Pertanika J. Trop. Agric. Sc. 42 (1): 89 - 101 (2019)

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Removal of Heavy Metals in Lake Water Using Bioflocculant Produced by *Bacillus subtilis*

Choong Chiou Dih, Nurul Amirah Jamaluddin and Zufarzaana Zulkeflee*

Department of Environmental Sciences, Faculty of Environmental Studies, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

ABSTRACT

Bioflocculant is the extracellular polymeric substances that are produced as by-products of microbial growth with flocculating capabilities. Accordingly, a potential bioflocculant-producing bacterium was isolated from lake water and had been identified as *Bacillus subtilis* (*B. subtilis*). The removal of heavy metals from synthetic wastewater and lake water samples that had been treated with bioflocculant produced by the strain was investigated. Synthetic wastewater samples were prepared by spiking the solution with known concentration of Cd, Cr, Cu whereas the lake water samples had been collected from Cempaka Lake, Bangi, Malaysia. Concentration of the metals in the samples before and after treatment by the bioflocculant was measured using ICP-MS. The removal of heavy metals in synthetic wastewater was found to be ineffective without further pH and dosage manipulations. On the other hand, heavy metals found in the lake water samples (Al, Zn, Fe, Cu) were effectively removed at 92.9%, 94.3%, 86.2% and 68.1% respectively; the treatment was optimised at pH 2 ($p<0.05$), while effects of varying dosages were proven insignificant ($p>0.05$). Bioflocculant produced by *B. subtilis* had been proven to be a good alternative to chemically-based solution in remediating heavy metal polluted waters.

Keywords: B. subtilis, bioremediation, bioflocculant, heavy metals

ARTICLE INFO

Article history:
Received: 03 December 2018
Accepted: 30 January 2019
Published: 26 February 2019

E-mail addresses:
cdee_03@hotmail.com (Choong Chiou Dih)
nurulamirahjamaluddinn@gmail.com (Nurul Amirah Jamaluddin)
zufarzaana@upm.edu.my (Zufarzaana Zulkeflee)
* Corresponding author

INTRODUCTION

Heavy metal pollution is a significant environmental problem. Chemical-intensive industries bearing heavy metals, such as lead (Pb), chromium (Cr), cadmium (Cd), copper (Cu), arsenic (As), zinc (Zn), nickel (Ni) and mercury (Hg) pose threats to human health and the environment through the untreated discharge of their wastewater

ISSN: 1511-3701
e-ISSN: 2231-8542

into the aquatic ecosystem (Barakat, 2011). The high solubility characteristics of heavy metals will lead to their absorption by living organisms and bioaccumulate in the food chain, where eventually large concentrations of heavy metals may enter the human body (Low et al., 2015). Ingestion of metals beyond the permitted limit can lead to adverse health side effects (Kooner et al., 2014). In Malaysia, occurrences of heavy metals pollution are being continuously reported all over the country. Sultan et al. (2011) had reported heavy metals pollution had been detected on the surface water of the tropical river watershed in Terengganu. In 2012, Prasanna et al. (2012) and Lim et al. (2012) had reported on the occurrences of heavy metals in the Curtin Lake in Miri and Langat River in Selangor, respectively. Similarly, reports on heavy metals pollutions in Gombak and Penchala River, Selangor had been highlighted by Ismail et al. (2013), while Idriss and Ahmad (2014) had reported on Juru River in Penang, and Al-Badaii and Suhaimi-Othman (2014) report was on Semenyih River, Selangor. Occurrences of heavy metals in the coastal areas and in storm water runoff were also reported by Sany et al. (2013) in Port Klang, Selangor and by Chow et al. (2013), respectively. The most hazardous metals that have been reported includes Pb, Cr, Cd, Cu, As, Cd, Pb, Zn, Ni and Hg (Barakat, 2011). Wastewater from the textile industries (Halimoon & Yin, 2010) and from industrial activities such as burning of fossil fuels, mining, cement manufacturing, paper and glass production and waste recycling (Sany et al., 2013) are among the sources of reported heavy metals pollution. These overbearing occurrences of the metal pollutions in the ecosystem lead to the imminent need for an effective solution (Gaur et al., 2014).

Particulate and soluble form of heavy metal ions can potentially be accumulated by live or dead bacterial cells as well as their by-products (Gupta & Diwan, 2017). Bioflocculants are metabolic by-products of microorganism during growth (Subramanian et al., 2009), and according to Lee and Chang (2018) over the past decades bioflocculant that is produced by many microorganism including bacteria, algae, actinomomyces, fungi and yeast are being used as substitutes for chemical flocculants in water and for wastewater treatment purposes. Bioflocculants have also been described in various studies as potential metal binders (Chen et al., 2016; Gomaa, 2012; Lin & Harichund, 2012; Pathak et al., 2017; Sajayan et al., 2017). In this study, removal of heavy metals by bioflocculants produced by *B. subtilis* that had been isolated from lake water was attempted both on synthetic heavy metal solutions and lake water samples.

MATERIALS AND METHODS

Potential bioflocculant-producing bacteria were isolated from water samples collected from three different lakes in Universiti Putra Malaysia and cultured onto tryptic soy agar (TSA) at 37°C for 24 hrs. Four pure colonies of mucoid and ropy strains were then selected and sub-cultured into tryptic soy broth (TSB), incubated on an orbital shaker at 150 rpm, 25°C, for 24 h and tested for flocculating activities through kaolin assay. Both the TSA and TSB contain enzymatic digests of casein and soybean as their main ingredients.

The strain with the highest flocculating performance was then chosen and further characterised and identified through 29 biochemical and enzymatic reaction tests (BBL Crystal Gram-Positive ID System). These tests include the hydrolysis of amide and glycosidic bonds with positive utilisation of arginine, positive utilisation of carbohydrates such as glycerol, sucrose and mannitol and positive release of several fluorescent coumarin derivatives.

Flocculation Assay Using Kaolin Clay

Kaolin assay for the determination of flocculating activity was conducted in accordance with the study by Zulkeflee et al. (2012). About 0.5 mL cultured broth and 4.5 mL 0.1% (w/v) $CaCl_2$ were pipetted into 50 mL of 5 g/L kaolin clay suspension. The mixture was then agitated at 200 rpm for 30 seconds and left to stand for 5 mins. The cleared upper phase of the suspension was then collected and measured using a spectrophotometer (Spectronic 20 Genesys, USA) at 550 nm. Flocculating activity is expressed based on the following formula:

$$\text{Flocculating activity (\%)} = [(A-B) / A] \times 100$$

where, A is the optical density of the control and B is the optical density of the sample.

Bioflocculant Source and Timeline of Bioflocculant Production

The selected strain was incubated in tryptic soy broth on an orbital shaker at 150 rpm at 25°C for three days, to determine the timeline of bioflocculant production during growth. The growth of the strain was monitored through optical density measurements of the cultured broth at 600 nm using a spectrophotometer (Spectronic 20 Genesys, USA). Flocculating activity were measured daily through the kaolin assay. The relationship between bacterial growth and flocculating activity was analysed using Pearson Product-Moment Correlation in SPSS.

According to the study by Buthelezi et al. (2010), microbial cultured broth can be used directly as bioflocculant source without further extraction. This was in agreement with the findings by Zulkeflee et al. (2016) who had proven that bioflocculants were excreted by bacteria into their surrounding culture broths with flocculating activities exhibited by the cultured broth and the cell-free supernatant (after centrifugation) but was not exhibited by the separated cell. Furthermore, Pathak et al. (2015) reported that both bioflocculant in broth and in its purified form had successfully removed selected metals with similar efficiency. Therefore, the subsequent heavy metal removal assays utilised batch culture broths of the strain that was at its highest flocculating performance, as the bioflocculant source (without extraction).

Pilot Test on Synthetic Heavy Metal Solutions

Synthetic heavy metals solutions of 500 µg/L Cd, Cr and Cu were prepared from their respective stock solutions. About 50 mL of each metal solution was poured into Erlenmeyer flasks and bioflocculant at varying dosage: namely 0.5%, 1%, 2% and 4% (v/v) were pipetted into their respective flasks. A control was also prepared by using sterile broth in place of the cultured broth. The pH of the mixtures were monitored but not controlled. The mixtures were then shaken on an orbital shaker at 150 rpm at 25°C, for 24 hrs (Gomaa, 2012). After 24 hrs the supernatant were collected and filtered through 0.45 µm filter syringes. Clear 4.0 mL filtered samples were then analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for heavy metal concentration. All analyses were done in duplicates.

The percentage removal was calculated based on the formula below.

Percentage removal (%)
$= [(A-B) / A] \times 100$

Where A is the initial concentration of heavy metal and B is the final concentration of heavy metal in the treated solutions.

Heavy Metals Removal from Lake Water Samples

Lake water samples were collected from three sampling points at Cempaka Lake in Bangi, Malaysia and were homogenised for heavy metal analyses. Cempaka Lake is a recreational lake surrounded by industrial, commercial and residential areas. The lake has previously been reported to be polluted by heavy metals due to the various anthropogenic activities surrounding the area (Gasim et al., 2017; Taweel et al., 2013). *In situ* water quality parameters including pH, temperature and turbidity were measured on site. The parameters were compared with the National Lake Water Quality Criteria and Standards (NLWQS) that was developed in 2015 by the National Hydraulic Research Institute of Malaysia (NAHRIM) for Category B lakes i.e. lakes used for recreational purposes that includes secondary body contact such as boating and cruising.

The experiment as described in the pilot test was conducted on the homogenised water samples that were taken from the lake polluted with heavy metals such as Al, Zn, Fe and Cu. These were measured as the highest metals that were present in the water samples. Additionally, the effect of pH on bioflocculant treatment was also investigated by varying the pH of the mixture to pH 2.0, 6.0 and 9.0.

For heavy metal concentrations the levels detected were compared with the NLWQS which also adopted the heavy metal standards of the National Water Quality Standards for Malaysia underlined by the Department of Environment (DOE), Malaysia in 2006.

RESULTS AND DISCUSSION

Bacterial Identification and Characteristics

A mucoid and ropy colony forming bacterial strain with the highest flocculating activity at 97% was chosen and was further characterised and identified. Mucoid forms a colony that has morphological characteristics bearing a slimy and glistening appearance, and is ropy i.e. the ability of colonies to form long strings when touched with a wire inoculating loop. These were reported as good indicators of bioflocculant-producing microorganism (Li et al., 2009). The BBL Crystal Gram-Positive ID System had biochemically identified the strain as *Bacillus subtilis* with a 99% similarity. Figure 1 shows the microscopic image of the Gram-stained strain captured at 4000x magnification using Nikon YS100 light microscope. The image shows rod-shaped Gram-positive bacteria.

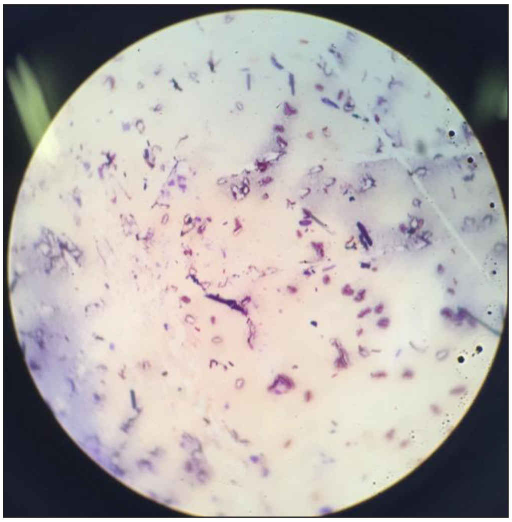

Figure 1. Purple stained rod-shaped bacteria identified as *B. subtilis*

Bioflocculant Production during Growth

Figure 2 shows the flocculating performances of the bioflocculant that was produced during a 72 hrs batch culture of *B. subtilis*. Under controlled condition, the growth slowly increased to 0.098 and hit its maximum growth at 0.904. The growth drastically declined by the third day.

Bioflocculant productions are reflected by the flocculating activities measured during growth (Ugbenyen & Okoh, 2013). Bioflocculant was observed to be produced throughout bacterial growth with a strong positive relationship (r = 0.81, $p<0.01$), with high flocculating activity being directly associated with the rapid growth of *B. subtilis.* The maximum flocculating activity measured was 81.96% at the peak of bacterial growth in 48 hrs before similarly declining together with bacterial growth to around 66%. Bioflocculant productions throughout growth could vary with different microorganisms depending on their respective growth rates. Liu et al. (2010) reported that bioflocculant production by *Chryseobacteriumdaeguense* occured during the death phase, while Gong et al. (2008) and Wang et al. (2007) had reported on the production of bioflocculants occurring parallel with logarithmic growth of their respective strains. On the other hand, Su et al. (2012) reported that bioflocculant production by *Arthrobacter* sp. occured parallel with growth but eventually declined by 72 hrs of incubation, which was similar in the case of *B. subtilis* bioflocculant production in this study.

The decline of bacterial growth was mainly due to insufficient provision of nutrient. This was also reflected by the declination of the flocculating activity reading. However, the flocculating activity had maintained above 50% towards the end, indicating that the bioflocculant that was produced had remained in the culture system while some might have been re-uptake by the strain as an alternative food source (Zulkeflee et al., 2016).

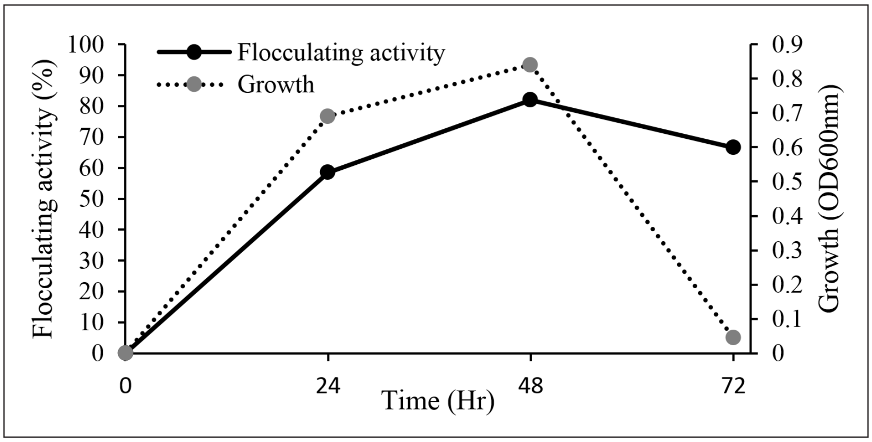

Figure 2. Timeline of bioflocculant production during growth of *B. subtilis*

Pilot Test of Heavy Metals Removal from Synthetic Solutions

A pilot test on the removal of 500 µg/L of Cd, Cr, Cu from synthetic solutions was conducted utilising 48 hrs old cultured broths of *B. subtilis,* which had been tested for its flocculating activity (Figure 3). The removal rate of different metals differed at different dosages of bioflocculant used. The highest removal of Cu (16.3%) was attained at 4.0% (v/v) of bioflocculant dosage while for Cd the highest removal (13.4%) was by using 2.0% (v/v) of bioflocculant dosage. At a higher or lower dosage, the removal of Cd was observed to be lower while for Cu a high removal rate (14.4%) was achieved at 0.5% (v/v) of bioflocculant dosage; however it dropped at the increase of subsequent two-fold bioflocculant dosage before the rate increased at a dosage of 4.0% (v/v). Cr removal was observed to be less than 5% at all applied dosages. The removal rate was also observed to be oddly negative at 1.0% (v/v) bioflocculant dosage for all metals. Nevertheless, all rates were lower compared to the metal bioflocculation reported by Gomaa (2012) and Lin and Harichund (2012).

According to Morillo et al. (2006) different removal rates of various metals by bioflocculants at different dosages were the results of different affinity, charge density and polymer conformation on adsorbed ions of the metal-bioflocculant interaction. This was agreed by Gomaa (2012) who reported on the different optimum dosages for Pb, Zn, As and Cd removal through bioflocculation by *Pseudomonas aeruginosa*. Hence, it explained the different removal rates that were achieved at different bioflocculant dosage for Cd, Cu and Cr (Figure 3). Effective removal of metals was also reported to favour lower bioflocculant dosages (Das & Santra, 2007). This was in agreement with Lin & Harichund (2012) where removal of Pb, Zn and Hg by bioflocculants from *Paenibacillus* sp. CH11, *Bacillus* sp. CH15 and *Halomonas* sp. increased when the dosages were reduced. However, Cd removal was reported to be highest at high dosages. Fundamentally, the basis of choosing the dosages in this study was in accordance to the low dosage theory; however, it was observed that fluctuations in bioflocculating metals by *B. subtilis* bioflocculant were sensitive even at two-fold dosage differences. This could be due to many other factors that affect bioflocculation performance.

One important factor is the pH, because pH value affects flocculation processes (El-Salam et al., 2017). The initial pH of the treatments were monitored but not controlled and were observed to be in the range of pH 3 to 4. Abdel-Ghani & El-Chaghaby (2014) reviewed that in acidic condition, H^+ competed with metal ions to adsorb onto the biosorption surface of bacterial biomass; the bioflocculant. Therefore, the removal of heavy metals tended to be low as presented in this pilot test because the heavy metal ions might compete with hydrogen ions for biosorption with the bioflocculant.

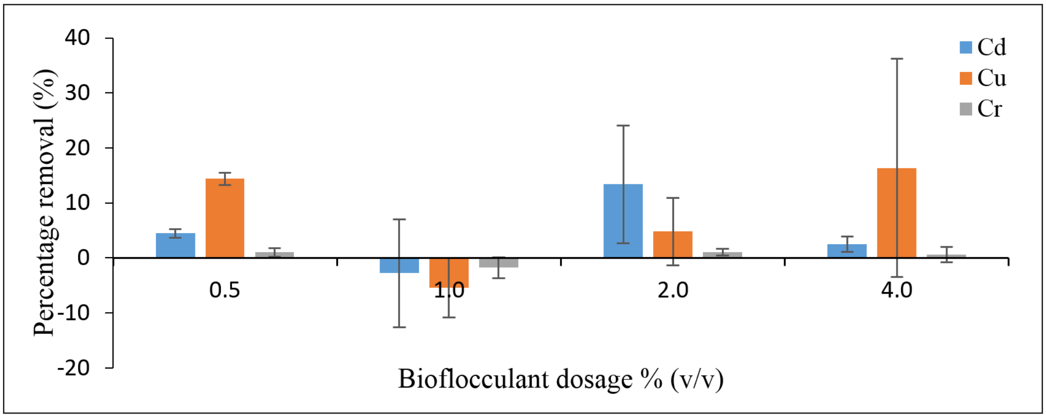

Figure 3. Percentage removal of Cd, Cu, Cr at different dosages of bioflocculant

Heavy Metals Concentrations in the Lake Water Samples

Water samples from Cempaka Lake had an average temperature of 28.9°C, a pH 6.86 and an average turbidity reading of 42.3 NTU. According to the NLWQS all of these parameters were within the range of standards for Category B lakes. Table 1 lists the ten heavy metals that have been measured from the homogenised water samples from the lake and their comparisons with standard values. The results revealed that Fe was found abundantly in Cempaka Lake with the highest concentration at 999.63±35.46 µg/L, followed by Al and Zn with concentrations of 506.22±25.42 µg/L and 129.22±4.09 µg/L respectively. In contrast, Cr and Ni were found to be absent in the lake with values below detection limit. The concentrations of Cu, Cd, Pb, As and Co were all found to be below the standard limit.

Table 1
Heavy metals concentration in Cempaka Lake and comparison with standard values

Heavy Metals	Concentration (µg/L)	NLWQS (µg/L)
Cd	0.09	2
Cu	3.95	20
Cr	BDL	50
Al	506.22*	100
Zn	129.22	3000
Pb	6.48	50
Ni	BDL	20
As	10.04	100
Co	0.28	50
Fe	996.63	1000

BDL – Below Detection Limit; *exceed the standard value

The concentration of Al in Cempaka Lake had exceeded the standard limit while Fe nearly reached the standard limit, thus both posed as threats to aquatic life as well as to human health. The high levels of Fe detected in the lake had similarly been reported by Gasim et al. (2017) with values exceeding the standard limit. Contradictorily, all other metal concentrations were reported to be below standard limits. In 2011, a similar study conducted by Said et al. (2011) had reported the high occurrences of Zn as compared to other metals. The common trend of detecting high levels of Zn in 2011 and Fe in 2017 proved that there was consistent input of Fe and Zn sources from anthropogenic activities that had increased throughout the years, with the addition of Al that was detected at exceeding levels in 2018.

According to a study by Shuhaimi-Othman et al. (2012), freshwater toxicity of metals on aquatic biota follows the order Cu>Cd>Fe>Mn>Pb>Ni>Zn>Al. Toxicity of Al were reported to be pH dependent where they may become more toxic to aquatic biota in acidic conditions. Thus, it was of interest to investigate the possible removal of heavy metals through bioflocculation.

Removal of Heavy Metals from Lake Water Samples through Bioflocculation

The removal rate of selected heavy metals (Al, Zn, Fe, Cu) by varying the bioflocculant dosages and pH of the lake water homogenised samples are summarised in Table 2. The overall highest percentage of removal was recorded by Zn at 94.3%, followed by Al at 92.9%, Fe at 86.2% and Cu at 68.1%, in an acidic medium of pH 2.0 ($p<0.05$). In an alkaline medium of pH 9.0, most percentages of removal were marked with negative values, indicating that bioflocculation of the selected metals were unfavourable in alkaline conditions. At pH 6, removal of Al, Zn and Fe were maintained to be high around 70-85%. Removal of Cu on the other hand were low in all conditions. Therefore, the removal of all metals was concluded to favour acidic conditions compared to alkaline conditions ($p<0.05$) with no significant contribution from the varied dosages used ($p>0.05$). Generally, at the optimal pH, bioflocculation of metals differed according to the different types of heavy metals that were being treated (Lin & Harichund, 2012).

According to Gupta and Diwan (2017) H^+ions tends to enhance bridging process that aids in flocculation. This is true based on the results since indication of pH 2.0 and pH 6.0 were more favourable for high bioflocculation of the selected metals due to the higher amount of H^+ ions present ($p<0.05$). At the optimum pH for bioflocculation of metals, efficiency of metal removal is high due to the negative charges saturating the adsorbing surfaces (Pathak et al., 2015). At pH higher than the optimum level, adsorption may be hindered by the formation of hydroxo species of the metals ion that do not bind to the bioflocculant (Lin & Harichund, 2012; Pathak et al., 2015). Furthermore, negatively charged sites and positively charged sites availability fluctuates with pH, with the latter commonly decreased with increasing pH, thus resulting in a lower metal removal at higher pH through bioflocculation (Zhao et al., 2017). Thus, this could explain the negative results obtained at pH 9.

Table 2
Percentage removal of Al, Zn, Fe and Cu from lake water sample at various dosages and pH

		Percentage Removal (%)		
Bioflocculant Dosage % (v/v)	**Heavy Metals**	**pH 2.0**	**pH 6.0**	**pH 9.0**
0.5	Al	52.9 ± 44.47	71.7 ± 24.26	-46.6 ± 88.85
	Zn	53.5 ± 43.66	85.6 ± 3.24	1.6 ± 22.90
	Fe	42.0 ± 55.30	71.3 ± 11.90	-24.6 ± 77.70
	Cu	47.8 ± 45.86	47.0 ± 3.71	-6.8 ± 1.94
1.0	Al	92.6 ± 4.17	73.0 ± 19.78	-58.0 ± 94.75
	Zn	94.3 ± 2.42**	84.7 ± 3.74	-16.8 ± 5.72
	Fe	53.7 ± 44.32	67.7 ± 10.86	-58.3 ± 91.50
	Cu	68.1 ± 26.05**	51.4 ± 1.15	-24.2 ± 18.99
2.0	Al	34.0 ± 63.1	51.2 ± 25.63	-29.4 ± 68.23
	Zn	32.4 ± 62.23	69.5 ± 4.70	-23.0 ± 12.05
	Fe	38.4 ± 60.16	64.3 ± 12.50	-19.8 ± 52.76
	Cu	38.8 ± 55.62	46.6 ± 2.61	-49.1 ± 8.37
4.0	Al	92.9 ± 14.05**	32.8 ± 11.61	-6.1 ± 45.71
	Zn	74.0 ± 43.32	60.6 ± 6.74	-11.7 ± 3.67
	Fe	86.2 ± 8.55**	60.6 ± 5.86	-6.8 ± 33.90
	Cu	40.9 ± 43.10	15.4 ± 2.09	-37.6 ± 5.90

**highest percentage removal for each metal ($p<0.05$)

CONCLUSION

Rod-shaped, Gram-positive and mucoid and ropy colony forming bacteria that had been isolated from lake water, and identified as *B. subtilis*, was proven to produce bioflocculants with high flocculating activities. Production of bioflocculant by the strain was found to be positively correlated with growth (p<0.01) with the highest flocculating activity measured within 48 hrs of incubation. Possible removal of selected metals (Cd, Cr and Cu) in synthetic wastewater was proven ineffective without pH and bioflocculant dosage manipulations. Further tests on lake water samples that had been polluted with Al, Zn, Fe and Cu showed promising results, with successful removal at 92.9%, 94.3%, 86.2% and 68.1% respectively, in an acidic condition of pH 2 with no significant effects despite the varying dosages that were used (p>0.05). Bioflocculant produced by *B. subtilis* was proven to be successful in removing selected metals with promising potential in wastewater treatment applications.

ACKNOWLEDGEMENTS

This research was funded by Putra IPM Grant GP-IPM/2016/9499500.

REFERENCES

Abdel-Ghani, N. T., & El-Chaghaby G. A. (2014). Biosorption for metal ions removal from aqueous solutions: A review of recent studies. *International Journal of Latest Research in Science & Technology, 3*(1), 24-42.

Al-Badaii, F., & Shuhaimi-Othman, M. (2014). Heavy metals and water quality assessment using multivariate statistical techniques and water quality index of the Semenyih River, Peninsular Malaysia. *Iranica Journal of Energy and Environment, 5*(2), 132-145.

Barakat, M. (2011). New trends in removing heavy metals from industrial wastewater. *Arabian Journal of Chemistry, 4*(4), 361-377.

Buthelezi, S., Olaniran, A., & Pillay, B. (2010). Production and characterization of bioflocculants from bacteria isolated from wastewater treatment plant in South Africa. *Biotechnology and Bioprocess Engineering*, *15*(5), 874-881.

Chen, H., Zhong, C., Berkhouse, H., Zhang, Y., Lv, Y., Lu, W., Yang, Y., & Zhou, J. (2016). Removal of Cd by bioflocculant produced by *Stenotrophonas maltophilia* using phenol-containing wastewater. *Chemosphere, 155*, 163-169.

Chow, M. F., Yusop, Z., & Shirazi, S. M. (2013). Storm runoff quality and pollutant loading from commercial, residential, and industrial catchments in the tropic. *Environmental Monitoring and Assessment, 185*(10), 8321-8331.

Das, S., & Santra, S. C. (2007). Microbial interactions with heavy metals and their applications in bioremediation of wastewater. *New Frontiers of Environmental Biotechnology Applications*, *3*, 1-10.

El-Salam, A. E., Abd-El-Haleem, D., Youssef, A. S., Zaki, S., Abu-Elreesh, G., & El-Assar, S. A. (2017). Isolation, characterization, optimization, immobilization and batch fermentation of bioflocculant produced by *Bacillus aryabhattai* strain PSK1. *Journal of Genetic Engineering &andBiotechnology, 15*(2), 335-344.

Gasim, M. B., Toriman, M. E., Barggig, A., Mutfah, S., Hairoma, N., & Endut, A. (2017). The evaluation of heavy metals concentrations in Cempaka Lake, Bangi, Selangor, Malaysia. *International*

Journal on Advanced Science, Engineering and Information Technology, 7(2), 453-459.

Gaur, N., Flora, G., Yadav, M., & Tiwari, A. (2014). A review with recent advancements on bioremediation-based abolition of heavy metals. *Environmental Science: Processes and Impacts, 16*(2), 180-193.

Gomaa, E. Z. (2012). Production and characteristics of a heavy metals removing bioflocculant produced by *Pseudomonas aeruginosa. Polish Journal of Microbiology*, *61*(4), 281-289.

Gong, W. X., Wang, S. G., Sun, X. F., Liu, X. W., Yue, Q. Y., & Gao, B. Y. (2008). Bioflocculant production by culture of *Serratia ficaria* and its application in wastewater treatment. *Bioresource Technology*, *99*(11), 4668-4674.

Gupta, P., & Diwan, B. (2017). Bacterial Exopolysaccharide mediated heavy metal removal: A Review on biosynthesis, mechanism and remediation strategies. *Biotechnology Reports,13,* 58-71.

Halimoon, N., & Yin, R. G. S. (2010). Removal of heavy metals from textile wastewater using zeolite. *Environment Asia, 3*(Special Issue), 124-130.

Idriss, A. A., & Ahmad, A. K. (2014). Concentration of selected heavy metals in water of the Juru River, Penang, Malaysia. *African Journal of Biotechnology, 11*(33) 8234-8240.

Ismail, Z., Salim, K., Othman, S. Z., Ramli, A. H., Shirazi, S. M., Karim, R., & Khoo, S. Y. (2013). Determining and comparing the levels of heavy metal concentrations in two selected urban river water. *Measurement, 46*(10), 4135-4144.

Kooner, R., Mahajan, B. V. C., & Dhillon, W. S. (2014). Heavy metal contamination in vegetables, fruits, soil and water–A critical review. *International Journal of Agriculture, Environment and Biotechnology, 7*(3), 603-612.

Lee, D. J., & Chang, Y. R. (2018). Bioflocculants from isolated stains: A research update. *Journal of the Taiwan Institute of Chemical Engineers*, *87*, 211-215.

Li, Z., Zhong, S., Lei, H. Y., Chen, R. W., Yu, Q., & Li, H. L. (2009). Production of a novel bioflocculant by *Bacillus licheniformis* X14 and its application to low temperature drinking water treatment. *Bioresource Technology*, *100*(14), 3650-3656.

Lim, W. Y., Aris, A. Z., & Zakaria, M. P. (2012). Spatial variability of metals in surface water and sediment in the Langat River and geochemical factors that influence their water-sediment interactions. *Scientific World Journal, 2012*, 1-14.

Lin, J., & Harichund, C. (2012). Production and characterization of heavy-metal removing bacterial bioflocculants. *African Journal of Biotechnology, 11*(40), 9619-9629.

Low, K. H., Zain, S. M., Abas, M. R., Salleh, K. M., & Teo, Y. Y. (2015). Distribution and health risk assessment of trace metals in freshwater tilapia from three different aquaculture sites in Jelebu Region (Malaysia). *Food Chemistry, 177*, 390-396.

Liu, W., Wang, K., Li, B., Yuan, H., & Yang, J. (2010). Production and characterization of an intracellular bioflocculant by *Chryseobacterium daeguense* W6 cultured in low nutrition medium. *Bioresource Technology*, *101*(3), 1044-1048.

Morillo, J. A., Aguilera, M., Ramos-Cormenzana, A., & Monteoliva-Sánchez, M. (2006). Production of a metal-binding exopolysaccharide by *Paenibacillus jamilae* using two-phase olive-mill waste as fermentation substrate. *Current Microbiology*, *53*(3), 189-193.

Said, K. S., Shuhaimi, M., & Kutty, A. (2011). The water quality and metal concentrations of Cempaka Lake, Selangor, Malaysia. *WIT Transactions on Ecology and the Environment*, *148*, 47-52.

Sany, S. B. T., Salleh, A., Sulaiman, A. H., Sasekumar, A., Rezayi, M., & Tehrani, G. M. (2013). Heavy metal contamination in water and sediment of the Port Klang coastal area, Selangor, Malaysia. *Environmental Earth Sciences, 69*(6), 2013-2025.

Shuhaimi-Othman, M., Nadzifah, Y., Umirah, N. S., & Ahmad, A. K. (2012). Toxicity of metals to an aquatic worm, *Nais elinguis* (*Oligochaeta, Naididae*). *Research Journal of Environmental Toxicology*, *6*(4), 122-132.

Su, X., Shen, X., Ding, L., & Yokota, A. (2012). Study on the flocculability of the Arthrobacter sp., an actinomycete resuscitated from the VBNC state. *World Journal of Microbiology and Biotechnology, 28*(1), 91-97.

Sultan, K., Shazili, N. A., & Peiffer, S. (2011). Distribution of Pb, As, Cd, Sn and Hg in soil, sediment and surface water of the tropical river watershed, Terengganu (Malaysia). *Journal of Hydro-Environment Research, 5*(3), 169-176.

Pathak, M., Devi, A., Bhattacharyya, K. G., Sarma, H. K., Subudhi, S., & Lal, B. (2015). Production of a non-cytotoxic bioflocculant by a bacterium utilizing a petroleum hydrocarbon source and its application in heavy metal removal. *RSC Advances, 5*(81), 66037-66046.

Pathak, M., Sarma, H. K., Bhattacharyya, K. G., Subudhi, S., Bisht, V., Lal, B., & Devi, A. (2017). Characterization of a novel polymeric bioflocculant produced from bacterial utilization of n-hexadecane and its application in removal of heavy metals. *Frontiers in Microbiology*, *8*, 1-15.

Prasanna, M. V., Praveena, S. M., Chidambaram, S., Nagarajan, R., & Elayaraja, A. (2012). Evaluation of water quality pollution indices for heavy metal contamination monitoring: a case study from Curtin Lake, Miri City, East Malaysia. *Environmental Earth Sciences, 67*(7), 1987-2001.

Sajayan, A., Kiran, G. S., Priyadharshini, S., Poulose, N., & Selvin, J. (2017). Revealing the ability of a novel polysaccharide bioflocculant in bioremediation of heavy metals sensed in a Vibrio bioluminescence reporter assay. *Environmental Pollution*, *228*, 118-127.

Sany, S. B., Salleh, A., Rezayi, M., Saadati, N., Narimany, L., & Tehrani, G. M. (2013). Distribution and Contamination of Heavy Metal in the Coastal Sediments of Port Klang, Selangor, Malaysia. *Water, Air and Soil Pollution, 224*(1476), 1-18.

Subramanian, S. B., Yan, S., Tyagi, R. D., & Surampalli, R. Y. (2009). Bioflocculants. In R. D. Tyagi, R. Y. Surampalli, S. Yan, T. C. Zhang, C. M. Kao, & B. N. Lohani (Eds.). *Sustainable sludge management* (pp. 146-167). Reston, USA: American Society of Civil Engineers.

Taweel, A., Shuhaimi-Othman, M., & Ahmad, A. (2013). Evaluation of Cu, Pb and As level in tilapia fish in Cempaka Lake (Bangi, Malaysia) and human daily/weekly intake. *Biologia, 68*(5) 983-991.

Ugbenyen, A. M., & Okoh, A. I. (2013). Flocculating properties of a bioflocculant produced by *Bacillus* sp. isolated from a marine environment in South Africa. *Chemical and Biochemical Engineering Quarterly*, *27*(4), 511-518.

Wang, S. G., Gong, W. X., Liu, X. W., Tian, L., Yue, Q. Y., & Gao, B. Y. (2007). Production of a novel bioflocculant by culture of *Klebsiella mobillis* using dairy wastewater. *Biochemical Engineering Journal, 36*(2), 81-86.

Zhao, G., Ji, S., Sun, T., Ma, F., & Chen, Z. (2017). Production of bioflocculants prepared from wastewater supernatant of anaerobic co-digestion of corn straw and molasses wastewater treatment. *BioResources*, *12*(1), 1991-2003.

Zulkeflee, Z., Aris, A. Z., Shamsuddin, Z. H., & Yusoff, M. K. (2012). Cation dependence, pH tolerance, and dosage requirement of a bioflocculant produced by *Bacillus* spp. UPMB13: Flocculation performance optimization through Kaolin assays. *Scientific World Journal*, *2012*, 1-7. doi: 10.1100/2012/495659

Zulkeflee, Z., Shamsuddin, Z. H., Aris, A. Z., Yusoff, M. K., Komilis, D., & Sánchez, A. (2016). Glutamic acid independent production of bioflocculants by *Bacillus subtilis* UPMB13. *Environmental Processes*, *3*(2), 353-367.