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Characterization of *Rhodopseudomonas* sp. for hexavalent chromium reduction in solution

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Abstract
The arising of anthropogenic activities leads to an excess discharge of heavy metal into the water that may give rise to water pollution. Therefore, one type of purple non-sulfur bacteria (PNSB) species, *Rhodopseudomonas* sp. isolated from Sungai Kim Kim has been chosen to reduce Cr (VI) to its less toxic form which is Cr (III) due to its ability to thrive in diverse conditions. In this study, *Rhodopseudomonas* sp. was characterized based on its morphology, growth profile and dry cell weight analysis. *Rhodopseudomonas* sp. was proven to be gram-negative bacteria using the Gram staining procedure. Factors such as temperature (20℃, 25℃, 30℃, 35℃ and 40℃), biomass size (2 mg DCW/mL, 4.5 mg DCW/mL, 6 mg DCW/mL, 8 mg DCW/mL and 10 mg DCW/mL), initial pH of Cr (VI) solution (pH 5, 6, 7, 8 and 9) and incubation time (5 min, 15 min, 30 min, 45 min and 60 min) were examined using the dead cell of *Rhodopseudomonas* sp. for Cr (VI) reduction process. Concentrations of Cr (VI) reduction was determined using diphenylcarbazide method before being analyzed quantitatively using UV-VIS Spectrophotometer at 546 nm. The results were calculated to obtain the percentage of Cr (VI) reduction and analyzed using statistical analysis. It showed that the maximum reduction of Cr (VI) was 23% with the optimal condition of *Rhodopseudomonas* sp. biomass is 6 mg DCW/mL which is incubated in Cr (VI) solution at pH 6 for 15 minutes at 35℃. As a conclusion, *Rhodopseudomonas* sp. has the potential to be applied in bioremediation for reducing the Cr (VI) to Cr (III) process.
Keywords: *Rhodopseudomonas* sp.; Cr (VI) reduction; heavy metal; diphenylcarbazide method

Introduction
Water pollution is one of the critical issues in ecological perspectives. The interminable of waste disposal into the river harms the aquatic life, consequently affecting the human and animal health through the food cycle (Ali et al., 2019). On March 2019, Sungai Kim Kim, Pasir Gudang, Johor has been heavily polluted with chemical waste that disturbs the community's activities around the area. It is a controversial crisis as it started with water pollution and ended up being air pollution as a result from the interaction between the chemical waste discharged with water and air (Lee Goi, 2020). Development of industrial, agricultural and economy worldwide contributed to the dumping of hazardous and harmful pollutants due to inadequacy in treating the effluent before discharge into the reservoirs (Ayele et al., 2021).

Heavy metal is the dominant contaminant in polluted water contributed from anthropogenic activities such as mining, phosphate fertilizers, industries and combustion of fossil fuels (Ali et al., 2019). It is highly toxic to living things as it can cause respiratory problem and inflammation to humans and animals and also affect the growth and photosynthesis process in plants if the concentration of heavy metals is higher than the permissible bio-available levels (Ayele et al., 2021; Yunus et al., 2020). Moreover, it is a non-biodegradable pollutant which makes it tenacious and accumulates in the environment and may later enter the food chain (Cui et al., 2020). Their unstable characteristics makes the remediation process of heavy metals demand a great effort and cost a lot of money (Upadhyay et al., 2017).

(Heikal, 2017).

This study aimed to enhance melon growth and quality through optimized nutrient utilization and effective nutrient promotion. Integrating zeolite into the growth medium has potential implications for agriculture, capitalizing on prior research affirming its benefits. Notably, this study strived to achieve a balanced fertilizer application for improved melon market quality, focusing on sweetness enhancement. This advancement could pave the way for zeolite's widespread adoption, offering slow-release fertilization and cost-effectiveness to enhance crop yields (Wen et al., 2022).

Therefore, there is an urgency to establish a brand-new, effective and inexpensive approach to treat polluted rivers. Nowadays, biological mechanisms have been extensively adopted in several fields by using microorganisms such as bacteria, yeast and fungi (Fulazzaky et al., 2014). A promising group of microorganisms used in treatment of sewage is the photosynthetic bacteria (Wang et al., 2016). Talaiekhozani et al. (2017) claimed that photosynthetic bacteria can eliminate toxic wastes from water. Moreover, photosynthetic bacteria can assist in dwindling carbon footprint as they are able to employ carbon dioxide (Idi et al., 2015). It is reported that bioremediation using chemotrophic microorganisms produces an immense quantity of microbial biomass which probably give bigger threat than the existing pollutants (Chitapornpan et al., 2013). Furthermore, photosynthetic bacteria have been exploited for remediation of various effluents from sugar production, food industries, chicken slaughterhouses and dairy sewage (Chitapornpan et al., 2013; Kaewsuk et al., 2010).

Materials and methods

Rhodopseudomonas sp. a locally isolated purple non sulfur bacteria (Shah, 2020) was grown in PNSBEM for 7 days under anaerobic light condition at 30℃. The growth was observed using UV-VIS spectrophotometer at 660 nm at every single day until day 7 of incubation. 3 mL of *Rhodopseudomonas* sp. from day 1 to 7 was collected from the culture using centrifuge at 4,400 rpm for 10 minutes and the cell pellet was resuspended in sterile distilled water and centrifuge it again. Next, the cell pellet was dried in the oven at 40℃ for 24 hours. The analysis was completed after constant weight is achieved. Gram staining was performed to characterize the morphology of *Rhodopseudomonas* sp.

Rhodopseudomonas sp. was grown separately on PNSBEM for 96 hours under anaerobic light condition. Next, the cells were harvested by centrifuge at 8,000 rpm for 15 minutes and the pellets obtained were washed three times with 0.1% peptone water at pH 7. 10 mL of 2 mg/L chromium solution was prepared at pH 7. Next, 4.5 mg of dry cell weight (DCW/mL) of the cell was added into the chromium solution. The mixture was incubated for 30 minutes in anaerobic light condition at 30℃. Then, the cell was centrifuged at 8,000 rpm for 15 minutes. Biosorption experiment was repeated by manipulating different factors such as temperature (20, 25, 35 and 40℃), biomass size (2.0, 6.0, 8.0 and 10.0 mg DCW/mL), pH (5, 6, 8 and 9) and contact time (5, 15, 45, 60 minutes).

1 mL of diphenylcarbazide solution and 1 drop of sulfuric acid was added into the supernatant. Then, the mixture was kept at room temperature for 10 minutes for color changes following the absorbance was measured using UV-VIS spectrophotometer at 540 nm (Congeevaram et al., 2007; Zahoor et al., 2009). The reduction of hexavalent chromium was evaluated using UV-VIS spectrophotometer and the percentage of reduction was calculated as stated in (Bharagava et al., 2018) using the following formula:

$$\text{Cr (VI) Reduction (\%)} = \frac{A-B}{A} \times 100$$

Where A: Initial concentration of Cr (VI)
B: Final concentration of Cr (VI)

Each set of experiments was done in triplicate (n=3). All data was presented in mean ± standard deviation. GraphPad Prism 9.3 software was used to check the normality of the data analytically using the Shapiro-Wilk test. Then, the results were analyzed via One-way Anova with Tukey's multiple comparison. The significance of the results was determined at ($p < 0.05$).

Results and discussion

A sigmoid graph was obtained which represents the growth of *Rhodopseudomonas* sp. after incubation under anaerobic light conditions for seven days. Based on Figure 1, *Rhodopseudomonas* sp. entered lag phase on day zero until day one and continued to logarithmically grow from day two to four before reaching stationary phase at day five. It was observed that day 4 is the best growth of PNSB in logarithmic phase, making it the most favourable day for biomass preparation of *Rhodopseudomonas* sp. Wang et al. (2015) discovered that PNSB has maximum metabolic activity throughout its logarithmic phase, making it suitable for further research such as heavy metal removal.

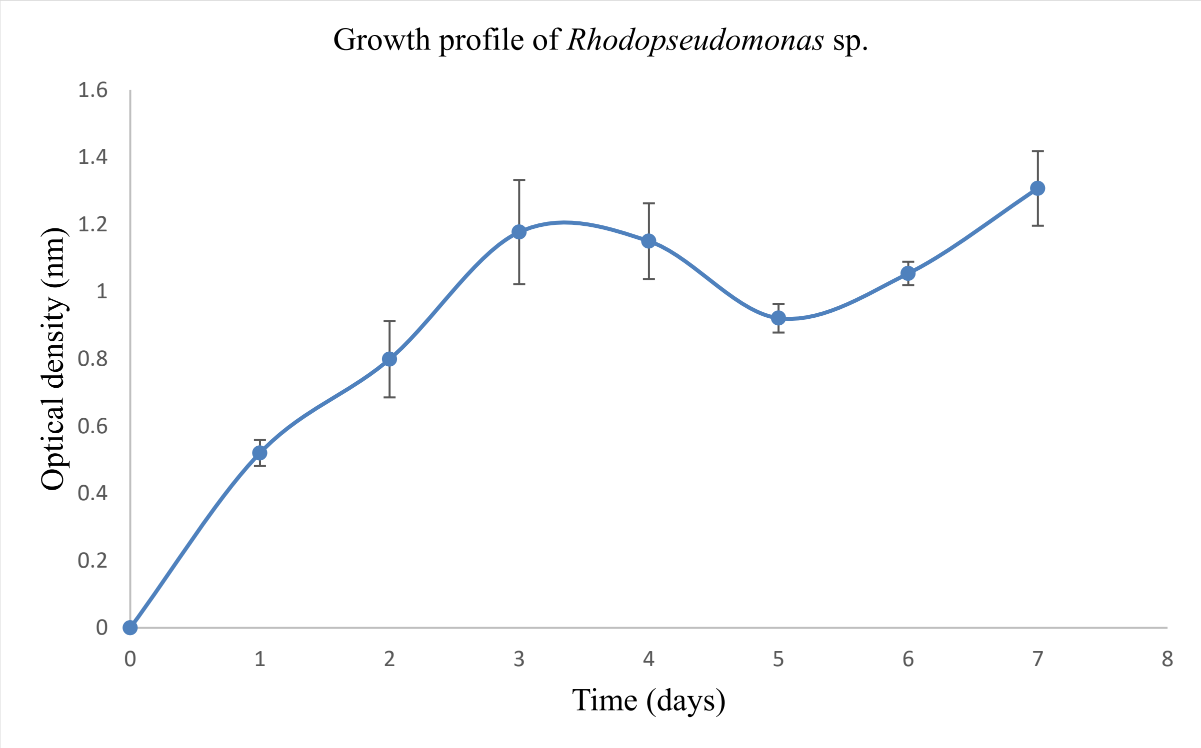

Figure 1 Growth profile of *Rhodopseudomonas* sp.

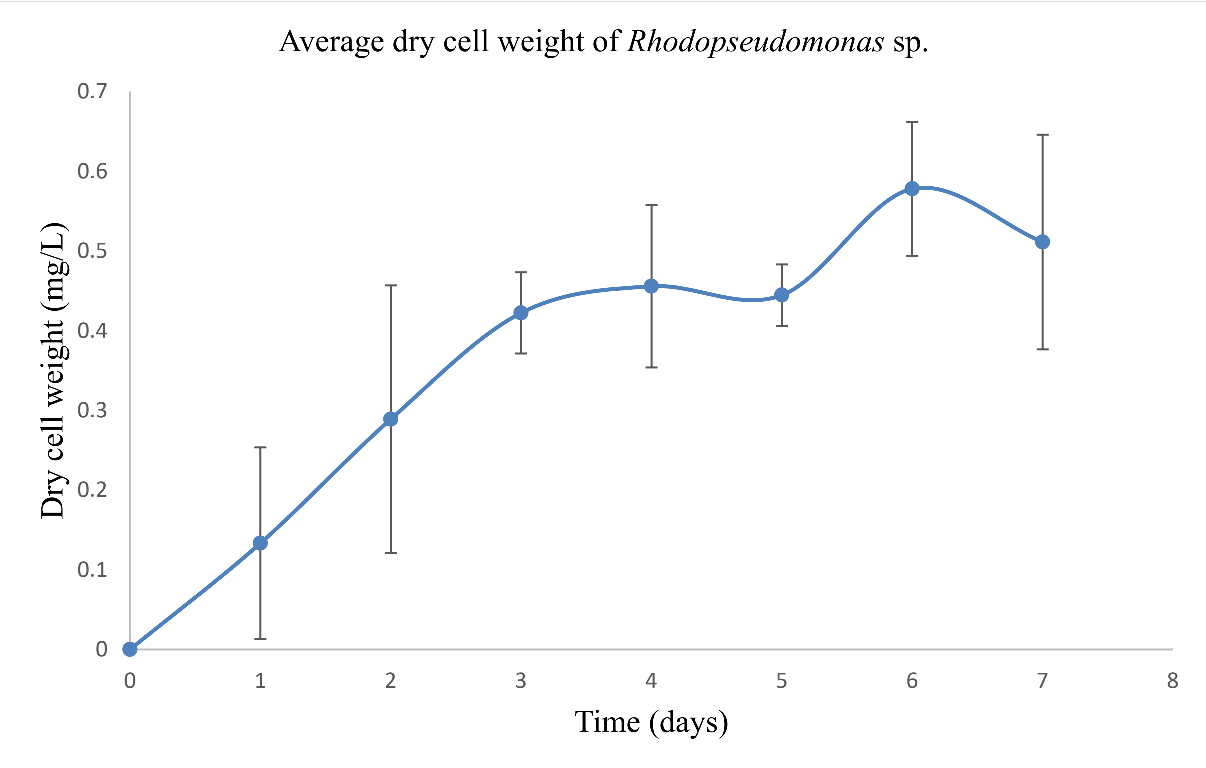

Figure 2 The average dry cell weight for *Rhodopseudomonas* sp.

Figure 2 presented the dry cell weight of *Rhodopseudomonas* sp. that was also evaluated along the seven days of incubation. Figure 3 depicted the linear relationship between the average optical density (OD) at 660 nm and the dry cell weight of this species. The value of R^2 for *Rhodopseudomonas* sp. is 0.7271 which is equivalent to 72.71% of certainty. Based on this value, it can be concluded that the data obtained was significant and conclusive.

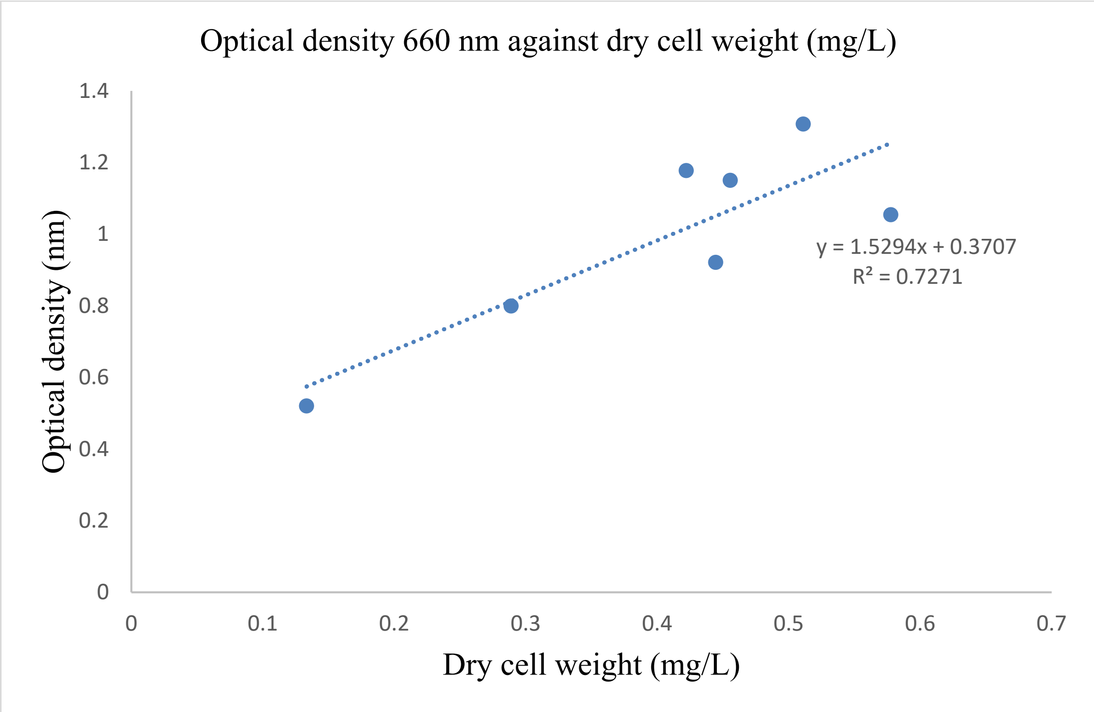

Figure 3 The correlation between growth profile at optical density 660 nm between average dry cell weight (mg/L) of *Rhodopseudomonas* sp.

Temperature is an important parameter that affects the thermodynamics of the biosorption process (Al-Homaidan et al., 2018). Temperature has been shown to influence the ionization of functional groups of cell walls, the stability and composition of bacterial cell walls (Malaviya et al., 2016). Figure 5 showed that as the temperature increases, the percentage of Cr (VI) reduction increases. It can be concluded that the biosorption mechanism involved an endothermic process which with increasing heat improved the reduction of Cr (VI) to Cr (III) (Ali Redha, 2020). The highest percentage of Cr (VI) reduction was observed at 35℃ which is 22.7%. This may be due to high kinetic energy and advanced surface activity that could maximize the biosorption activity of *Rhodopseudomonas* sp. (Mukkata et al., 2019). Additionally, higher temperature leads to low viscosity of solution therefore it has lower internal friction between Cr (VI) and the solution makes it easily bind to the microbial cell wall (Al-Homaidan et al., 2018).

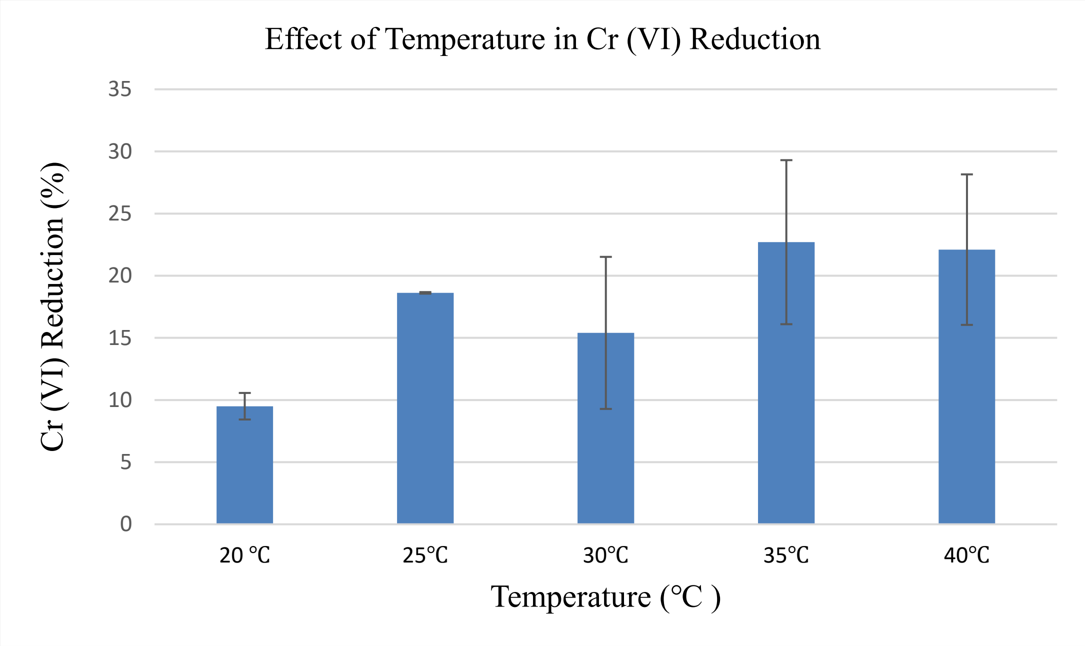

Figure 4.5 Effect of temperature in Cr (VI) reduction

Biomass of *Rhodopseudomonas* sp. has the tendency to be damaged especially at the binding sites at higher temperature than 35℃ (Fathollahi et al., 2021; Panwichian et al., 2010) which explains the decline of percentage of Cr (VI) reduction at 40℃. On the contrary, *Rhodopseudomonas* sp. remove lower percentage of Cr (VI) at 20℃ due to lack of kinetic force and minimum surface activity (Mukkata et al., 2019). The reduction of Cr (VI) with various temperatures found to be statistically non-significant by one-way Anova analysis. However, in this research, it was observed that at 35℃*, Rhodopseudomonas* sp. successfully reduced the Cr (VI) to Cr (III) at 22.7%. This is in accordance with biosorption studies by Paul et al. (2012) which showed similar optimum temperature of 35℃ for Cr (VI) reduction using gram-negative bacteria, *A.junii*.

Generally, biomass size has significant effects on the performance of biosorption due to the availability of binding sites offered for heavy metal ions (Cr^{6+}) attachment (Priyadarshanee et al., 2021). In fact, increasing the dosage of biosorbent may improve the biosorption capacity as it provides larger surface area at the bacterial cell wall (Razzak et al., 2022). This is proven at Figure 4.6 as it illustrates an upward trend of Cr (VI) reduction from 2 to 6 mg DCW/mL wet cell of *Rhodopseudomonas* sp. The highest percentage of Cr (VI) reduction was discovered at 6 mg DCW/mL with 17% of reduction while 2 mg DCW/mL only reduced 5% of Cr (VI) due to the high competitiveness among the hexavalent chromium ions as there are limited number of attaching sites on *Rhodopseudomonas* sp. cell wall (Ali Redha, 2020; Priyadarshanee et al., 2021).

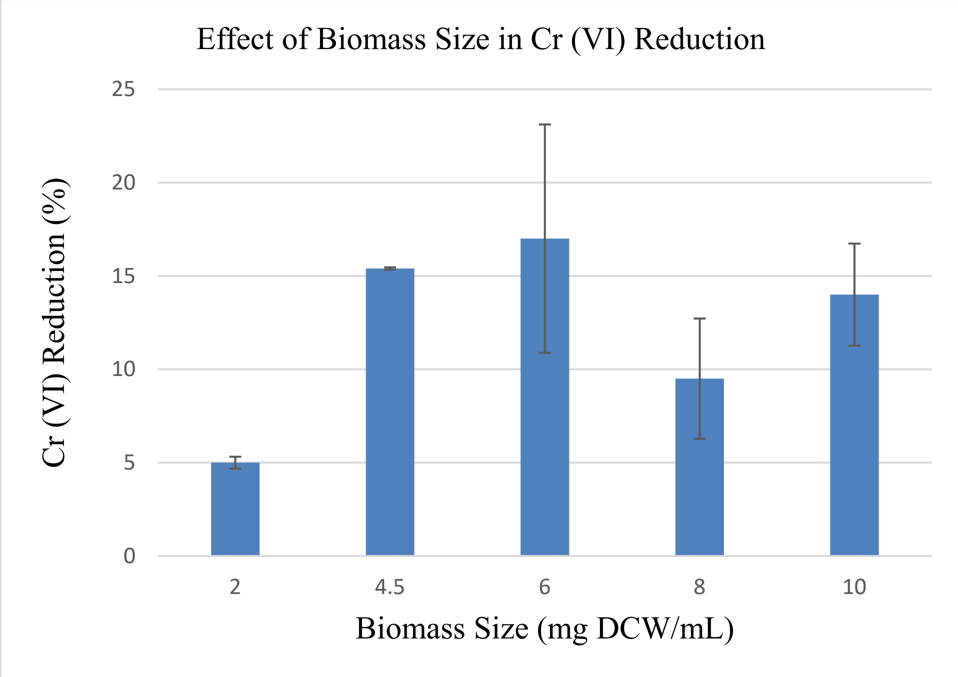

Figure 4.6 Effect of biomass size in Cr (VI) reduction

However, 8 and 10 mg DCW/mL of biomass size exhibited inconsistent reduction of Cr^{6+} to Cr^{3+} even though it consists of high cell density. Priyadarshanee et al. (2021) mentioned that the biosorption efficiency will rise up to a specific limit before decreasing along the increased supply of biomass. This is a result of a shell effect mechanism that prevents Cr^{6+} from conquering the active binding sites of *Rhodopseudomonas* sp. as well as the development of biomass clumps (Fathollahi et al., 2021; Razzak et al., 2022). Similar trend where the percentage of reduction increase at certain biomass size before drastically drop was observed in the case of biosorbent of *S.rimosus* for Cr (VI) reduction (Sahmoune, 2018).Anova showed no significant different between all biomass size for reduction of Cr (VI). In this study, it is observed that the best biomass size of *Rhodopseudomonas* sp. that can be used to reduce Cr (VI) is 6 mg DCW/mL.

pH is the most crucial physicochemical aspect that can regulate the biosorption rate owing to its capability in altering the chemistry of biomass surface and the charges of metal ions in aqueous solution (Wei et al., 2016; Ali Redha, 2020; Fathollahi et al., 2021; Priyadarshanee et al., 2021; Razzak et al., 2022). The effect of pH varies based on the metal ions involved in the biosorption process (Bilal et al., 2018). Therefore, pH amendment is critical to achieve the effective Cr (VI) reduction using bacteria.

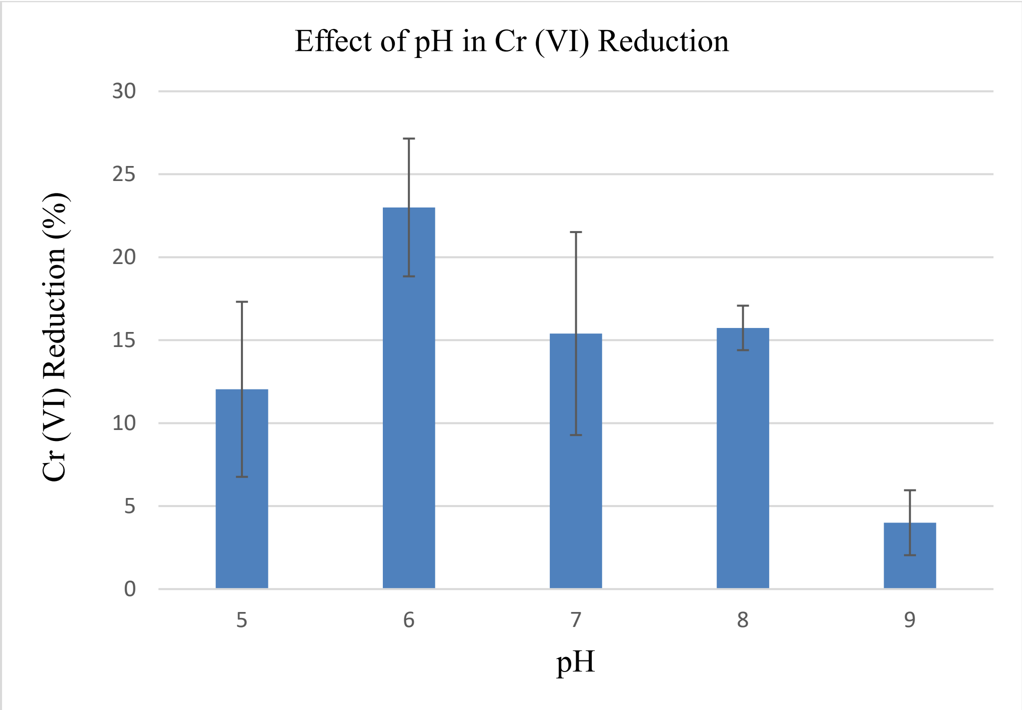

Figure 4.7 Effect of pH in Cr (VI) reduction

Cr^{6+} ionization differs in low and high pH. Hexavalent chromium ions exist as anions at acidic and alkali pH but in different species where at pH 2 to 6, Cr^{6+} ionize as $HCrO^{4-}$ and $Cr_2O_7^{2-}$ whereas above pH 8, Cr^{6+} form into CrO_4^{2-} in the existence of hydroxyl ions (Fathollahi et al., 2021; Silva et al., 2012). This condition allowed for the binding of the protonated functional groups of *Rhodopseudomonas* sp. (Ali Redha, 2020; Ayele et al., 2021). Based on Figure 4.7, pH 6 showed a significant rise for reduction of Cr (VI) from pH 5 where pH 6 effectively removed 23% of Cr (VI) while pH 5 removed only 12.04%. This is due to the attraction between the predominant form of chromium, ($HCrO^{4-}$ and $Cr_2O_7^{2-}$) and the positively charged surface of *Rhodopseudomonas* sp. via electrostatic interaction to reduce into Cr^{3+} (Dadrasnia et al., 2015).

As the pH increases, the reduction of Cr (VI) declines drastically at pH 9. *Rhodopseudomonas* sp. reduced Cr^{6+} merely at 4%. This can be attributed by the interaction between chromate ions and hydroxyl ions present in the solution that form chromium hydroxides and cause precipitation (Ali Redha, 2020). Moreover, the carboxyl and amino groups on the surface of *Rhodopseudomonas* sp. encounter deprotonation due to the presence of OH^- ions which results in repulsion of negatively charged chromium ions (Ayele et al., 2021). However, all pH was proved to be statistically non-significant by one-way Anova analysis. pH 6 is the most suitable pH for reducing Cr (VI) using *Rhodopseudomonas* sp. since most wastewater generally has pH in the range of 6-8 (Abdul Syukor et al., 2021). This result is well in agreement with the previous studies carried out by Kalola et al. (2020) using *Halomonas* sp. in Cr (VI) reduction at optimum pH 6.

Aside from temperature, biomass size and pH, contact time also plays an important role in the improvement of Cr (VI) reduction because it mostly affects the kinetics of biosorption and eventually the potency of biosorption (Razzak et al., 2022). A rapid initiation of the sorption rate was observed in most of the biosorption experiments before experiencing a decline in the process and reaching a state of equilibrium (Al-Homaidan et al., 2018; Ali Redha, 2020). Ali Redha (2020) explained that contact time functions as a limiting factor and does not directly influence the mechanism of biosorption. The optimum contact time also depends on the types of biosorbents (Bilal et al., 2018).

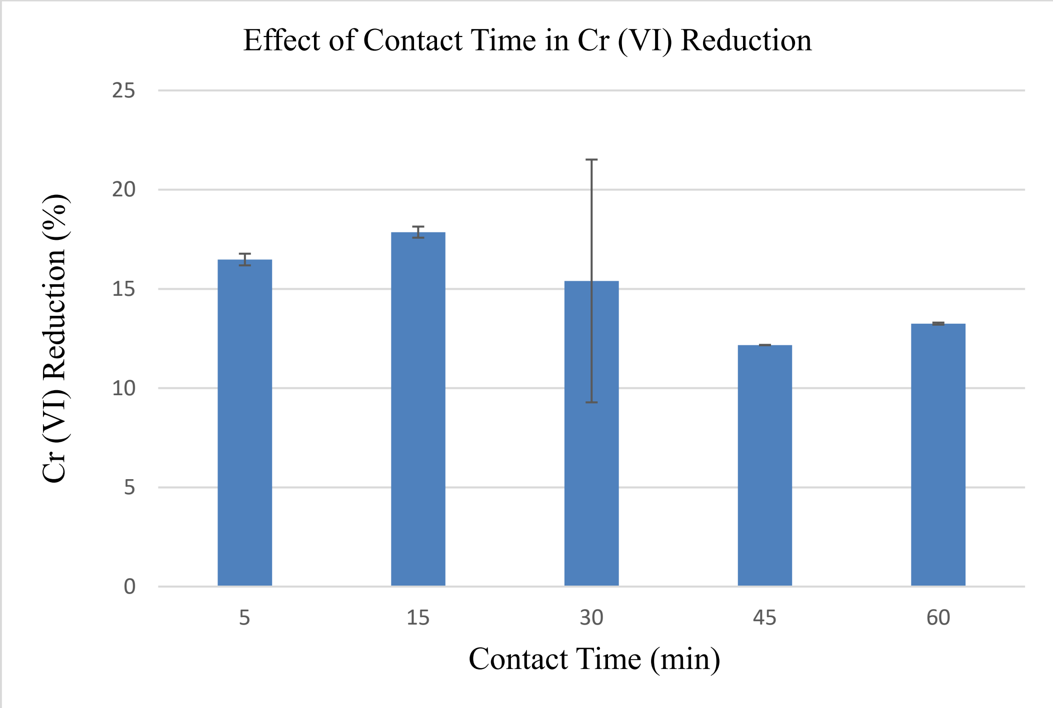

Figure 4.8 Effect of contact time in Cr (VI) reduction

In this study, the best percentage of Cr (VI) reduction by *Rhodopseudomonas* sp. demonstrated after incubating for 15 minutes under anaerobic light condition. This can be interpreted by the abundant presence of binding sites on the cell wall of *Rhodopseudomonas* sp. which allow for faster adsorption of Cr^{6+} (Bilal et al., 2018; Fathollahi et al., 2021). After 30 to 60 minutes of incubation, the reduction of Cr (VI) exhibited an inconsistent trend because all of the attaching sites have been occupied and became saturated with Cr^{6+} (Al-Homaidan et al., 2018; Ali Redha, 2020). Anova showed no significant difference between all contact time for reduction of Cr (VI). Therefore, it can be concluded that the optimum contact time for Cr (VI) reduction is 15 minutes with the reduction of 17.86% which is 1.47 times more than 45 minutes of incubation. The incubation time of 15 minutes is in corresponding with previous studies on PNSB species, *A.marina* that has the similar contact time recorded (Mukkata et al., 2019).

Conclusion

Rhodopseudomonas sp. that was isolated locally by Shah (2020) has been successfully characterized by its morphology, growth profile and dry cell weight analysis. It is a gram-negative prokaryote with bacilli shape that appears red in color under light microscope. The optimum condition for the reduction of Cr (VI) by *Rhodopseudomonas* sp. are 6 mg DCW/mL which is incubated in Cr (VI) solution at pH 6 for 15 minutes at 35℃ since it recorded the highest percentage of Cr (VI) reduction.

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