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ARTICLE

Influence of culture age on exopolymeric substances from common laboratory bacterial strains: a study on yield, profile and Cu(II) biosorption

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ABSTRACT Extracellular polymeric substances (EPS) produced by laboratory strains *Bacillus cereus* and *Pseudomonas aeruginosa* were extracted from cultures incubated at various incubation periods (24, 48, 72, 96 and 120 h). At each sampling time, the EPS were analysed for yield, quality, functional groups present, and their efficacies in copper (Cu(II)) biosorption (using 30 and 50 ppm EPS). Results revealed that EPS yield was influenced by incubation period, with 48-h culture of *B. cereus* and 96-h culture of *P. aeruginosa* producing the highest yield of EPS at 8.30 mg and 6.95 mg, respectively. The EPS produced at various incubation periods have similar characteristics in solubility, quality and major functional groups (C-O, CH3, C=C, O-H) present. Efficacy of Cu(II) biosorption was influenced by the amount of EPS used and the EPS-metal incubation time. Although Cu(II) removal was higher for EPS from 24-h *B. cereus* (18.96%) and 48-h *P. aeruginosa* (19.19%) when 30 ppm was used, application of 50 ppm EPS demonstrated no distinct differences in amount of Cu(II) removed. This suggested that higher biomass of EPS used and longer EPS-metal incubation period, superseded the efficacy of EPS from various incubation periods. **Acta Biol Szeged 65(2):221-232 (2021)**

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Introduction

Copper (Cu(II)) is one of the common inorganic metal cations that are extensively used in the manufacturing, electroplating and fertilizer industries (Sulaymon et al. 2013; Chen et al. 2018). By nature, Cu(II) is an essential metal required for physiological processes. However, high concentrations of Cu(II) in wastewater, can lead to toxicity, affecting living organisms and the environment. Prolonged exposure to high concentrations of Cu(II) can result in deleterious health implications affecting vital organs such as brain, heart, liver and kidney (Singh et al. 2011). The removal of Cu(II) from the environment is therefore pertinent to reduce hazards as Cu(II) cations are non-biodegradable and tend to accumulate in the environment. Several techniques have been adopted as approaches to remove Cu(II) from the environment. They include mostly physicochemical approaches such as chemical-metal precipitation, ion exchange, membrane filtration and adsorption (Cheah and Ting 2020). These approaches are useful, but are also costly and contributes to the generation of toxic sludge. As an alternative, bioremediation is investigated, in which biological agents are used to remove toxic metals from the environment. Bacteria, fungi and plants, are all capable of bioremediation via biosorption, biodegradation, bioaugmentation or bioflocculation.

In recent years, the application of biomolecules produced from microbes such as exopolymeric substances (EPS) has become an attractive alternative. The application of EPS eliminates the need to apply live microbial cells, addressing concerns of having microbial cell residues in the environment and potential transfer of resistance genes (Cheah and Ting 2020). EPS comprises of long chain polysaccharides and are naturally-produced by bacteria into the surrounding environment during bacterial growth, especially in response to stressful environmental conditions (Sivakumar et al. 2012; Ahmed et al. 2013; Cheah and Ting 2020). The typical composition of EPS includes macromolecules such as nucleic acids, proteins, lipids and carbohydrates (Sheng et al. 2010; Ozturk et al. 2014; Jia et al. 2017). These constituents contribute to the many functional groups present (e.g., carbonyl, carboxyl, hydroxyl, phosphoryl, sulfhydryl and amino groups) in the EPS, which assist in biosorption of metal cations (More et al. 2014; Crini and Lichtfouse 2018). While some of these EPS are well-known and used in various industries (i.e.,

xanthan, gellan, alginate, hyaluronan) (Messner 1997; De Philippis et al. 2011; Freitas et al. 2011); only scattered reports are available on microbial EPS for metal removal. For example, EPS from cyanobacteria have been found to be effective for the removal of Cu(II), Cr(III) and Ni(II) (Micheletti et al. 2007). Paniwichian et al. (2011) reported similar metal removal potential by EPS produced from *Rhodobium marinum* and *Rhodobacter sphaeroides,* which reduced levels of Cd(II), Cu(II), Pb(II) and Zn(II) from contaminated shrimp ponds. There is therefore a demand for more thorough investigations on EPS production, optimization of yield and quality, and their subsequent metal removal capacity.

The focus of this study is to investigate if EPS production is influenced by culture age. This is because EPS is organically produced by bacteria, and the EPS and possibly their constituents may differ depending on response of bacteria to incubation time, nutrient, and stress. To ascertain this, the influence of culture age on EPS production (yield), profile and subsequent Cu(II) removal efficacy, is determined. The influence of culture age encompasses the response of bacteria to incubation time as well as towards depletion of nutrients and build-up of toxic products (Brose and Eikeren 1990). As such, influence of culture age was the approach adopted in this study. The findings here would offer new understanding on the characteristics of the EPS from bacterial cultures of various incubation periods, and the integrity of their properties. It also outlines the consistency of the properties and quality of EPS to be used as an effective biomolecule for metal removal.

To achieve this, *Bacillus cereus* and *Pseudomonas aeruginosa* were selected as they are known to produce EPS. They also represent gram positive and gram negative bacteria, respectively. Both *B. cereus* and *P. aeruginosa* are laboratory strains, and has no prior exposure to metal stress or any other stress factors. The intention of using two laboratory strains are to eliminate pre-disposing factors that may induce EPS production other than under the typical controlled environment. The bacterial isolates were incubated at various incubation time to give rise to different culture ages, and the EPS produced was investigated for yield and quality (solubility, FTIR). The EPS produced were then studied (using different concentrations) for their efficacy for Cu(II) biosorption to remove Cu(II) from solutions.

Materials and Methods

Establishment of bacterial cultures

Two bacterial isolates, *Bacillus cereus* ATCC 14579 and *Pseudomonas aeruginosa* ATCC 10145 were selected as producers of EPS. Both isolates were from the Microbial Culture Collection, Microbiology Laboratory, School of Science, Monash University Malaysia. The bacterial isolates were cultured on Nutrient Agar (NA) (Merck 105450, Germany) and incubated for 24 h at 25 ± 2 °C and 35 ± 2 °C for *B. cereus* and *P. aeruginosa,* respectively, until use.

Bacterial growth and EPS production

Bacterial isolates were inoculated into 50 ml of Nutrient Broth (NB) (Merck 105443, Germany), and incubated with agitation (35 ± 2 °C, 100 rpm, 24 h) (Thermo Scientific, MaxQ 6000, USA). After incubation, the broth cultures were centrifuged at 4000 rpm (30 min, 25 ± 2 °C) (Thermo Scientific CL40R Centrifuge, USA) to obtain pellets (bacterial cells) (Chug et al. 2016). The pellets were weighed (fresh weight) (weighing balance, Ohaus® Pioneer™, USA) and the biomass recorded to represent bacterial growth. The supernatant was transferred into 250 ml flasks and three volumes of 95% ethanol (J. Kollin Chemical) were added. The mixture was incubated at 4 °C overnight to allow precipitation of EPS. After incubation, mixtures were centrifuged (4000 rpm, 45 min, 4 °C) and the supernatant discarded. The pellet (EPS) was mixed with double distilled water to dissolve the precipitate, followed by filtration using 0.45 μm membrane pore filters (Jet Biofil, China) to obtain cell-free EPS. The cell-free EPS was then lyophilized (freeze dryer Labconco Freezone 4.5, Missouri) to obtain powder-forms. The procedure was repeated to obtain EPS from both bacterial cultures incubated for 48, 72, 96 and 120 h.

Characterization of EPS

The physical properties of the lyophilized EPS was determined by characterizing based on colour and solubility in various solvents. The EPS was first weighed (Mettler Toledo, USA) and the colour of lyophilized EPS was assigned based on visual interpretation. The solubility of EPS was tested in distilled water and organic solvents, i.e. absolute ethanol (J. Kollin Chemical), ether (Friendemann Schmidt) and acetone (Friendemann Schmidt). Solubility test was performed by dissolving 0.5 mg EPS in 5 ml of the solvents (or distilled water). The procedure was repeated for EPS from both bacterial cultures incubated for 48, 72, 96 and 120 h.

The quality and purity of the EPS obtained was ascertained by detecting the presence/absence of DNA peaks. EPS (0.5 mg) was first mixed in 2 ml of distilled water, and the absorbance read within the range of 190-500 nm (UV-vis spectrophotometer, Perkin Elmer Lambda 365, USA). The absence of peaks within regions of 260 to 280 nm indicated absence of DNA in the EPS sample, suggesting high quality and purity of EPS (Lin et al. 2010; Liu et al. 2010). On the contrary, peaks detected at 260-280 nm regions would indicate contamination with DNA, concluding that EPS is of poor quality. The procedure was

repeated for EPS from both bacterial cultures incubated for 48, 72, 96 and 120 h.

The Fourier transformed infrared (FTIR) spectroscopy analysis was performed for EPS samples to determine the functional groups present on the EPS. EPS samples (0.5 mg) were analysed with the FTIR spectrometer (Perkin Elmer Spectrum Two, USA) with the spectrum measured at the resolution of 4 cm^{-1} in the range of 400-4000cm^{-1} (Chew and Ting 2016). The procedure was repeated for EPS from both bacterial cultures incubated for 48, 72, 96 and 120 h.

Cu(II) biosorption

The removal of Cu(II) by EPS was determined by first preparing the Cu(II) solution (10 ppm concentration) using Copper(II) nitrate trihydrate (Friendemann Schmidt) in 1000 ml of distilled water. The solution was then dispensed into 50 ml Falcon tubes (25 ml Cu(II) solution in each tube). The EPS (from 24-, 48-, 72-, 96-, 120-h culture) was then weighed (0.75 mg) and added into the Cu(II) solutions, to give a final concentration of 30 ppm of EPS for the treatment of 25 ml Cu(II) solution (Chug et al. 2016). The EPS-Cu(II) solution was incubated for 24 h, at room temperature (23 ± 2 °C), with agitation (120 rpm) (Lab Companion SI-600R, Korea). After incubation, the EPS-Cu(II) solutions were centrifuged (4000 rpm, room temperature, 45 min), and the supernatant collected while the pellet was discarded. The supernatant was subjected to Cu(II) analysis via Atomic Absorption Spectrometry (AAS) analysis using flame atomic absorption (Perkin Elmer AAnalyst 100, USA). The procedure was repeated for EPS-Cu(II) solutions incubated for 48 and 72 h. The removal of Cu(II) was recorded and calculated using Equation 1 (Eq.1), with C_0: initial concentration of Cu(II), and C_e: final Cu(II) concentration.

$$\% \text{ Cu(II) removal} = [(Co - Ce)/Co] \times 100\% \qquad \text{(Eq.1)}$$

A subsequent test was conducted using a higher concentration of EPS (50 ppm) and with longer EPS-Cu(II) solution incubation time (24, 48, 72, 96, and 120 h). For this test, 1.50 mg of EPS (from 24-, 48-, 72-, 96-, and 120-h culture) was added into 30 ml of 10 ppm Cu(II) solution to give rise to 50 ppm EPS for the test and incubated in similar conditions as described previously. At each sampling period (24, 48, 72, 96, and 120 h), aliquots were sampled and analysed for Cu(II) removal as previous.

Statistical analysis

Analysis for bacterial growth and EPS production was conducted using six replicates, while Cu(II) biosorption analyses were conducted in triplicates. Data was analysed using One Way Analysis of Variance (ANOVA) performed using SPSS Statistics version 21.0. Means were compared with Tukey's test ($p < 0.05$). Comparison between two variables were performed using T-test ($p < 0.05$).

Results and Discussion

Bacterial growth and EPS yield

The growth of *Bacillus cereus* was generally inferior to *Pseudomonas aeruginosa*, for all incubation periods (24-120 h) (Fig. 1). The growth of *B. cereus* was slower (184.83-449.66 mg) with highest biomass achieved at 120 h (449.66 mg). On the contrary, the biomass of *P. aeruginosa* was significantly higher than *B. cereus* (328.33-632.33 mg) for all incubation periods (Fig. 1), with highest biomass observed as early as 24 h (632.33 mg). It is presumed that both bacterial isolates achieved stationary phase by 96 h as there were no longer any significant increase in biomass thereafter (Fig. 1). Although 120-h culture of *B. cereus* and 24-h culture of *P. aeruginosa* had the highest biomass, keeping the cultures longer than 120 h may not necessary be beneficial. Bacteria will grow to a point where growth is then limited by the gradual depletion of nutrients and the possible build-up of toxic by-products (i.e. organic acids, carbon dioxide) (Brose and Eikeren 1990), which may have then inadvertently affected their growth in the later stages.

On the contrary, the production of EPS continued throughout the incubation period, suggesting that EPS production is possible exceeding beyond the optimum culture age of 120 h. Although maximum EPS yield was derived from 48-h culture for *B. cereus* (8.30 mg EPS) and 96-h culture for *P. aeruginosa* (6.95 mg EPS), there is an increasing trend in EPS production beyond the optimum culture age, more evidently for *P. aeruginosa* with 6.67 mg EPS recovered from 120-h culture (Fig. 1). For *B. cereus*, EPS production increased almost two-fold after 24 h, and subsequent 72-, 96- and 120-h cultures yield 7.81, 6.19 and 8.21 mg of EPS, respectively (Fig. 1). It is evident that cultures kept at prolonged incubation period beyond the optimum period, was able to sustain the production of high EPS yield.

When examined between growth and yield, 48-h culture of *B. cereus* appeared to produce more EPS (8.30 mg) despite the low biomass (184.83 mg). This gives a percentage of EPS production of 4.49% for B. cereus. In fact, *B. cereus* had lower biomass (poorer growth) (184.83-449.66 mg) compared to *P. aeruginosa* (328.33-632.33 mg), but the production of EPS by *B. cereus* was significantly higher than *P. aeruginosa*, especially from 48, 72 and 120-h cultures with 8.30, 7.81 and 8.21 mg of EPS obtained, respectively (Fig. 1). For *P. aeruginosa*, EPS production was highest from 96-h culture (6.945 mg) which had a

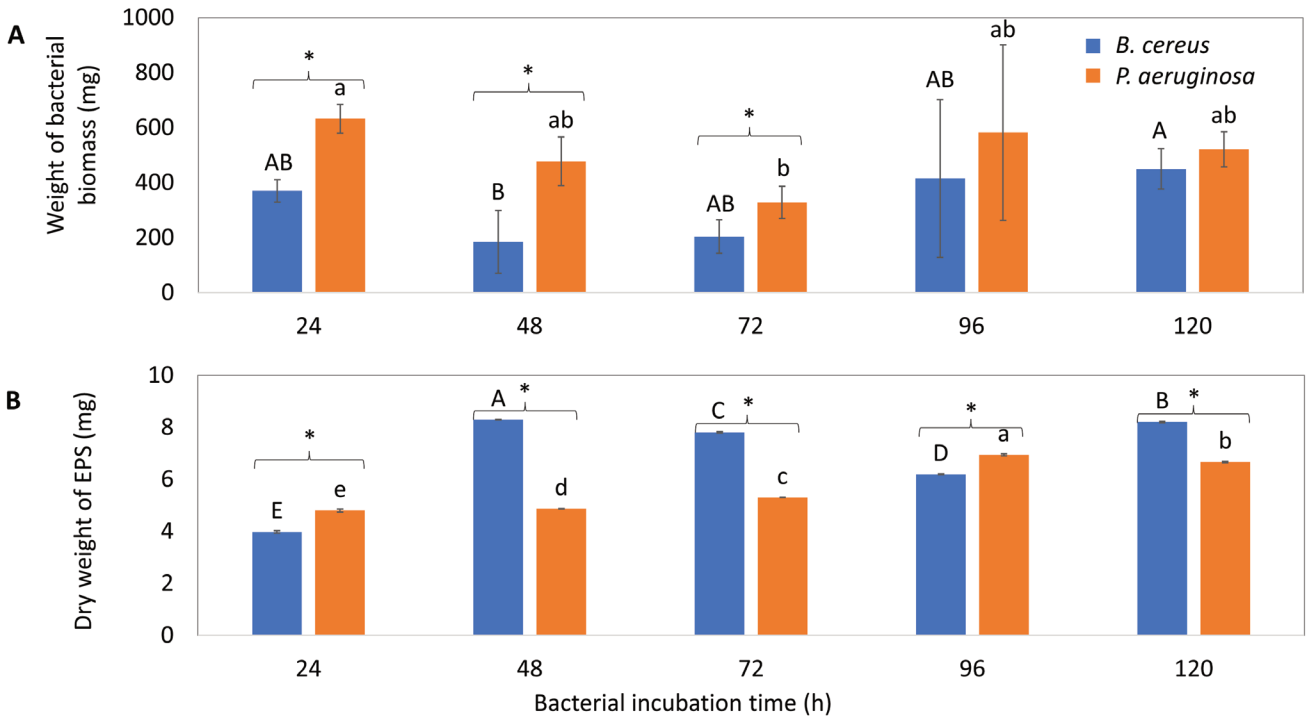

Figure 1. (a) Growth of *B. cereus* and *P. aeruginosa* expressed as weight of bacterial biomass (mg) from 24, 48, 72, 96 and 120 h cultures. (b) Production of exopolymeric substances (EPS, dry weight in mg) by 24, 48, 72, 96 and 120 h cultures of *B. cereus* and *P. aeruginosa*. Means with the same alphabets of the same caption are not significantly different (Tukey's Test, $p<0.05$). *indicates significant differences between the two bacteria as according to T-test ($p<0.05$). Bars indicate standard error.

corresponding biomass of 582 mg. This gives a percentage of EPS production of only 1.19%, This affirmed that *B. cereus* produced more EPS than *P. aeruginosa* although the growth of *B. cereus* was inferior to *P. aeruginosa*.

Clearly, EPS production differs in the two isolates tested. *B. cereus* have higher EPS production as early as 48 h incubation, suggesting EPS production is high during the early log (exponential) growth phase. On the contrary, *P. aeruginosa* produced more EPS at 96 h, coinciding with the stationary growth phase. The high EPS production during late log or stationary phase has been reported (Bragadeeswaran et al. 2011; Razack et al. 2011) and is associated with the various bacterial species and their response to limited nutrient conditions (Myszka and Czacyzk 2009) and release of EPS due to cell lysis (Zeng et al. 2016). Therefore, in some bacteria, growth is correlated to their production of EPS (Finore et al. 2014), whereas no correlation exists for others (Arunkumar et al. 2012). This study has shown that the two different bacteria responded to the incubation conditions differently which influenced growth. However, since EPS is typically produced at early or late log phase of growth between 48-96 h incubation, culture age does influence the EPS yield whereby older cultures (prolonged incubation) may produce high EPS yield irrespective of growth. The higher production of EPS by 48 and 96-h old cultures appeared typical for most bacteria (Kilic and Dönmez 2007; Sheetal and Gupte 2016).

Table 1. Properties (colour, solubility) of EPS produced from *Bacillus cereus* and *Pseudomonas aeruginosa*.

Physical properties	***Bacillus cereus***	***Pseudomonas aeruginosa***
Colour	Brown	Greyish white
Solubility in water	Yes	Yes
Solubility in absolute ethanol	No	No
Solubility in ether	No	No
Solubility in acetone	No	No

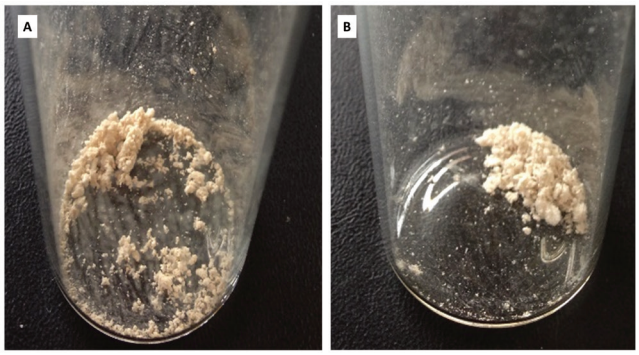

Figure 2. Lyophilized EPS produced by 120 h cultures of (a) *B. cereus* and (b) *P. aeruginosa*, bearing the distinct brownish and greyish white colours, respectively.

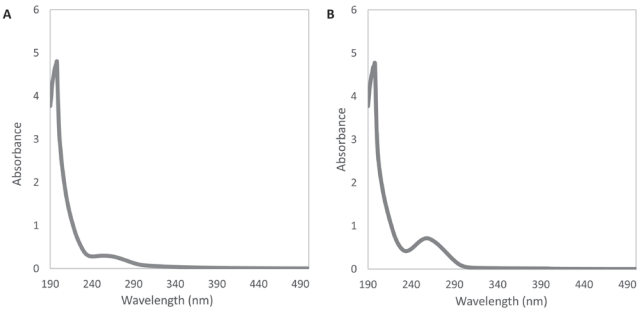

Figure 3. UV-Vis spectra of EPS derived from 24 h cultures of (a) *B. cereus* and (b) *P. aeruginosa*, measured within a wavelength of 190-500 nm.

Quality and purity of EPS yield

The quality of EPS produced from the various incubation periods was generally acceptable, as the integrity of physical properties was evident throughout. The colour of the EPS produced by *B. cereus* and *P. aeruginosa* were brown and white in colour, respectively (Fig. 2). EPS from both *B. cereus* and *P. aeruginosa* were soluble in water, but insoluble in the organic solvents (Table 1). The quality of EPS was consistent for all EPS sampled from all culture age of *B. cereus* and *P. aeruginosa* throughout the experiment.

The EPS produced by *B. cereus* and *P. aeruginosa* were considered pure, as no impurities (i.e. nucleic acids, proteins) were detected. Absence of peaks between 260 to 280 nm indicated absence of nucleic acids or proteins (Fig. 3). Minor shoulder peaks were however, detected at 190-210 nm and 250-260 nm. Peaks at 190-210 nm may be attributed to the n-σ* and/or π-π* transitions, associated with functional groups such as carboxyl, carbonyl or ester functional groups (Lin et al. 2010). Peaks at 250-260 nm wavelength may originate from the π-π* electron transitions of the aromatic and polyaromatic compounds (Liu et al. 2010; Lin et al. 2010). The EPS produced by both *B. cereus* and *P. aeruginosa* were concluded to have acceptable purity, and were not contaminated by nucleic acids or proteins, although aliphatic and aromatic compounds associated with the EPS were detected as inconspicuous shoulder peaks (Lin et al. 2010). This observation was consistent for all EPS derived from the various culture ages of *B. cereus* and *P. aeruginosa*.

The FTIR analysis showed that EPS from *B. cereus* and *P. aeruginosa* have similar spectral and major peak patterns, although the intensity of the peaks differed, especially within sections from wavenumber 500-1800 cm^{-1} (Fig. 4). In those regions, the peak widths for *B. cereus* were broader, whereas the peaks for *P. aeruginosa* were narrower. Broad strong bands of the O-H stretch was abundant in both bacteria (7) (3271.82-3258.98 cm^{-1}) (Fig. 4, Table 2 and 3). The primary functional groups detected were C-O stretching of alcohols (2) and carbonyl (3), alkanes (CH_3) (4,6), and C=C stretch of alkene compounds (5), each at their respective wavenumbers (Fig. 4; Table 2 and 3). These observations were consistent for all samples derived from EPS of *B. cereus* and *P. aeruginosa* of different culture age.

The FTIR spectra analysis confirmed the presence of polar and non-polar functional groups in EPS produced

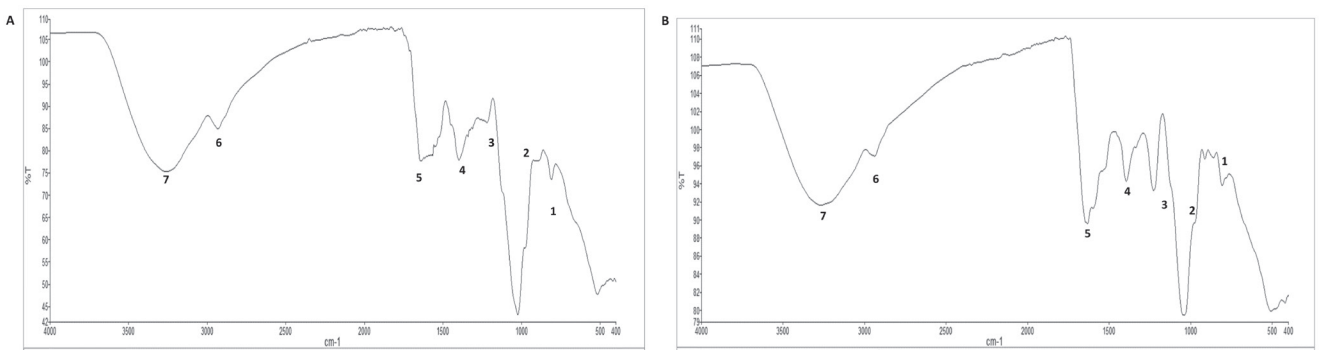

Figure 4. FTIR spectra of EPS derived from 24 h cultures of (a) *B. cereus* and (b) *P. aeruginosa*. Major peaks are labelled with numbers (i.e. 1-7), with corresponding functional groups listed in Table 2. and 3. for *B. cereus* and *P. aeruginosa*, respectively.

Table 2. Functional groups and their respective wavenumbers (cm^{-1}) detected from EPS samples produced by 24-h cultures of *Bacillus cereus*. Corresponding spectra peaks are in Figure 4.

No.	Wavenumber (cm^{-1})	Functional groups
1	810.78	C-Cl stretching of alkyl halides
2	1024.28	C-O stretching of alcohol
3	1220.45	C-O stretching of carbonyl; stretching vibration of C-O
4	1397.84	CH_3 (alkanes)
5	1636.28	C=C stretching of alkene compounds
6	2932.08	-CH_3 alkanes; symmetric and asymmetric CH_2- and CH_3- stretching vibrations
7	3258.98	O-H stretching of alcohol

by both gram-positive and gram-negative bacteria. The primary functional groups identified were alkyl halides, carbonyl, alcohol, alkane, and alkenes, which are negatively charged and capable of binding metal ions (Abraham and Le 1999; Wu et al. 2016). In this study, there were no distinct differences in major functional groups found in the EPS produced by *B. cereus* and *P. aeruginosa*. There were however, two peaks detected within 1210-1230 cm^{-1} (3) and 1630-1650 cm^{-1} (5) in *P. aeruginosa*, which had higher peak intensity than in *B. cereus* (Fig. 4). The intense peaks from EPS of *P. aeruginosa* correspond to phenol, amine and alkene, suggesting that more of the groups may be present in *P. aeruginosa* compared to *B. cereus*. FTIR analysis also revealed the presence of O-H stretching group (3000-3500 cm^{-1}), C-H stretching group (2930 cm^{-1}) and deviational vibration of C-H (1400-1200 cm^{-1}), which represent the polysaccharide constituent in the EPS (Ryder et al. 2007; Chen et al. 2017). This carbohydrate constituent (40-95% of the total composition of EPS) typically comprise of sugar molecules (e.g., hexose or uronic acids), homopolysaccharides, heteropolysaccharides, proteins and lipids (Messner 1997; Flemming and Wingender 2010; More et al. 2014; Gupta and Diwan 2017). Ionization of the functional groups releases the hydrogen ion, giving rise to negatively-charged groups that bind readily to metal cations (Wei et al. 2016; Gupta and Diwan 2017; Zhang et al. 2017). The differences in peak intensity were attributed to the gram positive and gram negative nature of the bacterial isolates, rather than to the culture age.

The quality and yield analyses indicated that both *B. cereus* and *P. aeruginosa* produced EPS of similar characteristics or properties throughout the experiment. EPS from both bacterial cultures, whether from 48, 72, 96 or 120-h cultures, all demonstrated similar characteristics and profile for quality (solubility, purity, functional groups). Older cultures may produce more EPS yield, given that it is within the late exponential or stationary phase of the bacteria growth cycle. This suggested that the EPS produced by both bacterial isolates has good integrity and consistency, although harvested from different culture ages.

Cu(II) biosorption by EPS

The initial Cu(II) removal test using 30 ppm EPS concentration for metal incubation from 24-72 h, indicated that a longer metal incubation time is required to benefit Cu(II) biosorption. The amount of Cu(II) removed was 2-3 fold higher when EPS-Cu(II) solution was incubated for 48 h and 72 h instead of 24 h (Fig. 5). This was evident for EPS derived from both *B. cereus* and *P. aeruginosa*. Application of EPS from *B. cereus* into EPS-Cu(II) solutions incubated for 48 h recorded 15.15 and 17.64% Cu(II) removal by EPS from 24- and 48-h cultures. The increasing trend

Table 2. Functional groups and their respective wavenumbers (cm^{-1}) detected from EPS samples produced by 24-h cultures of *Pseudomonas aeruginosa*. Corresponding spectra peaks are in Figure 4.

No.	Wavenumber (cm^{-1})	Functional groups
1	810.78	C-Cl stretching of alkyl halides
2	1041.40	C-O stretching of alcohol
3	1226.33	C-O stretching of carbonyl; stretching vibration of C-O
4	1394.91	CH_3 (alkanes)
5	1632.80	C=C stretching of alkene compounds
6	2939.45	-CH_3 alkanes; symmetric and asymmetric CH_2- and CH_3- stretching vibrations
7	3271.82	O-H stretching of alcohol

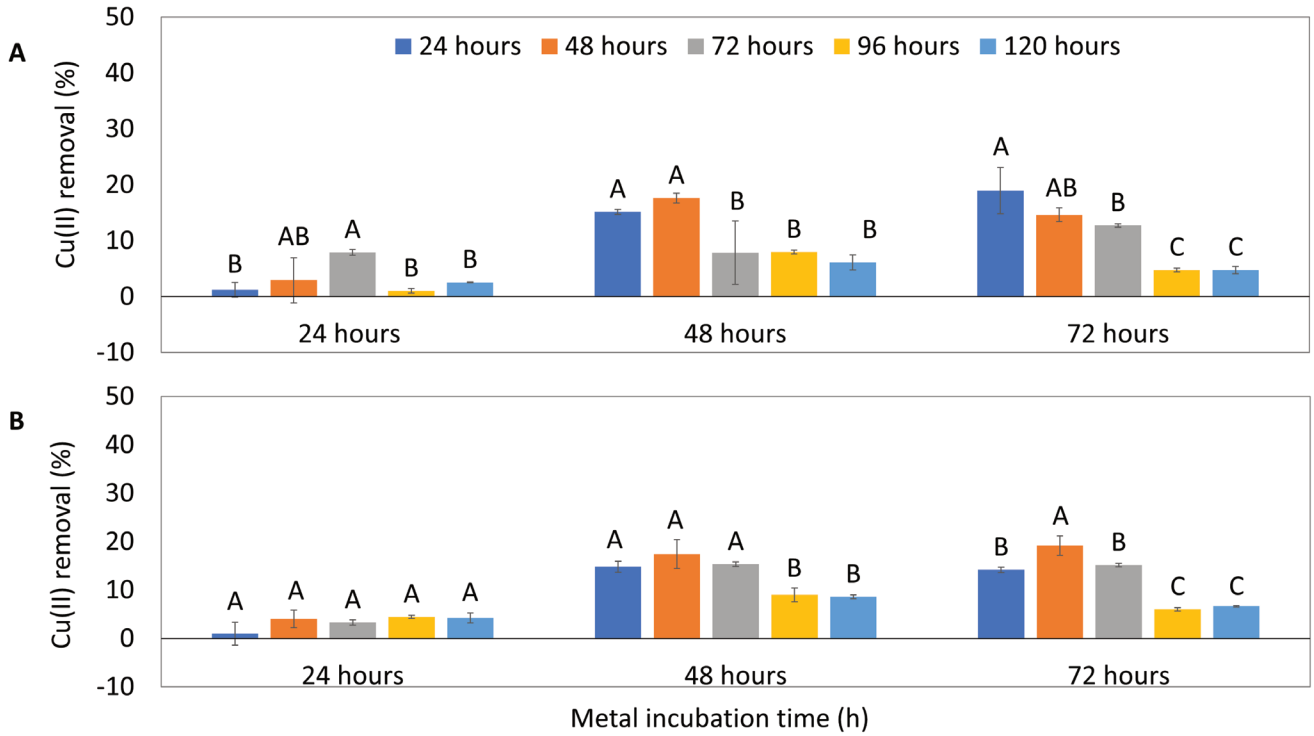

Figure 5. Cu(II) removal (%) by 30 ppm EPS produced by (a) *B. cereus* and (b) *P. aeruginosa* from 24, 48, 72, 96 and 120 h cultures. Cu(II) removal was assessed from EPS-Cu(II) incubation for 24, 48 and 72 h. Means with the same alphabets within a specific EPS-incubation period, are not significantly different (Tukey's Test, $p<0.05$). Bars indicate standard error.

continued with EPS-Cu(II) incubation for 72 h, in which 8.96%, 14.63% and 12.73% Cu(II) removal was achieved by EPS from 24-, 48- and 72-h cultures of *B. cereus*. These levels were higher than amount of Cu(II) removed when the EPS-Cu(II) solutions were incubated for 24 h. At 24 h of EPS-Cu(II) incubation, the highest Cu(II) removal was by EPS from 72-h cultures of *B. cereus* (7.92% Cu(II) removal) (Fig. 5).

Similarly, Cu(II) removal by EPS produced by *P. aeruginosa* were also higher when the EPS-Cu(II) solutions were incubated for 48 h and 72 h. At 48 h of EPS-Cu(II) incubation, Cu(II) removal was significantly higher by EPS derived from 24-, 48- and 72-h cultures, with 14.82%, 17.41% and 15.35% Cu(II) removal (Fig. 5). When EPS-Cu(II) solutions were incubated for 72 h, Cu(II) removal was relatively higher by EPS produced from 24-, 48- and 72-h cultures of *P. aeruginosa,* with 14.18%, 19.19% and 15.17% removal, respectively (Fig. 5). The EPS produced from 24-, 48- and 72-h cultures were therefore observed to have significantly higher Cu(II) removal potential than EPS produced from 96- and 120-h cultures (Fig. 5).

Comparing the efficacy of 30 ppm EPS from *B. cereus* and *P. aeruginosa*, the EPS produced by *P. aeruginosa* appeared to have significantly higher Cu(II) removal potential, particularly for EPS-Cu(II) solutions incubated for 48 and 72 h (Fig. 6). T-test analysis showed Cu(II) removal by EPS produced from 72-h (15.35%) and 120-h (8.60%) for EPS-Cu(II) incubation at 48 h, revealed significant differences. A more evident trend was observed for EPS-Cu(II) solutions incubated for 72 h. Cu(II) removal at this stage by EPS derived from 48-h (19.19%), 72-h (15.17%), 96-h (6.02%) and 120-h (6.67%) cultures of *P. aeruginosa* were significantly higher than EPS from *B. cereus* (Fig. 6). On the contrary, EPS from *B. cereus* removed only 7.84 (by 72-h culture) and 6.11% (by 120-h culture) of Cu(II) post EPS-Cu(II) incubation for 48 h. At 72 h of EPS-Cu(II) incubation, Cu(II) removal was only 14.63%, 12.73%, 4.75%, and 4.77%, by 48-, 72-, 96-, and 120-h cultures, respectively (Fig. 6). Interestingly, EPS from 72-h culture of *B. cereus* was more effective than *P. aeruginosa* for Cu(II) removal at 24 h EPS-Cu(II) incubation, with 7.92 compared to 3.31% of removal, respectively (Fig. 6).

The early indications here suggested that culture age may have influenced Cu(II) biosorption, with EPS from *B. cereus* and *P. aeruginosa* from specific culture age demonstrating more superior Cu(II) biosorption than others. This may be ascribed to the fact that EPS is organic and subtle variations in the EPS constituents may exist, particularly for EPS derived from a gram positive or gram negative bacteria. The production of EPS and their composition,

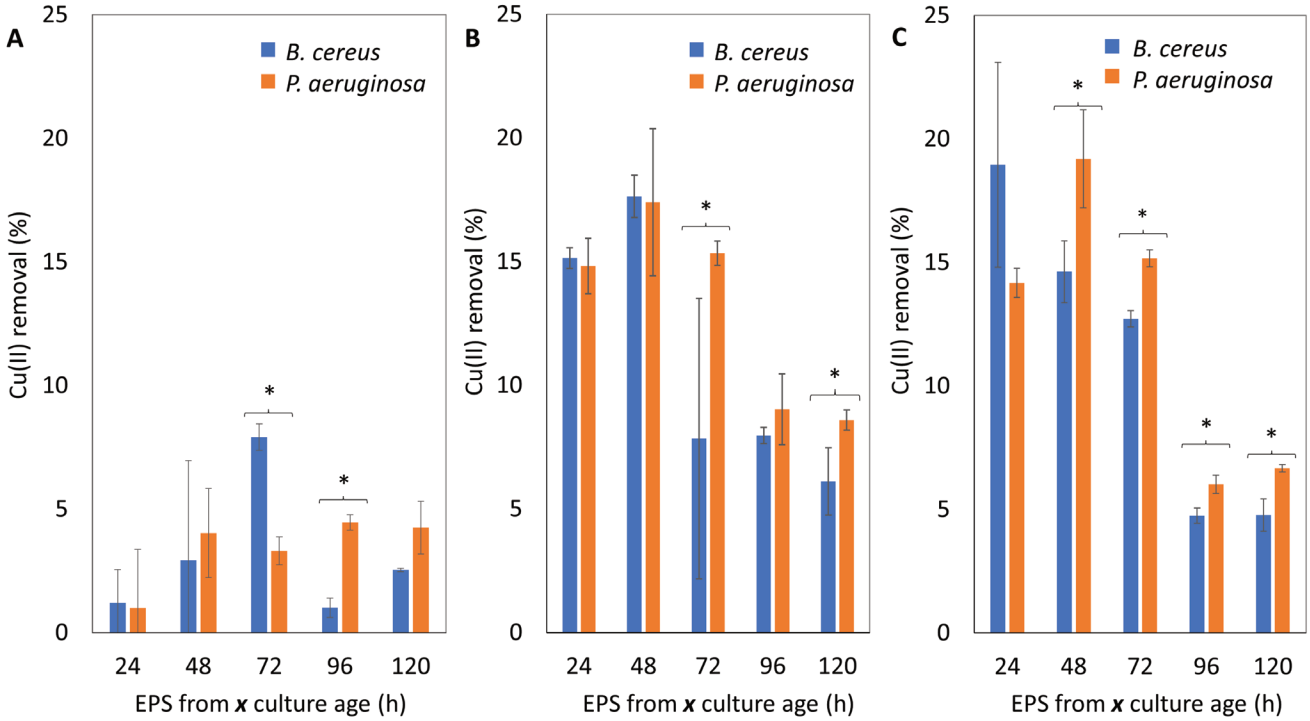

Figure 6. Comparison of Cu(II) removal (%) efficacy by EPS produced by *B. cereus* and *P. aeruginosa* from 24, 48, 72, 96 and 120 h cultures. Cu(II) removal was assessed using 30 ppm EPS incubated in EPS-Cu(II) solution for (a) 24, (b) 48 and (c) 72 h. *indicates significant differences between the two bacteria as according to T-test ($p<0.05$). Bars indicate standard error.

may have subtle differences in the carbohydrate and protein constituents, owing to their cell wall structures (Seltmann and Holst 2013). Gram-negative bacteria have an outer membrane that is rich in lipopolysaccharides, in addition to the thin inner layer of peptidoglycan. On the contrary, Gram-positive bacteria do not have an outer membrane; instead consists of several layers of peptidoglycan (Seltmann and Holst 2013). This may have influenced the composition in the EPS produced, thus influencing the metal adsorption capability of bacteria (Vu et al. 2009; More et al. 2014; Cheah and Ting 2020). This has also been shown in the FTIR results (in the earlier sections) in which peak intensity differs between *B. cereus* and *P. aeruginosa*. Nevertheless, there were no conclusive evidences on the significant influence of culture age on EPS and Cu(II) biosorption as EPS-Cu(II) incubation period and EPS concentration appeared to contribute to biosorption. As such, the EPS-Cu(II) incubation period

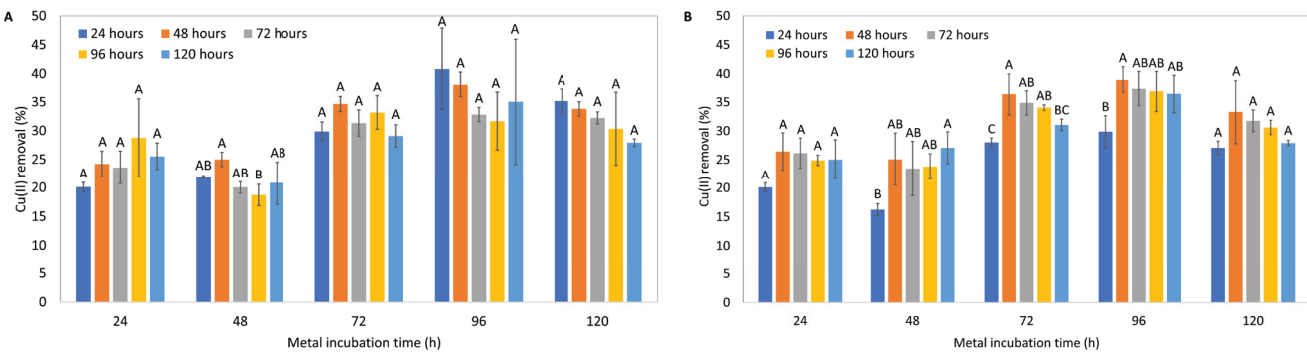

Figure 7. Cu(II) removal (%) by 50 ppm EPS produced by (a) *B. cereus* and (b) *P. aeruginosa* from 24, 48, 72, 96 and 120 h cultures. Cu(II) removal was assessed from EPS-Cu(II) incubation for 24, 48, 72, 96 and 120 h. Means with the same alphabets within a specific EPS-Cu(II) incubation period, are not significantly different (Tukey's Test, $p<0.05$). Bars indicate standard error.

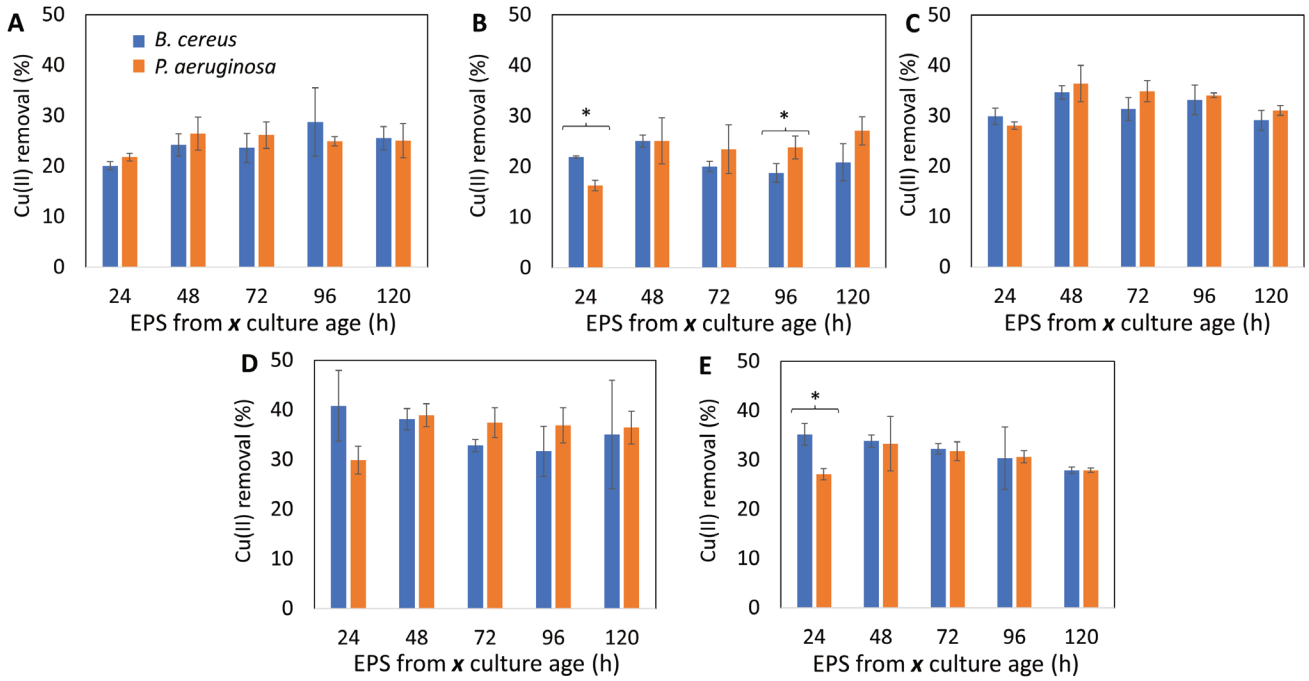

Figure 8. Comparison of Cu(II) removal (%) efficacy by EPS produced by *B. cereus* and *P. aeruginosa* from 24, 48, 72, 96 and 120 h cultures. Cu(II) removal was assessed using 50 ppm EPS incubated in EPS-Cu(II) solution for (a) 24, (b) 48, (c) 72 h, (d) 96, and (e) 120 h. *indicates significant differences between the two bacteria as according to T-test ($p<0.05$). Bars indicate standard error.

was prolonged and a higher concentration of EPS was used so that a clearer trend can be seen.

With higher concentration of EPS used (50 ppm) and prolonged incubation period beyond 72 h (to 96 and 120 h), higher amount of Cu(II) was removed (Fig. 7). As such, results here suggested that Cu(II) removal may now be independent of culture age, for both *B. cereus* and *P. aeruginosa*. When 50 ppm of EPS from *B. cereus* was used, the amount of Cu(II) removed by EPS from 24-, 48-, 72-, 96- and 120-h cultures were not significantly different, with the exception for EPS-Cu(II) incubated for 48 h (Fig. 7). With 48 h incubation, Cu(II) removal by EPS from 96-h culture (18.72%) was significantly lower than EPS derived from 48-h culture (25.03%) (Fig. 7). For *P. aeruginosa*, the EPS by 48- and 72-h cultures appeared to have an advantage in Cu(II) removal compared to 24-h cultures, particularly for EPS-Cu(II) incubations for 48, 72 and 96 h (Fig. 7). EPS from 24-h cultures of *P. aeruginosa* removed only 20.09%, 16.25%, 28.04%, 29.88%, and 27.08% of Cu(II) at 24, 48, 72, 96 and 120 h of EPS-Cu(II) incubation, respectively (Fig. 7). Clearly, with the higher amount of EPS used and prolonged EPS-Cu(II) incubation period, the Cu(II) removal efficacy has become less dependent on EPS derived from various culture ages of bacteria.

The efficacy of EPS from *B. cereus* was generally comparable to EPS from *P. aeruginosa* with the use of 50 ppm of EPS (based on T-test) (Fig. 8). Exceptions though, were observed for EPS from 24-h culture of *B. cereus*, which removed more Cu(II) when incubated for 48 (21.93%) and 120 h (35.18%) in EPS-Cu(II) solution, compared to *P. aeruginosa* (16.25 and 27.08%, respectively) (Fig. 8). On the contrary, EPS derived from 96-h cultures of *P. aeruginosa*, removed significantly higher Cu(II) when incubated in EPS-Cu(II) solution for 48 h, with 23.81% compared to 18.72% by EPS from *B. cereus* (Fig. 8). Notably, with a higher concentration of EPS used (50 ppm), the efficacy of Cu(II) removal is enhanced, and there were no longer significant discrepancies between the two EPS sources in removing Cu(II). Both *B. cereus* (gram positive) and *P. aeruginosa* (gram negative) were capable of producing EPS for Cu(II) removal. For *B. cereus*, the ability of EPS to remove Cu(II) conforms to reports of other *Bacillus* sp. in removing Cu(II), Pb(II) and Cd(II) (Shameer 2016). Similarly, gram negative bacteria are also known to produce EPS to remove metals. Ashruta et al. (2014) used EPS produced by a consortium of gram-negative bacteria, to effectively remove Zn(II), Pb(II), Cr(III), Ni(II), Cu(II) Cd(II) and Co(II). It is evident that Cu(II) removal by EPS is influenced significantly by the concentration of EPS used. It is anticipated that with increased concentrations of EPS used, Cu(II) removal would be enhanced by two to three-fold (Shameer 2016; Wei et al. 2016; Azzam and Tawfik 2015). In this study, the increase of Cu(II) removal

efficacy was observed when 50 ppm is used compared to 30 ppm. This is mainly attributed to having more available binding sites in EPS.

Observations here provided evidence that the influence of culture age on Cu(II) biosorption efficacy by EPS diminished with the use of higher EPS concentration (i.e. 50 ppm) and with prolonged EPS-Cu(II) incubation period. The age of the culture appeared to be more important when cells/biomass are used instead of EPS (Odokuma 2009). Although Pereira et al. (2009) suggested that composition of EPS produced by bacteria may have slight quantitative and qualitative differences attributed to age of culture, this was not clearly evident in this study. With prolonged incubation period, the influence of culture age diminished as EPS have more time to bind to the EPS (Kumar et al. 2009). This was demonstrated here whereby metal removal was enhanced two-fold (from < 20 to 40% removal efficacy) with incubation extended to 120 h. Nevertheless, metal binding may reach a saturation point where metal removal is gradually limited by the limited number of active binding sites (Pagnanelli et al. 2009).

Conclusions

To conclude, EPS produced from laboratory strains *B. cereus* and *P. aeruginosa* have potential use for Cu(II) removal. EPS production was higher in 48-h and 96-h cultures of *B. cereus* and *P. aeruginosa*, respectively, with acceptable quality and integrity across all culture ages. EPS produced from different culture ages also did not demonstrate varying metal removal capacity, especially when higher concentrations of EPS were used and with extended EPS-Cu(II) incubation time. There were also no clear distinctions between the efficacy of EPS produced from gram positive and gram-negative bacteria. This study revealed that culture age influenced the yield of EPS, but does not have a strong impact in determining the characteristics of the EPS (purity, solubility, functional groups) nor their Cu(II) biosorption efficacy.

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