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Removal of Iron (II) by *Burkholderia pseudomallei* in Brackish Environment

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Abstract

The activity of ship breaking is one source of iron metal pollution in coastal areas and sea water. This metallic pollution, in high concentrations, was a danger to the local organisms in the habitat. Hence, a means of detoxifying the accumulative harmful molecules of these heavy metals by bacteria to reduce the toxic level is required. This process is known as the bioremediation of heavy metals. Bacteria was isolated and identified as originating from the ship breaking areas contaminated with iron metal was *Burkholderia pseudomallei.* This research aimed at determining the percentage and kinetic rate of Iron (II) removal by *Burkholderia pseudomallei.* The variables in the research were salinity and pH of medium in laboratory scale. Salinity of medium was used at 15 and 20 ppt. The normal and optimum pH at acidic condition was chosen based on the pH effect test. The initial concentration of Iron (II) was 100 mg/L. The Iron (II) removal by *Burkholderia pseudomallei* was performed for 96 hr. The parameters measured were Optical Density (OD), pH, temperature, Iron (II) concentration, and a number of bacteria colonies. The results showed the iron (II) removal process is optimum at neutral pH. Reactor with pH of 7 showed the percentage of 57.8% and 58.5% at salinity of 15 and 20 ppt, respectively. Salinity affected the kinetics equation of the Iron (II) removal test. The model of *pseudo second order* kinetics equation in the reactor with neutral pH and salinity of 20 ppt showed well-fitted biosorption results (R^2 = 0.9668) with a constant kinetics rate *(k2)* of 0.00038 mg/g.hr.

Keywords: Bioremediation; *Burkholderia pseudomallei;* Heavy metal; Kinetics rate; pH; Salinity

1. Introduction

Ship breaking is one source of Iron contamination in the environment. Ship breaking causes problems such as damage and contamination in coastal zones, seawater, and sediments at the area (Kacar and Kocyigit, 2013). The average concentration of heavy metals found are in the order of Iron (Fe), Zinc (Zn), Cuprum (Cu), Manganese (Mn), Cadmium (Cd), Lead (Pb), Cobalt (Co), Nickel (Ni) and Chromium (Cr) (Basha *et al.*, 2006). Iron has the highest average concentration compared to other heavy metals contained in the ship breaking area. The average concentration found is between 0.33 mg/L and 160.5 mg/L (Hasan *et al.*, 2013). In seawater with a pH higher than 6, the permitted Iron content is less than 1 mg/L (Anzecc and Armcanz, 2000).

Iron is an essential heavy metal that exists in certain quantities needed by living organisms, but excessive amounts can have toxic effects (Ika *et al.*, 2012). High concentration of Iron (Fe) in some environments can pose a danger to organisms

residing there (Pamungkas and Zulaika, 2015). This relates to the properties of heavy metals that are difficult to degrade; therefore, it accumulates easily and tends to be challenging to remove. These heavy metals accumulate in aquatic biota, including shells, fish, and sediments having a high half-life in the body of marine biota, as well as having a large concentration factor value in the body of the organism (Supriantini and Endrawati, 2015). Iron content of more than 1 mg/L is considered harmful to aquatic microorganisms (Moore, 1991).

Although the physical and chemical methods of processing industrial wastes are quite efficient, they are not economical because it is relatively expensive, requiring a considerable amount of energy and chemicals (Atkinson *et al*., 1998; Dixit *at al*., 2015). In lieu of this, an easy, inexpensive, and effective processing alternative is developed and administered. This new method, the biological process, serves as an alternative or complement to the physical and chemical methods, and it prevents environmental pollution caused by accumulation of heavy metals such as Iron. Some alternative processes employ the Bioremediation system, using bacteria or other microorganisms. Bioremediation of heavy metals by microbes is the process of converting toxic molecules or metal ions to near toxicity levels (Yazid, 2007). It has proved to be effective and economically inexpensive to clean up soil and air contaminated by toxic chemical compounds. Bioremediation using bacteria is more practical since bacteria can perform transformation of heavy metals; examples of the mechanism used were biosorption and bioaccumulation (Vijayaraghavan and Yun, 2008). According to Dixit *et al.* (2015), the different mechanisms of heavy metals bioremediation are known to consist of biosorption, metal-microbe interactions, bioaccumulation, biomineralisation, biotransformation, and bioleaching.

In the isolation of bacteria from contaminated metal environments, it is essential to use bioremediation agents, due to the fact that the bacteria have resistance to the surrounding metal. Several genera of known aerobic and anaerobic Iron oxidizing bacteria were found. The *Burkholderia* genus uses four types of siderophore, namely pyochelin, ornibactin, cepabactin and cepaciachelin, to dissolve and transport Iron (Thomas, 2007). As Iron is an essential element for growth for *Burkholderia pseudomallei*, it produces hydroxamate siderophore and malleobactin types that can significantly exclude Iron (Alice et al., 2006). Siderophore has a low pressure that facilitates the dissolution and transport of Iron by *B. pseudomallei* (Crosa, 1989). According to Emerson et al. (2010), *Leptothrix, Gallionella, Mariprofundus, Sideroxydans paludicola, Gallionella ferruginea* were used as Iron oxidizing bacteria due to the fact that these bacteria utilize Iron as a chemosynthetic energy source.

Based on our previous research, bacteria isolated and identified from the ship breaking area was *Burkholderia pseudomallei.* The purposes of this research were to determine the percentage of Iron (II) removal by *Burkholderia pseudomallei* at brackish environment and to determine the kinetics of those removal processes.

2.Material and Methods

2.1 Effect of pH

The effect of pH on the growth of *B. pseudomallei* was conducted to determine the optimum pH value of bacteria growth. The optimum pH was used in the removal of Iron (II) test and the variations were 3, 3.5, 4, 4.5, and 5. The bacteria was inoculated aseptically into each Nutrient Broth (NB) medium (Merck, Germany) with a certain pH based on the variation. The pH adjustment was performed by adding a solution of H_2SO_4 or NaOH to obtain the desired value according to the pH variation. The NaOH and H2SO4 used to adjust the pH had molarity of 0.1M each. The NB medium that had been inoculated with the bacteria was put in a shaker with Jouan E82 model (Thermo, USA) and rotated at 150 rpm for 48 hrs. The measurement of bacteria colonies used the Colony Forming Unit (CFU) method based on Harley and Prescott (2002). The measurement of bacteria colonies was performed for 24 and 48 hrs. The normal pH and the value of optimum pH at acidic

conditions were used in the Iron (II) removal test. The value of pH was measured using pH-meter (Hanna model HI 98107, Germany), and the salinity was measured using salinometer (Yieryi model CT-3088, China).

2.2 Iron (II) removal test

The research variables used variation of salinity and pH. The salinity variation were 15 ppt and 20 ppt. The salinity medium was then prepared from pro analysis NaCl powder (Merck, USA) with calculation, 15 gr of NaCl powder added with 1 L of aquades for salinity of 15 ppt. The variation of pH at Iron (II) removal test was neutral (7) and based on the results of its effect determination on the growth of *B. pseudomallei.* All variations are shown in Table 1, and based on this table 1, the total reactor was six (6). The erlenmeyer flaks was used as the reactor, and it has a size of 250 mL (Pyrex, Germany). This reactor was selected because the volume of solution required was 200 mL in each. The concentration of Iron (II) was 100 mg/L and was chosen based on our previous study. Iron (II) stock solution was made from $(NH_4)_2FeSO_4)_2 \cdot 6H_2O$ (Merck, Germany). The medium for the Iron (II) removal test was Salt Base Solution (SBS) with formula $(NH_4)_2SO_4$. The solution contains 0.4 gram, KH_2PO_4 0.4 gram, $MgSO_4$ 0.8 gram, and 0.1 gram yeast extract in 1 litre of water (Irawati et al. 2017). The Iron (II) removal test was performed for 96 hr. Several parameters analysed during the Iron (II) removal test were optical density (OD), pH, temperature, number of bacteria colonies, and Iron (II) concentration. The parameters of OD, pH, temperature, and total concentration of Iron (II) were performed at 0, 24, 48, 72 and 96 hrs. However, those of the number of bacterial colonies were performed at 0, 72, and 96 hrs.

2.3 Kinetics of Iron (II) removal

The dry weight of the biomass bacteria was measured prior to calculating the kinetics rate. The objective was to obtain a graph of the relationship between Optical Density (OD), dry weight of biomass bacteria and bacteria colonies (CFU) over a period of time, and the medium employed was NB (Merck, Germany). The parameters determined were optical density, dry weight of the biomass bacteria, and the number of bacteria colonies. All measurements were performed at 0, 5, 20, 24, and 72 hr, and results were plotted on a graph. The OD measurement was conducted using UV Spectrophotometer (Genesys, Germany) with 550 nm. For the measurement of the bacteria colonies, the Colony Forming Unit (CFU) method based on Harley and Prescott (2002) was used. Meanwhile, the dry weight of biomass bacteria was carried out based on Bratbak and Dundas (1984) and Cavalca *et al.* (2010).

Tabel 1. Variables used in this research

Salinity	pH	
	P1	P2
Control	C1	C2
S1	S1P1	S1P2
S2	S2P1	S2P2

Notes:

C1 : SBS Medium added iron (II) in the absence of bacteria

C2 : SBS Medium added bacteria in the absence of iron (II)

S1 : SBS Medium with salinity of 15 ppt

S2 : SBS Medium with salinity of 20 ppt

P1 : SBS Medium with normal pH conditions (pH = 7)

P2 : SBS Medium with optimum pH conditions under conditions acid based on pH effect test

The dry weight of bacteria (mg/mL) can be determined by interpolating graphs of OD, bacterial colony (Log CFU/mL), and dry weight of biomass bacteria. The dry weight of bacteria (mg/mL) was used to calculate the kinetics rate. The kinetics rate was conducted using pseudo first order and pseudo second order, and the kinetics of pseudo first order was expressed in the equation (Lagergren, 1898) :

$$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303} t \quad \text{.....(1)}$$

Where q_e is the amount of Iron (II) adsorbed by bacteria biomass at equilibrium (mg/g). While qt is the amount of Iron (II) adsorbed by bacteria biomass at time t (mg/g). k_1 is the first order rate constant (hr) and k_2 is the second order rate constant (mg/g.hr).

The kinetics of pseudo second order kinetic rate model was expressed in the equation (Ho and McKay, 1999) :

$$\frac{t}{q_t} = \frac{1}{k_2 (q_e)^2} + \frac{1}{q_e} t \quad \text{.....(2)}$$

with $h = k_2 (q_e)^2$ can be described as the initial rate constant at the moment $t = nol.q_t$ is the number of metal ions that are sorption in equilibrium conditions (mg/g); k_2 is the pseudo second order kinetics rate constant applied (mg/g.hr), the plot $\frac{t}{q_t}$ to t will provide a linear line. If the plot is linear, then the sorption process can be described as chemisorption. The plot $\frac{t}{q_t}$ to t and linear relationships are observed. The value of sorption capacity in biomass q_e, pseudo-second order kinetics rate constant k_2, sorption rate constant h, and r^2 is evaluated from the plot (Ho and McKay, 1999).

2.4 Statistical analysis

The experimental data of Iron (II) removal percentages were subjected to an analysis of variance (ANOVA) using SPSS Statistics for Windows version 21.0 (SPSS, Inc., Chicago, IL). Statistical significance was defined as $p < 0.05$.

3. Results and Discussion

3.1 Effect of pH on growth of B. pseudomallei

Based on Table 2, the optimum acidic pH for the growth of *B. pseudomallei* occurred at pH 5. The population of B. pseudomallei was more than 250 x10^6 CFU/mL at 24 hrs and more than 250 x10^7 CFU/mL at 48 hrs. *B. pseudomallei* had an optimum pH in the range of 5 - 8 (Tong *et al.*, 1996). Bacteria can grow well at pH 4.5. Based on the observed results, *B. pseudomallei* cannot grow at pH 4, 3.5, and 3 for 24 hrs. However, *B. pseudomallei* began to grow at pH 4, 3.5, and 3 for 48 hrs. The bacterial growth process could be slow at a pH less than 5 (Sarbini, 2012).

Table 2. Number of bacterial colonies on medium with variations of pH

pH	Number of Bacteria Colonies (CFU/mL)	
	24 Hrs	48 Hrs
5.0	> 250 x 10^6 = TNTC	> 250 x 10^7 = TNTC
4.5	70 x 10^6	> 250 x 10^6 = TNTC
4.0	10 x 10^6	103 x 10^6
3.5	-	6 x 10^6
3.0	-	4 x 10^6

notes :

\- = There is no bacterial growth

TNTC = too numerous to count

3.2 Iron (II) removal test

Table 3 showed all monitoring parameter values during Iron (II) removal testing. Based on the table, OD value was observed to have increased, indicating that *B. pseudomallei* grow during removal process. The highest increase of OD, which reached 0.590, occurred in reactor of S2P2. Based on Figure 1, the percentage of Iron (II) removal in control 1 reactor only occurred in the precipitation process, and it reached 27.4% due to the fact that this reactor was not added with bacteria, and the medium was sterile. Hence, precipitation process of Iron (II) occurred physically due to the unstable pH value in the solution during removal testing. The range of value of pH was 4.2 to 7.0 in reactor of Control 1. The total highest percentage of Iron (II) removal was 80.6% at salinity of 20 ppt and pH 7 (reactor of S2P1) for 24 hrs of exposure. However, the percentage of Iron (II) removal by *B. pseudomallei* was 58.5%, after the value was reduced by removal of Iron (II) in the control reactor. The lowest Iron (II) removal percentage that occurred was 4.8% at salinity of 15 ppt and pH 5 (S1P2 reactor) for 24 hrs. However, the total of Iron (II) removal by *B. pseudomallei* was 3.5%.

The percentage of Iron (II) removal at salinity of 15 ppt and pH 7 (S1P1 reactor) was 79.6% for 96 hrs. However, it was 57.8% in the code reactor of S1P1. *B. pseudomallei* has a high efficiency in Iron (II) removal with an environment suitable for bacteria growth. It also has a unique metabolic activity that makes it a fit bioremediation agent (Coenye and Vandamme, 2003). The process of removal of Iron (II) by *B. pseudomallei* begins with the bacteria producing hydroxamate type siderophore compound. Then the siderophore compound binds the metal to form a complex compound of Iron siderophore. These compounds are

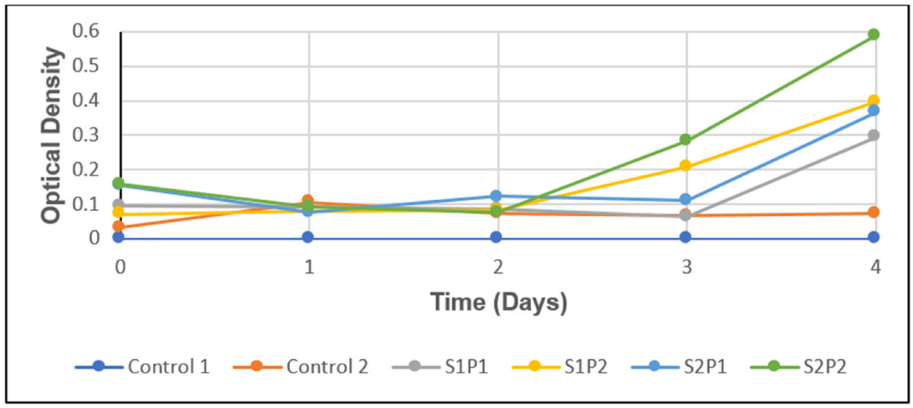


Figure 1. OD measurement during Iron (II) removal test

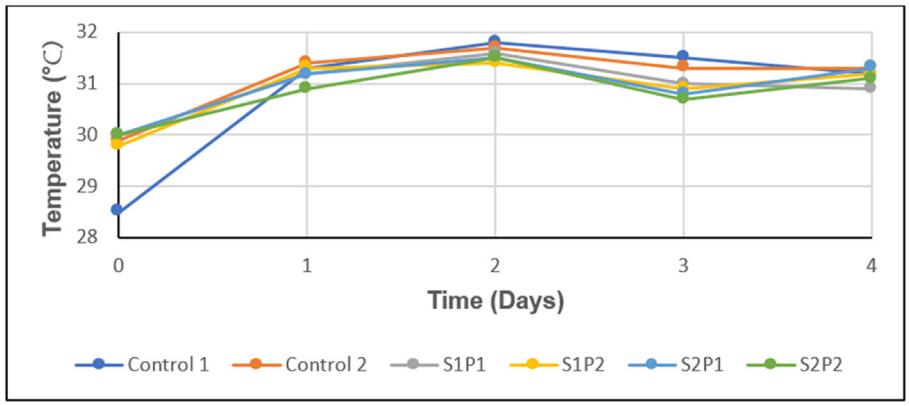


Figure 2. Temperature during Iron (II) removal test

then recognized by specific receptors on the outer membrane of the bacterial cells and sent to the ABC transporters in the cytosolic membrane by periplasmic binding proteins and then channelled into the cytosol. This transport is facilitated by the transduction of energy complexes of TonB-ExbB-ExbD protein (Andrews *et al*., 2003). Compounds of Iron siderophore complex are then separated by an Iron reducer. The separated siderophore was then recycled by bacteria cells (Guerinot, 1994).

Reactor with pH 7 (S1P1 and S2P1 reactor) showed high Iron (II) removal due to pH being optimum for bacteria. According to Tong et al., (1996), *B. pseudomallei* had an optimum pH in the range of 5 – 8, and Figure 1 shows the pH range of S1P1 and S2P1 reactors was at pH between 6.1 - 7.1, which was included the optimum pH range of bacteria. While at the condition of pH 5 (S1P2 and S2P2 reactor), there was very low Iron (II) removal by *B. pseudomallei*. It can be seen from Table 3, the pH value at the end of 24 hrs in S1P2 reactor was 4.3 and 4.5 in S2P2. There was a rapid inactivation of *B. pseudomallei* at pH ≤ 4.5 (Tong et al., 1996); however, this can cause low efficiency. Although the pH values were not optimal, they cannot cause disruption of the performance of enzymes. However, the disruption of enzymes performance can cause bacterial growth within the optimal pH (Pelczar and Chan, 1986).

The pH was one factor that influences the biosorption process, also influencing the load on the biosorbent active site, the species of copper, and the level of ionization of the biosorbent during the reaction (Peng *et al*., 2010). Based on Figure 1, the increasing pH value showed increasing percentage of Iron(II) removal, and the highest percentage removal was at neutral pH compared with acidic pH. The effect on metal removal by bacteria indicated the metal biosorption increased with increasing pH, i.e., from 2 to 6. This condition, due to the active sites on the cell surface wall of the microorganisms, has a positive charge with a pH value less than 3. However, it has a negative charge when the pH value was more than 3. Thus, the interaction between active sites of cell surface walls of microorganisms and Iron ions can have different charges.

The pH also affected other species of metals in the solution. First, metal ions contained in the solution were hydrolized before the adsorption process by the adsorbent, thus producing protons as in the following equation.

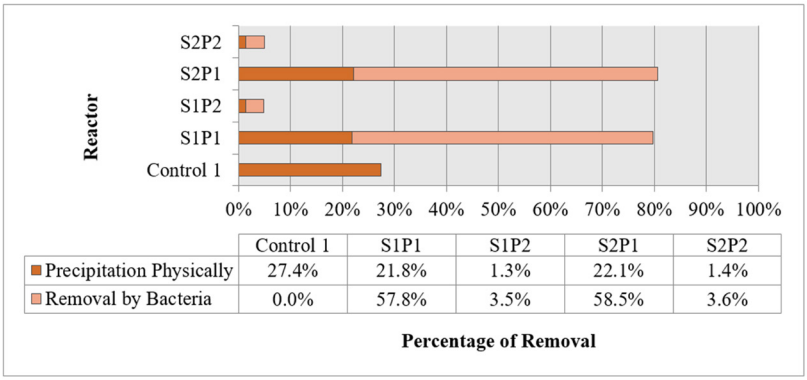
$$M^{2}+ + nH_2O \rightleftharpoons [M(OH)_n^{2-n}]+ + nH^{+} \quad .....(3)$$

The complex of hydroxo $[M(OH)_n^{2-n}]^{+}$ formed from the equation would be easier to adsorb compared to free metal cations (M^{2+}). The equation moves to the left, which would cause the number of metal hydroxo complexes to decrease and the number of free metal cations to increase in the acidic pH conditions. The biosorption process increases at relatively high pH compared to acidic conditions. Due to the fact that the metal hydroxide complex increases and the surface of the biosorbent become negatively charged, the attractive pull occurs such that it occurs through electrostatic forces, which causes an increase in adsorption (Sembiring *et al*., 2009).

Based on Figure 3, salinity has no significant effect on the percentage of Iron (II) removal by *B. pseudomallei*. The salinity level of 15 ppt and 20 ppt did not show a significant decrease in bacterial survival. Based on Table 3, CFU in reactor of *B. pseudomallei* can survive exposure to a solution containing less salinity than 25 ppt. The survival of *B. pseudomallei* will decrease significantly in salinity above 25 ppt (Inglis and Sagripati, 2006).

Emerson et al. (2010) reported genera *Pseudoalteromonas*, *Pseudomonas* and gammaproteobacterium *(Marinobacter aquaeolei)* suggested that those heterotrophs bacteria may play a role in precipitation of iron oxides in marine systems. According to Muller *et al*. (2012), *Marinobacter aquaeolei* plays a major role in iron oxidation under circumneutral conditions. However, besides bacteria, the fungi can be used to remove iron. *Aspergillus restrictus* has shown the Fe removal of 64% and Fe removed from the liquid media by obligate halophilic fungi (*A. flavus)* reached average of 85% (Bano *et al.* 2018).

Based on the results of the statistical analysis (ANOVA), the percentages of iron (II) removal were significantly different ($p < 0.05$) between control and all treatment. It indicated that B. pseudomallei has ability to remove iron (II) with high removal in normal pH.



	Control 1	S1P1	S1P2	S2P1	S2P2
Precipitation Physically	27.4%	21.8%	1.3%	22.1%	1.4%
Removal by Bacteria	0.0%	57.8%	3.5%	58.5%	3.6%

Figure 3. Percentage of Iron (II) removal by *B. pseudomallei*

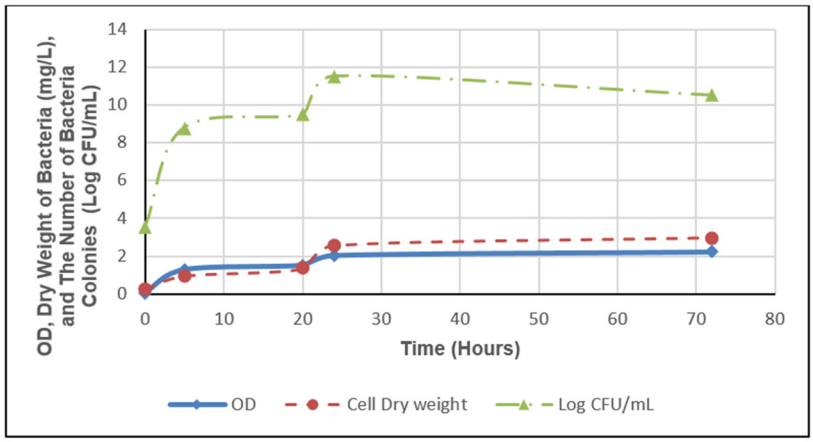


Figure 4. Graph of Biomass Concentration of Bacterial Cells

The results of kinetics rate was performed in reactor with a high percentage of iron (II) removal by *B. pseudomallei* (S1P1 and S2P1 reactors). The kinetics equation model was shown in Table 4, Figure 5 and Figure 6. Based on Table 4, S1P1 reactor with salinity of 15 ppt and S2P1 reactor with salinity of 20 ppt had a different q_e, k_1, k_2, and R^2 values. This implied that salinity affects the values of q_e, k_1, *k2,* and R^2 on the kinetic equation model. Salinity affected the osmotic pressure that occured in bacterial cell. The osmotic pressure occurred as a result of the ratio of solute in the cell and outside the cell was not the same. Bacteria can grow well in substrates that have slightly lower pressure than the osmotic pressure inside their cells (Sari *et al*., 2011).

Based on Table 4 and Figure 5, using the *pseudo first order* kinetics equation model on S1P1 reactor obtained q_e (49.04 mg/g), k_1 (0.123/hr), and R^2 (0.7909). While those value in S2P1 reactor obtained q_e (67.89 mg/g), k_1 (-0.018/hr), and R^2 (0.7139). By analyzing based on the value of R^2, there was a good sorpsi process by bacteria to iron (II) because the value of R^2 was great. In the biosorption process, there are several parameters that determine the rate of biosorption, including the structural properties of both sorbate and biosorbent. The presence of biosorbent, the initial concentration of metal ions and the presence of other ions (which may compete in active biosorption sites) also affect the rate of biosorption (Tuzun *et al.*, 2005). The biosorption of heavy metal ions in various microorganisms through two stages, i.e, a rapid initial uptake step on the surface of adsorption of cell wall components and subsequently decreased the rate of uptake at the transport of metal ion membranes to the cell cytoplasm. On the surface of bacterial cells contain polysaccharides, proteins and lipids that have the ability to bind to metal ions (Susanti and Nofdianto, 2014).

Table 3. Kinetics Equation Model

Reactors	Iron (II) Concentration (mg/L)	*Pseudo First Order*			*Pseudo Second Order*		
		q_e (mg/g)	k_1 (/hr)	R^2	q_e (mg/g)	k_2 (mg/g.hr)	R^2
S1P1	15.83	49.04	0.123	0.7909	49.04	- 0.00018	0.1048
S2P1	15.83	67.89	-0.018	0.7139	67.89	0.00038	0.9668

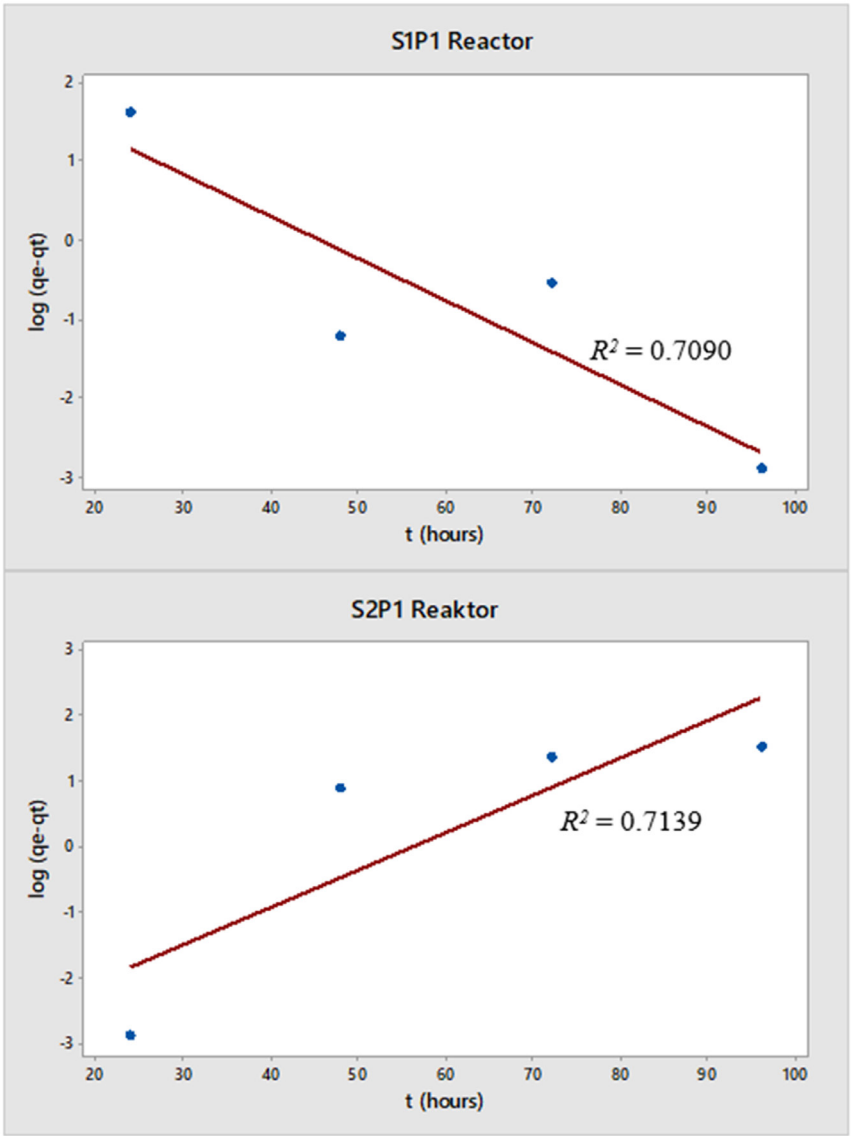


Figure 5. The Pseudo First Order Kinetics Equation Model

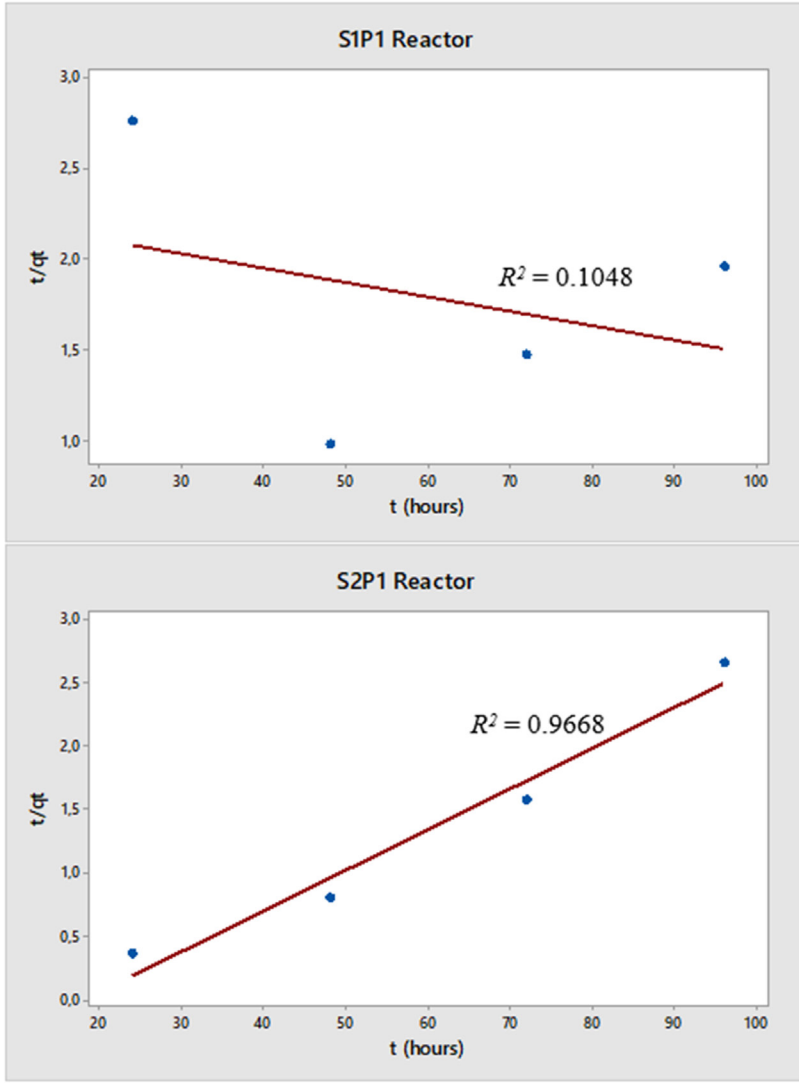


Figure 6. The Pseudo Second Order Kinetics Equation Model

Based on Table 4 and Figure 6, using the pseudo second order kinetic equation model on S1P1 reactor obtained q_e (49.04 mg/g), k_2 (-0.00018 mg/g.hr), and R^2 (0,1048). While in S2P1 reactor obtained q_e (67.89 mg/g), k_2 (0.00038 mg/g.hr), and R^2 (0.9668). Based on the value of R^2, S1P1 reactor had a very small R^2 value, while S2P1 reactor has a high of R^2 value (R^2 approaches 1). The value of R^2 was 0.7909 in S1P1 reactor using pseudo first order showed a better result compared to pseudo second order kinetics equation model (R^2 = 0.1048) because R^2 is higher in *pseudo first order.* The value of R^2 in reactor S2P1using pseudo second order kinetics equation model was 0.9668. It showed better results compared to the model of pseudo first order kinetics equation (R^2 = 0.7139). Some states that in many cases, *pseudo second order* kinetics equation model illustrates the accuracy of a high kinetics model based on the value of R^2 when compared to the value of R^2 derived from pseudo first order kinetics model (Mishra *et al*., 2013). The model was based on the assumption that the rate-limiting step may be chemical sorption, or chemisorptions involving valence forces through sharing or exchange of electrons between sorbent and sorbate (Ho and McKay, 1999). In the research, sorption process suggested chemical sorption involving valence forces through sharing or exchange of electrons between sorbent i.e bacteria and sorbate i.e Iron (II).

4. Conclusion

Considering the pH variable, it was seen that the percentage of Iron (II) removal by *B. pseudomallei* was optimum at neutral pH. This was evident from its removal percentage being 57.8% at 15 ppt and 58.5% at 20 ppt salinity. Hence, the salinity affected the kinetics equation model. Similarly, the removal of Iron (II) at pH 7 and salinity of 15 ppt correlates with the model with constant rate (k_1) of 0.123/hr and R^2 of 0.7909. However, doing the same at salinity of 20 ppt showed agreement with pseudo second order kinetics equation, and the constant rate (k_2) of 0.00038 mg/g.hr and R^2 of 0.9668 were observed. In conclusion, the kinetic removal of Iron (II) by *B. pseudomallei* showed it complied with the pseudo second order kinetics model.

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