



The potential of mercury resistant purple nonsulfur bacteria as effective biosorbents to remove mercury from contaminated areas

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ABSTRACT

This study aimed to investigate the possibility to remove mercury (Hg) using the live or dead biomass of Hg resistant purple nonsulfur bacteria (PNSB) for their potential application to clean up shrimp ponds and during cultivation. The efficiency of Hg²⁺ removal by three PNSB strains; *Rhodovulum sulfidophilum* SRW1–5, and *Aiffella marina* strains SSS2-1 and SSW15-1, grown with 2 different growth conditions (microaerobic light and aerobic dark conditions) were tested (2 mg/L HgCl₂, 4.5 mg dry cell weight/mL, 30 min). It was found that the efficiency to remove Hg²⁺ by the dead cells of all strains was significantly higher than for live cells; and the most effective strain was SSS2-1. The highest Hg²⁺ removal under the optimum conditions using a 4 mg/L initial HgCl₂ concentration by live and dead cells grown with both incubation conditions were approximately 87% and 95%, respectively. Under both incubation conditions, Hg²⁺ biosorption by strain SSS2-1 fitted the Freundlich model for live cells and the Langmuir model for dead cells. The kinetics of Hg²⁺ biosorption by both cell types of strain SSS2-1 suggests the process as a pseudo-second order kinetic model. This study demonstrates that biomass of the strain SSS2-1 has great potential for its biosorption to remove Hg²⁺ from contaminated areas like shrimp ponds.

1. Introduction

Shrimp farming is one of the important aquaculture businesses in Thailand; and the worldwide export of shrimp from Thailand is normally reprocessed into value added products (FAO, 2018). However, the residues of chemicals and antibiotics used during shrimp cultivation have become a major concern due to their persistence and toxicity in shrimp ponds (Gräslund et al., 2003; Visuthismajarn et al., 2005; Lacerda et al., 2011). Mercury (Hg) is one of the toxic elements that has been recently reported to be present in aquatic animals such as shrimp, fish, shellfish, crab, and mollusks (Brambilla et al., 2013; Li et al., 2013). Our previous studies found that Hg contamination in water and sediment samples from various shrimp ponds located in the south of Thailand (Mukkata et al., 2015a, 2015b). Hence, Hg contamination can be one critical problem for shrimp cultivation that should be dealt with,

because it might accumulate in shrimp through the food web and through contaminated shrimp consumption poses risks to human health.

As a response to toxic Hg in either the inorganic or organic forms present in the contaminated environment, microorganisms have developed some resistance mechanisms for detoxification (Barkay et al., 2003; Mukkata et al., 2015b). To deal the problem of Hg contaminated areas, Hg resistant microorganisms have been isolated from contaminated environments to investigate their resistance mechanisms to assist in bioremediation (Glendinning et al., 2005; Zhang et al., 2012; Yu et al., 2014; Mukkata et al., 2015a, 2015b). Bioremediation has received increasing attention in recent times to help to clean up polluted environments, because it is efficient, eco-friendly and cost effective (Vijayaraghavan and Yun, 2008). Of the many bioremediation methods, the use of live or dead biomass as biosorbents for

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bioaccumulation and/or biosorption has been demonstrated to possess good potential to remove metals from an aqueous phase (Hlihor et al., 2016). Biosorption is a non-metabolic mediated passive process of removing toxicants by adsorption on dead or inactive biomass, while bioaccumulation is the metabolic driven uptake of toxicants by live cells (Vijayaraghavan and Yun, 2008; Chojnacka, 2010; Panwichian et al., 2010a). The capacity to remove metals by microbes depends on the biomass types and their properties that are related to the functional groups of the binding sites on cell wall and also the parameters associated with the sorption systems such as the maximum concentrations of the biosorbent and the contaminant metal ions, the pH and temperature (Abbas et al., 2014). Moreover, the isotherm and kinetic sorption parameters are also investigated to determine optimum conditions (Lesmana et al., 2009; Chojnacka, 2010) for the highest potential of the biosorption systems.

Purple nonsulfur bacteria (PNSB) are the most versatile group of phototrophic bacteria that gain their energy from light by anoxygenic photosynthesis process, while using CO₂ or organic compounds as a carbon source (Madigan and Jung, 2009). In addition, they can grow by using organic compounds for energy and carbon sources using aerobic dark conditions via fermentation processes that include anaerobic respiration in the dark (Kantachote et al., 2005; Madigan and Jung, 2009). Normally, these bacteria are widely distributed in various natural habitats that are enriched with nutrients and exposed to sunlight including manmade environments such as shrimp ponds and paddy fields (Mukkata et al., 2015a; Khuong et al., 2017). Several research groups have reported that PNSB are relatively resistant to heavy metals and have the potential to remove them (Bai et al., 2008; Panwichian et al., 2010b, 2012; Nguyen et al., 2018).

It should be noted that PNSB are so versatile that they can be potential probiotics and are also used to clean up water in shrimp cultivation (Shapawi et al., 2012; Chumpol et al., 2017a, 2017b). Hence, they have the potential to be used for many purposes in shrimp cultivation. Our previous studies demonstrated that PNSB are part of the normal flora in shrimp ponds. A few PNSB strains can volatilize Hg and are therefore Hg resistant (Mukkata et al., 2015b). In addition to removing Hg from contaminated shrimp ponds by biovolatilization, the selected PNSB should be investigated for their potential to decontaminate Hg in shrimp ponds through bioaccumulation and biosorption. There have been no previous reports on the removal of Hg from shrimp ponds by PNSB. The objectives of this work were to determine the potential of live and dead PNSB biomass to remove Hg including factors that affect Hg biosorption prior to their application in removing Hg and bioremediation in shrimp ponds during shrimp cultivation for producing safe shrimp.

2. Materials and methods

2.1. Inoculum and biomass preparations

The Hg resistant PNSB; *Rhodovulum sulfidophilum* strain SRW1–5, and *Aififella marina* strains SSS2-1 and SSW15-1 isolated from shrimp ponds by our previous study (Mukkata et al., 2015b) were used in this work. To obtain an active inoculum, each strain was subcultured twice by inoculation into glutamate acetate medium (GA) containing 3% NaCl and incubated under microaerobic light conditions (ca. 3000 lx) for 48 h prior to their use in further studies. To obtain biomass, all selected PNSB strains were grown with GA broth containing 3% NaCl with a half volume of the test tubes covered with aluminium foil to provide aerobic dark conditions in a shaker at 150 rpm, at 35 °C for 48 h. At the same time microaerobic light conditions were set by filling the same medium with a full volume of the test tubes and providing an illumination of roughly 3000 lx for 48 h. Culture broths were centrifuged at 8000 rpm for 15 min and washed three times with 0.1% peptone water (pH 7.0) for preparing live cells, whereas dead biomass was prepared from similarly grown live cells but dried at 60 °C in an

oven for 10 h (Wang et al., 2010).

2.2. Biosorption experiments

Each wet cell pellet and dead biomass that was equivalent to 4.5 mg dry cell weight (DCW)/mL (Panwichian et al., 2010b) was added into 10 mL of a 2 mg/L HgCl₂ solution, at a pH 7.0 and incubated with both conditions of aerobic dark and microaerobic light as previously described for 30 min depending on the growth conditions i.e. cells harvested from aerobic dark used aerobic dark conditions. Cell suspensions were centrifuged at 8000 rpm for 15 min and the supernatant was mixed with 5% HNO₃, and the remaining Hg concentration was then determined by a Flow Injection Mercury Systems (FIMS). The potential of live and dead cells of three PNSB strains to remove Hg²⁺ was calculated and compared with control sets without biomass under both sets of incubation conditions.

2.3. Factors affecting the removal of Hg²⁺ by a selected PNSB strain

For growth studies, a ten percent inoculum of a selected strain was grown with GA broth containing 3% NaCl under both optimal growth conditions as previously described. Cell growth was observed every 6 h. Bacterial cells were harvested from their various phases (log, late log, and stationary). The cell pellets for biosorption were prepared as previously described to study the optimal growth phases and the following factors; biomass doses by collecting cells from the optimum growth phase for Hg²⁺ removal and a varying cell density of 3.5, 4.0, 4.5, 5.0 and 5.5 mg DCW/mL; initial Hg concentrations by using HgCl₂ solution at 1–8 mg/L; varying pH values of 5.0, 6.0, 7.0, 8.0 and 9.0; varying temperatures of 25, 30, 35 and 40 °C; and varying contact times of 0–120 min. In addition, the presence of other cationic ions; 3.5% NaCl (Na⁺), 85 mg/L CaCl₂ (Ca²⁺), 160 mg/L MgSO₄ (Mg²⁺) and 0.75 mg/L CdCl₂ (Cd²⁺) were also investigated by following the methods as described by Panwichian et al. (2010b). Values for the pH and temperature were designed according to the conditions of the shrimp cultivation. The Hg concentrations were set to match the contamination levels of Hg found in the shrimp ponds (Mukkata et al., 2015b).

2.4. Biosorption isotherm models

The biosorption isotherms for removing Hg by a selected PNSB strain SSS2-1 were obtained with varying Hg concentrations of 1 and 8 mg/L HgCl₂ solutions, a pH of 7.0, and 5.0 mg DCW/mL of live or dead cells that had been grown with both incubation conditions of microaerobic light and aerobic dark for a 60 min exposure. Each biomass was separated from the solutions containing HgCl₂ by centrifugation at 8000 rpm for 15 min, and then the remaining Hg concentration was determined by FIMS. The remaining Hg concentrations were used to determine the Hg sorption isotherms. The amount of metal ions (Hg²⁺) sorbed by the biomass (q_e , mg/g) was calculated as follows:

$$q_e = \frac{V(C_0 - C_e)}{m} \quad (1)$$

Where C_0 and C_e are the initial and equilibrium solution concentrations (mg/L), respectively, V is the volume of the solution (L) and m is the amount of adsorbent or biomass (g).

The results were fitted to the Langmuir and Freundlich models that were described by formulae (2) and (3), respectively (Abbas et al., 2014):

$$q_e = \frac{1 + K_L C_e}{q_m K_L C_e} \quad (2)$$

$$q_e = K_F C_e^{1/n} \quad (3)$$

Where q_e and q_m are the final amount and the maximum of Hg²⁺ per unit mass of adsorbent (mg/g), C_e is the equilibrium Hg concentrations

(mg/L), K_L is the Langmuir equilibrium constant, and K_F and n are Freundlich equilibrium constants.

A plot of C_e/q_e versus C_e yielded a straight-line with a slope and intercept that corresponded to $(1/q_m)$ and $(1/K_L \cdot q_m)$, respectively, from which q_m and K_L were calculated from Eq. (2) of the Langmuir model. Meanwhile, a plot of $\log q_e$ versus $\log C_e$ yielded a straight line with a slope and intercept that correspond to $(1/n)$ and $(\log K_F)$, respectively, from which n and K_F were calculated assuming the adsorption process follows Eq. (3) of the Freundlich model. Then, a regression co-efficient (R^2) was calculated and compared to the linear regression equations of the Langmuir and Freundlich model graphs. The model that fitted better was considered for Hg sorption by PNSB (SSS2-1) biomass.

2.5. Biosorption kinetic models

In the biosorption kinetic models for Hg^{2+} by a selected PNSB strain, a 5.0 mg DCW/mL was suspended in 4.0 mg/L $HgCl_2$ solution, pH 7.0. Samples were taken from the solutions at contact times of 0, 5, 15, 30, 45, 60, 75, 90 and 120 min, centrifuged at 8000 rpm for 15 min and the supernatants were collected. The remaining Hg^{2+} concentrations were determined by FIMS and then q_e (mg/g) was calculated using Eq. (1). The results were adjusted to the pseudo-first and pseudo-second order models. The pseudo-first and pseudo-second order models were calculated by the Eqs. (4) and (5), respectively (Abbas et al., 2014):

$$\frac{dq}{dt} = K_1(q_e - q_t) \quad (4)$$

$$\frac{dq}{dt} = K_2(q_e - q_t)^2 \quad (5)$$

Where q_e and q_t are the values of the amount adsorbed per unit mass at equilibrium and any times, respectively (mg/g) and K_1 (min^{-1}) and K_2 (g/mg min^{-1}) are the pseudo-first and pseudo-second order adsorption rate coefficient, respectively. The plot of $\log(q_e - q_t)$ versus t gives a straight-line graph with a slope and intercept that were used to calculate the K_1 and q_e for the pseudo-first order kinetics. Meanwhile, the plot of (t/q_t) versus t provided a straight-line graph with a slope and intercept that were used to calculate q_e and K_2 for the pseudo-second order kinetics. Then, R^2 was calculated and compared to the linear regression equations of the graphs of the pseudo-first and second order models. If the R^2 values of any of the models are higher than another model, the kinetics of Hg^{2+} sorption by the PNSB biomass is based on that model.

2.6. Statistics

All experiments in this study were conducted in triplicate and data presented as means with their standard deviation. For analysis of the data one way ANOVA was performed and significant differences among means were analyzed using the Duncan's multiple range tests at a P -value < 0.05 .

3. Results

3.1. The potential of selected PNSB strains to remove Hg^{2+} by biosorption

The potential of the three selected PNSB strains following their growth under microaerobic light and aerobic dark conditions were tested for their ability to remove Hg^{2+} from a 2 mg/L $HgCl_2$ solution using 4.5 mg DCW/mL of live or dead cells of each strain for 30 min using the same conditions as for the growth. The efficiency of Hg^{2+} removal by live cells of strains SRW1-5, SSS2-1 and SSW15-1 under conditions of microaerobic light were 81.11%, 76.31% and 79.29%, while from aerobic dark conditions they were 71.67%, 78.93% and 77.14%, respectively (Fig. 1A). In contrast, the removal percentage of Hg^{2+} by dead cells was 92.94%, 89.90% and 92.92% for SRW1-5,

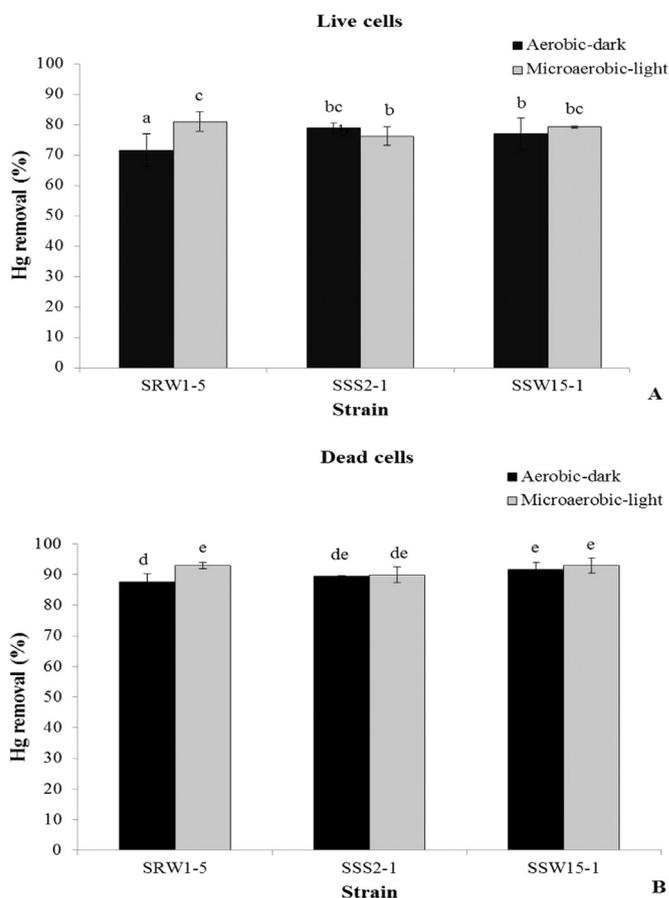


Fig. 1. The potential of PNSB strains to remove Hg^{2+} at 2 mg/L $HgCl_2$, pH 7.0 for 30 min by 4.5 mg DCW/mL of live (A) and dead (B) cells under microaerobic light and aerobic dark conditions. Different lowercase letters above bars indicate significant differences among the means of both incubating conditions ($P < 0.05$). Error bars indicate standard deviations of three determinations.

SSS2-1 and SSW15-1 under microaerobic light and 87.50%, 89.45% and 91.61% under aerobic dark conditions (Fig. 1B). A higher Hg^{2+} biosorption by all selected PNSB strains was observed using dead cells, particularly strain SRW1-5 when compared to the live cells (Fig. 1). Strains SSS2-1 and SSW15-1 showed no significant difference for their efficiency to remove Hg^{2+} under both incubation conditions suggesting their potential for bioremediation in shrimp ponds. However, the strain SSS2-1 was selected for further studies due to its higher resistance to Hg^{2+} compared to strain SSW15-1 (Mukkata et al., 2015b).

3.2. Factors affecting Hg removal by a selected PNSB strain

Based on the previous experiment, *A. marina* SSS2-1 was selected for further studies as its removal of Hg^{2+} by both live or dead cells from both incubation conditions was very high (Fig. 1). Results of the cell growth phases from log, late log and stationary phases on Hg^{2+} removal by live or dead cells of strain SSS2-1 are shown in Fig. 2A, B. The percentage Hg^{2+} removal of live cells from their log and late log phases from both incubation conditions were similar of roughly 80%, while biosorption of cells from their stationary phase decreased to 61% and 69% for the microaerobic light and aerobic dark conditions, respectively (Fig. 2A). In contrast, dead cells obtained from both incubation conditions demonstrated no significant difference in their ability to remove Hg^{2+} as both were in the range of 92–96% removal (Fig. 2B).

The effects of increasing the biomass dose from 3.5 to 5.5 mg DCW/mL of live or dead cells grown under both incubation conditions were tested using a 4 mg/L $HgCl_2$, a pH of 7.0 solution for 30 min and

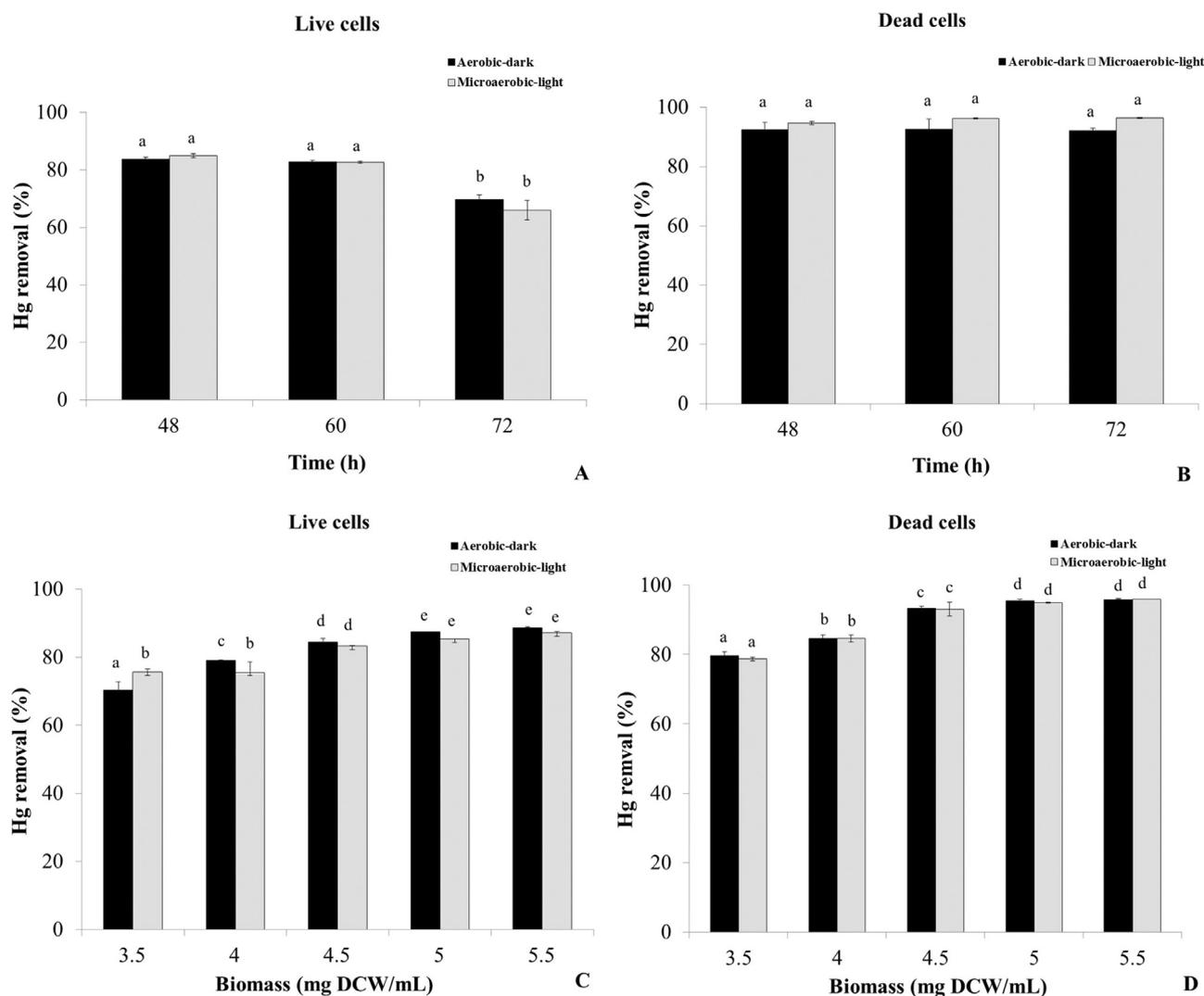


Fig. 2. Effects of cell ages (log, late log and stationary phases) of live (A) and dead (B) cells, and biomass doses of live (C) and dead (D) cells of *Afifella marina* SSS-2-1 on Hg^{2+} removal at 4 mg/L HgCl_2 , pH 7.0 for 30 min under microaerobic light and aerobic dark conditions. Different lowercase letters above bars indicate significant differences among means for each subfigure ($P < 0.05$). Error bars indicate standard deviations of three determinations.

incubated with the same conditions as for growth. The removal percentage of Hg^{2+} by both cell types significantly increased with increasing biomass from 3.5 to 5.5 mg DCW/mL. Therefore, the optimum biomass dose for Hg^{2+} removal was 5.0 mg DCW/mL but there was no significant difference for the 5.0 and 5.5 mg DCW/mL. This biomass dose was therefore selected for further studies. Under both incubating conditions of microaerobic light and aerobic dark at a dosage of 5.0–5.5 mg DCW/mL, the maximum Hg^{2+} removal of live cells were 87% and 88%, respectively, but it was up to 95% using dead cells (Fig. 2C, D).

Increasing the initial HgCl_2 concentrations from 1 to 8 mg/L produced different effects on the percentage Hg^{2+} removal by live or dead cells; however, no significant differences were found between both incubation conditions (Fig. 3A, B). Live cells showed a dose-dependent decrease in their ability to remove Hg^{2+} (Fig. 3A). On the other hand, the dead cells did not show any significant difference in their Hg removal ability at 1–3 mg/L HgCl_2 concentrations although a marginal decrease (up to 20%) in their Hg^{2+} removal efficiency was observed between 4 and 8 mg/L HgCl_2 concentrations (Fig. 3B).

Removal of Hg^{2+} under both incubating conditions with the use of 5.0 mg DCW/mL of live or dead biomass significantly increased over a range of pH from 5 to 7 and thereafter significantly decreased at pH values between 8 and 9 (Fig. 3C, D). The optimum pH to remove Hg^{2+}

for both cell types under both incubation conditions was 7.0 followed by 6 and 8; and thus the optimum pH of 7.0 was selected for further studies.

Increasing contact times from 5 to 120 min increased Hg^{2+} removal by live or dead cells (5 mg DCW/mL, pH 7.0) with a marked effect on live cells than dead cells under both incubation conditions (Fig. 4A, B). For live cells, the removal percentage of Hg^{2+} significantly increased from about 60–89% with increasing contact times from 5 to 90 min (Fig. 4A). However, the biosorption of dead cells increased suddenly after 5 min of contact time (roughly 82–88%), but there was little further increase up to 60 min. Furthermore, the removal percentage of Hg^{2+} significantly decreased at higher contact times (75, 90 and 120 min) (Fig. 4B). According to these results 60 min of contact time was selected for further studies of both cell types under both incubation conditions because the objective was to investigate Hg^{2+} removal by biosorption although over the longer time of 90 min the efficiency of live cells was similar to the dead cells at 15–60 min.

The effect of temperature (25–35 °C) on Hg^{2+} removal by live or dead cells under both incubation conditions was investigated using conditions of 5.0 mg DCW/mL of live or dead cells, pH 7.0 for 60 min (Fig. 4C, D). Under both incubation conditions, the removal percentage of Hg^{2+} by live cells significantly increased from 75% to 87% with an increase of the temperature up to 35 °C, but at a higher temperature of

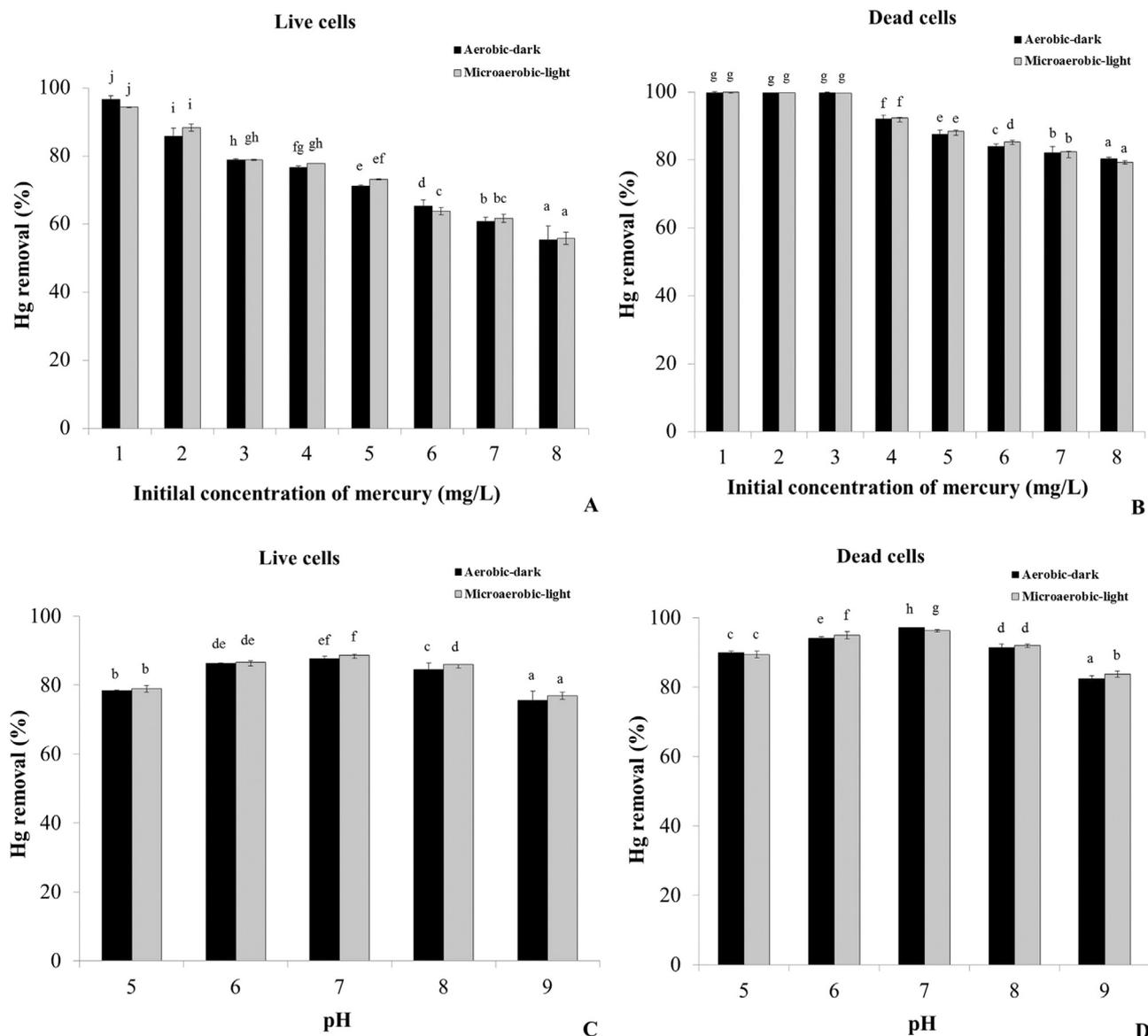


Fig. 3. Effect of initial concentrations of Hg^{2+} on its removal at pH 7.0 by live (A) and dead (B) cells, and the effect of pH values at 4 mg/L HgCl_2 by live (C) and dead (D) cells of *A. marina* SSS2-1 (5.0 mg DCW/mL) under both incubating conditions for 30 min. Different lowercase letters above bars indicate significant differences among means for each subfigure ($P < 0.05$). Error bars indicate standard deviations of three determinations.

40 °C, there was a significant reduction of 76% (Fig. 4C). However, the removal percentages of Hg^{2+} using dead cells increased from 82% to 95% with an increase of the temperatures from 25 °C to 40 °C with a significant increase at 30 °C (Fig. 4D). Hence, 35 °C was selected as the optimum temperature for further studies for both cell types.

The effect of the presence of other cationic ions; 3.5% NaCl (Na^+), 85 mg/L CaCl_2 (Ca^{2+}), 160 mg/L MgSO_4 (Mg^{2+}) and 0.75 mg/L CdCl_2 (Cd^{2+}) (Panwichian et al., 2010b) on the Hg^{2+} removal by live or dead cells (5.0 mg DCW/mL) was evaluated at a pH of 7.0 and temperature of 35 °C for 60 min. The presence of other ions significantly decreased the removal percentage of Hg^{2+} when compared with the control sets (Fig. 5). There was a significant reduction of the percentage of Hg^{2+} removed under both incubation conditions by both cell types in the order of Cd^{2+} and Mg^{2+} (28–31%) > Ca^{2+} (24–25%) > Na^+ (18–20%); however, the presence of these other light and heavy metal ions had more effect on live cells than on dead cells.

3.3. Isotherms of Hg sorption by a selected PNSB biomass

The adsorption isotherms of Hg^{2+} biosorption by live or dead cells of *A. marina* SSS2-1 grown with either microaerobic light or aerobic dark conditions were analyzed using the Langmuir and Freundlich models. Both models have been commonly used for interpretation of biosorption processes. The linear regressions of the data of the Langmuir and Freundlich models are presented in Fig. 6. The values of constants from both models, and the linear correlation coefficients (R^2) are displayed in Table 1. The prediction of Hg biosorption by the Langmuir model of live cells was less precise with lower R^2 values of 0.954 and 0.970 when grown with microaerobic light and aerobic dark conditions, respectively, while the prediction by the Freundlich model gave R^2 values of 0.994 and 0.991 under microaerobic light and aerobic dark conditions, respectively. In contrast, the values of R^2 from the dead cells (0.986 and 0.993) that were predicted by the Langmuir model were higher than the predicted from the Freundlich model (0.824 and 0.712). Therefore, the Hg^{2+} sorption of live cells under both incubation conditions was in agreement with the Freundlich model. On

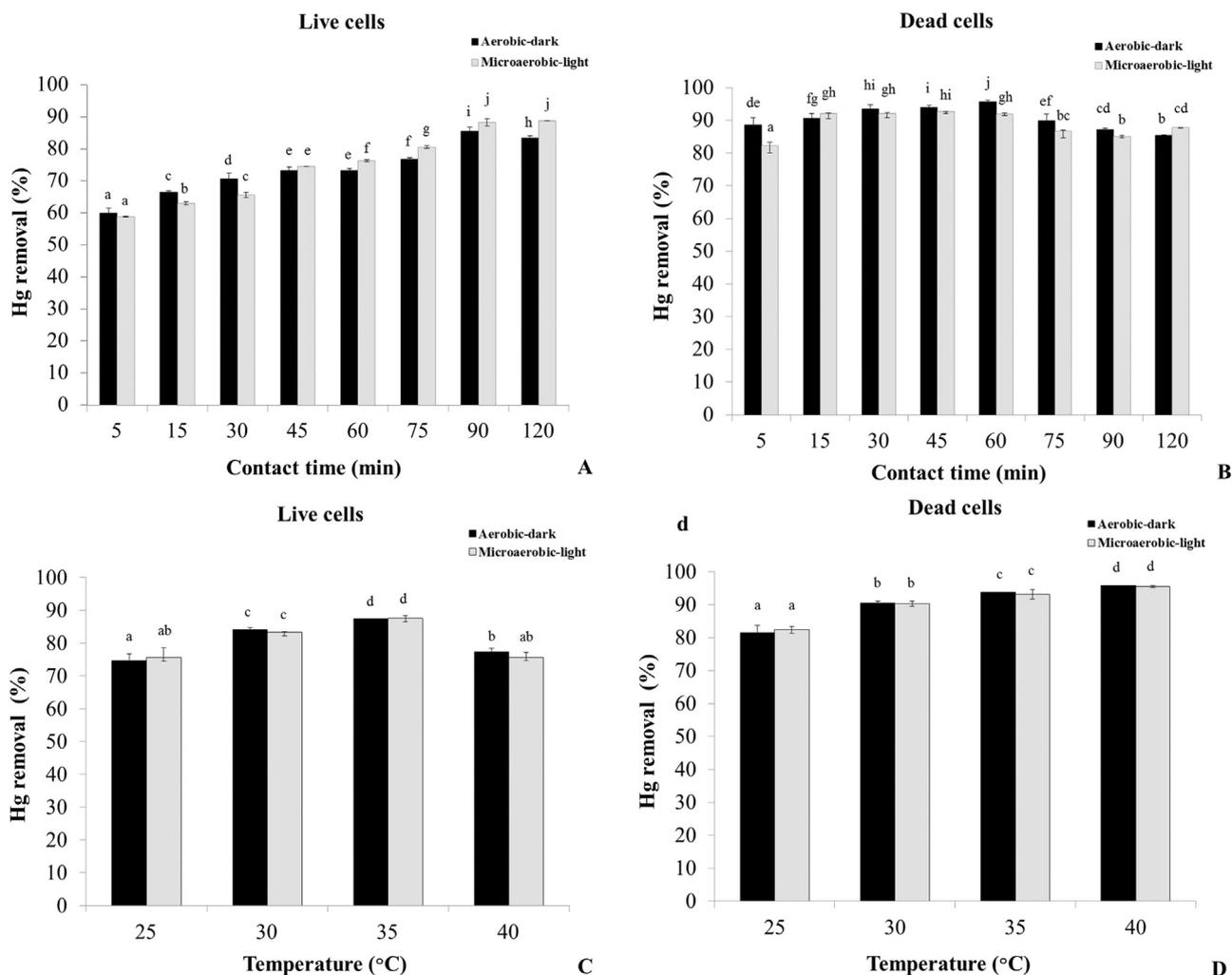


Fig. 4. Effects of contact time and temperature on Hg²⁺ removal by live (A, C) and dead (B, D) cells of *A. marina* SSS2-1 (5.0 mg DCW/mL) under both incubating conditions at 4 mg/L HgCl₂, pH 7.0 for 30 min. Different lowercase letters above bars indicate significant differences among means for each subfigure (*P* < 0.05). Error bars indicate standard deviations of three determinations.

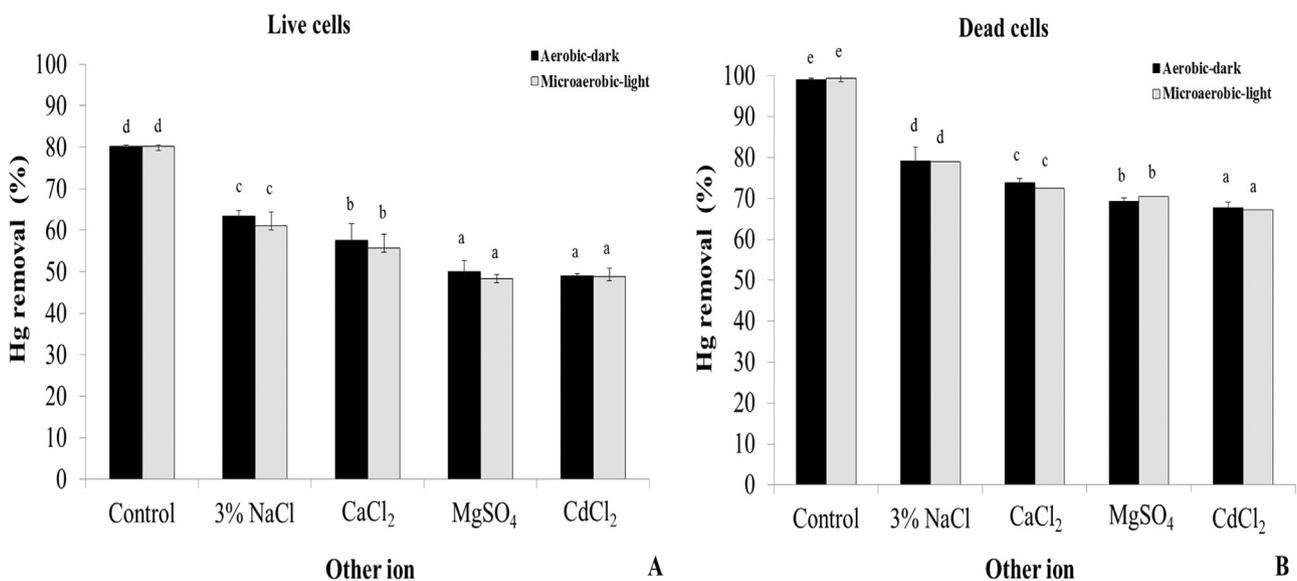


Fig. 5. Effect of other ions on Hg²⁺ removal at 4 mg/L HgCl₂, pH 7.0, 35 °C for 60 min by live (A) and dead (B) cells of *A. marina* SSS2-1 (5.0 mg DCW/mL) under both incubating conditions. Different lowercase letters above bars indicate significant differences among means (*P* < 0.05). Error bar indicates standard deviations of three determinations.

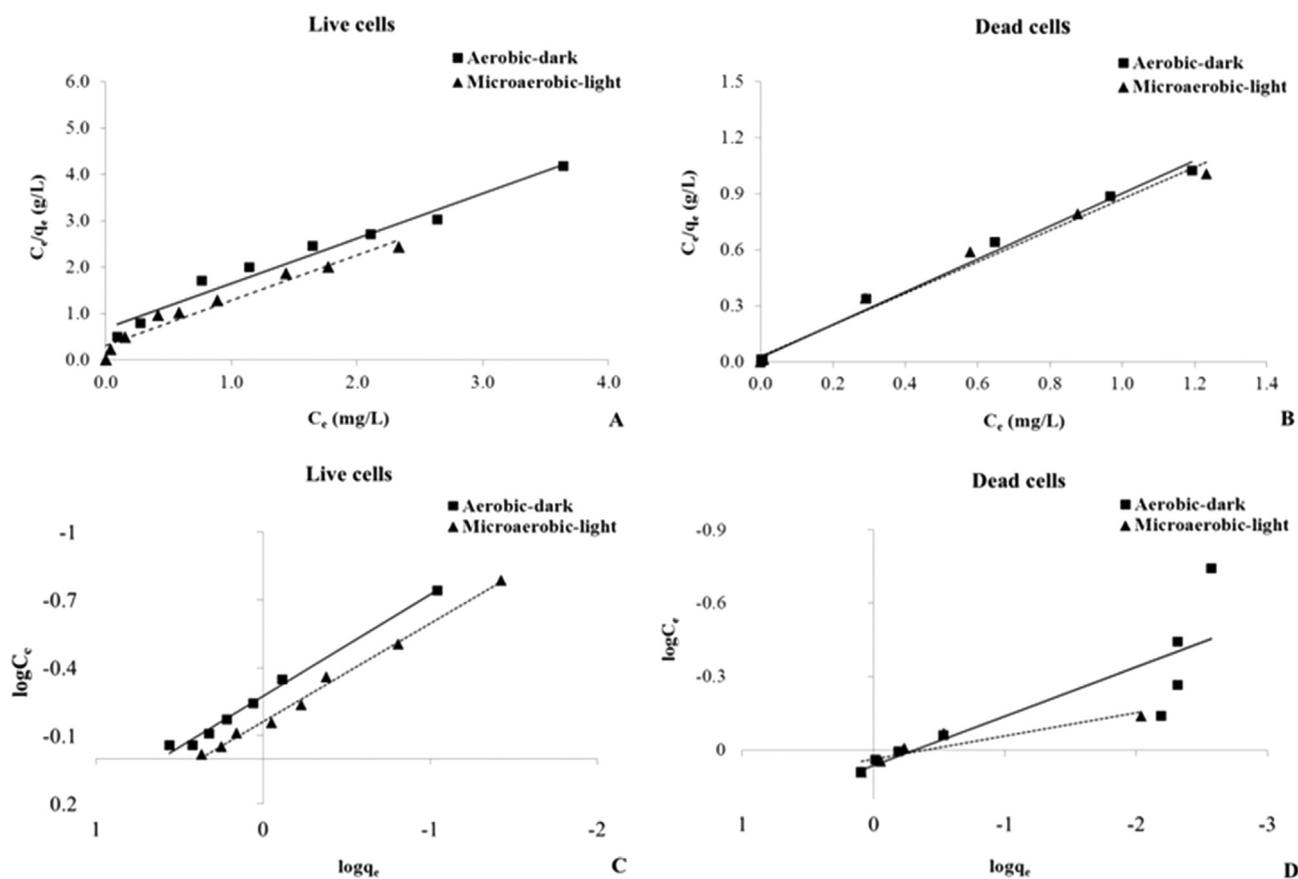


Fig. 6. Langmuir model of Hg^{2+} biosorption by live (A) and dead (B) cells, and Freundlich model by live (C) and dead (D) cells of *A. marina* SSS2-1 under both incubating conditions.

the other hand, the biosorption of dead cells collected from both incubation conditions was in agreement with the Langmuir model.

From the prediction with the Freundlich model, the K_F value of live cells were 0.686 and 0.531, and n was 2.312 and 2.237 with microaerobic light and aerobic dark conditions, respectively. While the predicted maximum adsorption capacities (q_m) by the Langmuir model for the dead cells were 1.184 and 1.139 mg/L, and their K_L values were 29.323 and 36.596 L/mg with growth under microaerobic light and aerobic dark conditions, respectively (Table 1).

3.4. The kinetic modelling of Hg^{2+} removal by a selected PNSB biomass

The adsorption kinetics of Hg^{2+} biosorption by live or dead cells of *A. marina* SSS2-1 with microaerobic light and aerobic dark conditions were analyzed using the pseudo-first and pseudo-second order models. The linear regressions of the data of the pseudo-first and pseudo-second order models by the live and dead cells are presented in Fig. 7. The kinetic constants and correlation coefficient values of the pseudo-first and pseudo-second order models are given in Table 2. The correlation

coefficient of the first-order kinetics of live and dead biomass under both incubation conditions was lower than in the case of the second-order kinetic model. The lower of the R^2 values from the regression lines of the pseudo-first order kinetic model in live cells and dead cells were calculated to be 0.930 and 0.295 for the microaerobic light conditions, and 0.892 and 0.165 for the aerobic dark conditions. On the other hand, the R^2 values from the regression lines of the pseudo-second order kinetic models in the live and dead cells were 0.988 and 0.999 with microaerobic light conditions, and 0.987 and 0.996 with aerobic dark conditions (Table 2). This shows that the kinetics of Hg^{2+} biosorption by the strain SSS2-1 biomass was better described by pseudo-second order kinetic model rather than by a pseudo-first order kinetic model. From the pseudo-second order kinetic model, the q_e value (mg/g) for the live cells were 0.678 and 0.672, and 0.691 and 0.707 for the dead cells grown with microaerobic light and aerobic dark conditions, respectively. While the K_2 ($g/mg \text{ min}^{-1}$) for the live cells were 0.220 and 0.195, and 1.182 and 1.030 for the dead cells grown with microaerobic light and aerobic dark conditions, respectively (Table 2).

Table 1

Langmuir and Freundlich isotherm constants and values of linear correlation co-efficient (R^2) for Hg^{2+} biosorption by live and dead cells of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions.

Biomass	Conditions	Langmuir			Freundlich		
		q_m (mg/L)	K_L (L/mg)	R^2	K_F (L/g)	n	R^2
Live	Light	1.253	3.224	0.954	0.686	2.312	0.994
Live	Dark	0.982	13.483	0.970	0.531	2.237	0.991
Dead	Light	1.184	29.323	0.986	1.087	10.460	0.824
Dead	Dark	1.139	36.596	0.993	1.153	4.987	0.712

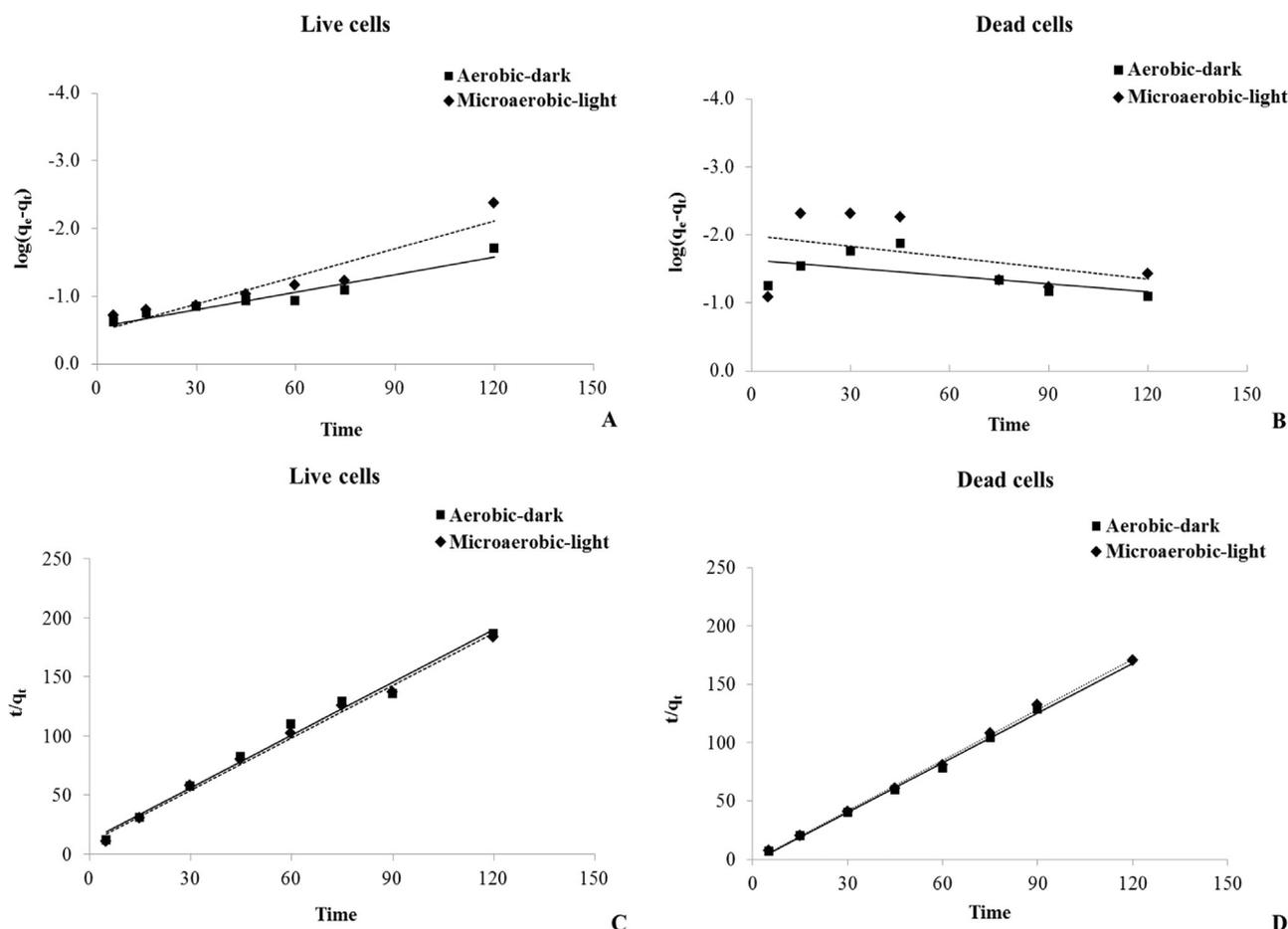


Fig. 7. Pseudo-first order model of Hg^{2+} biosorption by live (A) and dead (B) cells, and pseudo-second order model by live (C) and dead (D) cells of *A. marina* SSS2-1 under both incubating conditions.

4. Discussion

4.1. The potential of selected PNSB strains to remove Hg^{2+} by biosorption

This study explored the potential use of either live or dead selected Hg resistant PNSB strains as a biological approach to remove contaminating heavy metals, especially Hg^{2+} in shrimp ponds. Such a bioremediation process would provide many advantages such as being cost effective, eco-friendly and provide opportunities for metal recovery, including the advantage that the removal of the metals can be applied in situ at a contaminated site (Jafari and Cheraghi, 2014). Some previous studies have suggested that the biosorption capacity by dead cells can be higher than for live cells (Kaçar et al., 2002; Bayramoğlu and Arica, 2008; Velásquez and Dussan, 2009), because biosorption by dead cells occurs passively in the absence of metabolism so binding of metals occurs rapidly and mainly at the cell surface. In contrast, bioaccumulation by live cells is a metabolically active process and accumulation may be limited by toxicity (Chojnacka, 2010; Panwichian

et al., 2010b). The results obtained in this study support this possibility as the percentage removal of Hg^{2+} from a solution containing 2 mg/L HgCl_2 was significantly higher using dead cells of the three PNSB strains tested, in both incubation conditions when compared with their live cells (Fig. 1B). There were no significant differences for the percentage removal of Hg^{2+} by cells obtained from the 2 sets of incubation conditions, except for strain SRW1–5 for which Hg^{2+} binding by cells from the aerobic dark conditions was lower for both live and dead cells (Fig. 1A, B). It was also clear that the growth of the strain SRW1–5 (*R. sulfidophilum*) with microaerobic light conditions provided more suitable binding conditions compared to those provided by the other strains SSS2-1 and SSW15-1 (*A. marina*).

Normally, the biomass consists of many functional groups on the cell surfaces such as carboxyl, sulfonate, phosphoryl, amido, amino and imidazole (Chojnacka, 2010; Nguyen et al., 2018). These functional groups play an important role in metal binding to the cell biomass. The Hg^{2+} removal capacity by strains SSS2-1 and SSW15-1 were similar because their surface biomass functional groups are similar as they

Table 2

Rate constants for kinetic models of the Hg^{2+} biosorption by live and dead cells of *A. marina* SSS2-1 under microaerobic light and aerobic dark conditions.

Biomass	Conditions	Pseudo-first order			Pseudo-second order		
		q_e (mg/g)	K_1 (min^{-1})	R^2	q_e (mg/g)	K_2 (g/mg min^{-1})	R^2
Live	Light	0.568	4×10^{-5}	0.930	0.678	0.220	0.988
Live	Dark	0.567	6×10^{-5}	0.892	0.672	0.195	0.987
Dead	Light	0.595	3×10^{-5}	0.295	0.691	1.182	0.999
Dead	Dark	0.548	3×10^{-5}	0.165	0.707	1.030	0.996

belonged to the same species. On the other hand, the percentage of Hg^{2+} removal by both cell types of strain SRW1–5 when grown with microaerobic light conditions was significantly higher than with aerobic dark conditions. This could be explained by this strain's capacity to produce slime or other extracellular polymeric substances (EPS) when grown under microaerobic light conditions (data not shown). It is well recognized that EPS is secreted by many microbes in response to some physiological stresses such as heavy metals and osmotic salts such as Na^+ (Panwichian et al., 2011; Nunkaew et al., 2015; Nguyen et al., 2018); and EPS can contain rich functional groups for binding metal ions such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups (Liu and Fang, 2002; Joshi and Juwarkar, 2009). Hence, the production of EPS by strain SRW1–5 when growing under microaerobic light conditions enhanced the percentage of Hg^{2+} that could bind to the cell surface. There is no significant difference in the Hg^{2+} removal by dead cells of all three PNSB strains (Fig. 1); the efficiency of live cells under both incubation growth conditions was considered for shrimp cultivation and/or bioremediation. Therefore, strain SSS2-1 was chosen because it had a higher efficiency to remove Hg^{2+} using both incubation conditions in addition to its Hg^{2+} resistance as previously described (Mukkata et al., 2015b).

4.2. Factors affecting of Hg^{2+} removal by a selected PNSB strain

The efficient removal of Hg^{2+} by bacterial biomass depends on many factors such as the growth phases, biomass dose, environmental conditions such as the initial Hg concentration, pH, contact time, temperature and the presence of other ions (Abbas et al., 2014). With both sets of incubation conditions, the percentage of Hg^{2+} removal by live cells from the log and late log phases was significantly higher than by cells from the stationary phase; however, the amounts of Hg^{2+} bound by dead cells was not significantly different from all three growth phases (Fig. 2A, B). Bioaccumulation involved with removal of Hg^{2+} by live cells is a metabolically driven detoxification processes (Chojnacka, 2010). Hence, cells in the log and late log phases had a higher capacity for bioaccumulation of Hg^{2+} than cells from the stationary phase. During the stationary phase the composition and flexibility of the cell wall changes and binding sites may not be so accessible (Abbas et al., 2014). All three growth phases of dead cells produced a similar result for removal of Hg^{2+} ; this is because only the sorption of Hg^{2+} on preformed binding sites on the cells surface occurs with dead cells. This is also why the binding of Hg^{2+} by dead cells under both sets of growth conditions was always similar and slightly greater than by live cells.

The dosages of the biomass strongly influenced Hg^{2+} removal because an increase in biomass dose increased the surface area and numbers of binding sites for Hg ions (Vijayaraghavan and Yun, 2008) (Fig. 2C, D). The initial Hg^{2+} concentration also influenced the percentage removal of Hg ions. At the low concentration of Hg^{2+} , the removal percentage of Hg^{2+} was extremely high, especially at 1–3 mg/L by dead cells (Fig. 3A, B). In this case the biosorption sites on cell surface were sufficient for binding most of the Hg^{2+} ; hence all of Hg^{2+} could bind to the cell surface (5 mg DCW/L) of dead cells. In contrast, an increase of the initial concentration of Hg^{2+} significantly reduced the removal percentage of Hg^{2+} because of a decrease in binding sites. These results were similar to those of previous studies (Mo and Lian, 2010). For live cells, the initial Hg^{2+} concentration did not only affect the sorption sites on the cell surface but were toxic to the cells; therefore the percentage removal of Hg^{2+} was significantly decreased with an increase of the initial Hg^{2+} dose (Fig. 3A, B).

The pH plays an important role in metal removal because of its influence on the metal ion solubility and the total charges of the functional binding groups such as carboxylate, phosphate and amino groups on the biomass (Abdel-Ghani and El-Chaghaby, 2014; Nguyen et al., 2018). The percentage Hg^{2+} biosorption capacity of both cell types increased with increasing pH up to a pH of 5–7 (Fig. 3C, D). This

could be due to an increase in pH values providing more negative charges on the biomass surface for binding with positive charges like Hg^{2+} (Aryal and Liakopoulou-Kyriakides, 2015). However, at pH values higher than 7, hydroxo species of the metals can be formed; and these will not bind to sorption sites on the biomass surface (Kaçar et al., 2002; Green-Ruiz, 2006). This causes the reduction of metal ion binding such as Hg^{2+} ; so the Hg removal capacity by both types of biomass at a pH of 9 was significantly decreased (Fig. 3C, D). Many previous studies have reported that the optimum pH for Hg^{2+} sorption by live and dead cells of bacteria was pH 6 (Kaçar et al., 2002; Green-Ruiz, 2006; Jafari and Cheraghi, 2014). For strain SSS2-1 it was between 6 and 7 with a small decrease at pH 8; this provided the opportunity to use this bacterium in shrimp farm either for cultivation or bioremediation as the pH of shrimp ponds is in the range of 6–8 (Cheung and Wong, 2006).

The results of this study are in agreement with many previous studies in that the percentage of metals removed is most rapid at the beginning of the sorption time because of the larger surface area of biomass for the sorption of metals until it reached the equilibrium (Mo and Lian, 2010; Panwichian et al., 2010b). Hence, the percentage of Hg^{2+} removal by dead cells was very high within the first 5 min and reached a maximum at 15 min and then was stable until 60 min after exposure; but the Hg^{2+} removal declined afterwards for up to 75 min of contact time (Fig. 4A, B), as the Hg^{2+} desorbed (Fowle and Fein, 2000). However, the percentage removal of Hg^{2+} by live cells gradually increased until it reached equilibrium at 90 min (Fig. 4A). The results indicate that in addition to biosorption, live cells also used bioaccumulation for detoxification as they continually increased Hg^{2+} uptake into the cells.

The capacity for the biosorption of heavy metals also depended on the temperature as increases of temperature enhanced surface activity and kinetic energy leading to an increase in the biosorption capacity (Panwichian et al., 2010b; Aryal and Liakopoulou-Kyriakides, 2014). This is the reason why the percentage of Hg^{2+} removal increased following an increase of the temperature from 25 up to 40 °C for dead cells (Fig. 4C, D). However, higher temperatures can destroy biomass, and reduce the biosorption capacity (Aryal and Liakopoulou-Kyriakides, 2014, 2015); so this might be one reason for the reduction of Hg^{2+} removal by live cells as the temperature increased up to 40 °C (Fig. 4A). The optimum temperature of 35 °C for the binding of Hg by *A. marina* SSS2-1 was in agreement with Jafari and Cheraghi (2014) who studied the Hg sorption by *Vibrio parahaemolyticus*; both these organisms are aquatic Gram negative bacteria.

The effect of other ions (Na^+ , Ca^{2+} , Mg^{2+} and Cd^{2+}) normally present in shrimp ponds on Hg^{2+} removal was associated with an increase of positive charges; and the positive charges in an aqueous environment will compete with the positive charges of the Hg ions for binding with negative charges on the surface of the cells (Vijayaraghavan and Yun, 2008). Hence, the Hg^{2+} removal was reduced in the presence of other ions with Mg^{2+} and $\text{Cd}^{2+} > \text{Ca}^{2+} > \text{Na}^+$ (Fig. 5). The amount of reduction was dependent on the numbers and types of available binding sites and their relative binding affinities for the different metal ions.

The investigation of the several parameters that affected Hg^{2+} removal based on biosorption showed that dead cells had a higher efficiency than live cells (Figs. 2–5). However, the highest removal percentage of Hg^{2+} under the optimum condition by live cells under both incubation conditions was quite high (87%) and could become as high as the dead cells by increasing the contact time from 30 min to up to 90 min (Fig. 4A). Also, live cells of strain SSS2-1 could remove Hg^{2+} via different mechanisms during shrimp cultivation such as by biovolatilization, biosorption and bioaccumulation (Mukkata et al., 2015b; this study), whereas biosorption was the only method used by dead cells. On the other hand, dead cells would be an alternative way when the focus was to remediate Hg from contaminated shrimp ponds before/after cultivation. In addition, the versatile growth modes of PNSB allow the possibility of their application in remediation of various Hg

contaminated areas. It should be noted that the feasibility for removal of Hg ions from water collected from shrimp ponds and other contaminated areas by the PNSB species, particularly *A. marina* strain SSS2-1 as an effective biosorbent, should be tested in laboratory scale prior to field applications, for successful bioremediation. Hence, future research work will be aimed to confirm the potency of strain SSS2-1: whether it still is an effective biosorbent in various water samples acquired from Hg contaminated areas including shrimp ponds.

4.3. Isotherms for Hg²⁺ sorption by a selected PNSB biomass

There are several isotherm equations that have been used to provide information on sorption processes such as cell surface properties and biosorption mechanisms. However, two models have been frequently used; the Langmuir and Freundlich models because both models are simple, well-established and have physical meaning, and are easily interpretable (Vijayaraghavan and Yun, 2008). The Langmuir model assumes that the sorption process is based on a monolayer of a homogeneous surface with constant sorption energy and with no interactions between adsorbed molecules. The Freundlich model assumes that the sorption process is based on a monolayer with a heterogeneous surface and molecules that attach to a surface site will have an effect on the neighboring sites (Kaçar et al., 2002; Plaza et al., 2011; Jafari and Cheraghi, 2014).

The Hg²⁺ biosorption by dead cells corresponded to the Langmuir model (Fig. 6B); while live cells corresponded to the Freundlich model (Fig. 6C). These results indicate that the biosorption process of live cells occurred on a monolayer with a heterogeneous surface that means the biosorption of Hg²⁺ was not only on the cell surface, but also Hg²⁺ accumulated intracellularly (Kaduková and Virčíková, 2005; Li et al., 2010). In contrast, the biosorption process of dead cells occurred only on a monolayer with a constant sorption energy as previously described. These results are in agreement with previous studies for the appropriate isotherms of metal sorption by live and dead biomass (Li et al., 2010; Mo and Lian, 2010; Jafari and Cheraghi, 2014)

In the case of the Freundlich model of live cells, the *n* value indicated formation of strong bonds between the biosorbent and heavy metal with a value of between 1 and 10 (Li et al., 2010; Jafari and Cheraghi, 2014). In this study, the *n* values of Hg²⁺ sorption were 2.312 and 2.237 for microaerobic light and aerobic dark conditions (Table 1); this means that the SSS2-1 biomass is a good biosorbent. The *n* and *K_F* values for the SSS2-1 live biomass were not high; however, the *n* values were a little higher than found in previous studies for Hg²⁺ biosorption by *Bacillus* sp., and *Vibrio parahaemolyticus* that were 1.51 and 1.53–1.68 (Jafari and Cheraghi, 2014). The values of *q_m* and *K_L* from the Langmuir model were calculated for description and the maximum monolayer biosorption capacity of the biosorbent (mg/g) and the affinity of the binding sites, respectively. Although the high value of *q_m* and *K_L* indicate that biomass provided good biosorption, the low values of *q_m* and high *K_L* were found, when biosorption was carried out at low metal concentrations (Li et al., 2010). This could be used to explain the results of the dead cells SSS2-1 that were tested on low concentration of Hg (1–8 mg/L) and showed a low *q_m* (1.184 and 1.139 mg/L) and a high *K_L* (29.323 and 36.596 L/mg) under microaerobic light and aerobic dark conditions, respectively (Table 1). Moreover, *R_L* values that are normally used to predict if an adsorption system is favorable or unfavorable (Rasmussen et al., 2000) were calculated from the Eq. (7):

$$R_L = \frac{1}{1 + K_L C_0} \quad (7)$$

Where *C₀* is the highest metal concentration (mg/L). The *R_L* value can indicate unfavorable (*R_L* > 1), linear (*R_L* = 1), favorable (0 < *R_L* < 1) and irreversible (*R_L* = 0) adsorption (Abdel-Ghani and El-Chaghaby, 2014). From our results, the *R_L* values of the dead cells grown with microaerobic light and aerobic dark conditions were 0.004

and 0.003 (data not shown), and indicated that the Hg²⁺ biosorption by dead biomass SSS2-1 was favorable.

4.4. The kinetic models of Hg²⁺ removal by a selected PNSB biomass

The two kinetic models namely, pseudo-first and pseudo-second order models, were investigated for their ability to describe the transport mechanisms of the Hg ion in the adsorbent (Lesmana et al., 2009). It was very clear that the kinetic models for Hg²⁺ removal by both the live and dead PNSB biomass fitted to a pseudo-second order model rather than to a pseudo-first order model (Fig. 7). Our results agreed with several literature studies that have reported the prevalent kinetic models of metals biosorption by microbes is more often associated with the pseudo-second order model rather than the pseudo-first order model (Bayramoğlu and Arica, 2008; Khambhaty et al., 2008a; Jafari and Cheraghi, 2014; König-Péter et al., 2014). The pseudo-second order model was used to demonstrate that the rate-limiting step was by chemisorption, not limitation by mass transport (Lesmana et al., 2009). It also assumed that the reaction of biosorption included two reactions; the first one was fast and reached equilibrium quickly; and the second was a slower reaction that continued for long time periods (Khambhaty et al., 2008b). This assumption correlated well with the results in this study that showed that the Hg²⁺ removal of live and dead cells was high within the first 5 min and then the biosorption rates were slow until they reached equilibrium at about 90 min and 15 min, respectively (Fig. 4A, B). Furthermore, the calculated *q_e* values for both types of biomass and incubation conditions agreed with the experimental data (data not shown) and these supported the view that the biosorption of Hg with SSS2-1 biomass fitted to the pseudo-second order kinetic model. In comparison the rate parameter *k₂* (g/mg min⁻¹) as shown in Table 2 indicate that the biosorption with microaerobic light and aerobic dark conditions by the dead biomass (1.182 and 1.030) was faster than that of the live cells (0.220 and 0.195).

Overall the results indicate that biosorption by the dead cells had higher efficiency to remove Hg²⁺ than that of the live cells, with many of the reasons described previously. However, the removal efficiency of Hg²⁺ by live cells under the optimum conditions was close to that of dead cells, particularly with the longer exposure times up to 90 min (Fig. 4A). In addition, live cells have more mechanisms to remove Hg ions, such as biosorption, bioaccumulation and biovolatilization, as previously stated. In general, during the use of live cells some cells die, but as dead cells they still have the ability to remove Hg ions by biosorption. This improves the efficiency of removing Hg ions from contaminated water. Based on the above, live cells of PNSB should be used to remove Hg ions from various contaminated waters, including shrimp ponds.

5. Conclusions

Overall these results have proven that the live or dead biomass of *A. marina* SSS2-1 grown with either microaerobic light or aerobic dark conditions have the potential to remove Hg²⁺ by biosorption; and their optimal conditions under both incubating conditions of both cell types were similar to conditions of shrimp cultivation. Dead cells showed higher efficiency for Hg²⁺ removal through biosorption; however, live cells also should be considered as they may have alternative mechanisms to remove Hg²⁺ from contaminated areas. The strain SSS2-1 has the great potential as the effective biosorbent to remediate Hg contaminated shrimp ponds.

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