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# Effective Iron-Accumulating Bacteria Isolated from Chemical Laboratory Drainage for Iron Removal

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**ABSTRACT**

Improperly treated heavy metal wastewater discharged into water sources could cause a serious issue for the environment. The aim of this study was to bioaccumulate iron (Fe) using native bacteria isolated from the laboratory drainage water containing a high concentration of iron. The experiment was conducted in 250 mL conical flasks containing 150 mL Fe solution in concentrations of 25, 100, and 250 mg/L, respectively. Approximately 10% of bacteria inoculum was cultivated in each Fe concentration for 24 and 48 hours. The results showed that *Pseudomonas hibiscicola* was identified as an effective iron-accumulating species of bacteria. The species could remove Fe up to 82% (25 mg/L), 77.8% (100 mg/L) and 32% (250 mg/L). This promising result indicates that the native bacteria isolated from the environment pose a great potential for the remediation of wastewater containing iron.

**Keywords:** Iron-accumulating bacteria; heavy metal; bioaccumulation; effective microbe; *Pseudomonas hibiscicola*

## INTRODUCTION

An experiment conducted in the laboratory, whether for educational purposes or research, will contribute to the generation of waste, mostly in the form of solid or liquid waste. Between these two types of waste generated, liquid waste (laboratory liquid waste [LLW]) is generated in abundance, compared to solid waste. It is the responsibility of all researchers who perform experiments in the laboratory to ensure the safe and correct disposal of all wastes produced during the course of their work. Although prevention measures are implemented, via the safe and correct disposal of waste, some breakthrough of waste (including the waste containing heavy metals) into the drainage systems of laboratories may occur. Heavy metals are harmful to the environment due to their persistence, toxicity, bioaccumulation and biomagnification in the food chain (Khan et al. 2018). Therefore, this study was conducted to monitor the water quality in the drainage system of a chemical laboratory while identifying the potentially effective bacteria for heavy metal bioaccumulation in the wastewater, particularly iron.

In wastewater treatment systems, three treatment processes are often chosen to treat wastewater, namely physical, chemical and biological processes (Wang et al. 2019). Nowadays, the combination of such processes can also be observed. The physical-chemical methods such as adsorbent (Bakar et al. 2016), coagulation-flocculation (Bakar et al. 2015), precipitation (Wang et al. 2019), electro-Fenton (Ghosh et al. 2011) and reverse osmosis (Reilly et al. 2019), for example, have been widely applied for the removal of metal ions from industrial wastewater. Unfortunately, these methods are overly expensive and require skilled

technicians (Basha & Rajaganesh 2014; Hanafiah et al. 2020). Therefore, other treatment processes that are inexpensive, easily operated and yield increased performance are urgently required.

Currently, the activity of microorganisms has been extended to environmental management and microbes have superseded the conventional techniques of remediation (Vidali 2001). The biological methods such as biosorption and bioaccumulation, seem to provide promising alternatives to the chemical methods (Hasan et al. 2012 & 2016; Zainudin et al. 2016). Bioaccumulation is a process by which chemicals are taken up by organisms directly from the exposure to a contaminated medium. The heavy metal bioaccumulation studies on the *Pseudomonas* bacteria in the wastewater from agricultural land showed that the organism was capable of removing zinc and copper from pollutants (Ahmad & Malik 2012). Previous studies also reported that a species of *Bacillus* isolated from soil, water and marine sediment could reduce $Fe^{3+}$ (Liu 2011; Lovley 2000; Scheid et al. 2004). Moreover, *Bacillus subtilis* was easily manipulated and had no or low levels of pathogenicity and possessed the biotechnological potential for bioaccumulation (Diderichsen et al. 1991). Bioaccumulation, particularly the microbial bioaccumulation, is an effective, eco-friendly, and affordable technology for the removal of heavy metals from laboratory wastewater.

Iron is the fourth most abundant element found naturally in the Earth's crust, potentially making it the largest acceptor of electrons present in the environment (Dong et al. 2009; Marschner et al. 2011; Stuckl et al. 2006). Although Fe is categorised as an essential mineral, diseases such as Alzheimer's disease, arteriosclerosis, diabetes mellitus, hepatic necrosis and others have been linked to excessive Fe intake (George 2009; Maurya et al. 2019). In drinking water, iron in the quantities greater than 0.3 mg/L, can produce an unpleasant taste and a rusty colour.

This study was conducted to isolate the iron-accumulating bacteria from a chemical laboratory drainage system and to test for the bioaccumulation of Fe in concentrations of 25 mg/L, 100 mg/L and 250 mg/L. Prior to the isolation of the bacteria, the characteristics of the chemical laboratory discharge were determined. In addition, the growth rate of the isolates under different concentrations of Fe was evaluated.

## MATERIALS AND METHODS

### Bacteria isolation and identification

The water samples from laboratory drainage were collected and their quality was analysed. The water samples were serially diluted from $10^{-1}$ to $10^{-5}$ in sterile saline water (0.9% NaCl). Approximately 0.1 mL of each dilution was spread on nutrient agar plates and incubated in a growth chamber (GC 1050, Protech, Malaysia) at 37°C for 48 hours. Afterwards, the bacterial colonies were isolated on new agar plates using a plastic loop to obtain pure isolates. Characterisation of the isolates was conducted via Gram staining, colony characterisation and a biochemical test.

Identification of the isolates was performed using the 16S DNA sequencing method. The bacterial DNA was extracted from the bacterial suspension in a nutrient broth using a Wizard® Genomic DNA Purification Kit (Promega, USA) which included a protocol for the isolation of genomic DNA from the Gram positive and negative bacteria. The universal primers 8F (5'-AGAGTTTGATCMTGG-3') and 1492r (5'-ACCTTGTTACGACTT-3') were used to amplify the 16S DNA gene according to the polymerase chain reaction (PCR) amplification protocol provided by the Promega manufacturer (USA). PCR was performed using a Mastercycler (Epgradient S, Eppendorf, Version 3.608). Next, the PCR-amplified product was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The PCR product was sent to First BASE Laboratories Sdn. Bhd (Kuala Lumpur, Malaysia) for sequencing. Finally, the 16S DNA sequences of the isolates were compared to those of other microorganisms by way of BLAST through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

### The growth rate of the bacteria

The growth of each isolate was determined by measuring the weight of biomass. The growth rate was then calculated using the following equation:

$$\ln \frac{x}{x_0} = \mu t \quad (1)$$

where: $x_o$ is the initial biomass (mg/L),
$x$ is the biomass (mg/L) at time,
$\mu$ is the specific growth rate (hour$^{-1}$),
$t$ is time (hour).

### Iron bioaccumulation experiment

Approximately 4.84 g of iron (III) chloride ($FeCl_3$) (Systerm, Malaysia) was diluted in 1000 mL of distilled water to represent a 1000 mg/L concentration of $Fe^{3+}$. The stock solution was diluted with distilled water to simulate 25, 100 and 250 mg/L of $Fe^{3+}$, respectively. The remaining stock solution was preserved at 4°C prior to use.

The respective isolated bacteria were cultivated in 250 mL conical flasks containing 100 mL nutrient broth and different concentrations of $Fe^{3+}$. The $Fe^{3+}$ bioaccumulation was conducted in an incubator shaker at 37°C for 24 hours at 130 rpm. The $Fe^{3+}$ concentrations were measured at 24 hours using an atomic absorption spectrophotometer (Perkin Elmer AA400).

### Analytical methods

Heavy metals such arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and manganese (Mn) were measured using inductively coupled plasma mass spectrometry (ICP/MS) (Perkin Elmer ELAN 9000) at 560 nm absorption, while Fe was measured using atomic absorption spectroscopy (AAS) (Perkin Elmer AA400). Chemical oxygen demand (COD) was analysed using low-range (0–150 mg/L) COD reagent vials. Approximately 2 mL of water was heated in a digestion reactor (HACH DRB200, USA) at 150°C for 2 hours. The COD reading was then obtained via a HACH spectrophotometer (HACH DR3900, USA) at a wavelength of 420 nm. The ammonia-nitrogen ($NH_3$-N) concentration was determined using a Nessler reagent with measurement at a wavelength of 425 nm using a HACH spectrophotometer (HACH DR3900, USA). The pH and dissolved oxygen (DO) were measured using a pH meter (Metrohm 827 pH Lab, USA) and a DO meter (YSI 550A, USA). Total suspended solids (TSS) was measured through the gravimetric method. Approximately 20 mL of laboratory drainage water was filtered using a 0.45 µm cellulose nitrate membrane (Whatman™, UK). The filtered sample was then dried for 1 hour at 105°C prior to weighing. Equation 2 was applied to calculate the TSS content.

$$\text{TSS} = \frac{M_1 - M_0}{V} \quad (2)$$

where: $M_1$ is the final mass of the filter paper (g),
$M_0$ is the initial mass of the filter paper (g)
$V$ is the volume of the sample (mL).

## RESULTS AND DISCUSSION

### Characterisation of laboratory drainage water

The characterisation results of the water discharged from the laboratory drainage are tabulated in Table 1. Sampling was conducted once per month for seven consecutive months to monitor the changes in the quality of the laboratory drainage wastewater. Table 1 illustrates that the highest pH readings observed were recorded in the fifth month of monitoring (pH 9.44). This may be due to many laboratory and research activities involving highly alkaline materials being conducted at that time, normally involving the dilution of alkaline materials via washing before entering the drainage system. The pH values recorded were still within the safe limit, since the pH values that are lower than 5 or exceed 11.5 may cause damage to the piping system.

Conversely, the highest DO value observed (4.62 mg/L) was recorded in the fourth month of monitoring, and the lowest DO values were observed in the first and sixth months. The highest TSS value (5.10 mg/L) was recorded in the second month, whereas the lowest TSS value (0.6 mg/L) was found in the third month, with the second-lowest value (1.00 mg/L) recorded in the fifth month. The COD values were lowest in the fifth (14 mg/L) and second (21 mg/L) months. However, a fluctuation in the COD value was observed in the fourth month of sampling with 88 mg/L of COD being recorded. This occurred due to the disposal of chemical into the drainage system during the laboratory activities. Meanwhile, the values of $NH_3$-N ranged from 0.25 mg/L to 0.77 mg/L and were stable throughout the sampling period.

The analyses of heavy metals indicated that nine types were present in the water samples (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, and Pb). These toxic heavy metals are common in wastewater (Akpor et al. 2014). Fe was observed at the highest concentrations, followed by Mn. The average value of Fe for the seven months of sampling was 704.05 µg/L with the highest value (1850.20 µg/L) recorded in the seventh month of sampling. The average

**Table 1**. Characterisation of laboratory drainage water

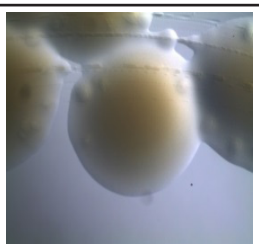
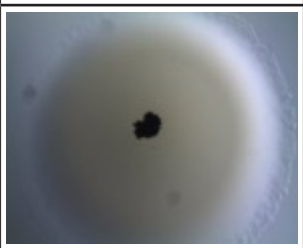
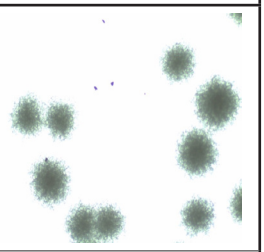
| Parameters | Months | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Water Quality Analysis | | | | | | | |
| pH | 7.33 | 7.32 | 7.68 | 6.93 | 9.44 | 7.03 | 7.90 |
| DO (mg/L) | 2.83 | 2.97 | 4.59 | 4.62 | 4.33 | 2.83 | 2.97 |
| TSS (mg/L) | 4.50 | 5.30 | 0.60 | 1.50 | 1.00 | 3.40 | 5.10 |
| $NH_3$-N (mg/L) | 0.25 | 0.64 | 0.47 | 0.31 | 0.77 | 0.23 | 0.45 |
| COD (mg/L) | 31.00 | 21.00 | 30.00 | 88.00 | 14.00 | 30.00 | 22.00 |
| Heavy Metals | | | | | | | |
| As (µg/L) | 0.56 | 1.38 | 0.63 | 0.77 | 0.47 | 0.72 | 1.80 |
| Cd (µg/L) | 0.21 | 22.77 | 7.67 | 10.21 | 0.19 | 0.71 | 23.50 |
| Co (µg/L) | 0.36 | 2.63 | 0.36 | 1.54 | 0.35 | 1.09 | 2.73 |
| Cr (µg/L) | 1.68 | 6.86 | 5.32 | 4.66 | 1.56 | 1.57 | 6.09 |
| Cu (µg/L) | 3.48 | 3.69 | 3.14 | 3.64 | 3.23 | 3.36 | 3.45 |
| Fe (µg/L) | 102.28 | 1801.34 | 120.11 | 850.76 | 99.87 | 103.80 | 1850.20 |
| Mn (µg/L) | 68.08 | 44.04 | 67.45 | 12.08 | 67.41 | 65.02 | 44.92 |
| Ni (µg/L) | 1.57 | 3.28 | 1.67 | 2.34 | 1.43 | 1.67 | 3.30 |
| Pb (µg/L) | 0.25 | 0.76 | 0.24 | 0.25 | 0.45 | 0.56 | 0.80 |

concentration of Mn was 52.71 µg/L with the highest value (68.08 µg/L) recorded in the first month of sampling. The heavy metal with the lowest concentration in the laboratory wastewater was Pb with an average concentration of 0.47 µg/L. From the results of the characterisation, Fe was chosen as a model for the bioaccumulation experiment using native bacteria isolates.

## Characterisation and identification of bacterial strains

A total of three pure strains were isolated from the laboratory drainage sludge. It was found that three of the isolates were Gram negative (NAJ1, NAJ3, and NAJ5). The isolates possessed a thin peptidoglycan layer and had an outer lipid membrane (Steward 2019). Three of the isolates were rod-shaped bacilli. Table 2 shows the morphological characteristics (diameter, colour, arrangement, shape, margin and elevation) and biochemical characteristics (oxidase activity). The isolated colonies showed a difference in colour (Table 2). The NAJ5 isolate was white in colour and NAJ1 was light white, while the NAJ3 isolate was light yellow. Moreover, the NAJ1 isolate was observed to grow in a circular-shaped colony on the nutrient agar; it was slightly convex and had a thorough

**Table 2**. Morphology characterisation of bacterial isolates

| Morphology | | *Cupriavidus pauculus* NAJ1 | *Stenotrophomonas maltophilia* NAJ3 | *Pseudomonas hibiscicola* NAJ5 |
|---|---|---|---|---|
| Microscopic observation | |  |  |  |
| Morphology | Diameter (mm) | 3 | 1 | 0.25 |
| | Color | White light | White light | White |
| | Morphology | Long and thin rod | Short rod | Long and thin rod |
| | Arrangement | Irregular | Gather | Irregular |
| | Shape | Circular | Circular | Circular |
| | Margin | Entire | Curled | Filamentous |
| | Elevation | Convex | Convex | Raised |
| Biochemical test | Catalase test | + | + | + |
| Gram Staining | | - | - | - |

colony edge. Meanwhile, the NAJ3 isolate was also observed to grow in a circular-shaped colony and was slightly convex but had a wavy edge. Conversely, the NAJ5 isolate was observed to grow in a circular-shaped colony but had an upward elevation and a filamentous edge. The catalase test showed positive results for all isolates despite the differences in the morphological characteristics.

The homology searches of the 16S DNA gene sequence of strain NAJ1 in GenBank by BLAST revealed that it had high similarity to the sequences of the *Cupriavidus pauculus* species (NR_116147.1) with 99% identity. Meanwhile, the NAJ3 and NAJ5 strains had high similarity to the sequences of the *Stenotrophomonas maltophilia* (NR_040804.1) and *Pseudomonas hibiscicola* species (NR_024709.1) with 95% and 99% identity, respectively. Therefore, it can be concluded that NAJ1, NAJ3 and NAJ5 belonged to the *Cupriavidus pauculus, Stenotrophomonas maltophilia* and *Pseudomonas hibiscicola* species, respectively.

### The growth rate of the isolated strains

Figure 1 shows the relationship between the dry weight of each bacterial isolate and the incubation time. The dry weight was preferred to represent the growth profile of each isolate instead of the real number of cells, because it was easily obtained (Mauerhofer et al. 2019). On the basis of the results obtained, the dry weight of each isolate increased with incubation time. NAJ5 showed the lowest dry weight with the longest lag phase, while NAJ3 had the highest dry weight. Table 3 summarises the specific growth rate (μ) for each bacterial isolate. NAJ5 had the lowest dry weight but the highest specific growth rate (0.2577 $hr^{-1}$) compared to the other isolates, where NAJ3 and NAJ1 had specific growth rates of 0.1215 $hr^{-1}$ and 0.0902 $hr^{-1}$, respectively.

## BIOACCUMULATION OF FE

### Colony forming units of isolates

Colony forming units (CFU) represented the ability of the isolated bacteria to grow in the presence of Fe at different concentrations. Figure 2 shows that Fe, at different concentrations, either promoted or demoted the ability of the respective isolates to grow. NAJ5 showed the greatest tolerance towards Fe, while NAJ3 showed the least tolerance towards Fe concentration, as the CFU decreased as Fe increased from 25 to 250 mg/L.

It was also observed that the CFU value of for all strains decreased as the Fe was increased to 250 mg/L indicating that 250 mg/L of Fe was toxic to the bacterial growth. NAJ1 and NAJ5 showed the same pattern of CFU and demonstrated growth at 25 and 100 mg/L of Fe but showed decreased growth as the Fe concentration increased to 250 mg/L. According to de Silva et al. (2012), the Gram negative bacteria have better tolerance for heavy metals compared to the Gram positive bacteria. This supports the finding in this study where a higher tolerance of Fe was observed in the Gram negative strains, particularly NAJ5.

### The effect of different Fe concentrations

The removal of Fe was monitored to identify the bioaccumulation potential by the respective

**Table 3**. Specific growth rate (μ) for each isolate

| Isolates | Specific growth rate, μ ($hr^{-1}$) | $R^2$ |
|---|---|---|
| *C. pauculus* (NAJ1) | 0.0902 | 0.9703 |
| *S. maltophilia* (NAJ3) | 0.1215 | 0.9659 |
| *P. hibiscicola* (NAJ5) | 0.2577 | 0.9333 |

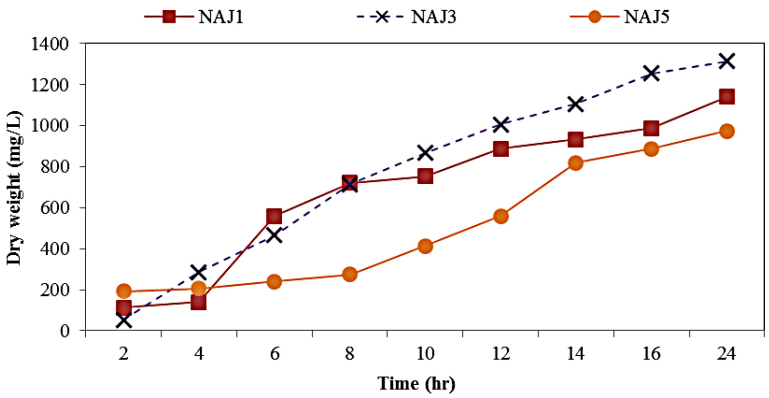



**Figure 1.** Growth profile of isolated strains

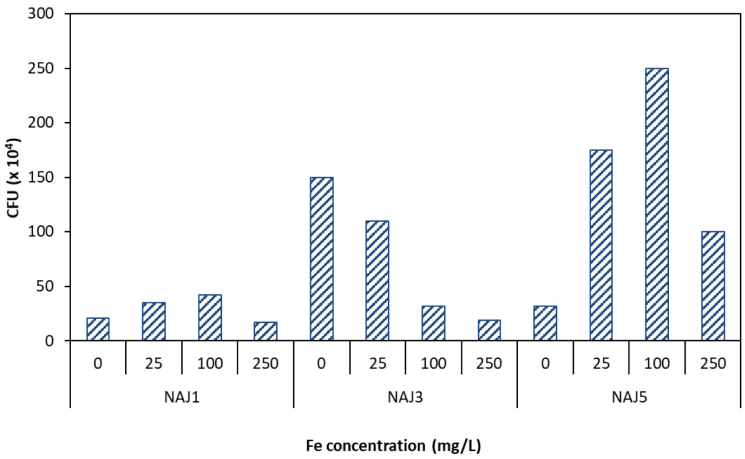



**Figure 2.** CFU of each isolate at different concentrations of Fe

isolates since bioaccumulation potential can be measured as the difference between the initial and the final concentration (after 24 hours exposure of Fe with the cells). Figure 3 summarises the Fe concentrations in the nutrient broth after 24 hours of exposure while Table 4 summarises the Fe removal by NAJ1, NAJ3 and NAJ5. Figure 3 shows that the Fe concentrations decreased with time, indicating that Fe was bioaccumulated by all isolates.

NAJ1, NAJ3 and NAJ5 showed similar Fe removal patterns as the Fe concentrations increased. This may be because higher concentrations of Fe cause conformational alterations in nucleic acids and polypeptides and also cause a disturbance in the oxidative phosphorylation and osmotic balance of the isolates (Nanda et al. 2019). NAJ5 showed the highest Fe removal for all concentrations, where the

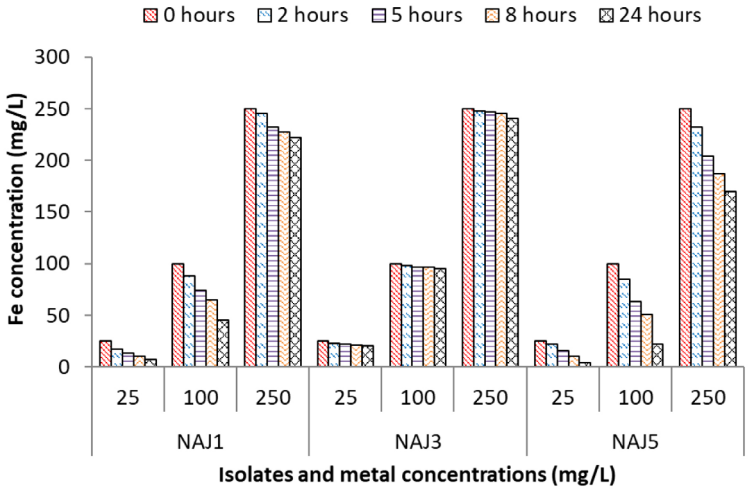



**Figure 3.** Fe concentrations at different sampling times

**Table 4**. Summary of Fe removal by each isolate after 24 hours of exposure

| Isolates | Initial Concentration (mg/L) | Final Concentration (mg/L) | Removal (%) |
|---|---|---|---|
| *C. pauculus* NAJ1 | 25 | 7 | 72.3 |
| | 100 | 45 | 54.2 |
| | 250 | 222 | 10.2 |
| *S. maltophilia* NAJ3 | 25 | 20 | 16.4 |
| | 100 | 95 | 4.1 |
| | 250 | 241 | 3.6 |
| *P. hibiscicola* NAJ5 | 25 | 4 | 82.1 |
| | 100 | 22 | 77.8 |
| | 250 | 170 | 32.0 |

removal percentages were 82.1% (25 mg/L), 77.8% (100 mg/L) and 32% (250 mg/L). NAJ1 was the second effective isolate with the removal values of 72.3% (25 mg/L), 54.2% (100 mg/L) and 10.2% (250 mg/L). NAJ3 had the lowest bioaccumulation potential of all the isolates. The removal of Fe by NAJ3 was 16.4%, 4.1% and 3.6% at 25, 100, and 250 mg/L Fe, respectively. On the basis of the results, NAJ5 was the most effective isolate for the bioaccumulation of Fe.

### Comparison of Fe removal by other species

A comparison of Fe removal through bioaccumulation by other species is tabulated in Table 5. Microorganisms such as *Bacillus licheniformis, Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae*, *Desulfovibrio halophilus* sp., *Bacillus subtilis*, *Bacillus sphaericus* OT4b31 and *Bacillus sphaericus* IV(4)10 were studied for Fe bioaccumulation and showed good performance. NAJ5 showed the highest Fe removal compared to all species except for *D. halophilus* sp. (85.3%) and *B. subtilis* (100% removal). In this study, NAJ5 was identified as a Gram negative bacteria which was similar to *D. halophilus*. Conversely, the Gram positive bacteria (*B. subtilis*) had a greater tendency to accumulate more heavy metals on its cell wall, compared to the Gram negative bacteria, as shown by Karakagh et al. (2012).

## CONCLUSION

Three species of bacteria were isolated from the laboratory wastewater were identified as *C. pauculus* (NAJ1)*, S. maltophilia* (NAJ3) and *P. hibiscicola* (NAJ5). As the Fe concentrations increased, the removal of Fe by all isolates decreased. *P. hibiscicola* (NAJ5) was identified as an effective isolate for bioaccumulating Fe. The specific growth rate of this species was observed at 0.2577 $hr^{-1}$ and up to 82.1% removal was achieved at 25 mg/L of Fe, compared to the other isolates. This bioaccumulation method using native isolates has great potential for the removal of heavy metals from wastewater.

### Acknowledgements

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**Table 5**. Bioaccumulation of Fe by other species

| Microorganisms | Fe removal (%) | Reference |
|---|---|---|
| *Bacillus licheniformis* | 52.0 | Karakagh et al. (2012) |
| *Zygosaccharomyces rouxii* | 35.7 | Li et al. (2013) |
| *Saccharomyces cerevisiae* | 15.6 | |
| *Desulfovibrio halophilus* | 85.3 | Torbaghan & Torghabeh 2019 |
| *Bacillus subtilis* | 100 | Reis et al. 2014 |
| *Bacillus sphaericus OT4b31* | 34.4 | Velásquez & Dussan 2009 |
| *Bacillus shpaericus IV(4)10* | 26.5 | |
| *P. hibiscicola* (NAJ5) | 82.1 | This study (2020) |

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