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# Isolation, Characterization and Identification of Purple Non Sulfur Bacteria for Cadmium Removal from Aqueous Solution

[a]Balqis Nurul Huda Armadi, [a]Siti Halimah Hasmoni, [a]Shafinaz Shahir, [a]Wahid Ali Hamood Al Towayti, [a]Zaharah Ibrahim

[a] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.

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*Corresponding author
sitihalimah@utm.my

**Abstract**

Cadmium (Cd) contamination in aquatic ecosystems poses serious environmental and health challenges due to its high toxicity and non-biodegradable nature. Conventional methods for Cd removal are often expensive and environmentally unsustainable, highlighting the need for alternative bioremediation approaches. Purple Non-Sulfur Bacteria (PNSB) have shown potential in heavy metal biosorption, but their application in Cd removal requires further exploration.This study is focused on the isolation, characterization and identification of purple non sulfur bacteria (PNSB) isolated from Kim Kim River, Pasir Gudang, Johor and its ability in removal of Cadmium from aqueous solution. PNSB was isolated and identifies based on morphological, pigment analysis and 16S rRNA approaches. *Rhodobacter* sp. was isolated and identified and was then studied for its ability to remove Cd in different environmental conditions such as various temperature, initial Cd solution pH and microbial growth conditions. The results showed that the best condition for Cd biosorption by *Rhodobacter* sp. was at 25°C, initial cadmium pH of 5.5 and facultative light incubation condition with 37.77% removal of Cd from aqueous solution. In conclusion, *Rhodobacter s* sp. isolated from Kim Kim River has the potential to remove cadmium from aqueous solution.

*Keywords* Purple Non-Sulfur Bacteria (PNSB); Rhodobacter sp.; biosorption; cadmium removal

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## 1.0 INTRODUCTION

The frequent use of agrochemicals such as fertilizers, herbicides (Ahmed et al., 2015; Mshair et al., 2020) and sewage disposal or discharges from industries that are not properly equipped with outflow treatment facilities contaminate the freshwater resources with toxic elements such as lead, zinc, mercury, and cadmium. Due to the impact of the pollution, the ecological biotic and abiotic components in the water are disrupted and affect human's health. Regular consumption of polluted water by these heavy metals leads to accumulation and could lead to vomiting, skin lesions, poor blood circulation, and damage to the human heart, kidneys, and nervous system (Afroz et al., 2016). Therefore, an increased interest is in developing a heavy metal removal system from aqueous solution.

Bioremediation using purple non-sulfur bacteria (PNSB) is one of the microorganisms frequently studied for heavy metal removal via biosorption or bioaccumulation. PNSB is a photosynthetic bacteria that is packed with bacteriochlorophylls and carotenoids and has been known for its potential to remove heavy metals from water. Their diverse metabolic traits help them to utilize organic and inorganic substances for their growth (Ashokkumar, 2015), depending on the environment in which

they are grown. Other than that, PNSBs are also known for their potential in many other biotechnology applications, such as in the production of plant growth hormones and biodegradable plastics. PNSB such as *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* has been shown to remove a wide range of heavy metals such as aurum, zinc, cadmium and plumbum ( Li et al., 2017; Mukkata et al, 2019).

In March 2019, Kim Kim River was polluted with chemical waste which was caused by illegal dumping by an irresponsible party. The massive water pollution in Kim Kim River had significant health impacts on nearby residents, especially children. This incident has made Pasir Gudang the most polluted area in Johor. PNSB is known to be a versatile microorganism that can use any organic compound as their carbon or energy source which makes it possible to isolate PNSB from this river. To date, locally isolated PNSB and the application of locally isolated PNSB for heavy metal removal are scarce. Thus, this research will study and characterize locally isolated PNSB for its capability to remove cadmium from aqueous solution. The PNSB was isolated from the upper stream of Kim Kim River in Pasir Gudang. The PNSB will be characterized on its morphology, pigments, and growth profile and its availability in removing cadmium from aqueous solution. The ability of PNSB to remove cadmium from aqueous solution was analyzed quantitatively and the factors affecting PNSB in removing cadmium were also investigated in this study.

## 2.0 EXPERIMENTAL

### 2.1 Bacterial Isolation

#### 2.1.1 Media Preparation

The prepared enrichment media were purple non-sulfur bacteria enrichment medium (PNSBEM) adapted from Feng et al. (2007). PNSBEM was prepared with 1 g/L $NH_4Cl$, 0.5 g/L $Na_2HPO_4$, 0.2 g/L $MgCl_2$, 2 g/L NaCl, 2 g/L yeast extract, 6 mL of 80% sodium lactate and distilled water up to 1000 mL. The medium was then adjusted to pH 7 and sterilized via autoclaving.

#### 2.1.1 Sampling and Isolation of Purple Non-Sulfur Bacteria

Water samples from the upper stream area of Kim Kim River (1°28’42.0’’N 103°56’11.5’’E) were collected at a depth of approximately 0.5 m from the water surface. The water sample was then inoculated 10% (v/v) into PNSBEM. The media were then incubated under anaerobic condition along with light intensity between 2000 to 3000 lux at room temperature until the inoculum culture turn to purple or red.The purple inoculum was harvested by serial dilution and spread plate technique and the bacteria was streaked on fresh agar plates until single colony was obtained.

### 2.2 Characterization of PNSB

#### 2.2.1 Gram Staining

A loopful of the bacteria colony was picked and stirred into a drop of water using an inoculating loop and mixed with distilled water on the glass slide. The glass slide was air dried before proceeding to Gram staining method by adding a few drops of crystal violet and flooded the glass slide with iodine. The cell shapes and the presence of specialized structures were observed under a light microscope of 100 x magnification.

#### 2.2.2 Photopigment analysis

Photopigment analysis was done on day 7 of the incubation period. 2 mL of the culture was centrifuged at 3,300 rpm for 15 minutes before resuspending in 60% sucrose solution. The spectra absorbance of the cells in sucrose solution was measured by Hach DR 5000 UV- Vis spectrophotometer at 300-1000 nm (Hiraishi et al. 2020).

#### 2.2.3 Growth Profile and Dry Cell Weight Analysis

The isolated bacteria was grown in PNSBEM and incubated for seven days at 30°C under anaerobic light condition. Bacterial growth (optical density, OD) was done in triplicates and was analyzed from day 1 to day 7 of incubation using spectrophotometry at 660 nm.

The dry cell weight analysis was done in triplicate from day 1 to day 7. The isolated bacteria of 10 mL from the culture was harvested using 4,400 rpm centrifuge for 20 minutes at 4°C and the bacterial pellet was resuspended using sterile distilled water and centrifuged again. Then, the bacterial cells were dried in the oven at 60°C for 24 hours.

## 2.3 Bacterial identification

### 2.3.1 DNA extraction

The DNA from the bacterial culture was extracted using the Wizard genomic DNA extraction kit (Promega, Wisconsin, USA) for PNSB identification. Then, PCR amplification of the 16S rRNA gene was performed with 25 µL reaction volume consisting of 10 µM of each 16S rRNA primer, fD1_0815 (5’-AgA gTT TgA TCC Tgg CTC Ag-3’) and rD1_0815 (5’-AAg gAg gTg ATC Cag CC-3’), 1.5 µL of DNA template and 12.5 µL of GoTaq® Green Master Mix in a Eppendorf™ Mastercycler™ Nexus Thermal Cycler, with conditions, 5 minutes at 94˚C for initial denaturalization, 30 cycles of 1 minute at 94˚C for denaturalization, 1 minute at 55˚C for annealing, 4 minutes at 72˚C for extension and 10 minutes at 72˚C as the final extension. The 1% agarose gel electrophoresis was used to visualize the PCR products by using ethidium bromide as stain to confirm the presence of DNA (Kantachote *et al*., 2005). The PCR products were run through gel electrophoresis for 55 minutes using 425 ampere at 80 V.

### 2.3.2 DNA sequencing analysis

The PCR product was sent to Apical Scientific Sdn Bhd for purification and sequencing. Sequence differentiation was performed using the Finch TV, BioEdit and online BLASTn program at (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The result obtained from BLASTn was then clustered using MEGA X software to construct the phylogenetic tree using the neighbor joining method. The sequences were later submitted to NCBI.

## 2.4 Biosorption experiments

Bacterial culture was grown in PNSBEM media for four days under anaerobic light condition at 30°C. Then, it was centrifuged at 3,500 rpm for 15 minutes and washed thrice with pH 7 0.1% peptone water. 13.5 mg of wet cell was added into 30 mL of 2 mg/ L cadmium solution at pH 7. Then, the cell was incubated under anaerobic light conditions for 30 minutes at 30°C, and the cell suspensions were centrifuged at 8,000 rpm for 30 minutes. The cadmium concentration was analysed using Perkin Elmer AA-6300 atomic absorption spectrophotometer and the cadmium removal was calculated quantitatively using the following formula: The experiments were done in triplicate.

$$\% \text{ of Cd Removal} = [(C_o - C_e)/ C_o] \times 100 \quad \text{(Gupta and Kumar, 2019)} \tag{1}$$

where $C_o$ = Initial Cd concentration (mg/ L)
$C_e$ = Final Cd concentration (mg/ L)

## 2.5 Factors Affecting PNSB Ability in Cd Removal

The biosorption experiment was carried out in three different conditions to determine the factors affecting the identified PNSB ability to remove Cd and was analyzed as previously described.

The cell pellets for biosorption were prepared as previously described but with varying factors that affects the metabolism of PNSB and the capability of biosorption at different temperatures, pH and growth conditions. The factors are as follows: temperature (20°C , 25°C, 35°C, 40°C and 45°C), pH (pH 5.5, 6, 6.5, 7.5 and 8) and oxygen-light incubation conditions (anaerobic dark, facultative light and facultative dark).

## 2.6 Statistical analysis

All experiments in this study were conducted in triplicate, and the data was analyzed using one-way ANOVA, and the significance difference among means were analyzed using Duncan’s multiple range tests at a P-value <0.05.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Growth profile and average dry cell weight analysis of PNSB

The growth profile of isolated bacteria in Figure 1 shows a sigmoid curve obtained after seven days of incubation under anaerobic light condition. The lag phase started from day zero until day one and followed by a log phase from day two until day 3. Stationary phase was observed starting from day 3 until day seven. The dry cell weight of *Rhodobacter* sp. was observed throughout the seven days of incubation period under light anaerobic condition alongside its growth profile (OD, 660 nm). The linear regression in Figure 2 shows that the $R^2$ value of the average dry cell weight and growth profile is equivalent to 0.7163 with 71.63% accuracy. The data proves that it is significant and reliable for biomass preparation.

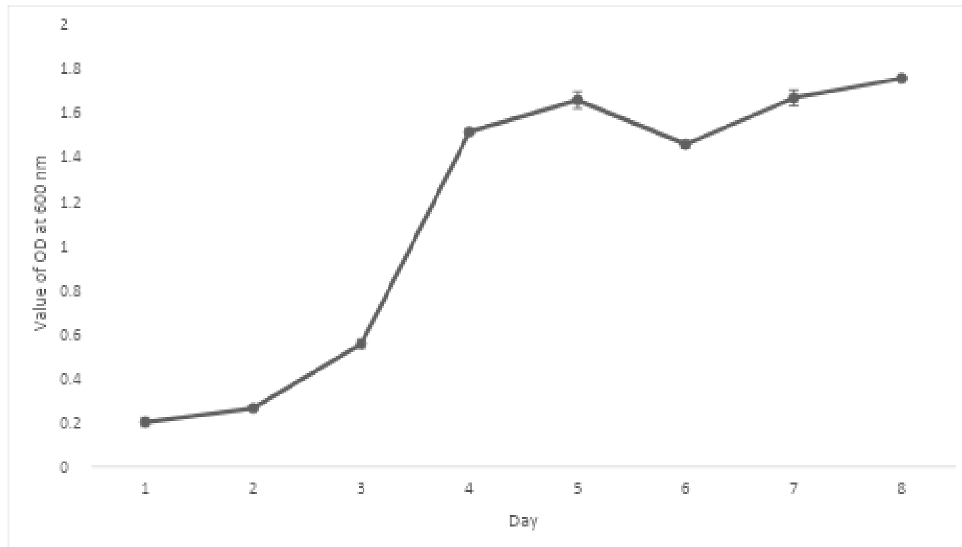


**Figure 1** Growth curve of isolated PNSB.

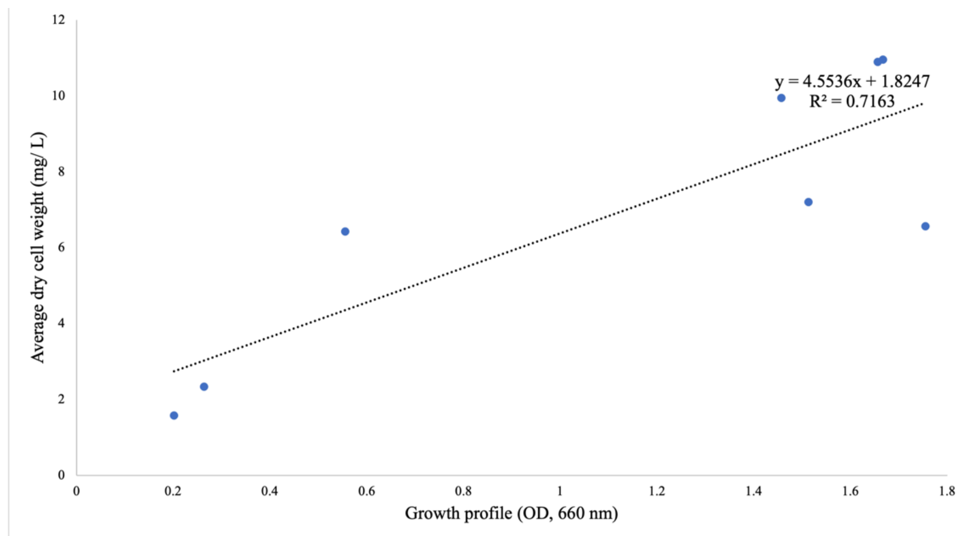


**Figure 2** Linear regression of average dry cell weight against growth profile of isolated PNSB over seven days.

### 3.2 Characterization of isolated PNSB

After 7 days of incubation under anaerobic light, the inoculated bacteria's colour changed from cloudy to dark purple in both PNSBEM and basal media.

**Figure 3** PNSB culture after 7 days of incubation.

### 3.2.1 Gram staining

The Gram staining of isolated PNSB showed that the cells were Gram-negative and rod-shaped.

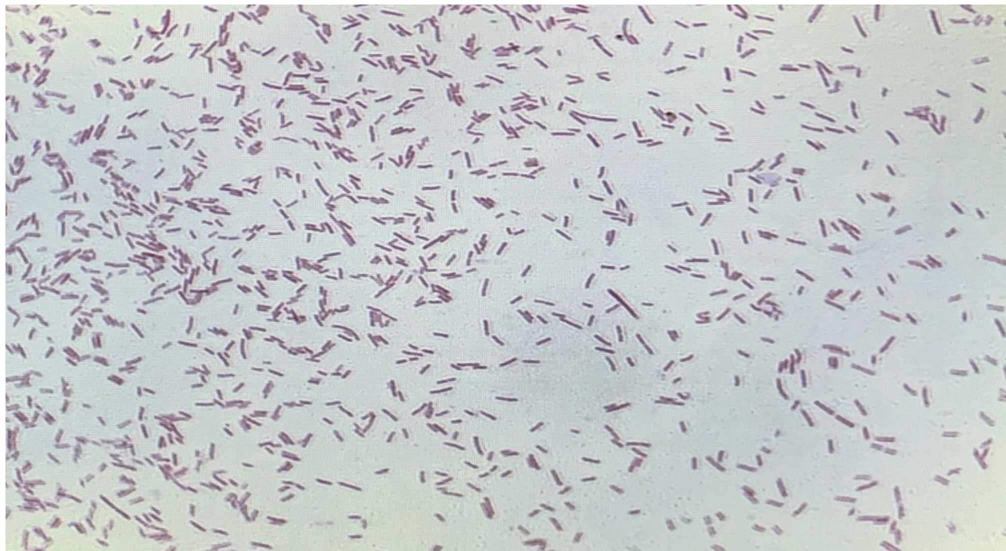

**Figure 4** Isolated PNSB observed under 40 x magnification

A rod-shaped gram-negative prokaryote is observed. PNSB is known to have a rod-shaped structure, which can be observed through the Gram staining method (Chen et al., 2020).

### 3.2.2 Photopigment analysis of PNSB

Based on Figure 5, few peaks were observed between 300 to 900 nm. Peak 350 nm indicates the presence of carotenoid, 500 and 600 as bacteriochlorophyll *b* indicators while peak 800 nm indicates bacteriochlorophyll *a*. According to Achenbach et al. (2001), PNSB consists of bacteriochlorophyll *a* or *b* and carotenoid as their primary pigments.

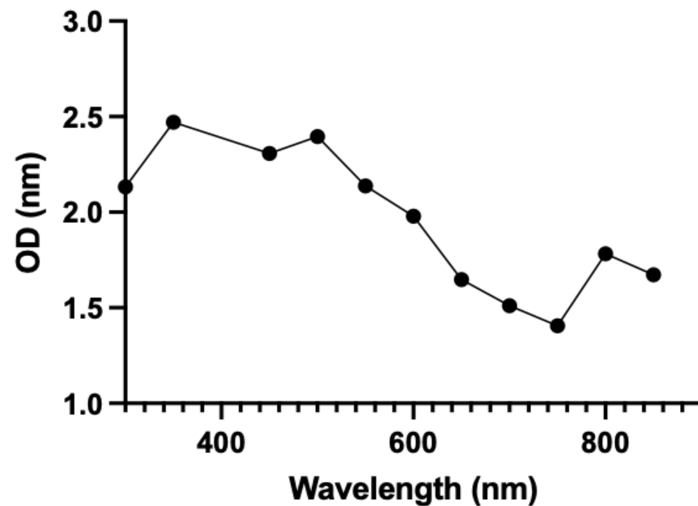


**Figure 5** Photopigment analysis of PNSB ranging from 300 to 900 nm.

## 3.3 Bacterial identification

Genomic DNA from the isolated PNSB was successfully extracted and 16S rRNA was successfully amplified. The DNA sequence data was obtained, and the verification was done via BLASTn. The bacteria were 98.19% identical to *Rhodobacter capsulatus* strain HS-1, with 98% query coverage. In addition, based on the BLASTn best hits, the expected value (E-value) of bacteria were found to be '0.0' which means the bacteria is closely related to the sequences found in the database. The bacteria, *Rhodobacter* sp. strain A1, was then uploaded into the database with the accession number MT605396.1. The phylogenetic tree was done by using the neighbor-joining method in MEGAX software. Based on Figure 6 below, the bacteria were labeled as A1, and it showed that it belongs to the family of *Rhodobacteraceae* and the sister taxa of *Rhodobacter capsulatus* strain HS-1 and *Rhodobacter capsulatus* strain DBNRh31.

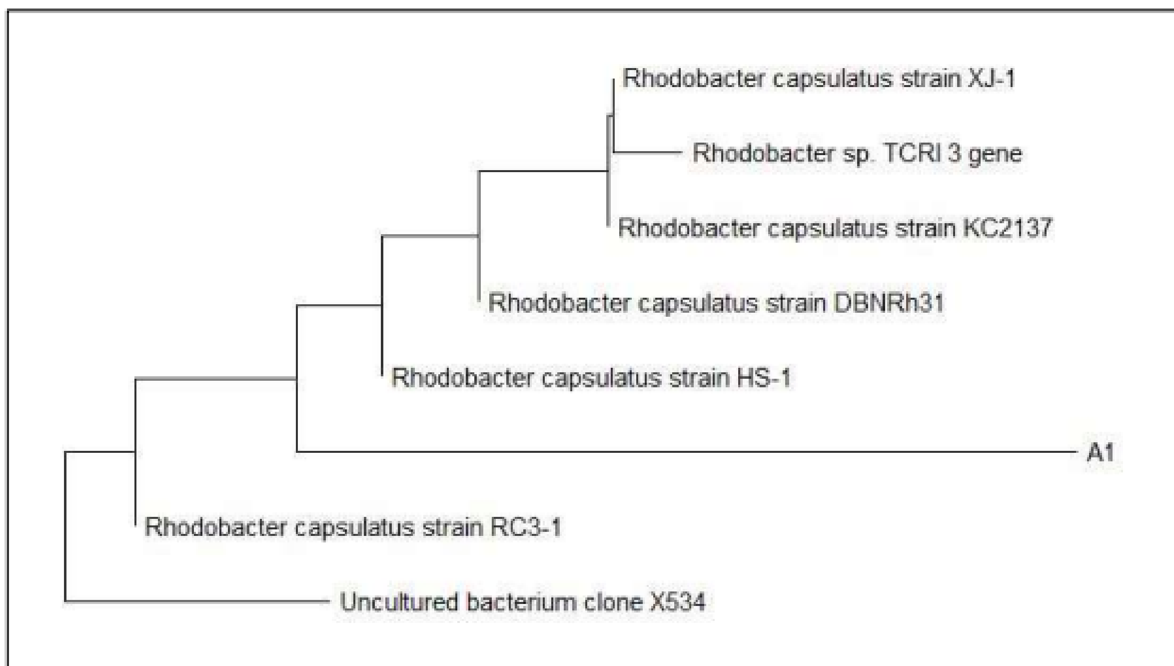


**Figure 6** Phylogenetic Tree of *Rhodobacter* sp. strain A1

## 3.4 Factors affecting *Rhodobacter* sp. strain A1 ability in Cd removal

Day four of the growth profile was chosen for biomass preparation and is the highest point of the log phase or late log phase of microbial growth. During this time, rapid cellular division and metabolic activity occurs and exhibit optimal physiological conditions, including an actively growing and metabolically robust cell wall, which is crucial for efficient heavy metal binding.

The late log phase is particularly advantageous for $Cd^{2+}$ removal because the cell wall and associated binding sites are at their most functional and accessible state. This is in contrast to the stationary phase, where cellular metabolism slows, and structural changes in the cell wall, such as thickening or reduced permeability, can limit the availability and efficacy of binding sites for $Cd^{2+}$ attachment (Abbas et al., 2014; Mukkata et al., 2019). Furthermore, the nutrient-depleted conditions of the stationary phase may induce stress responses that alter the chemical composition of the cell surface, potentially decreasing its affinity for heavy metals.Choosing biomass at the late log phase ensures maximum efficiency in $Cd^{2+}$ adsorption or uptake, as the cells retain their structural and functional integrity, making them more suitable for applications in bioremediation or metal recovery

### 3.4.1 Effect of temperature on Cd removal

The incubation temperature of 25°C offers the highest $Cd^{2+}$ uptake and has the highest removal percentage of 38.11%, which may be because of the enhanced surface activity by the temperature that leads to an increase in the biosorption capacity (Aryal and Liakopoulou- Kyriakides, 2013; Mukkata et al., 2019). An incubation temperature of 30°C removed the least amount of $Cd^{2+}$ by 36.25%. The difference between each incubation temperature is insignificant, with a p-value more than 0.05 indicating that the temperature does not substantially affect the biosorption efficiently within the tested range.

PNSB is known to have strong temperature adaptability of 10 – 40°C (Aryal and Liakopoulou-Kyriakides, 2013; Mukkata et al., 2019), but the temperature could still affect the permeability of the membrane components (Chen et al., 2020). High temperatures can easily destroy the biomass, which will then reduce the biosorption capacity of *Rhodobacter* sp. strain A1(Aryal and Liakopoulou-Kyriakides, 2013, ; Mukkata et al., 2019) but it can increase the metal solubility when the temperature gets higher (Gupta and Kumar, 2019), this could be one of the reasons when the percentage of removal starts to fluctuate from 30°C to 45°C. However, *Rhodobacter* sp. strain A1 removed a relatively low percentage of $Cd^{2+}$ when the incubation temperature was at 20°C by 36.43% due to low surface activity. Therefore, while temperature-related differences exist, they are minimal and do not significantly impact the biosorption capacity of *Rhodobacter sp.* strain A1 under the experimental conditions.

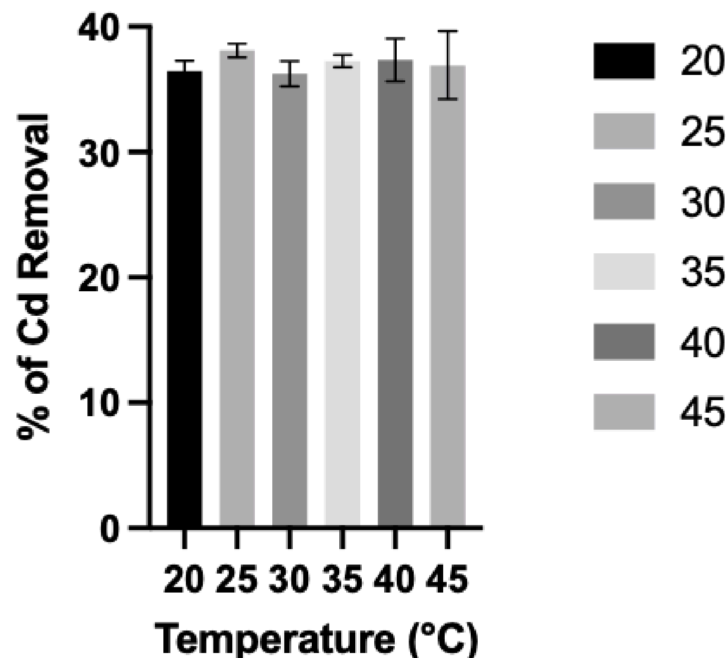


**Figure 7** Effect of temperature on Cd removal by *Rhodobacter* sp. strain A1

### 3.4.2 Effect of pH on Cd removal

The effect of pH on the percentage of Cd removal by *Rhodobacter* sp.strain A1. pH 5.5 removed the highest percentage of Cd, followed by pH 8 and 7.5]. The difference of Cd removal between all the pH conditions tested is statistically significant with a p-value less than 0.05.

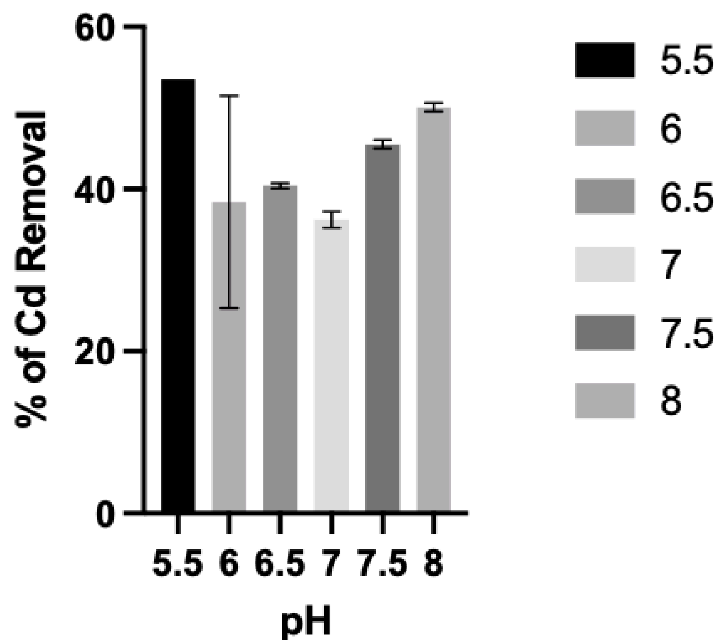


**Figure 8** Effect of initial pH on Cd Removal.

pH is considered one of the most crucial parameters in this experiment because it affects the metal ions ($Cd^{2+}$) solubility (Gupta and Kumar, 2019) and concentration of counter ions on the functional groups of the surface of the cell wall of *Rhodobacter* sp. (Li et al., 2017). The alteration of pH values can disrupt the removal of $Cd^{2+}$ ions, damage the biomass, and collapse the system (Chen et al., 2020). Thus, different initial pH of Cd solutions values was tested to study the effect of pH on Cd removal by *Rhodobacter* sp. strain A1.

*Rhodobacter* sp. strain A1 cell wall consists various types of polysaccharide and proteins that offers several active sites to bind $Cd^{2+}$ ions (Feng et al., 2007) as they are mostly negatively charged surface (Das et al., 2008; Aryal and Kiakopoulou- Kyriakides., 2013; Li et al., 2017) and acidic pH has a significant impact towards the biosorption capacity of Cd by *Rhodobacter* sp. strain A1 as huge amount of $H^+$ ions will protonate the functional groups on the adsorbent surface (Cheng et al., 2016; Elaigwu et al., 2014; Liu and Zhang, 2011; Teng et al., 2020) and will cause $Cd^{2+}$ ions to compete to bind at the active sites on the cell wall (Lestari and Windyartini, 2020). However, pH 5.5 has the highest amount of Cd removed by 53.58%, proving that there was less competition between $Cd^{2+}$ ions and $H^+$ ions to bind to the cell wall when the pH is 5.5.

Furthermore, at low pH, $Cd^+$ ions were hydrolyzed to $CdOH^+$ ions and will hinder the other $Cd^+$ ions to interact with the functional groups on the cell wall of *Rhodobacter* sp strain A1 when higher pH is reached (Lestari and Windyartini, 2020). pH 6, 6.5, and 7 only removed 38.42%, 40.41%, and 36.25% of Cd, respectively, showing that CdOH+ ions were blocking the Cd2+ ions in the solution to interact with the functional groups on the cell wall of *Rhodobacter* sp. which has already been impede by $H^+$.

When the pH is raised, the affinity for metal ions also increases (Gupta and Rastogi 2008; Panwichian and Kantachote, 2010). As shown in Figure 8, pH 7.5 and pH 8 can remove 45.53% and 50.1% of Cd. Although the presence of $CdOH^+$ ions can interrupt the interaction of $Cd^{2+}$ ions and the functional groups, the presence of $OH^-$ may also help to allow $Cd^{2+}$ ions to bind to the cell wall as there will be no competitions between $Cd^{2+}$ and $OH^-$.

### 3.4.3 Effect of oxygen-light on Cd removal

Figure 9 shows the effect of oxygen-light incubation condition on percentage of Cd removal by *Rhodobacter* sp. strain A1. Facultative light has the highest $Cd^{2+}$ ions uptake and percentage of Cd removal which is 38.02%. The presence of oxygen helps to prevent damage to the pigment system (Lu et al., 2011; Lu et al., 2021) and allows *Rhodobacter* sp. strain A1 to

proliferate while the unlimited light contributes to *Rhodobacter* sp. strain A1 to convert light energy into chemical energy abundantly, which help to increase the biological activity and remove the Cd. The importance of oxygen towards *Rhodobacter* sp. strain A1 can be seen when facultative dark removed 37.76% of Cd. At the same time, anaerobic dark and anaerobic light can only remove Cd by 37.75% and 36.25%, respectively. The difference is statistically not significant between all of the growth conditions with a p-value of more than 0.05. This indicates that the growth condition of oxygen-light tested of this strain does not affect the biosorption efficiency.

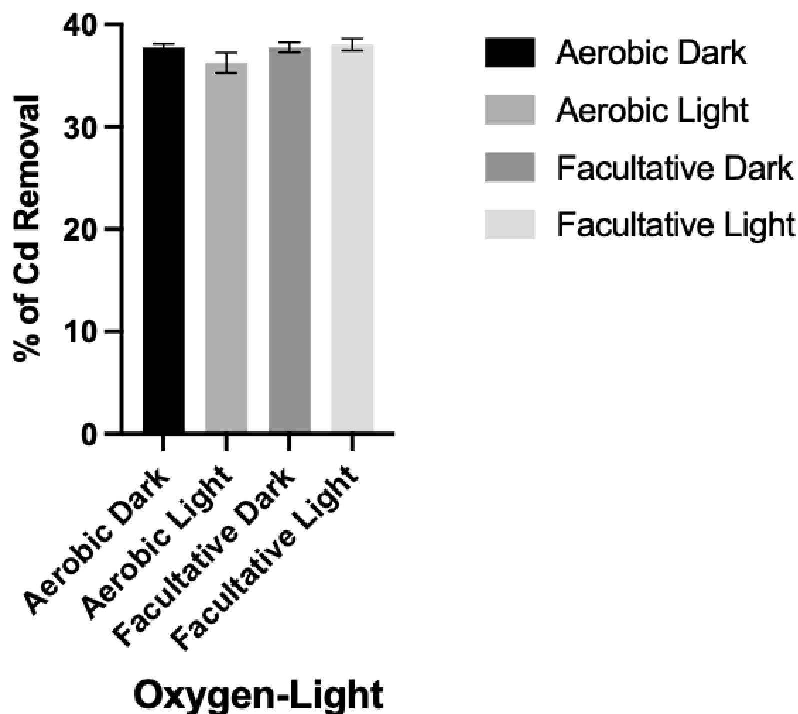


**Figure 9** Effect of PNSB growth condition on Cd removal

*Rhodobacter* sp. strain A1 is known to have a unique metabolism in which oxygen and light are the general factors influencing its growth, and it is able to adjust its metabolism as needed (Chen et al., 2020). According to Lu et al. (2011), oxygen plays a major role, more than light, in Cd removal, macromolecule degradation, and biomass production. It is believed that oxygen has the advantage caused by three important factors in the PNSB metabolic pathways: the final electron acceptor, carbon source, and biomass synthesis pathway. Still, with the presence of light it will help to induce photosynthesis which will provide energy and be beneficial for *Rhodobacter* sp. strain A1 growth and metabolism (Lu et al., 2019; Lu et al., 2021). The effect of oxygen-light condition was studied under four different incubation conditions: facultative dark, facultative light, anaerobic dark, and anaerobic light. Oxygen and light also regulate biological pigment (Chen et al., 2020).

Oxygen will stimulate respiration while light induces photosynthesis for the cell, and the final dominant metabolism depends on the ratio of light and oxygen. According to Chen et al. (2020), generally, CdOH decomposes into intermediate products through the glycolysis pathway and enters the tricarboxylic acid cycle (TCA), taking oxygen as the final electron acceptor, which will result in the completion of Cd to be mineralized. However, in anaerobic-light conditions, *Rhodobacter* sp. strain A1 will perform anoxygenic photosynthesis (Madigan and Jung, 2009). The energy is produced by substrate-level phosphorylation and photophosphorylation (Chen et al., 2020), causing Cd to mineralize as the electron acceptors would differ incompletely. This highlights the versatility of PNSB as a microorganism capable of adapting and biosorption of Cd occurs at a wide range of oxygen conditions, whether facultative or aerobic, and thriving in both light and dark environments.

## Conclusion

In this study, PNSB, identified as *Rhodobacter* sp. Strain A1, was successfully isolated and characterized for its cadmium biosorption potential. The findings show that the temperature of between 25°C to 40°C and the growth condition of oxygen-light of Rhodobacter sp. strain A1 does not significantly affect its ability for biosorption of Cd. However, pH showed a significant effect towards *Rhodobacter* strain A1 in biosorption of Cd indicating pH 5 has the highest Cd removal. These

characteristics make *Rhodobacter sp.* strain A1 a promising candidate for sustainable cadmium remediation strategies in polluted aquatic systems.

Future studies should explore optimizing environmental parameters and scaling up biosorption processes to enhance efficiency. Additionally, the genetic and molecular mechanisms underlying cadmium uptake by *Rhodobacter sp.* strain A1 necessitates further investigation to maximize its application in bioremediation technologies.

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