PAOs. Subsequently, in the aerobic zone PAOs use PHA as a source of carbon and energy for metabolism and cell growth. PAOs will also restore their supplies of glycogen and poly-phosphate (Ahn et al., 2007; Oehmen et al., 2007; Ye et al., 2016).

The mechanism of P recovery via biological process (Modified from Ye et al., 2016) by Phosphorus Accumulating Organisms (PAOs)

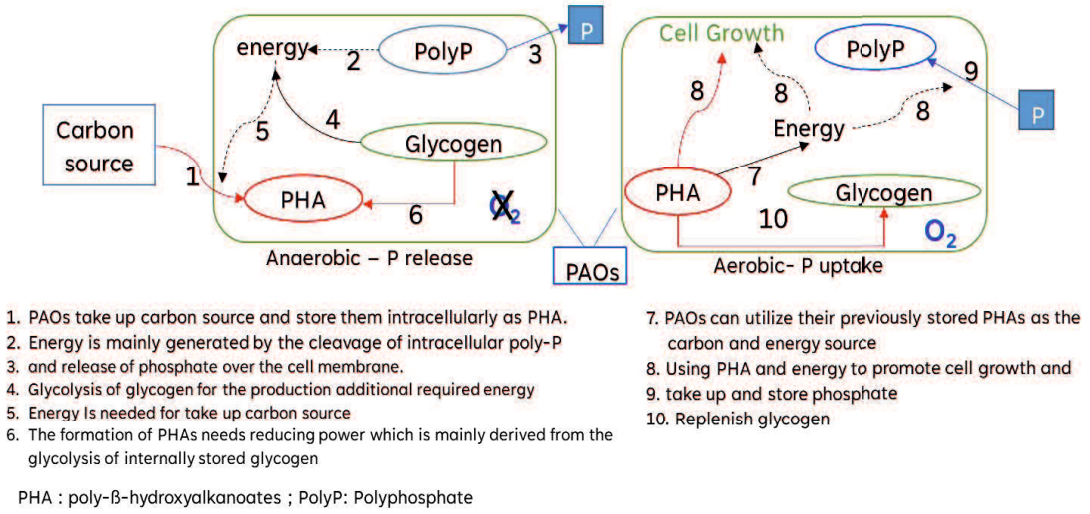

Figure 2.3 The mechanism of P recovery via biological

Understanding of microbiology in the early years was hampered by the lack of available techniques to isolate or identify PAO. later in the early 2000s the availability of molecular microbiology tools has helped in identifying some of the major organisms and this proved that the previous description of organisms was incorrect. Precise isolation is not yet possible and the identification of multiple organisms that may play a role in the EBPR process makes it difficult to establish the major changes observed in a single organism (Bunce et al., 2018).

The problem of salt in the wastewater treatment process is not new in the scientific papers (Lay et al., 2010), with research focused on the impact of salinity on various wastewater processes being published on a regular basis (de Graaff et al., 2020)

Several studies have dealt with the treatment of certain types of saline wastewaters. Most of them have evaluated the performance of complex bioreactor systems consisting of dual anoxic and aerobic filters (Glass and Silverstein, 1999; Wang et al., 2009), and most times these studies are carried out by specialized cultures of microorganisms, where

sterilization of the influent stream is required to maintain microbial community composition (Wang et al., 2018; Zhao et al., 2004).

An major consequence of high salinity influent on WWTPs is an increase in osmotic pressure in the mixed liquor sludge, which leads to a reduction in the metabolism of the microorganisms present in the medium, or possibly their mortality (Cortés-Lorenzo et al., 2014; Luo et al., 2015).

According to Yuan et al., (2020) the findings of this study, salinity (0.5-0.75 wt%) enhanced the generation of AGS (aerobic granular sludge) enriched with AOB (ammonia oxidizing bacteria) in the anaerobic SPNDPR (simultaneous partial nitrification, denitrification, and phosphorus removal) process. Under 0.5 wt% salinity, the P removal performance was high, but it was limited at 0.75wt% salinity. Salinity causes an increase in EPS (extracellular polymeric substance) secretion, which speeds up granulation. Under salinity pressure, the relative activity and abundance of AOB was substantially greater than that of NOB (nitrite oxidizing bacteria), with a 98.9 percent observed NAR (nitrite accumulation ratio). GAO (Glycogen accumulating organisms) prefers salt water to PAO (polyphosphate accumulating organisms/DPAO) (denitrifying polyphosphate accumulating organisms).

PAO are reported to be more sensitive to salinity than other heterotrophs (Bassin et al., 2011; Uygur, 2006). In fact, a number of previous studies reported a decrease in the phosphorus removal rate when treating wastewater with a salt content as low as 0.5% (Bassin et al., 2011; Lu et al., 2016; Luo et al., 2015).

Al-shammari et al., (2015) study biological P removal using sequential anoxic/aerobic bioreactors system in saline wastewater showed phosphorus removal was not very high with ranged between 38.2% and 63.5% with an average value of 49.6%. The incomplete phosphorus removal may be resulted from carbon substrate competition between Phosphorus Accumulating Organisms (PAOs) and the denitrifying bacteria.

Many attempts have been made to isolate PAOs responsible for EBPR, the table 1 show some bacteria that contribute for phosphorus removal from various sources.

Sources of bacteria	Bacteria	References
Activated sludge	Candidatus Accumulibacter Phosphatis	Seviour et al (2003)
Catfish ponds in the Mekong Delta, Vietnam	*Bacillus* sp *Burkholderia vietnamiensis* TVT003L *Acinetobacter radioresistens* TGT013L *Arthrobacter protophomiae* VLT002L	Khoi and Diep (2013)

Sludge reduction	*Devosia* sp and *Bdellovibiro* sp	Zuo et al (2016)
Brewery wastes	*Aeromonas* sp., *Pseudomonas* sp., and *Bacillus* sp.	Oljira et al (2018)
Marine wastewater	*Bacillus* sp. KGN1 and *Vibrio* sp. KGP1	Cho et al (2018)

2.3 Environmental Factor

The growth and development of microorganisms is stimulated by external stimuli, namely environmental factors. Microorganisms have a relatively large tolerance range to changing environmental conditions. microorganisms reproduce very well under the right conditions (Stanaszek-Tomal, 2020). Different microorganisms (microbes) need different factors in order to grow and survive. These factors are physical or chemical properties that define the environment of the microbe. Understanding which environmental factors influence the microbial community is a key goal of microbial ecology. Essentially, the microbial environment consists of nutrient availability, pH, temperature, and salinity (Green et al., 2008; Jin and Kirk, 2018; Lennon and Jones, 2011). In Enhanced Biological Phosphorus Removal (EBPR) process certain environmental condition it is important for achieving removal efficiency by microorganisms.

(a) carbon sources: All living creatures need energy, carbon and nutrients (nitrogen, phosphorus, Sulphur, potassium, calcium, magnesium, etc.) for functions of growth, locomotion, reproduction and others In the terms of the carbon sources, there are two basic organism types: **autotrophic organisms** (carbon source: carbon dioxide (CO_2)), **heterotrophic organisms** (carbon source : organic matter) (Marcos, 2007). Nutrient availability (carbon source) is needed under anaerobic conditions, whereas P-removing bacteria transport volatile fatty acid (VFA, e.g. acetate) into the cell and subsequently convert and store these as poly-hydroxyalkanoates (PHA, e.g. poly-beta-hydroxy-butyrate; PHB) The energy for this transport and storage is supplied by the hydrolysis of intracellularly stored polyphosphate (poly-P) to ortho-phosphate, which is released from the cell to the liquid. Under aerobic conditions, carbon sources are used to generate energy for cell growth, poly-P synthesis, glycogen formation and maintenance, resulting in the uptake of phosphate (Fuhs and Chen, 1975; Hollender et al., 2002; Hrenović, 2001; Shen and Zhou, 2016).

(b) pH : The effect of pH, the incidence and distribution of microorganisms, the growth rate varies with pH along the curve is bell-shaped or triangular, which reflects the pH range of the structural integrity of cells and pH interference with cell metabolism (Jin and Kirk, 2018). In the EBPR process, it has been reported that pH plays an important role. The increase in pH causes increased phosphorus release in the acetate wastewater due to the increased energy requirements for acetate transport. A pH of around 7.5 has been found to

be the overall working optimum pH for an EBPR process with propionate as the sole carbon (Pijuan et al., 2004; Smolders et al., 1994).

(c) temperature: Temperature is a fundamental factor that affects all living organisms. It influences the rates of enzymatically catalyzed reactions and the rate of diffusion of substrate into cells. In biological nutrient removal systems, an optimum temperature condition is essential for the efficiency of the treatment processes. This is because temperature is known to have influence on water chemistry and biological activities. Also, microbial growth is known to be strongly influenced by temperature (Akpor et al., 2014; Brdjanovic et al., 1997; Gomez et al., 2020). Temperature is considered one of the most important factor that effecting the performance in an EBPR process (Mulkerrins et al., 2004). There were many studies regarding the effects of temperature on phosphate removal in biological way. Studies conducted by (Akpor et al., 2014; Panswad et al., 2003) observed that temperature between 20 ℃ and 35 ℃ gave effect the microbial community of the EBPR system. In some other studies the aerobic phosphorus uptake rate showed a maximum in the interval between 15 ℃ and 20 ℃ (Baetens et al., 1999; Boontian, 2012; Chen et al., 2014).

(d) salinity: in order for all living organisms to survive, they must be able to sense the environmental parameters that define their habitat and react according to the changes that come with various adaptive mechanisms (Omotoyinbo, 2016; Rubiano-Labrador et al., 2015). The performance of the saline wastewater biological treatment process is usually low due to the detrimental effect of the salt on the microbial flora. High salt concentration in wastewater causes plasmolysis and loss of cell activity resulting in low COD removal efficiency (Kargi and Dincer, 1996). High salinity poses unbalance osmotic stress across the cell wall an even leads to cell plasmolysis (He et al., 2017; Wu et al., 2013; Zhou et al., 2020). Saline wastewater is usually treated through physico-chemical means, as conventional biological treatment is known to be strongly inhibited by salt (mainly NaCl) Nowadays, alternative systems for the removal of organic matter are studied, most of them involving anaerobic or aerobic biological treatment (Al-shammari et al., 2015; Lefebvre and Moletta, 2006).

2.4 Cultivation of Bacteria

Microorganisms exist in different natural environments such as the soil, the animal intestine, the air, and water. Each of these habitats has peculiar characteristics to which the organisms living therein must adapt (Yates et al., 2014). Cultivation is the process of propagating organisms by providing the proper environmental conditions (Salcher and Šimek, 2016). Cultivation of environmental microorganisms in pure culture usually consist of the following steps: (1) isolation : separation of single cell from the other microbial cells coexisting in the sample; (2) inoculation: inoculating single cell into a nutrient medium; (3)

incubation: allowing the cell to propagate; (4) detection: visually recognizing growth; and (5) recovery and archiving; replicating growth and storing thus obtained biomass (Yates et al., 2014). Conventional culture-dependent techniques are based on the isolation of pure cultures, typically in solid medium (or agar plate), followed by the phenotypic identification (morphological and physiological characterization) of the specifically targeted microbes. Obtaining a pure culture of bacteria is usually accomplished by spreading bacteria on the surface of a solid medium so that a single cell occupies an isolated portion of the agar surface. This single cell will go through repeated multiplication to produce a visible colony of similar cells, or clones (Zimbro et al., 2009). Critical conditions now exist for tracing bacterial growth on solid media, particularly where certain difficulties can be overcome in terms of ensuring the reliability and readiness of the obtained single-cell cultures (Ørskov, 1922). One of the basic principles of the plate method is to provide a nutrient or medium that favors the growth of particular microorganisms or inhibits the growth of unwanted ones (usually known as selective media) (Uthayasooriyan et al., 2016). Bacterial counts are usually carried out by viable cell counts using plate culture and identified by typical colony morphology. This method requires skilled technique; therefore, the results can be influenced depending on different analysts. Moreover, it is well known that the method which relies on cultivation largely underestimates the diversity or the number of microorganisms because of the difficulty of cultivating most of the microorganisms by conventional methods (Bhatia, 2009; Hatamoto et al., 2017; Vartoukian et al., 2010).

2.5 Polymerase Chain Reaction (PCR)

PCR permits the rapid amplification of defined sequences of DNA. Basically, PCR is used to amplify small amounts of targeted sequences, usually many copies of DNA with exactly the same sequence. PCR uses oligonucleotide primers which are usually short sequences of DNA (about 20-30 bp) used as starting point for nucleic acid synthesis (Peake, 1989). Polymerase Chain Reaction (PCR) is a method that can be used to multiply the DNA of an organism. PCR-based methods are often used in the identification of organisms, through DNA fingerprinting as well as through DNA barcoding. The genetic identification method uses the PCR method has been widely developed, and has been done on various microorganisms, including bacteria, archaea, and fungi (Khoi and Diep, 2013; Mahansaria et al., 2015; White et al., 2006).

In the PCR process, several main components are needed, namely DNA prints, Primary oligonucleotides, deoxyribonucleotide triphosphate (dNTP), DNA polymerase enzymes, and another supporting component is a buffer compound. In the PCR process using tools thermocycle. A machine that has the ability to heat as well as cool test tube and set the temperature for each stage of the reaction. There are three important stages in the PCR process that always repeats itself in 30-40 cycles and takes place quickly, namely denaturation where the DNA is melted to convert double-stranded DNA to single stranded

DNA. This is achieved by subjecting the DNA template to temperatures between 94 ℃ and 95 ℃ for between 30 to 60 seconds, annealing requires a lower temperature in the range 72 ℃ for usually 1 minute, and final step DNA strand lengthening (extension) this process is usually carried out at a temperature of 76 ° C. The duration of this stage is usually 1 minute. PCR products can be identified by their size by using agarose gel electrophoresis (Atawodi et al., 2011; Jagtar Singh , Niti Birbian, 2014; Peake, 1989; Rahman et al., 2013).

2.6 *Bacillus* sp.

The genus *Bacillus* represents a very large and diverse set of bacteria that have one thing in common but are different Characteristics: the ability to create aerobically inactive endospores when faced with unfavorable growing conditions. *Bacillus* sp. are gram-positive, spore-forming aerobic or facultative anaerobic bacteria and belong to the phylum Firmicutes. Some strains of *Bacillus* sp. exhibit a suppressive effect on phytopathogenic microorganisms, and thus, *Bacillus* sp. are one of the well-studied biocontrol agents against Fusarium wilt (Goldman and Green, 2008). *Bacillus* species can be found in nature and some of them have high wastewater purification capabilities to break down very concentrated organic substances in a short time and also release large amounts of enzymes that can break down excess sludge (Taguchi et al., 2017). In recent years there has been much literature on *Bacillus* sp. has been used in maintenance to remove nutrients in wastewater, especially to remove phosphorus. wherein the bacteria are able to reduce phosphate levels in wastewater, both individually or in combination (Eom et al., 2018; Khoi and Diep, 2013; Krishnaswamy and Muthuchamy, 2011; Li et al., 2003; Oljira et al., 2018)

2.7 *Thioclava* sp.

The genus *Thioclava*, a member of the family Rhodobacteraceae was first described by (Sorokin et al., 2005) the characteristics Gram-negative rods of varying size from long filaments with swollen ends, clustered in aggregates, to single small rods, rarely motile by one polar flagellum. Obligately aerobic and facultatively autotrophic, sulfur-oxidizing bacteria. Grow chemoautotrophically with thiosulfate and heterotrophically with simple organic compounds. At the time of writing, the genus Thioclava comprises four species with validly published name, *Thioclava pacifica* (Sorokin et al., 2005), along with *Thioclava dalianensis* (Zhang et al., 2013), *Thioclava atlantica* (Lai et al., 2014), *Thioclava indica* (Liu et al., 2015), and the newly described *Thioclava nitratireducens* (Y. Liu et al., 2017) and *Thioclava electrotropha* that capable of chemolithoautotrophic growth with poised solid-state electrodes, sulfur species and hydrogen. This organism can also grow as an organoheterotroph (Chang et al., 2018). Until now a large number of isolates of this genus were obtained from diverse marine environments and preliminary identified by 16S rRNA gene sequencing. The current research by Chen et al. (2018) that *Thioclava* could improve nitrogen and phosphorus removal treatment for the treatment of saline wastewater.

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CHAPTER 3

SCREENING MICROORGANISMS FOR PHOSPHORUS REMOVAL IN SALINE WASTEWATER

3.1 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, Keller, 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, Salmassi, et al., 2005; Aydin, 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, Ekama, 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, Muthusamy, 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, Xing, 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, Murgui, et al., 2017; Jiang, Wang, 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, LI, et al., 2007; Jiang, Wang, 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,

Lopez-Vazquez, 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, Pronk, et al., 2011; Wang, Sun, et al., 2017; Welles, Lopez-Vazquez, 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.

3.2 Materials and methods

3.2.1 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.

3.2.2 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.

3.2.3 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. The substrate was passed over the sponge surface to acclimatize the microorganisms growing on sponge surface as well as within its pores, ensuring sufficient growth surface. The cultivation duration was 112 days.

3.2.4 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

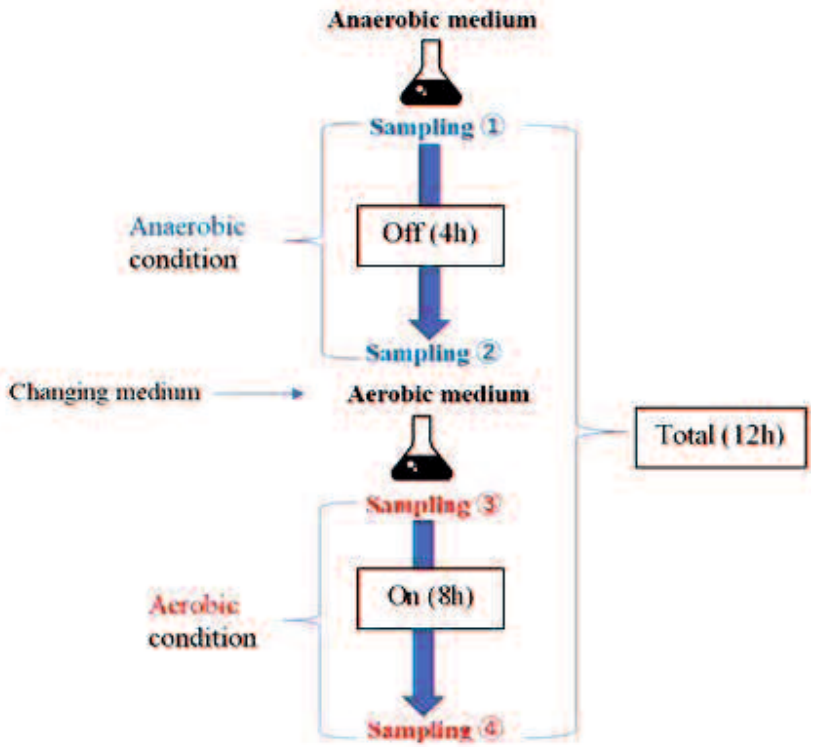

Fig.3. 1 Schematic Diagram of the Experimental Setup.

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.

3.2.5 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 the anaerobic conditions the plate was given anaero pack to avoid the entry of oxygen during incubation. Isolation and inoculation of bacteria were carried out following the method described in (Smith and Hussey, 2005). 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.

3.2.6 Microscope examination

Bacteria cells were Gram-stained according to (Smith and Hussey, 2005), and their 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.

3.2.7 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), 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, Stecher, 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.

3.3 Results and discussion

3.3.1 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 aerobic 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, Du, et al., 2013). Poly-p hydrolysis releases orthophosphate into the solution. The accumulated substrate is then transported and stored as polyhydroxyalkanoate (PHA) (Bunce, Ndam, 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 v., Loewenthal, 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 transfer (Li, Xing, et al., 2003)(Li, Xing, et al., 2003). The ability of PAOs to remove P decreases with increasing salinity (>2 %) owing to the reduction of the microbial community (He, Wang, 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, Huang, et al., 2016; Wu, Hao, 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

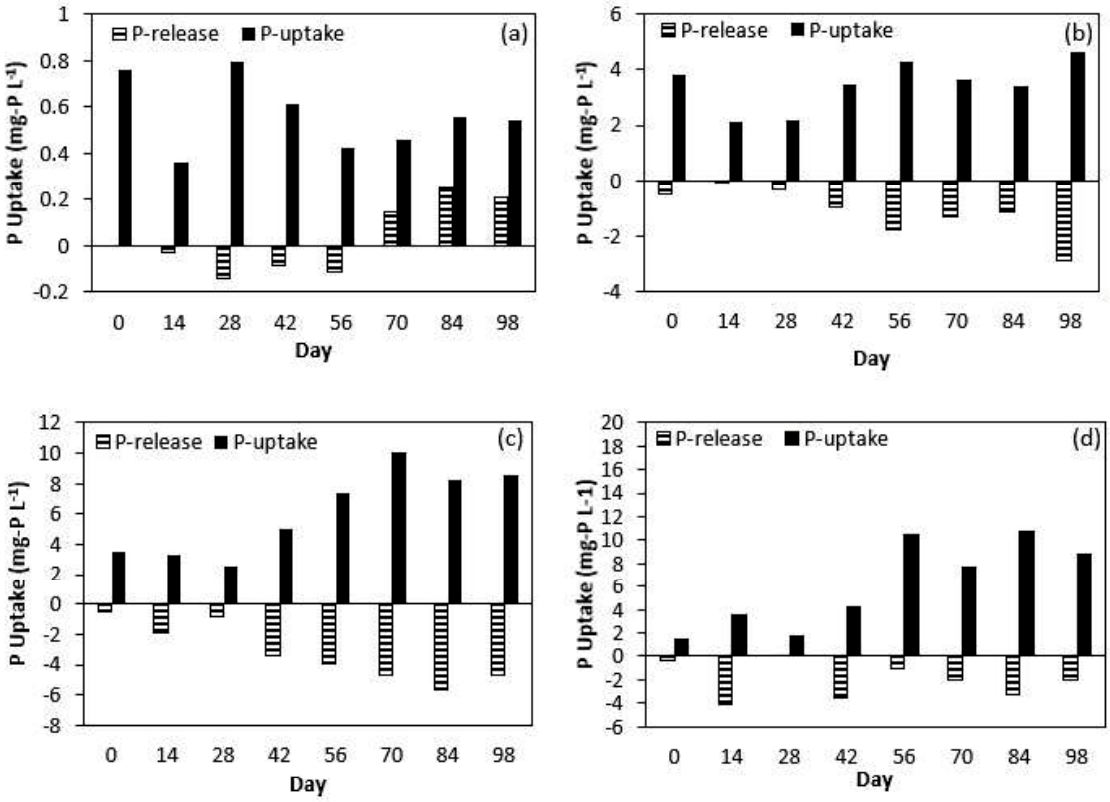

Fig. 3.2 P uptake and P release during 98 days in various P concentrations: (a) 1 mg-P/L, (b) 5 mg-P/L (c) 10 mg-P/L, and (d) 20 mg-P/L.

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,

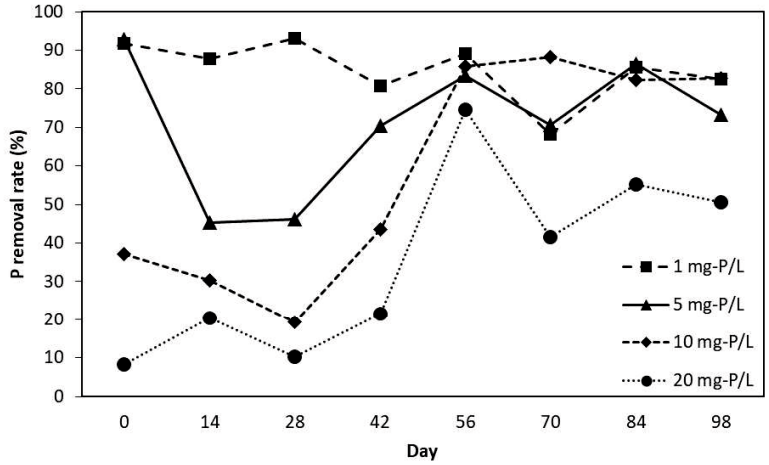

Fig. 3.3 Net P removal during 98 days in various P concentrations.

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. This signified that PAOs were able to adapt efficiently at concentrations of 1, 5 and 10 mg-P/L as indicated by uptake of phosphate which was over 70% these concentrations, whereas at 20 mg-P/L PAOs only uptake half of it. Besides, 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.

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. We could compare the ability of microorganism (with same amounts) for phosphate uptake, where condition of carbon source, pH and temperature remained the same. However, we cannot show exact amounts of microorganism because we did not measure the number of the biomass. In the future it is important to measure amounts of microorganisms in order to know its relationship with the phosphate uptake and release ability. 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.

3.3.2 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. While the strain MA3 formed colonies that are in punctiform, cream in color, with smooth surfaces and raised

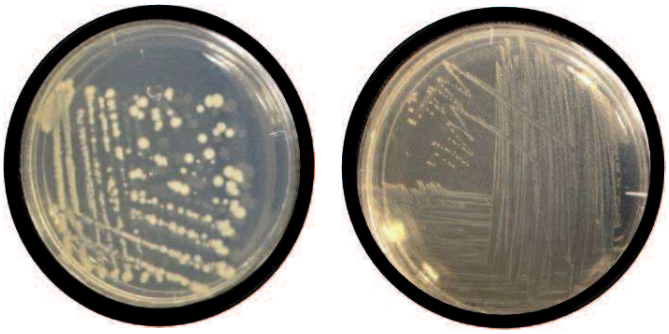

Fig. 3.4 Morphology of (a) strain TR1 colonies on R2A agar and (b) strain MA3 colonies on Marine agar 2216.

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.

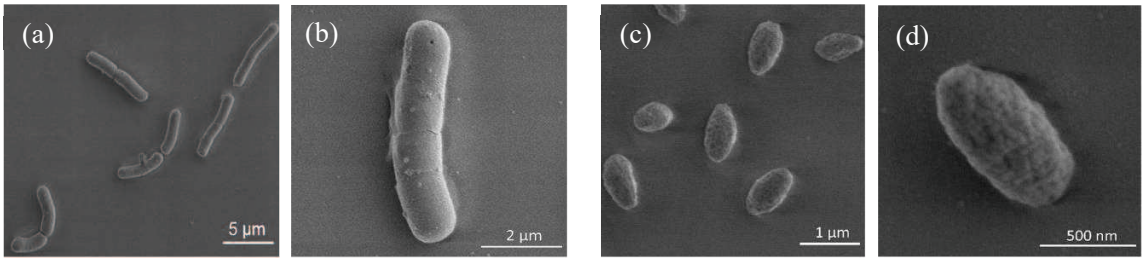

Fig. 3.5 Scanning electron microscopic micrographs of (a-b) strain Tr1 and (c-d) strain MA3.

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.

3.3.3 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 Genebank for all strains provided the percentage similarity between the cultivated microorganisms and those detected in Genebank for three most similar representatives as shown in Table 1.

Moreover, the neighbor-joining tree base don 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.

Table 3.1. Similarity percentage of cultivated strains toward representative species in BLAST search

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

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, Hong, et al., 2002). Two bacterial strains, *Bacillus aryabhattai* and *Bacillus* sp., has been found in the water of intensive catfish-

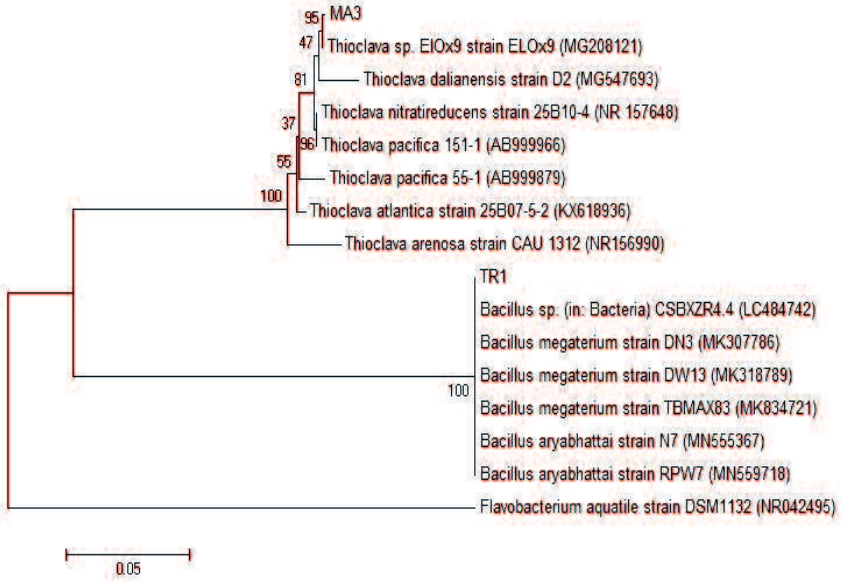

Fig. 3.6 Neighbor-joining tree showing the phylogenetic position of strain MA3, TR1, and related species, based on 16s rRNA gene sequence. *Flavobacterium aquatile* DSM1132 was used as an outgroup.

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), where a wastewater treatment system in Korea is operated with dominant bacilli strains which has capability of efficiently removing P.

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, He, et al., 2018). *Thioclava* sp. can found everywhere in the marine environment and are distributed in various ecological niches, which confirms their ability to live in saline environments (Liu, Lai, 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, Tourova, et al., 2005). It is suggested that *Thioclava* can improve the removal of N and P from saline wastewater. The study of *Thioclava* to remove P in saline wastewater requires further research.

3.4 Conclusions

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.

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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.

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CHAPTER 4

EFFECT OF ABIOTIC FACTORS ON *BACILLUS* SP. AND *THIOCLAVA* SP. FOR PHOSPHORUS REMOVAL FROM SALINE WASTEWATER

4.1. Introduction

Phosphorus (P), an essential element for all life forms, is a structural constituent of several cell components and a functional component of all organisms (Acevedo et al., 2017). However, under specific environmental conditions and high concentrations, it can be considered as a pollutant. Wastewater containing 1–10 g/L salt is usually defined as saline wastewater; otherwise, it is regarded as hypersaline or brine and necessitates treatment. Phosphorus-rich saline wastewater is often found in aquaculture, food processing, and saline farmlands. Excessive discharge of P into water bodies triggers eutrophication, which causes excessive growth of algae and other planktons, accelerating the depletion of dissolved oxygen (DO) that is fatal to the fish and other organisms (Zhimiao et al., 2019). Therefore, P removal from wastewater is of immediate concern, given its environmental impact; it can be detrimental and poses a threat to aquatic systems, requiring efficient treatment methods that is cost-effective in the long term (Krishnaswamy et al., 2009).

The specification set by the US Environmental Protection Agency (1986) recommends that total phosphorus concentrations should be <0.10 mg/L in streams that do not discharge directly into reservoirs and should not exceed 0.05 mg/L in streams that do not discharge directly into reservoirs. The limits of phosphorus in the effluent of wastewater treatment plant (WWTP) discharging to aquatic systems in other countries vary from 0.5 to 2.0 mg/L. Presently, the concentration of inorganic and organic forms of phosphorus in municipal wastewater is usually from 5 to 20 mg/L (United States Environmental Protection Agency (USEPA), 1986). Meanwhile, the concentration of phosphate levels are 0.015 mg/L for water supplies, 0.025 mg/L for aquatic life, 0.05 mg/L for lakes, and 0.02 mg/L for mountain lakes (Kotoski, 1997). In addition, the phosphate content in the seawater surface was 0.049 mg/L and deep seawater contained phosphate levels from 0.082 to 0.188 mg/L (Watanabe et al., 2000). Phosphates are not toxic to people or animal unless they are present in very high levels. Phosphate level greater than 1.0 mg/L can cause digestive problems, and can interfere with freezing in water treatment plants (DebRoy et al., 2012).

The enhanced biological phosphorus removal system (EBPR) is a sewage treatment method for removing P from wastewater. In this process, microorganisms such as bacteria, yeast, protozoa, microalgae, and fungi are used to accumulate phosphate in wastewater (Delgadillo-Mirquez et al., 2016) In general, all bacteria contain a small portion (1%–2%) of phosphorus, as a part of their cellular components, such as phospholipid membranes and DNA. Therefore, in the wastewater treatment plant, the bacterial cells grow by consuming the nutrients in the wastewater, thereby, accumulating

phosphorus in their biomass. Phosphorus is used by microorganisms for cell maintenance, nucleic acid synthesis, cell membrane construction (as phospholipids), and chemical energy transfer reactions with cells (such as ATP). Additionally, a quantity of phosphorus is also stored by cells for future use. The EBPR process uses specific bacterial metabolism, which in certain circumstances, accumulates large quantities of intracellular polyphosphate. In the anaerobic phase, carbon source is taken up by the bacteria and stored as polyhydroxyalkanoate (PHA) and is accompanied by the degradation of poly-P, which consequently releases orthophosphate. In the aerobic phase, bacteria grow aerobically and requires orthophosphate to restore poly-P levels using the stored PHA as a source of carbon and energy. Since PHA is a reduced polymer, its synthesis requires reducing power (Jiang et al., 2018).

Abiotic conditions such as water quality, carbon sources, pH, temperature, and salinity can affect the EBPR system. Consequently, the operation and management of these parameters are essential for removing P from wastewater treatment (Welles et al., 2014). The bacterial involvement in removing nutrients from wastewater and the removal efficiency has been studied. Bacterial communities are important in the activated sludge systems and are responsible in stabilizing the effluent entering the treatment plant (Zilles et al., 2002). Microbes play a fundamental role in the remediation of polluted water. Microbial activity is an essential parameter in understanding the ecological role of bacteria for pollutant removal processes in aquatic ecosystems (Shahid et al., 2020). Since microbial activity is a crucial parameter for the functioning of aquatic ecosystems, tracing the significance of various habitat changes for the entire environment is a serious research challenge. The marine bacteria are able to adapt and live in extreme abiotic conditions (such as high salinity and pressure, low temperature and nutrients) and play an essential role in P transformation in the oceans (Shi et al., 2012). However, the literature on the effect of seawater on EBPR is lacking. The effect of saline wastewater has been studied in NaCl-supplemented wastewater processes or NaCl-based enrichment culture of *Ca*. Accumulibacter phosphatis (Wang et al., 2015). Various studies have shown that *Bacillus* sp. is used in combination with *Pseudomonas* sp., *Aeromonas* sp., and *Enterobacter* sp. (Krishnaswamy et al., 2009; Oljira et al., 2018). Meanwhile, current research on *Thioclava* sp. is related to sulfur oxidation (Chang et al., 2018; Sorokin et al., 2005). Therefore, it is essential to understand the specific role of *Bacillus* sp. and *Thioclava* sp. in increasing phosphorus removal in saline wastewater that is adapted to seawater. In this study, the adaptability of *Bacillus* sp. and *Thioclava* sp. to abiotic factors such as carbon sources, pH, temperature, and salinity variations was investigated, and their individual and combined effects for P removal performance were examined at different P concentrations in saline wastewater, which was similar to that of seawater.

4.2. Materials and methods

2.1 Chemical and media

Chemicals used in this study were of analytic grade. The 216 L medium as described by (Liu et al., 2015) (Liu et al. 2015) contained (g/L): CH_3COONa (1), tryptone (10),

yeast extract (2), sodium citrate (0.5), NH_4NO_3 (0.2), synthetic sea salt (35) (Gex, Japan), and agar (15) . The salinity wastewater contained: (g/L): CH_3COONa (1.0), sodium citrate (0.5), NH_4NO_3 (0.2), and synthetic sea salt (35) (Gex. Japan). A specified amount of KH_2PO_4 was added to the medium to achieve the desired P concentration (1, 2.5, 5, and 10 mg-P/L). The medium pH was adjusted to 7 using 0.1 M NaOH and 0.1 M HCl.

4.2.2 Source of strains

The marine bacteria were isolated from sediments and seawater in Yamaguchi Bay, Japan. They were enriched and cultivated in a fed-batch process during100 days. The marine bacteria with high P removal ability were isolated and screened by sequencing 1500-bp 16S rRNA. The screening results identified the bacteria as *Bacillus* sp. (TR1) and *Thioclava* sp. (MA3), which are gram-positive and gram-negative respectively, as previously reported (Hasanah et al., 2021). The experiments were performed daily, and the cultures were maintained on plates at pH 7 using 216 L medium. In the present study, the bacteria were re-isolated and re-purified.

4.2.3 Carbon sources utilization on bacterial growth

The medium to assess the effect of carbon source contained different carbon sources (glucose, sucrose, and CH_3COONa), KH_2PO_4 and NH_4Cl with a C:P:N ratio of 100:5:1, used for acclimation purpose, were added to the medium in separate flasks. The pH of the medium was adjusted to 7 using 0.1 M NaOH and 0.1 M HCl before sterilization. The medium (200 ml) was dispensed in a 300 ml Erlenmeyer flask and sterilized in an autoclave (Hirayama; HA-300 MIV. Japan) at 121 °C and 15 psi for 15 min. After sterilization, a known concentration of the respective bacterial species was inoculated into individual flasks with different carbon sources. Each flask was mixed on a gyratory incubator shaker (145 rpm) (Eyela Multishaker MMS; Tokyo Rikakikai Co. Ltd. Japan) at 35 °C for 24 h. The optical density of the cell suspension was measured at 600 nm (OD_{600}) to determine cell growth using a UV-visible spectrophotometer (UV-VIS U2900; Hitachi Co., Ltd. Japan). All experiments were performed in triplicates.

4.2.4 Effect of pH, temperature, and salinity on bacterial growth

To determine the effect of optimum abiotic factors on the growth of selected strains, various pH values (4–10), temperature (25–50 °C), and salinity (0 %–10% w/v) were evaluated in this study. The flasks with 216 L medium were placed on a shaker for 24 h at 145 rpm. After 24 h, the cells in the suspension were estimated.

4.2.5 Estimation of phosphorus removal and bacterial growth

Phosphorus removal was performed in a salinity wastewater medium. Initially, 200 ml media was dispensed in a 300 ml Erlenmeyer flask and sterilized in an autoclave at 121 °C and 15 psi for 15 min. Bacteria were inoculated using an inoculating wire loop that was sterilized by flaming to redness and cooled by oscillating briefly in air. Three loopfuls of individual bacteria TR1 and MA3 and for the combined bacteria in the ratio of 1: 1 were inoculated into each marked flask at 25 °C, following the method by Yusuf

et al. (2013). Each flask was mixed on a gyratory incubator shaker (145 rpm) (Eyela Multishaker MMS; Tokyo Rikakikai Co. Ltd. Japan) at 35 °C for 24 h. The P removal ability was estimated by measuring the soluble phosphate content in the culture medium within 24 h of incubation. Within 24 h, 5 ml of agitated sample was drawn from the individual flasks and transferred to a centrifuge tube (15 ml) under aseptic conditions, which were centrifuged (Kokusan centrifugal machine H-103N. Japan) at 3500 rpm for 10 min, and the clear supernatant was used. The P concentration (as PO_4-P) was estimated 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. To determine cell growth within 24 h, the optical density of the cell suspension was measured using UV-visible spectrophotometer at 600 nm and expressed as OD_{600}

4.3. Results and discussion

4.3.1 Carbon sources utilization on bacterial growth

Carbon is a vital nutrient for microbial growth. Cell growth in the synthesized medium was investigated for different carbon sources. The effect of carbon sources on the cell growth of TR1 and MA3 after cultivating in shake flasks for 24 h is shown in Figure 1. From the figure, the standard deviation bars shows that the OD 600 results of MA3 using sucrose was more significant in the other results. The results showed that both TR1 and MA3 favored a medium with glucose, sucrose, or CH_3COONa as the carbon source. However, the rate of cell growth varied with different carbon sources. Glucose and sucrose exhibited the most prominent effect on *Bacillus* sp. (TR1), as shown in Figure 4.1

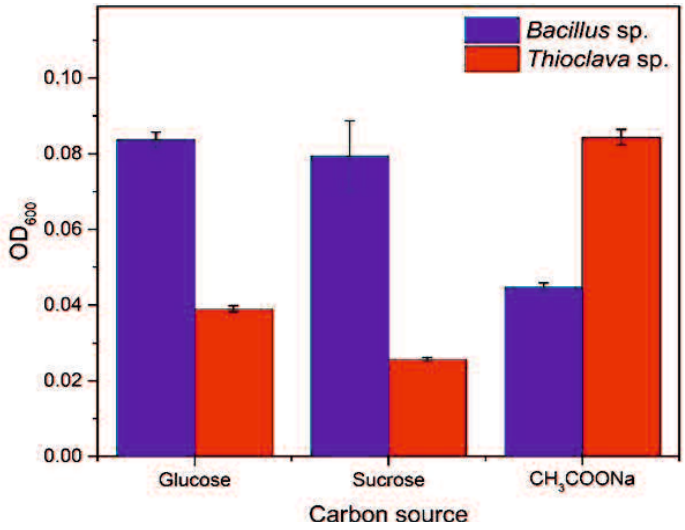

Figure 4.1 Effect of carbon sources on the growth of TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) at 35 ℃, salinity of 3.5 % w/v, and pH 7 for 24 h.

The growth of TR1 was approximately similar in glucose and sucrose, indicating that the bacteria preferred sugar. In contrast, MA3 shows that growth increased in CH_3COONa than in glucose and sucrose. Carbon sources play a significant role in cell growth, and the

synthesis of various metabolites during cultivation. Certain species prefer specific carbon sources, while others can utilize several carbon sources (Lindh, 2007). The gram-positive bacterium such as *Bacillus* sp is capable of using numerous carbohydrates for example glucose as the most preferred sources of carbon and energy (J.Stulke and W.Hillen, 2000). For *Thioclava* sp this bacterium the family of Rhodobacteraceae and the vast majority of the species contained in the family are aquatic, and many of them require sodium ion or combined salts for growth. Their cells are Gram negative and multiply by binary fission or by budding, following monopolar growth (Wolfe, 2014). Based on the available literature (Zhou et al., 2020) showed that *Bacillus cereus* that used glucose added NaF and $NaNO_2$ showed optical density below 0.1.The low value of OD (below 0,1) in the study of the effect of carbon sources on bacterial growth was probably caused by the absence of phosphate addition in the sample during the experiment. As we know that phosphate is one of the nutrients needed by bacteria to grow, this may be a limiting factor for bacteria to grow more.

4.3.2 Effect of pH, temperature, and salinity variations on bacterial growth

Evidently, pH affects microbial growth and enzymatic reactions (Cao and Zhang, 2014). Bacteria are pH-sensitive, with each species comprising an optimum growth pH.

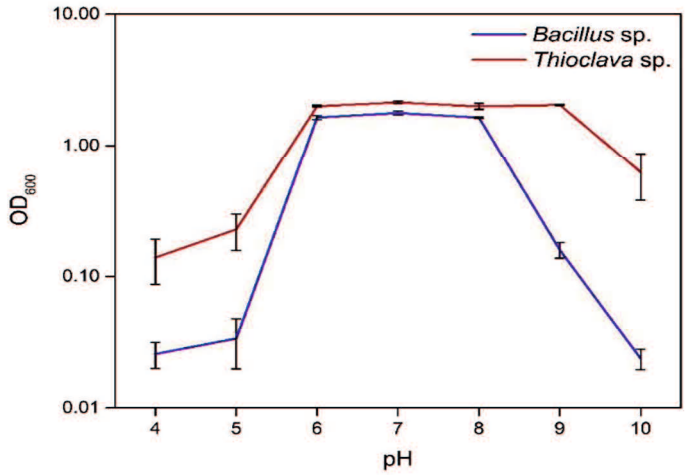

Figure 4.2 The effect of pH on the growth of TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) at 35 ℃ and salinity of 3.5% w/v for 24 h.

Bacteria also have a tolerable pH range and are unable to survive beyond this range. pH plays a significant role in biological P removal systems. The influence of pH (4.0–10.0) on cell growth was studied and the results are shown in Figure 4.2. Based on statistical analysis the OD 600 in the pH range of 6-8 for TR1 and 6-9 for MA3 were more significant than below and above those ranges. Strain TR1 exhibited good survival in the pH range of 6–8. Meanwhile, the biomass concentration of strain MA3 was high (>1.0) at pH 6–9, which was more than the optimum pH range of TR1. These results are similar to those of Jiang et al. (2018), who reported that an ideal P removal performance could only be obtained at a pH between 7.0– 8.0. This study showed that the optimal pH range suitable for P removal differed in strains TR1 and MA3 .

Figure 4.3 shows the effect of temperature, ranging between 25–50 °C, on the cell growth of TR1 and MA3. The statistical analysis of standard deviation of both TR1 and MA3 exhibited more significant results in the range of 25- 35 °C than 40-50 °C. Both strains TR1 and MA3 could grow at temperatures in the range of 25 to 40 °C. When the temperature increased to 50 °C, the growth decreased significantly, suggesting that the bacteria were unable to grow at high temperatures (>40 °C). The results indicated that the growth of TR1 and MA3 was affected by environmental temperature. Additionally, the optimum temperatures for phosphate removal using bacteria studied by Akpor et al. (2014) were 30–40 °C .

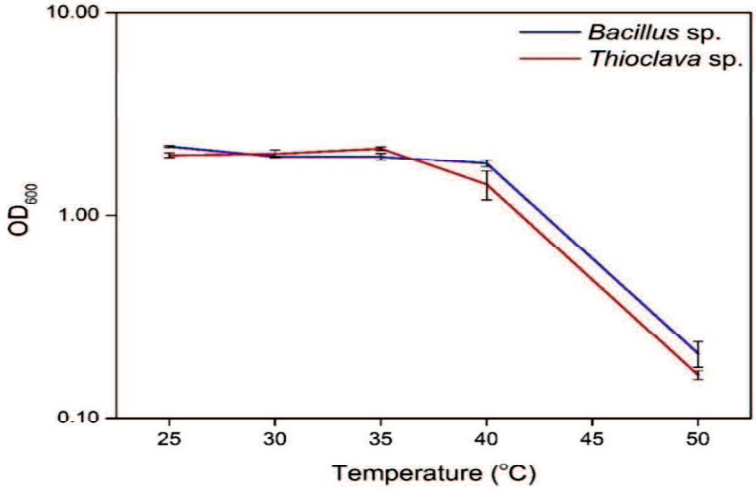

Figure 4.3 The effect of temperature on the growth of TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) at pH 7 and salinity of 3.5% w/v for 24 h.

Salinity has selective effects on the microbial community structure and influences the degradation rate by inhibiting microbial or enzyme activity . According to Munn (2011), the major ionic components of seawater are sodium, chloride, sulfate, magnesium, calcium, and potassium, including key nutrients such as nitrate, phosphate, silica, and iron. The concentration of each component is crucial in determining the growth of marine

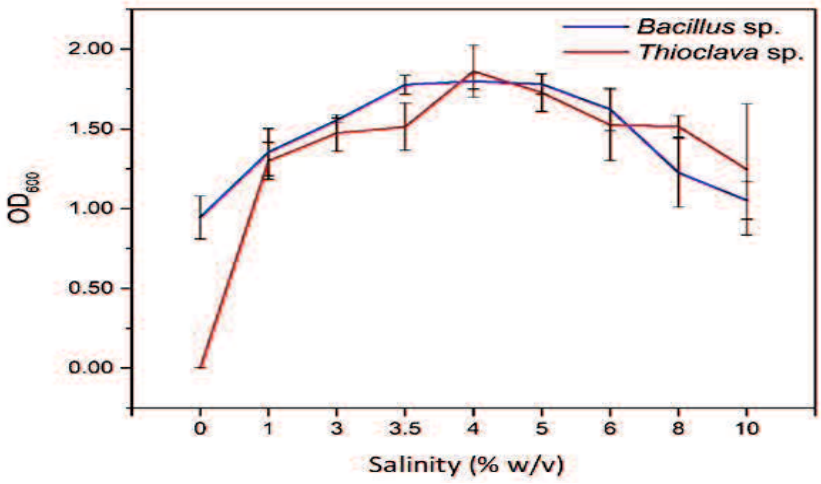

Figure 4.4 The effect of salinity variations on the growth TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) at 35 °C and pH 7 for 24 h.

microbes (Zilles et al., 2002). The effect of salinity on the growth of TR1 and MA3 cells is shown in Figure 4.4.

The results showed that both TR1 and MA3 exhibited a certain adaptability to survive in different salinities from 0 to 10%. In the optimum salinity condition range (3-5 % w/v), standard deviation showed that the calculation of OD 600 of *Bacillus* sp was more significant than *Thioclava* sp. Figure 4. shows that TR1 prefers the salinity range of 0%–10% and peaked at 3.5% to 5% salinity. However, MA3 indicates can grow from 1 to 10% salinity, and was absent in non-saline water, as opposed to TR1. The maximum growth of MA3 was recorded at a salinity of 4%. This result indicated that salinity had a significant influence on the growth of these two phosphate reducers.

4.3.3 Phosphorus removal capability and bacterial growth of the isolated strains of the isolated strains

Figure 4.5 shows the results of phosphorus removal by TR1 and MA3 at different P concentrations. The performance of the strains differed in batch experiments designed to estimate phosphorus removal. Each strain, individually and combined, removed phosphorus at various concentrations after 24 h. Phosphate is consumed by the cells to grow and modify polyphosphates under aerobic conditions (Krishnaswamy et al., 2009). Figure 4.5 shows that TR1 had a higher P removal efficiency than MA3 at every experimental P concentration.

Based on the standard deviation, the calculation of the removal of phosphorus concentration by TR1 and the combination showed more significant results compared to other calculations. The results showed that 1 mg-P/L of P in synthetic saline wastewater could be completely removed by TR1. However, MA3 could only reduce P to 38.2%. The percentage of phosphorus removal decreased as the P concentration increased. The

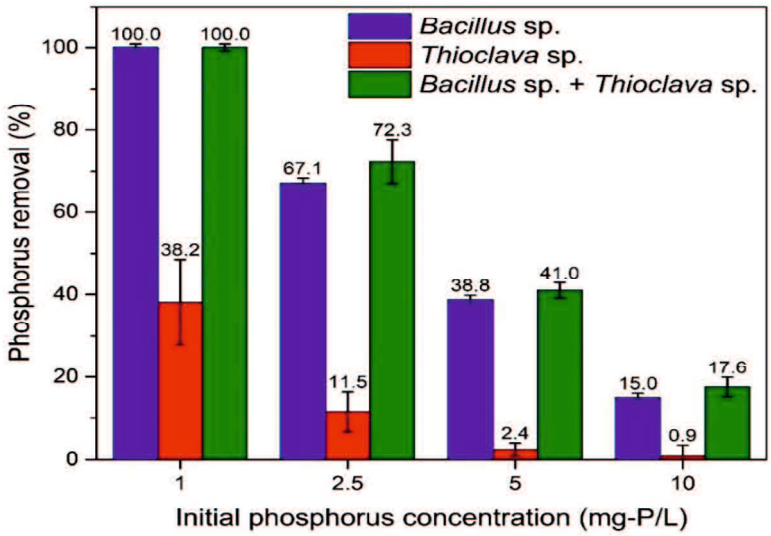

Figure 4.5 The effect of initial phosphorus concentration on phosphorus removal of TR1 (*Bacillus* sp.), MA3 (*Thioclava* sp.), and the combination of strain TR1 and MA3, under aerobic condition at 35 ℃, pH 7, and salinity 3.5% w/v.

phosphorus removal percentages of TR1 decreased from 67.1% to 15.0% as the P concentration increased from 2.5 to 10 mg-P/L. Meanwhile, MA3 could only remove 11.5% and 2.4% when the P concentration were 2.5 and 5 mg-P/L, respectively. Furthermore, a very small portion of P was reduced by MA3 at a P concentration of 10 mg-P/L. This is supported by Zhang et al. (2018), who found that an initial low phosphorus concentration creates conditions conducive for the growth of *Bacillus* sp. PK1 and stimulates enzyme activity induced by phosphoric acid that increases the total phosphorus uptake, but does not increase the rate of phosphorus removal, but a decreasing trend with an increase in the initial phosphorus concentration is observed (Li et al., 2012). According to Choi et al. (2013), conventional biological methods are effective in reducing wastewater phosphate levels to ~1 mg/L, and necessitates long durations of microbial adaptation for effective phosphate removal . In addition, perhaps the loopful of each strain inoculated in this experiment was insufficient for removing phosphorus at concentrations of 5 and 10 mg-P/L. The number of bacteria was not measured for this study, so in order to determine the relationship between phosphate uptake and the number of microorganisms, it will be necessary to measure it in the future.Thus, we attempted the plate count method using a calculation approach using the McFarland standard. Based on a calculation approach using the McFarland standard the amount of P uptaken 1 x 1011 CFU at concentration 1 mg-P/L is 0.22 mg-P for TR1, 0.16 mg-P for MA3 and 0.15 mg-P for combination, respectively.

Figure 4.6 shows The effect of initial phosphorus concentration on bacterial growth of TR1, MA3, and the combination under aerobic conditions. The figure shows that the calculation of OD 600 of the combination was more significant than the others. The lower P accumulation may be associated with the slower growth rate of TR1 and MA3 at cell density (OD_{600} of TR1 0.304 and 0.204; MA3 0.041 and 0.038, respectively) concurring

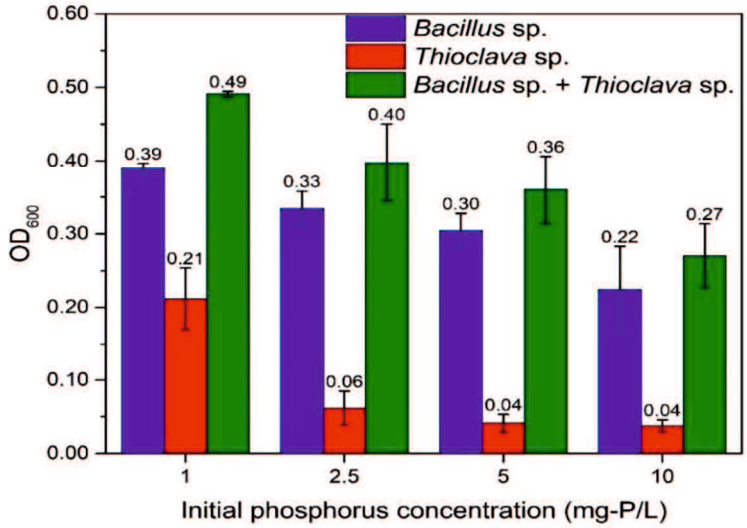

Figure 4.6 The effect of initial phosphorus concentration on bacterial growth of TR1 (*Bacillus* sp.), MA3 (*Thioclava* sp.), and the combination of strain TR1 and MA3 under aerobic condition at 35 ℃, pH 7, and salinity 3.5% w/v for 24 h.

with the result that the removal efficiency of this method is relatively poor at higher phosphorus concentrations, as shown in Figure 4.6. Besides, the lower P accumulation of MA3 compared to TR1 was related to the slower growth rate of MA3 at the salinity used (OD_{600} of TR1 and MA3 at salinity 3.5% are 1.8 and 1.5, respectively).

In this research, we did not measure the amount of bacteria, but we measure the cell densities of each bacterium (TR1, MA3, and their combination). *Bacillus* sp. (TR1) with a cell density of 0.39 can completely remove phosphate with an initial concentration of 1 mg-P/L. However, the increase in phosphate concentration, from 2.5 to 10 mg-P/L decreased the percentage of phosphate uptake of *Bacillus* sp. (TR1) and *Thioclava* sp. (MA3) as well as their combination (TR1-MA3), indicating that the amount of bacteria used in this study was not enough to remove phosphate with concentrations higher than 1 mg-P/L. To use the bacteria in high concentration effectively, in the future, it is important to find an effective ratio between bacteria and phosphate concentration. Moreover, the fact that *Bacillus* sp. is gram-positive and *Thioclava* sp. is gram-negative can also be a reason for the low P uptake in MA3. Several studies reported that gram-positive organisms exhibit a reasonably higher phosphate accumulation than gram-negative organisms (Oljira et al., 2018; Pasayeva et al., 2011). Another possibility for the low P uptake is that MA3 may be classified as a low phosphate accumulator similar to the strain YG-24 (*Pseudomonas stutzeri*), which accumulates low P concentration to maintain its growth (Li et al., 2012). Additionally, the P removal by combining the two strains (TR1 and MA3) was studied, and the results are shown in Figure 4.5. At a concentration of 1 mg-P/L, P was completely removed by this combination. Moreover, the combination of TR1 and MA3 at initial P concentrations of 2.5, 5, and 10 mg-P/L were 72.4%, 41.0%, and 17.6%, respectively, which was higher than their (TR1 and MA3) individual removal abilities. A study by Krishnaswamy and Muthuchamy (2011), and Oljira et al. (2018) showed that the combination of *Bacillus* sp. and *Pseudomonas* sp. efficiently removed phosphate from synthetic wastewater, suggesting that the combination of bacteria can encourage the increase in growth for a greater phosphate uptake capacity. This was attributed to an increase in the nutrient utilization rate of the polyphosphate organisms (Krishnaswamy et al., 2009; Oljira et al., 2018). This is consistent with our study of combined *Bacillus* sp. (TR1) and *Thioclava* sp. (MA3), which effectively removed phosphorus from saline wastewater. In addition, probably that the number of bacterial cell densities (Figure 4.6) that grow is greater for phosphorus removal of the combined bacteria in the ratio of 1: 1 compared to individual bacteria. Moreover, Roller and Schmidt (2015) stated that changes in cellular P demand can affect how efficiently cells grow, or how much biomass is produced per unit nutrient. This study allowed us to confirm that P uptake/P accumulation can be achived with single-stage aerobic process. Whereas, Figure 4.7 shows the capability of P uptake by baceria in the aerobic condition by utilizing sodium acetate as a carbon source. This carbon source is crucial for their metabolisms and cell growth, producing new bacteria and the other products such as, carbon dioxide, water, and energy (Seviour et al., 2003; Yuan et al., 2012).

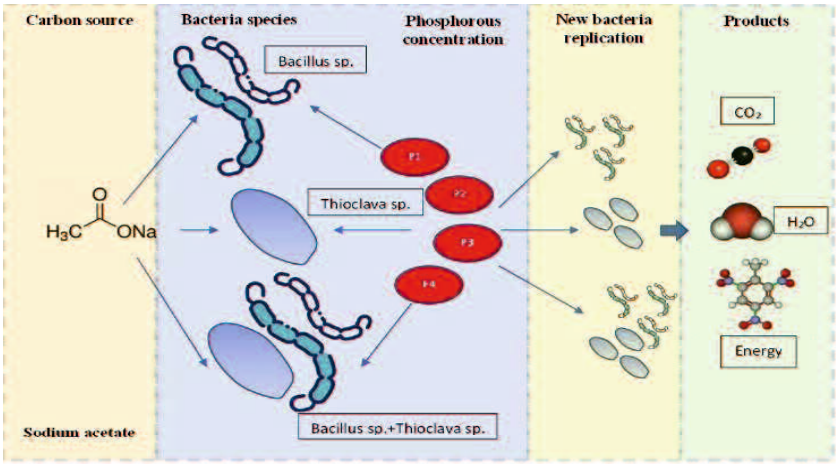

Figure 4.7 Phosphorus uptake mechanisms.

Furthermore, the efficient removal of P by the combination as compared to the single culture of bacteria may be due to the synergistic activity between the individual strains in a combination (Cho et al., 2018; Krishnaswamy and Muthuchamy, 2011; Oljira et al., 2018). This could imply that the nutrient composition of the media used for P uptake experimentation is of importance due to the fact that nutrient availability affects growth rates of organisms.

Table 4.1 compares the phosphorus removal efficiency of TR1 and MA3 in this study with prior studies strains KGN1 (*Bacillus* sp.), KGP1 (*Vibrio* sp.) (Eom et al., 2018), YG-16 (*Sphingomonas* sp.), BL-21 (*E. coli*) (Li et al., 2012), and recombinant *E. coli* (Choi et al., 2013; Li et al., 2012). The P accumulating abilities of YG-16, BL-2, and recombinant *E. coli* were evaluated in non-saline synthetic wastewater, while KGN1 and KGP1 were investigated in saline wastewater. It was demonstrated that TR1 has a significantly higher removal efficiency than *Sphingomonas* sp., *E. coli*, and recombinant *E. coli* at a P concentration of 1 mg-P/L; TR1 could completely remove P, whereas *Sphingomonas* sp., *E. coli*, and recombinant *E. coli* eliminated only 50%, 55%, and 58% of P, respectively. In addition, TR1 also displayed a higher P removal efficiency (38.8%) than *Sphingomonas* sp. (30%) and *E. coli* (26%) at initial P concentrations of 5 mg-P/L. Furthermore, the initial concentration was increased to 10 mg-P/L, *Sphingomonas* sp. and *E. coli* removal efficiencies (20% and 19%, respectively) slightly outperformed the P removal efficiency of TR1(15%). However, the P uptake activity of strain TR1 and MA3 seems not to be high enough comparing with that reported by Eom et al., (2018). On the other hand, *Thioclava* sp. (MA3) showed the least P removal ability, as shown in Table 4.1.

Table 4.1.Comparison table of the phosphorus removal efficiency of TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) in this study with KGN1 (*Bacillus* sp.), KGP1 (*Vibrio* sp.), YG-16 (*Sphingomonas* sp.), BL-21 (E. coli), and recombinant E. coli.

Initial P concentration (mg-P/l)	P removal efficiency (%)						
	TR1	MA3	KGN1 *Bacillus* sp.	KGP1 *Vibrio* sp.	YG-16 *Sphingomonas* sp.	BL-21 *E. coli*	Recombinant E.coli

	Bacillus sp. (This study)	*Thioclava* sp. (This study)	(Eom et al., 2018)	(Eom et al., 2018)	(Li et al. 2012)	(Li et al. 2012)	(Choi et al., 2013)
1	100	38.2	-	-	50	55	58
5	38.8	2.4	90.6	84.6	30	26	-
10	15	0.9	-	-	20	19	-

Table 4.2 shows the comparison of P removal efficiency (%) between combination of TR1 *Bacillus* sp. and MA3 *Thioclava* sp, and KGN1 *Bacillus* sp. and KGP1*Vibrio* sp. From the table, Cho et al. (2018), used the combination of *Bacillus* sp. KGN1 and *Vibrio* sp. KGP1 isolated from marine sediment, which showed high removal efficiency of 99.9% phosphorus with a total P concentration of 1.0 mg-P/L in saline wastewater. This indicates the ability of combination TR1 and MA3 had the similar results with KGN1 and KGP1 in removing P in saline wastewater.

Table 4.2.Comparison table of the phosphorus removal efficiency of combination TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) in this study with KGN1 *Bacillus* sp. and KGP1 *Vibrio* sp. in saline wastewater

Initial P concentration (mg-P/l)	P removal efficiency (%)	
	TR1 *Bacillus* sp. and MA3 *Thioclava* sp. (This study)	KGN1 *Bacillus* sp. and KGP1 *Vibrio* sp. (Cho et al., 2018)
1	100	99.9

Using biological treatment technology to remove phosphorus in saline wastewater may be difficult. Many investigations on the biological treatment of saline wastewater have discovered that salt has a considerable negative impact on biological system performance. It might, for example, increase the concentration of suspended particles in effluent, diminish the effectiveness of organic removal, and inhibit bacterial metabolism (Bassin et al., 2011; Pronk et al., 2014). The marine bacteria in this study are likely to be useful in removing phosphorus from saline wastewater. The high P removal efficiency of TR1 in saline wastewater was also confirmed by a study conducted by Eom et al. (2018) using *Bacillus* sp. to remove phosphate from saline wastewater. Meanwhile, current research on *Thioclava* sp. deals with the sulfur-oxidizing genus, which has demonstrated chemoautotrophic growth on intermediate sulfur compounds, including thiosulfate and heterotrophic growth on simple organic matter, including glucose (Acevedo et al., 2017; Sorokin et al., 2005). In addition, Chen et al. (2018) suggested that adding *Thioclava* sp. could improve nitrogen and phosphorus removal for the treatment of saline wastewater. However, our research showed that these bacteria (TR1 and MA3) had the ability to reduce phosphorus, both in pure culture and in combination. Both marine bacterial strains (TR1 and MA3) are suitable for phosphorus removal in saline wastewater, where TR1 had a higher phosphorus removal than MA3 based on the amount of phosphorus accumulated. The results of this study provide information on the removal of low

concentrations of P, notably by the pure strain PAOs. This study revealed a promising bacterial strain and a potential approach for removing P from saline water containing around 1.0 mg L1 of TP. In China, for example, effluent water in most wastewater treatment plants in total P(TP) concentrations range from 0.5 to 1.0 mg/L, which is much higher than the minimal quantity (0.03 mg/L), resulting in eutrophication (Li et al., 2012). When P in the discharge water is reduced to less than 0.03 mg/L, it is effective in controlling eutrophication (Dodds et al., 2002). According to Australian and New Zealand standards for fresh and marine water quality, phosphate concentrations from primary industries should be less than 0.1 mg/L in freshwater and less than 0.05 mg/L in salt water (ANZECC & ARMCANZ., 2000). For that reason, removing P from water with low P concentrations (1.0 mg/L) is crucial. Strain TR1and MA3 is a good choice to apply in low concentration of P removal in saline wastewater. Strain TR1and MA3 is a good choice to apply in low concentration of P removal. This indicates that both TR1 and MA3 can be used for phosphorus removal in marine environments.

4. Conclusion

The results of this study indicated that strains TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) utilized different carbon sources, such as glucose, sucrose, and CH_3COONa, for their growth. A study on environmental pH adaptation showed that the strain TR1 prefers pH 6–8 and MA3 favors pH 6–9 for growth. Both bacteria can grow in the temperature range of 25−40 °C. Strain TR1 preferred a salinity range of 0%–10 % with the optimum growth observed at 3.5%-5%, while MA3 favored a salinity range of 1%–10% and grew optimally at a salinity of 4%. *Thioclava* sp. (MA3) growth was undetectable in non-saline water. Phosphorus removal studies in various phosphorus concentrations at 3.5% salinity revealed a higher phosphorus removal efficiency of TR1 than the MA3. Furthermore, a combination of TR1 and MA3 showed P removal efficiency of 100% at an initial phosphorus concentration of 1 mg-P/L that showed similar values to TR1. The initial phosphorus concentration of 2.5 mg-P / L showed a slightly higher 72.35% P removal efficiency compared to the individual strains. The combination possibly built a synergistic activity between the individual strains to remove phosphorus. However, phosphorus removal did not increase, but showed a downward trend with increasing at initial phosphorus concentration of 5 and 10 mg-P/L. Therefore, the loopful of culture inoculated into the batch process must be increased by varying the ratios, and long duration for microbial adaptation should be provided. These experimental results indicate that two marine bacteria can be utilized as an economically and environmentally viable biological treatment method for developing phosphorus removal processes, especially in removing low concentrations of P from saline wastewater.

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CHAPTER 5

CONCLUSIONS AND FUTURE WORKS

5.1 Conclusions

The conclusions gathered from this study are as follows:

1. The batch test in saline wastewater with various concentrations of P showed that p removal by mix culture (PAOs) was > 70 % in 1, 5, and 10 mg-P/L and ~50 % in 20 mg-P/L.
2. Bacterial screening by isolation and sequence analysis illustrated that *Bacillus* sp. (TR1) and *Thioclava* sp. (MA3) were identified in the saline wastewater as phosphorus accumulating organisms (PAOs).
3. These experimental results indicate that TR1 (*Bacillus* sp.) and MA3 (*Thioclava* sp.) can be utilized different carbon sources (glucose, sucrose, sodium acetate).

Strain	pH	Temperature (°C)	Salinity (% w/v)
TR1	6-8	25-40	3.5 - 5
MA3	6-9	25-40	3.5 - 6

4. *Bacillus* sp. and bacterial combination (TR1 and MA3) showed similar values for phosphorus removal efficiency (100%) at 1.0 mg-P/L total P compared to *Thioclava* sp. (38.2%). However, phosphorus removal did not increase, but decreased as the initial phosphorus increased.

5.2 Future Works

Suggestions for future research topics concerning the application of phosphorus accumulating organisms (PAOs) are listed below:

1. To enable future research on the functional diversity, reliable selection methods and medium for isolation of PAOs should be developed to obtain highly enriched or pure cultures with specific PAOs
2. The loopful of culture inoculated into the batch process must be increased by varying the ratios.
3. In order for phosphorus removal to be effective, a long duration of microbial adaptation could be offered.
4. The investigation of the effectivity of enhanced biological phosphorus removal from saline wastewater by using sodium acetate as a carbon source in this experiment did not show high performance. Therefore, it is necessary to do further research using other carbon sources to know whether these other carbon sources can increase the phosphate removal of the bacteria higher than using sodium acetate or not.

APPENDIX

LIST OF PUBLICATIONS

1. **Hasanah R**., Imai T., Kanno A., Higuchi T., Sekine M., Yamamoto K. (2021). Screening Microorganisms for Phosphorus Removal in Saline Wastewater. Pollution Research. 40,2,526-534.
2. **Hasanah R**. & Imai T. Effect of abiotic factors on *Bacillus* sp and *Thioclava* sp. for Phosphorus removal in saline wastewater. Asia-pacific Journal of Science and Technology. (Under review)

LIST OF PRESENTATIONS

1. **Hasanah R**. & Imai T. Screening Microorganisms for Phosphorus Removal in Saline Wastewater. *Water and Environment Technology conference 2020-online. Online meeting*. November 7-8, 2020. (Oral and poster presentation)
2. **Hasanah R**. & Imai T. Biological phosphorus removal performance by microorganisms in saline-wastewater. *The 17th Young Scientist Seminar "Establishment of International Research Network for Bioresources and Their Utilization"*. Online Meeting. December 28-29, 2020. (Oral presentation)
3. **Hasanah R**. & Imai. T. Biological phosphorus removal performance using salt-tolerant phosphorus accumulating organisms in seawater. *Water and Environment Technology Conference 2019*. Osaka University, Japan. July 13-14, 2019. (Oral and poster presentation)
4. **Hasanah R**. & Imai. T. Biological phosphorus removal performance using salt-tolerant phosphorus accumulating organisms in seawater. *The 16th Young Scientist Seminar "Establishment of International Research Network for Tropical Bioresources and Their Utilization"*. Yamaguchi Prefectural Seminar Park, Japan. October 12-13, 2019. (Oral presentation)
5. **Hasanah R**. & Imai. T. Screening of marine bacteria for phosphorus removal in saline wastewater. The 5th International Symposium "Green and Smart Technologies for a Sustainable Society". Faculty of Engineering, Yamaguchi University. March 25-27, 2019. (Poster presentation)
6. **Hasanah R**. & Imai. T. Screening of marine bacteria for phosphorus removal in saline wastewater. *The 15th Young Scientist Seminar "Establishment of International Research Network for Tropical Bioresources and Their Utilization"*. Yamaguchi Prefectural Seminar Park, Japan. November 13-14, 2018. (Oral presentation)