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Co-cultivation, metal stress and molasses: strategies to improving exopolymeric yield and metal removal efficacy

Caleb Cheah, Yuen Lin Cheow and Adeline Su Yien Ting* 

Abstract

This study investigated and compared several improvement strategies to increase the yield and quality of exopolymeric substances (EPS) from *Bacillus cereus*. This includes co-culturing of *B. cereus* with *Trichoderma asperellum*, cultivation in media with metal (Zn) stress and supplementation with molasses. EPS is subsequently extracted from these different cultures and subjected to characterization and metal removal tests in single-metal systems (Cu, Pb, Zn, Cd, Cr). Results indicate that co-cultivation of *B. cereus* and *T. asperellum* produced EPS which have attributes differing from single cultivation. These changes were detected via functional group changes using Fourier-Transform Infrared Spectroscopy, as well as the increase in carbohydrate and protein content. However, the interaction of these two microbes were merely additive and did not result in improved EPS yield nor the subsequent metal removal efficacy in comparison to single cultivation (control). By contrast, supplementation of Zn (metal stress with 50 mg L⁻¹ Zn) improved EPS quality and metal removal, but decreased EPS yield. The application of 1% molasses was the only strategy demonstrating high yield and efficient metal removal. EPS quality and yield (0.45 mg mL⁻¹) and metal removal efficacy (Cu: 58%, Pb: 98%, Zn: 83%, Cd: 73%, Cr: 96%) were improved significantly. This study showed that among the three improvement strategies (co-cultivation, metal stress, molasses), supplementation with molasses was the most effective as it improved both yield and quality of EPS significantly, suggesting that this approach may be adopted for future production of bulk EPS for up-scaling of wastewater treatment.

Keywords: Biosorption, Co-cultivation, Exopolymeric substances, Metal stress, Molasses, Wastewater treatment

* Correspondence: adeline.ting@monash.edu

School of Science, Monash University Malaysia, 47500 Bandar Sunway, Malaysia



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1 Introduction

Metals are commonly found in effluents from various industries and their rampant discharge into waterways pollute the environment [1, 2]. Metals do not biodegrade but accumulate over time, resulting in metal poisoning of aquatic plants, animals and humans [3–5]. Metal poisoning in humans causes interference to protein folding and enzyme regulation [6], which leads to vascular diseases, kidney damage, and cancer [7, 8]. Removal of metals from the environment is therefore critical. Several approaches have been adopted with physicochemical techniques such as ion exchange and chemical precipitation as the most typical techniques applied. However, they are costly and also produce toxic wastes [7]. Adsorption using commercial adsorbents such as activated carbon and silica gel is also effective for metal removal, but they are costly and less sensitive to low metal concentrations [1, 4]. Therefore, bio-based techniques are preferred as they are cheaper and more environmentally friendly.

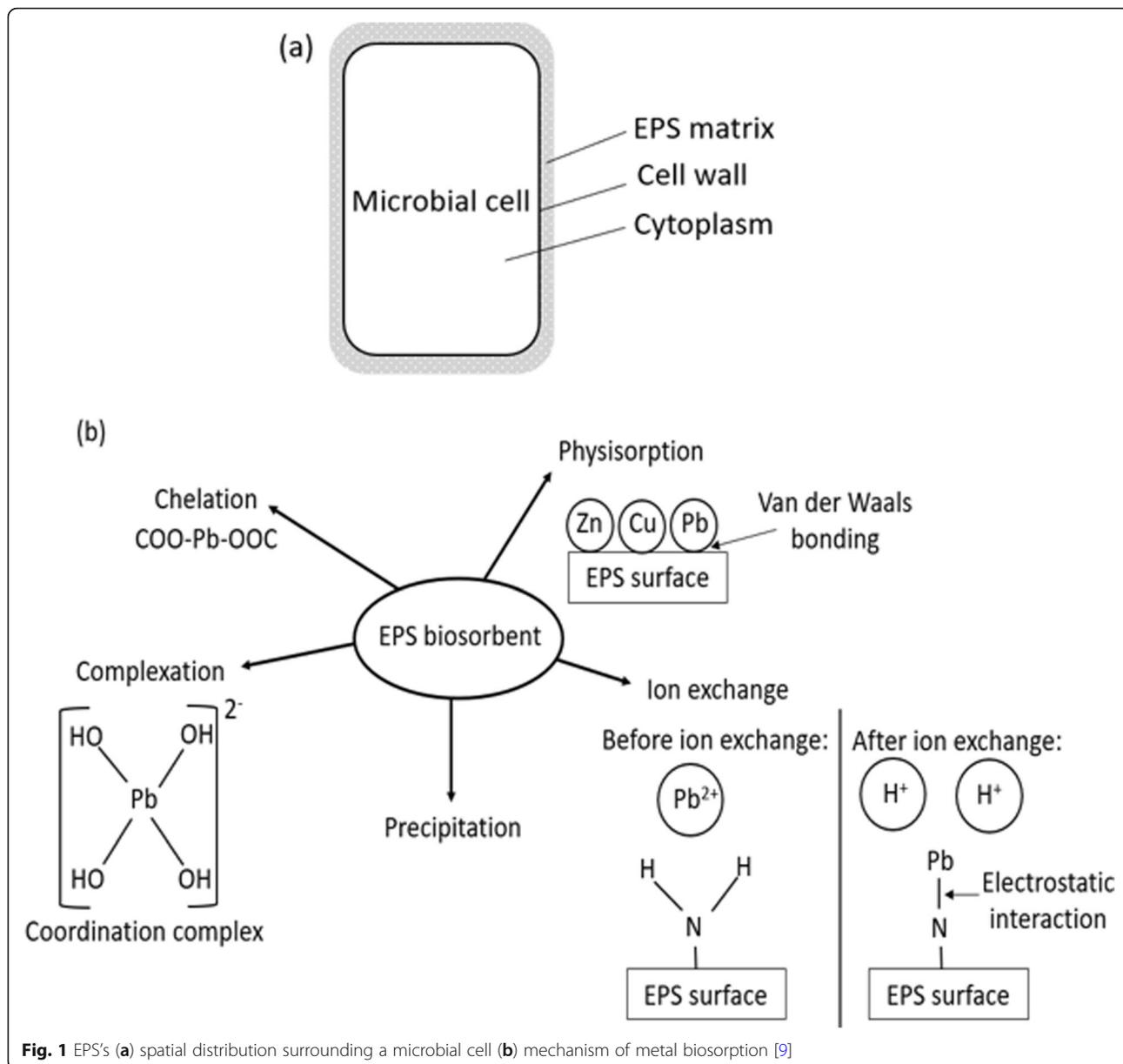
Bio-based techniques utilises bio-organic sources such as industrial and agricultural wastes, as well as microbial cells [9]. Of the various sources, microbial cells are preferred as they have several distinct advantages. Microbial cells can be easily cultivated, making it a cost-effective resource [10]. Microbial cells are also known to have a wide range of metal adsorption capabilities, allowing it to be applied for the removal of various metals [10]. Nonetheless, there are several challenges in utilising microbial cells. The primary challenge is the difficulty in maintaining live cells as they are susceptible to high concentrations of metal [11]. Furthermore, the introduction of microbial cells into the environment may create a potential health threat, particularly when opportunistic pathogens are used, as well as the unintentional possible transfer of resistance genes [12]. To overcome these challenges, biomolecules from microbial cells such as exopolymeric substances (EPS) are explored as they are known to have roles in metal biosorption [13].

EPS exists as a matrix surrounding the microbial cells [9] (Fig. 1). The main constituents of EPS are macromolecules such as carbohydrates, proteins, lipids and nucleic acids [14, 15]. EPS are formed or produced via cellular lysis, hydrolysis of macromolecules or even secreted by microbial cells as a response to environmental stress [16]. Recent studies have shown that bacterial EPS has the potential use for the bioremediation of metals [17]. Kalpana et al. [18] reported that EPS from *Bacillus cereus* was capable of removing 80 µg of Hg in 20 min. EPS generally contains a variety of functional groups (carbonyl, carboxyl, hydroxyl, phosphoryl, sulfhydryl, thiol, amino), causing its matrix to be negatively charged, attracting the positively charged metal cations in wastewater [13, 17] (Fig. 1). EPS can therefore remove

metals by ion exchange, complexation, precipitation, chelation and physisorption [9].

Several factors are known to influence the production of EPS by microbial cells, which can be manipulated to induce production of EPS. Primary strategies proposed here include co-cultivation, metal stress and by supplementing molasses. Co-cultivation is the co-existence of the EPS-producing microbe with other microorganisms during the growth phase. Co-cultivation of microbial cells (bacteria and fungi) has been reported to improve EPS production as microbial cells may secrete certain compounds or enzymes that support the growth of other microbes [16]. For example, the combined cultivation of *Penicillium frequentans* and *Bacillus mycoides* leads to the synthesis of a novel EPS with increased bioremediation potential [19]. Another example is the co-culturing of *Trichoderma asperellum* with *Bacillus* spp. (e.g., *T. asperellum* and *Bacillus subtilis*, *T. asperellum* and *Bacillus amyloliquefaciens*) in which improved metal removal efficacy was attributed to the many functional groups present in the complex glycopeptides produced [20–22]. The second approach is to induce EPS production by providing metal stress. Sheng et al. [14] discovered that the protein content in the EPS matrix increased significantly in conditions where metal stress was present, as structural proteins are often produced in the EPS matrix to remove metal pollutants via active or passive transport mechanisms. Among the various toxic metals, Zn has been reported to be among the most effective in inducing metal stress and the gradual production of EPS in *Bacillus* spp. [23]. This may be species specific as other metals such as Cu and Pb have also been found to trigger metal stress and improve EPS yield in other bacterial species. The third approach involves supplementing additional nutrient source such as sugarcane molasses during microbial growth. Molasses have been reported to improve EPS production as they are rich in carbon and nitrogen sources, which form the basic carbohydrate and protein structures and their functional groups within the EPS matrix [24]. Sugarcane molasses are better compared to other supplements such as rice bran, fructose and beet molasses, as sugarcane molasses reportedly has the optimal amount of nutrients which are vital for EPS production [18, 25].

In this study, the EPS of *B. cereus* is explored for its metal removal capabilities. *B. cereus* was selected as the isolate is easy to cultivate to produce EPS that has high bioremediation potential [18]. The EPS production by *B. cereus* was induced to improve the EPS yield and metal removal efficacy [18, 26, 27]. The strategies employed include co-cultivation techniques, inducing metal stress and supplementing sugarcane molasses during the cultivation phase of *B. cereus*. The effectiveness of these



strategies are assessed based on the yield of EPS and their subsequent metal removal efficacy. The metal removal efficacies of EPS were tested on five common toxic metals (Cu, Pb, Zn, Cd, Cr) in single metal systems. The characteristics of the improved EPS were also determined via carbohydrate (phenol-sulphuric acid) assay, protein (Bradford) assay and FTIR (Fourier-Transform Infrared Spectroscopy) analysis. The changes in characteristics of EPS would provide an understanding on improvements or changes to the quality of the EPS. It is expected that the findings in this study would contribute to a better understanding on yield and quality improvement of EPS as a strategy for future wastewater treatment.

2 Materials and methods

2.1 Culture preparation and production of EPS under various strategies

2.1.1 Co-cultivation

B. cereus and *T. asperellum* were obtained as lab cultures and stored as stock cultures on agar slants until use. Co-cultivation was established by inoculating *B. cereus* with *T. asperellum* in tryptic soy broth (25 °C, 120 rpm, 7 d) [20–22]. This co-culture of microbial cells (bacteria and fungi) is expected to potentially improve EPS quality and yield [14]. The broth was then centrifuged (7000 rpm, 25 °C, 30 min) and the resulting supernatant was collected. The supernatant was then mixed with 95% ethanol (ratio of 250 mL: 750 mL) and

incubated overnight (4 °C) [28]. This mixture was then centrifuged (7000 rpm, 4 °C, 30 min) and the pellet formed is dissolved in ultrapure water, frozen (−20 °C, 3 h) and lyophilized (−60 °C, 48 h) [28]. The yield of EPS after lyophilisation was then recorded by measuring their weight. Separate individual cultures of *T. asperellum* and *B. cereus* were also established as controls, and the EPS were collected and weighed.

2.1.2 Metal stress

B. cereus was inoculated into nutrient broth media (25 °C, 120 rpm, 2 d) that was supplemented with ZnSO₄ (Merck) at various concentrations (10, 20, 30, 40, 50 mg L^{−1}) [18, 23]. The various metal-supplemented broth cultures were then centrifuged (7000 rpm, 25 °C, 30 min) and the resulting supernatant was collected. The supernatant was then mixed with 95% ethanol (ratio of 250 mL: 750 mL) and incubated overnight (4 °C) [28]. This mixture was then centrifuged (7000 rpm, 4 °C, 30 min) and the pellet formed was dissolved in ultrapure water, frozen (−20 °C, 3 h) and lyophilized (−60 °C, 48 h) [28]. The yield of EPS after lyophilisation was then recorded by measuring their weight. A separate individual culture of *B. cereus* without metal stress was also established as control, and the EPS from this metal-free culture was also collected and weighed.

2.1.3 Supplementation of molasses

B. cereus was inoculated into nutrient broth media that was supplemented with sugarcane molasses at various concentrations (1, 2, 3, 4, 5%), and incubated (25 °C, 120 rpm, 2 d) [18]. The various molasses-supplemented broths were then centrifuged (7000 rpm, 25 °C, 30 min) and the resulting supernatant was collected. The supernatant was then mixed with 95% ethanol (ratio of 250 mL: 750 mL) and incubated overnight (4 °C) [28]. This mixture was then centrifuged (7000 rpm, 4 °C, 30 min) and the pellet formed was dissolved in ultrapure water, frozen (−20 °C, 3 h) and lyophilized (−60 °C, 48 h) [28]. The yield of EPS after lyophilisation was then recorded by measuring their weight. A separate individual culture of *B. cereus* without molasses was also established as control, and the EPS produced from this molasses-free culture was collected and weighed.

2.2 Characterisation of EPS

The EPS collected after undergoing improvement strategies described in Section 2.1 were characterised based on their colour and solubility. The solubility test was carried out using 1 mg of EPS in 2 mL of solvents (i.e., ethyl ether, absolute ethanol, acetone) and with water.

The EPS collected were also subjected to carbohydrate and protein (Bradford) assays to determine the changes in carbohydrate and protein content of the EPS. To determine carbohydrate content (phenol sulphuric assay), 1 mg of EPS was dissolved in 150 µL of sulphuric acid and 30 µL of ultrapure water, mixed well and then dispensed into a 96-well plate and incubated (90 °C, 15 min). Phenol (30 µL) was then added into the wells and the plate was agitated (120 rpm, 5 min). The absorbance was then measured (490 nm) via a microplate reader (Tecan Infinite Series) [29]. For the Bradford assay, 1 mg of EPS was dissolved in 100 µL of Coomassie Blue solution and 100 µL of 0.15 M NaCl water, and dispensed into a 96-well plate. The plate was then agitated (120 rpm, 5 min). The absorbance was then measured (595 nm) via a microplate reader (Tecan Infinite Series) [30]. The carbohydrate and protein content of EPS collected after implementation of the cultivation strategies were compared to the original EPS to determine the changes induced by the strategies.

The functional groups present on EPS were also characterised via FTIR (Perkin Elmer FT-IR C106361). The FTIR spectrum was obtained by scanning 5 mg of EPS using a resolution of 4 cm^{−1} with 16 scans over a spectral range of 4000–400 cm^{−1} [20]. The functional groups present were then identified based on the peaks detected in the spectra. The functional groups of EPS collected after implementation of the cultivation strategies were compared to the original EPS to determine the changes induced by the strategies.

2.3 Metal removal efficacy of EPS produced from co-cultivation, metal stress and molasses

Cu, Pb, Zn, Cd and Cr metal solutions (5 mg L^{−1} concentration) were prepared by dissolving 5 mg of Cu (NO₃)₂, Pb (NO₃)₂, Zn (NO₃)₂, Cd (NO₃)₂ and Cr (NO₃)₂ each (Merck), in 1000 mL solutions, respectively. The metal removal process was performed using both the modified and control EPS collected from the co-cultivation strategy. EPS (20 mg) was used to remove metals in 30 ml solutions (5 mg L^{−1}, pH 5), incubated with agitation (25 °C, 120 rpm). The amount of metal remaining in the solution at 2, 4, 6, 8, 10 and 24 h were determined using the Atomic Absorption Spectrophotometer (AAS) (Perkin Elmer Analyst 100) [20]. The AAS wavelengths for measuring the metal ions of Cu, Pb, Zn, Cd and Cr were 325, 283, 214, 229 and 358 nm, respectively [20]. This metal removal experiment was repeated using EPS produced under the influence of metal stress and molasses. The amount of metal removed from the solution is calculated based on Eq. (1):

$$\% \text{ of metal removal} = \frac{\text{Initial Concentration of metal (mg L}^{-1}\text{)} - \text{Final metal concentration (mg L}^{-1}\text{)}}{\text{Initial Concentration of metal (mg L}^{-1}\text{)}} \times 100\% \quad (1)$$

2.4 Statistical analysis

All the experiments were conducted in triplicates. The data obtained was analysed with One-Way Analysis of Variance using the Statistical Package for the Social Sciences version 23.0. The mean values were subjected to Tukey's Honestly Significance Test ($p < 0.05$). The t-test ($p < 0.05$) was also used where applicable to evaluate the significant difference of carbohydrate and protein content between control and modified EPS.

3 Results and discussion

3.1 Characterisation of EPS produced from co-cultivation, metal stress and molasses

3.1.1 Co-cultivation

The EPS collected from single cultures of *B. cereus* and *T. asperellum*, and from the co-culture were all white in colour. They were all soluble when added into water, but were not soluble in organic solvents (ethyl ether, absolute ethanol, acetone). The similarities in EPS properties indicated that co-culturing did not alter the physical properties of the EPS. EPS from co-culturing retained their highly hydrophilic nature as their functional groups form hydrogen bonds with water [14]. By contrast, the EPS yield differed when compared among the two single cultures and co-cultivation, and is suggestive of strain dependency. The EPS yield from co-cultivation were significantly higher compared to the yield derived from single culture of *B. cereus* (0.21 mg mL^{-1}). However, there was no significant difference in EPS yield from co-cultivation of *B. cereus* and *T. asperellum* (0.40 mg mL^{-1}) compared to the EPS yield of the single culture of *T. asperellum* (0.43 mg mL^{-1}) (Table 1). This suggested that co-cultivation did not necessarily lead to higher EPS yield, and that the interaction between isolates in the co-culture may play a crucial role in EPS production. In one study, co-culturing *Saccharomyces cerevisiae* and *Pichia stipitis* demonstrated improved growth and yield as each species synthesised and shared precursor metabolites required for their growth [28, 31]. However, co-culturing of *B. cereus* and *T. asperellum* in this study, did not lead to higher EPS yield for *T. asperellum*,

indicating that possibly the precursor metabolites from *B. cereus* may not induce production of EPS by *T. asperellum*. It is also postulated that *T. asperellum* may not have induced production of EPS from *B. cereus* as well. Although EPS yield from co-culture was higher than single-culture of *B. cereus*, this may have been contributed primarily by *T. asperellum*.

The carbohydrate content ($153 \text{ } \mu\text{g mg}^{-1}$) of the co-culture EPS is significantly lower compared to the EPS from single cultures of *B. cereus* ($258 \text{ } \mu\text{g mg}^{-1}$) and *T. asperellum* ($182 \text{ } \mu\text{g mg}^{-1}$) (Table 1). The difference in the carbohydrate and protein content identifies the co-culture EPS as novel EPS, which refers to the EPS produced by mixed isolates and a widely accepted concept in co-cultures. The novel EPS have been reported to be beneficial. For example, co-culture of *Penicillium frequentans* and *Bacillus mycoides*, resulted in a novel EPS that was better at bioremediation compared to EPS from single cultures [19]. Similarly, Zhang et al. [32] reported that a co-culture of *Candida tropicalis* and *B. subtilis* improved bioremediation of oil due to improved enzymatic interactions. Clearly, novel EPS are synthesised from microbial cells in a co-culture as a strategy for improvement owing to the mixture of compounds and enzymes [19, 33].

In this study, the novelty of the EPS produced from co-cultivation was further supported by the FTIR results. The co-culture EPS displayed sharper peaks, indicating they have more functional groups compared to EPS from single cultures of *B. cereus* and *T. asperellum*. The co-culture EPS have sharper peaks at $1500\text{--}1320 \text{ cm}^{-1}$ range, indicating additional methylene ($-\text{CH}_2$) groups, which were detectable by additional C-H stretching observed at $3000\text{--}2850 \text{ cm}^{-1}$ (Fig. 2) [34]. Furthermore, there is also a sharper peak appearing at $1680\text{--}1600$ and $1600\text{--}1450 \text{ cm}^{-1}$, reflecting the additional alkene ($\text{C}=\text{C}$) and aromatic groups ($\text{C}=\text{C}$) (Fig. 2) [34]. Although protein content was detected to be present in EPS, the FTIR spectra did not show the presence of any amine groups

Table 1 The characteristics of EPS from *B. cereus*, *T. asperellum* and co-culture

EPS sample	EPS yield (mg mL^{-1})	Carbohydrate content ($\mu\text{g mg}^{-1}$)	Protein content ($\mu\text{g mg}^{-1}$)
<i>B. cereus</i>	0.21 ± 0.07	258 ± 18	43 ± 5
<i>T. asperellum</i>	0.43 ± 0.10	182 ± 12	87 ± 6
Co-culture	0.40 ± 0.06	$153 \pm 9^*$	43 ± 9

*indicates a significant difference in EPS properties from co-culture compared to single cultures (*B. cereus*, *T. asperellum*) according to T-test ($p < 0.05$)

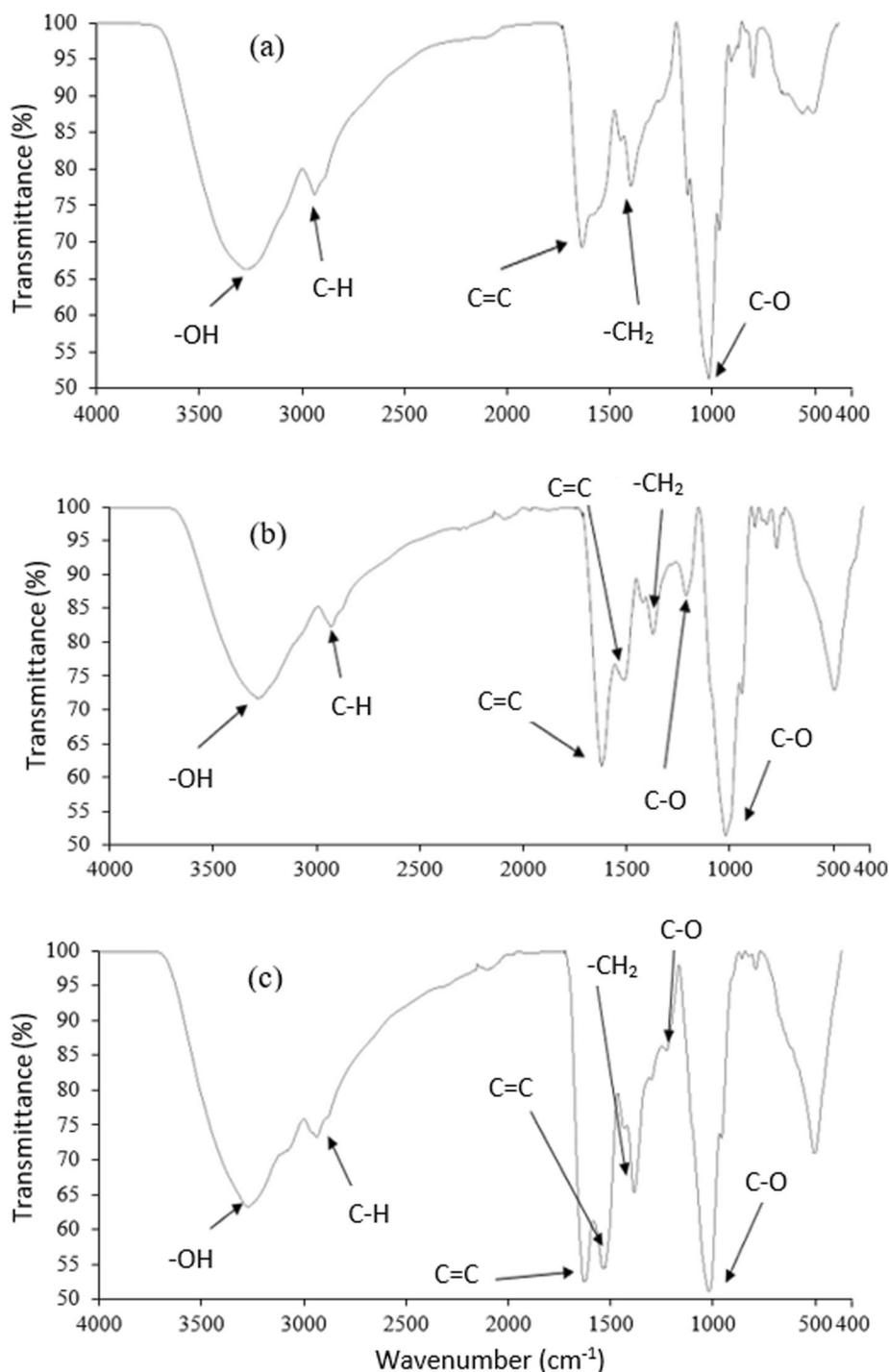


Fig. 2 FTIR spectrum for EPS from (a) *B. cereus* (b) *T. asperellum* (c) co-culture, indicating functional groups detected. The peaks indicate O-H stretching ($3650\text{--}3200\text{ cm}^{-1}$), C-H stretching ($3000\text{--}2850\text{ cm}^{-1}$), C=C stretching ($1680\text{--}1450\text{ cm}^{-1}$), -CH_2 bending ($1500\text{--}1320\text{ cm}^{-1}$) and C-O stretching ($1300\text{--}1000\text{ cm}^{-1}$)

(-NH_2). It is hypothesised that the amine groups present were either secondary amine stretching groups ($3500\text{--}3100\text{ cm}^{-1}$) which overlapped with the O-H stretching peaks ($3650\text{--}3200\text{ cm}^{-1}$), or tertiary amines which

cannot be detected in the FTIR spectra [34]. A co-culture study on the EPS of *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* showed that there were increased gene expressions of alkene (C=C), aromatic (C=

C) and methylene ($-\text{CH}_2$) groups [35] in microbial structures, that may probably explain the increase of functional groups in the EPS collected from co-culture in this study.

3.1.2 Metal stress

The EPS collected from the control culture of *B. cereus* and the cultures of *B. cereus* subjected to various concentrations of Zn (10, 20, 30, 40, 50 mg L^{-1}) appeared white in colour. These EPS were also soluble when added into water, but were not soluble in organic solvents (ethyl ether, absolute ethanol, acetone). This suggested that metal stress did not alter the physical properties and hydrophilic nature of the EPS produced. The yield of EPS for *B. cereus* under metal stress were similar for cultures exposed to lower levels of metal stress (10–30 mg L^{-1}), with 0.19 to 0.29 mg mL^{-1} of EPS produced (Table 2). However, at higher metal stress levels (40–50 mg L^{-1}), the yield decreased significantly (0.14–0.15 mg mL^{-1}) compared to the control (without metal stress- 0.21 mg mL^{-1}) (Table 2). This may be attributed to the impact of metal stress on enzyme inactivation within bacterial cells, causing many cells to die off and impeding EPS production [14, 23]. Furthermore, a decrease in carbohydrate content but an increase in protein content in EPS was also observed, more evidently with the increase in metal stress (Table 2). The highest metal concentration (50 mg L^{-1} of Zn) supplemented to *B. cereus*, resulted in lower carbohydrate content (119 $\mu\text{g mg}^{-1}$) and higher protein content (49 $\mu\text{g mg}^{-1}$) as compared to control (258 and 43 $\mu\text{g mg}^{-1}$, respectively). The reduction in carbohydrate content but increase in protein content under metal stress conditions has been reported by Zeng et al. [36]. The bacterial cell is pressured to produce more glycoproteins to act as barriers against the metal pollutants and to synthesise additional extracellular transport proteins to pump excess metals out of the cell [36]. The FTIR spectrum provided further evidence on this change of carbohydrate and protein content. A lower peak detected at 3650–3200 cm^{-1} indicated decreased of hydroxyl groups ($-\text{OH}$), probably due to the decrease of polysaccharide

chains (Fig. 3). This is also supported by the reduced C-H stretching and methylene groups ($-\text{CH}_2$) detected at 3000–2850 and 1500–1320 cm^{-1} , respectively. There is also a new peak appearing at 1600–1450 cm^{-1} , which indicates the addition of a new aromatic group (C=C) (Fig. 3), which is hypothesised to be caused by the increase of transport proteins used to pump out metal pollutants.

3.1.3 Supplementation with molasses

The EPS collected from the control culture of *B. cereus* was white in colour, while the EPS collected from the cultures of *B. cereus* subjected to various concentrations of sugarcane molasses (1, 2, 3, 4, 5%) were dark brown in colour. All EPS collected were soluble in water, but were not soluble in organic solvents (ethyl ether, absolute ethanol, acetone). The sugarcane molasses altered only the colour of EPS while the solubility and hydrophilicity to water remained. The EPS yield for *B. cereus* increased when cultivated with low concentrations of molasses (1–3%), but decreased significantly with higher concentrations (4–5%, Table 3). With 1% molasses, the EPS yield was 0.45 mg mL^{-1} , which was significantly higher than EPS yield derived from the highest concentration of molasses (5%) (0.17 mg mL^{-1}). It is postulated that at low concentrations of molasses, bacteria cells were able to utilise the additional carbon source to grow rapidly and produce more EPS; whereas at higher concentrations of molasses, catabolite repression of oxidative pathways in bacteria cells will occur, hindering the growth rate of bacteria [18, 37].

Lower concentrations of molasses (1–3%) resulted in an increase in the carbohydrate content in the EPS, whereas higher concentrations of molasses (4–5%) resulted in lower carbohydrate content (Table 3). The carbohydrate content of EPS from 1 to 3% molasses was 302, 254 and 271 $\mu\text{g mg}^{-1}$ respectively, while the carbohydrate content of EPS at 4 and 5% molasses was significantly lower at 233 and 203 $\mu\text{g mg}^{-1}$, respectively (Table 3). This matches the hypothesis that at low molasses concentration, there is increased EPS production (increased carbohydrate content) due to increased carbon source from molasses; whereas higher levels of molasses

Table 2 The characteristics of EPS under various Zn stress (10, 20, 30, 40, 50 mg L^{-1})

EPS sample	EPS yield (mg mL^{-1})	Carbohydrate content ($\mu\text{g mg}^{-1}$)	Protein content ($\mu\text{g mg}^{-1}$)
<i>B. cereus</i>	0.21 ± 0.07	258 ± 18	43 ± 5
<i>B. cereus</i> with Zn 10 mg L^{-1}	0.24 ± 0.05	148 ± 12*	51 ± 4*
<i>B. cereus</i> with Zn 20 mg L^{-1}	0.29 ± 0.11*	182 ± 10*	52 ± 6*
<i>B. cereus</i> with Zn 30 mg L^{-1}	0.19 ± 0.06	166 ± 15*	51 ± 4*
<i>B. cereus</i> with Zn 40 mg L^{-1}	0.15 ± 0.04*	145 ± 17*	49 ± 8*
<i>B. cereus</i> with Zn 50 mg L^{-1}	0.14 ± 0.04*	119 ± 14*	49 ± 6*

*indicates a significant difference for EPS properties from *B. cereus* exposed to various Zn concentrations compared to control (*B. cereus* only, without Zn stress) according to T-test ($p < 0.05$)

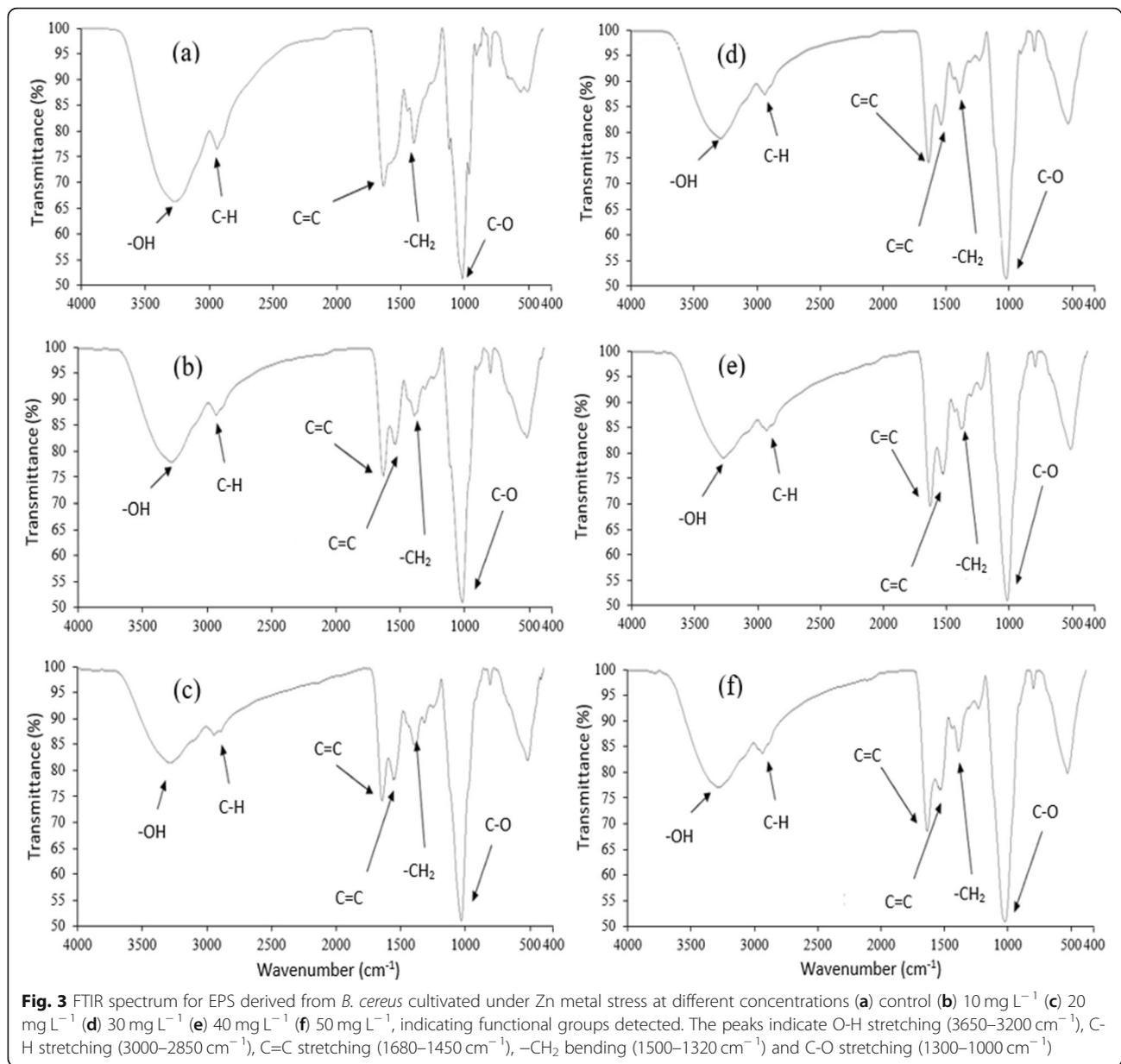


Table 3 The characteristics of EPS under various concentrations of sugarcane molasses (1, 2, 3, 4, 5%)

EPS sample	EPS yield (mg mL ⁻¹)	Carbohydrate content (μg mg ⁻¹)	Protein content (μg mg ⁻¹)
<i>B. cereus</i>	0.21 ± 0.07	258 ± 18	43 ± 5
<i>B. cereus</i> with 1% molasses	0.45 ± 0.05*	302 ± 19*	39 ± 6
<i>B. cereus</i> with 2% molasses	0.52 ± 0.03*	254 ± 13	41 ± 7
<i>B. cereus</i> with 3% molasses	0.54 ± 0.08*	271 ± 16	32 ± 5*
<i>B. cereus</i> with 4% molasses	0.22 ± 0.03	233 ± 9*	46 ± 3
<i>B. cereus</i> with 5% molasses	0.17 ± 0.03*	203 ± 19*	48 ± 4*

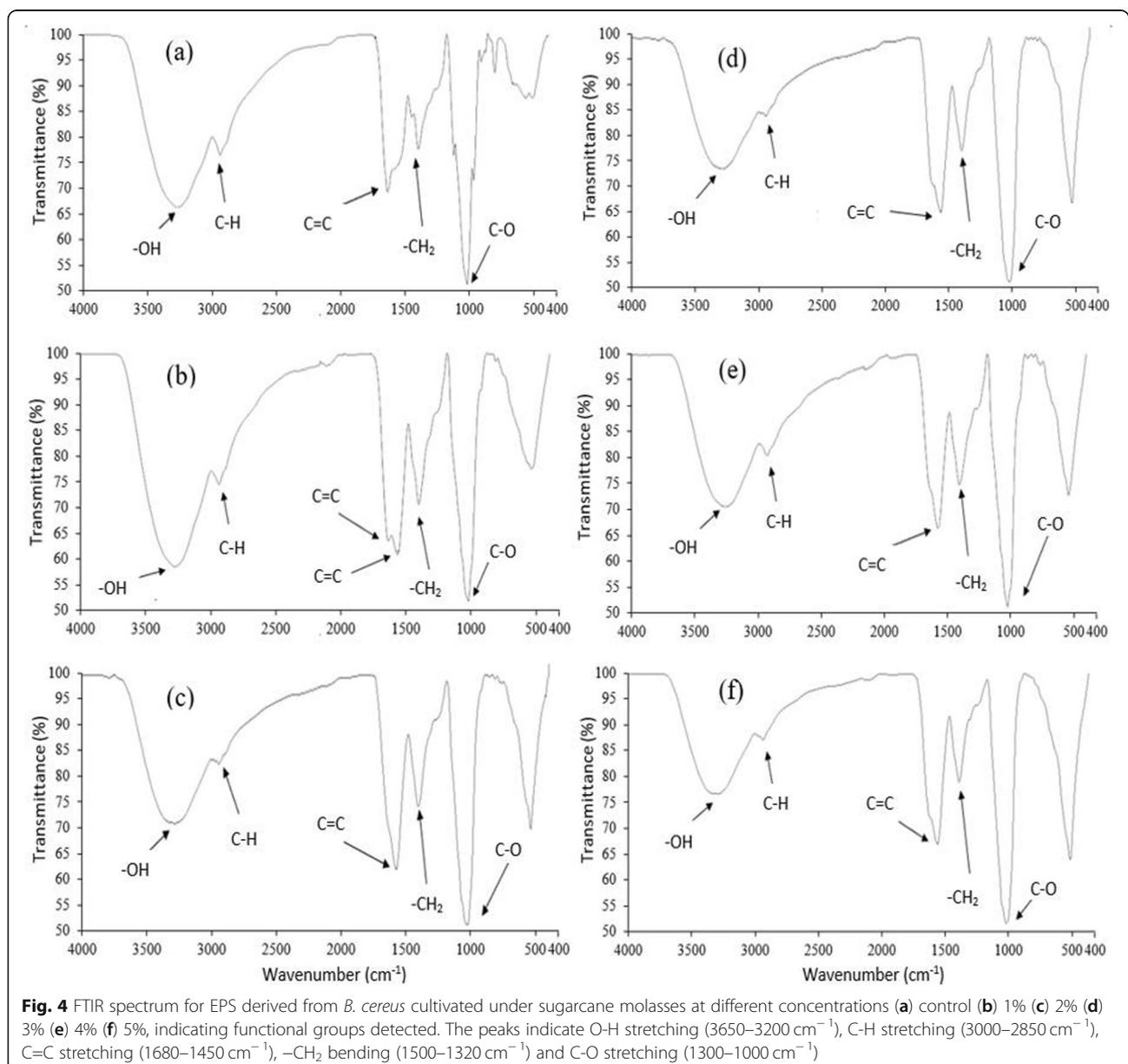
*indicates a significant difference for EPS properties from *B. cereus* cultured in various concentrations of molasses compared to control (*B. cereus* only, without molasses) according to T-tests ($p < 0.05$)

hinder the production of EPS (decreased carbohydrate content) [18, 37].

The protein content for EPS supplemented with 1 and 2% of molasses were 39 and 41 $\mu\text{g mg}^{-1}$ respectively, which were not significantly different from EPS of *B. cereus* without supplementation of molasses (control) (43 $\mu\text{g mg}^{-1}$) (Table 3). However, with 5% molasses, higher protein content (48 $\mu\text{g mg}^{-1}$) was detected compared to EPS from control (43 $\mu\text{g mg}^{-1}$) (Table 3). This is because at higher concentrations of molasses, bacterial growth may be impacted as bacterial cells attempt to pump out excess molasses present in the cells [18, 37]. Transport proteins may have been produced to remove molasses, resulting in the increased protein content [36].

At lower concentrations of molasses (1–2%), molasses are not present in excess, thus the protein content for EPS remains relatively unchanged, with levels similar to EPS from control.

This is supported by FTIR analysis whereby with low concentrations of molasses (i.e., 1%), the FTIR spectrum of the EPS revealed a sharper peak at 3650–3200 cm^{-1} and 1500–1320 cm^{-1} , indicating increased hydroxyl groups (–OH) and methylene groups (–CH₂) (Fig. 4b). There were also additional aromatic groups (C=C) appearing at 1600–1450 cm^{-1} (Fig. 4b). These changes are hypothesised to be caused by the synthesis of polysaccharide chains attributed to the carbon source from molasses [18]. Conversely, at high concentrations (i.e.,



5%), the FTIR spectrum showed a smaller peak at 3650–3200 and 3000–2850 cm^{-1} , which indicates decreased hydroxyl groups (–OH) and C–H stretching (Fig. 4b). These changes were ascribed to the probable occurrence of catabolite repression of oxidative pathways, which hindered polysaccharide formation in the EPS matrix [18, 37].

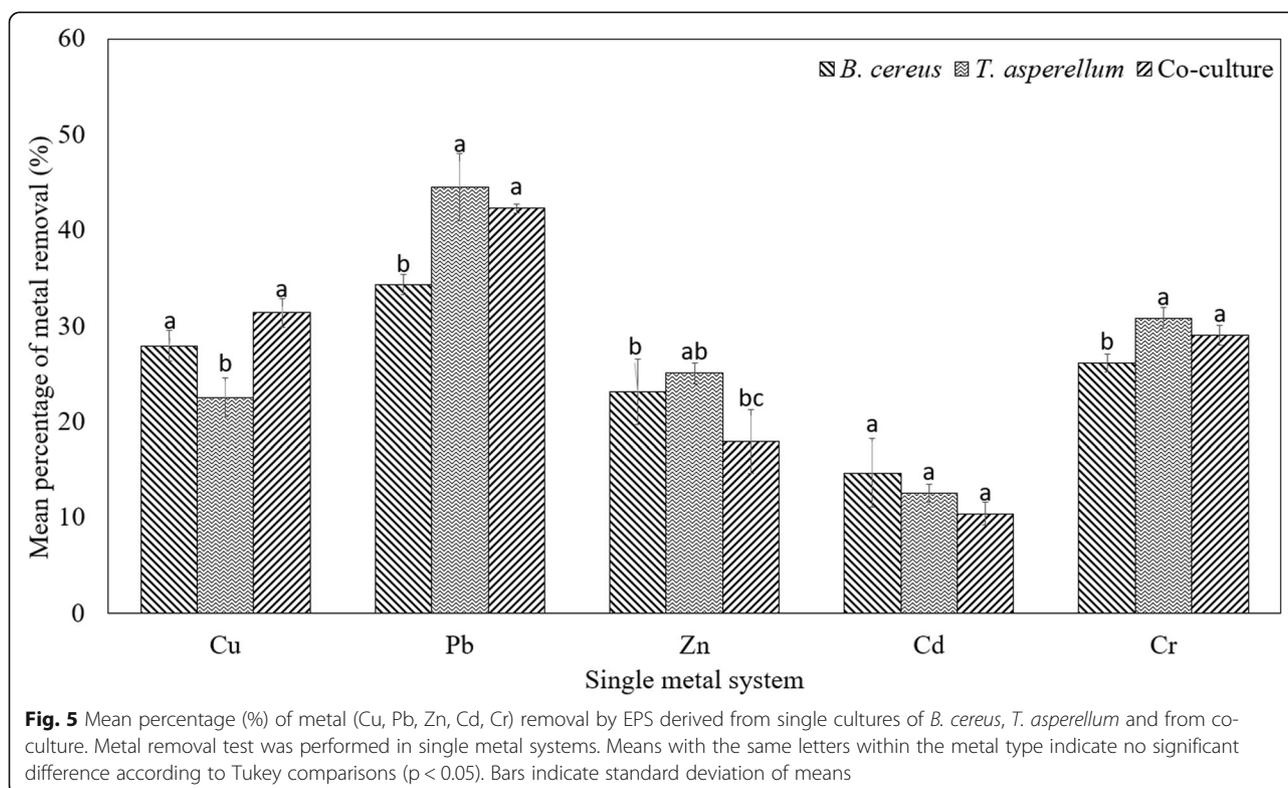
3.2 Metal removal efficacy for EPS produced from co-cultivation, metal stress and molasses

3.2.1 Co-cultivation

The metal removal efficacies for all three EPS from single cultures of *B. cereus* and *T. asperellum*, and a co-culture of *B. cereus* with *T. asperellum*, were not significantly different from each other. This suggested that co-cultivation of *B. cereus* with *T. asperellum* did not render benefit as the quality of EPS was not significantly enhanced and metal removal was not promoted. Results demonstrated that the EPS from co-culture (Cu: 31%, Pb: 42%, Zn: 18%, Cd: 10%, Cr: 29%) showed no significant improvement in metal removal compared to EPS from individual cultures of *B. cereus* (Cu: 28%, Pb: 34%, Zn: 23%, Cd: 15%, Cr: 26%) and *T. asperellum* (Cu: 23%, Pb: 45%, Zn: 25%, Cd: 13%, Cr: 31%) (Fig. 5). This is contrary to the original hypothesis, which proposed that novel EPS produced from the co-cultivation of *B. cereus* and *T. asperellum* could have the potential to produce better quality EPS. Co-culturing involves the function of

various signalling molecules such as N-acyl-homoserine lactones and autoinducers, which are responsible for cell-to-cell communication between different microbes in a co-culture [19, 38]. These signalling molecules can induce gene expression, which allows different microbes to work together to produce new biomolecules (EPS) for their shared benefit (mutualism), particularly in scenarios when microbes are facing toxic pollutants [38]. In this study, we did not further examine the signalling molecules, but it is presumed that the signalling molecules of *B. cereus* and *T. asperellum* are ineffective in activating genes to enhance production of EPS that enhanced metal removal. Although the mixture of enzymes and compounds secreted by both microbes successfully produced a novel EPS, this novel EPS was not optimal for metal biosorption.

Taking all into consideration, this interaction of *B. cereus* and *T. asperellum* in a co-culture is determined as additive to each other, whereby the novel EPS produced from the co-culture is equal in quality compared to the individual EPS from *B. cereus* and *T. asperellum*. This additive interaction indicates that although enzymes and compounds secreted by both microbes influenced the synthesis of their EPS matrix, it did not significantly improve their quality of EPS [19, 38]. This is supported by previous studies, which reported that co-cultures may have synergistic, antagonistic and additive interactions, which results in the production of EPS with greater,



lower or equal qualities, respectively, when compared to their individual components [20, 39]. In short, there is no notable advantages in using the co-culture strategy as it does not significantly improve EPS quality (metal removal efficacy) or yield as stated in Section 3.1.1.

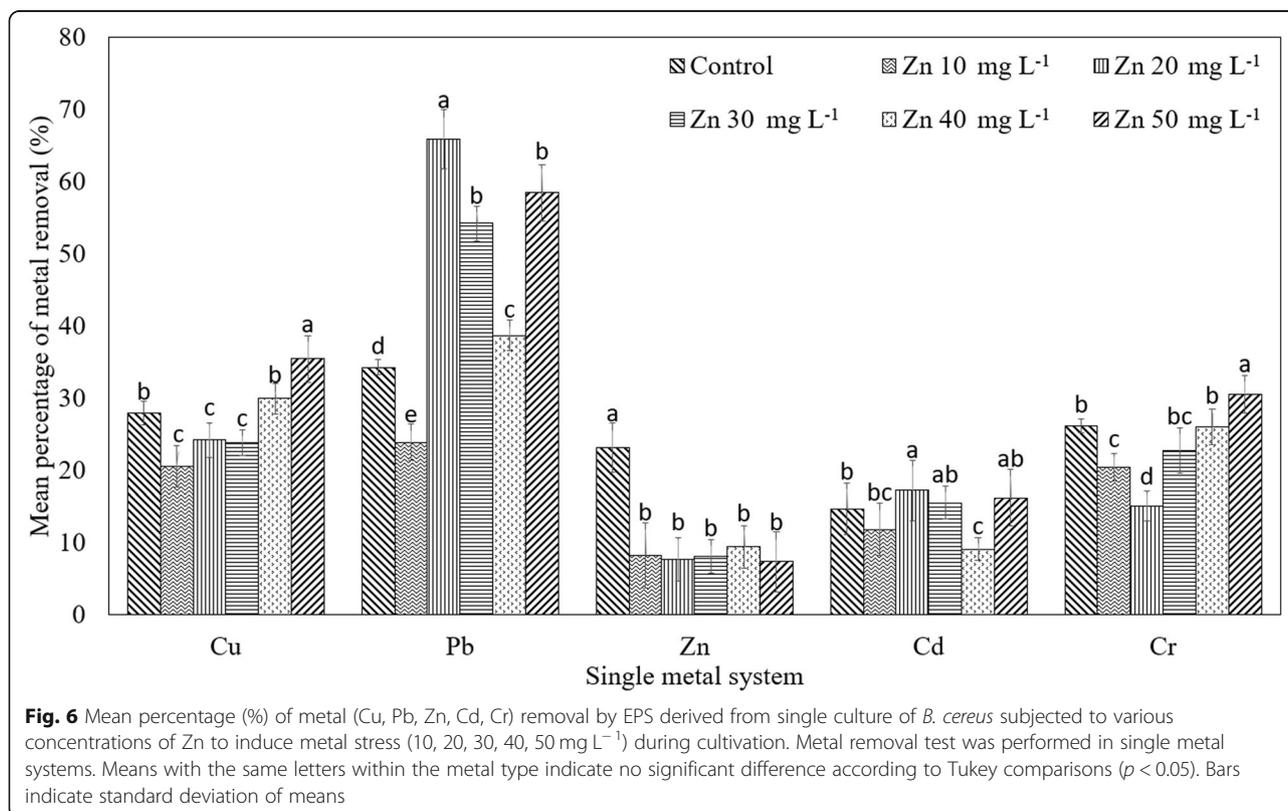
3.2.2 Metal stress

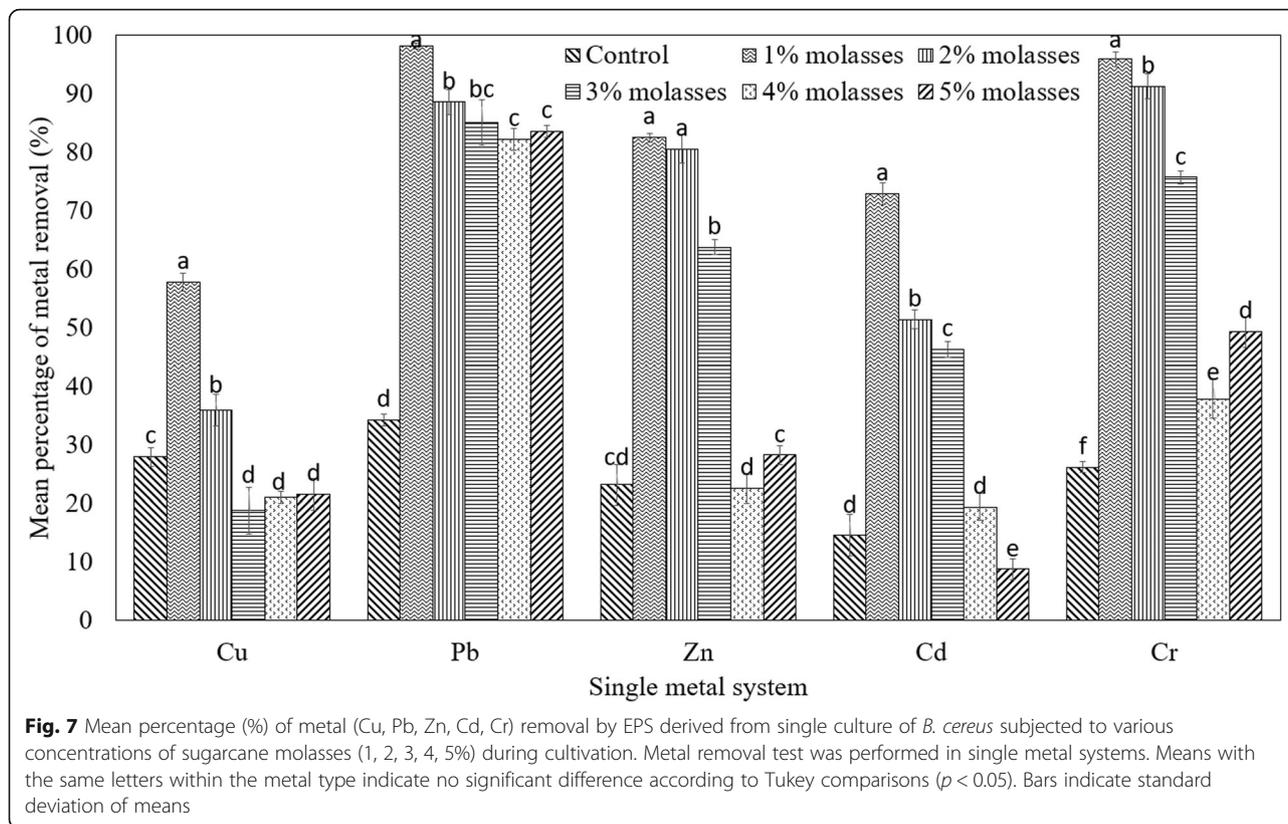
It was evident that inducing Zn metal stress during bacterial growth improved the quality of EPS (efficacy of metal removal). EPS cultivated under the highest metal stress (50 mg L^{-1}) showed significant improvements to metal removal efficacies (Cu: 35%, Pb: 58%, Zn: 7%, Cd: 16%, Cr: 31%) compared to the EPS of *B. cereus* from control (Cu: 28%, Pb: 34%, Zn: 23%, Cd: 15%, Cr: 26%), with the exception for Zn removal (Fig. 6). However, it is noted that Zn removal decreased significantly across all concentrations ($10\text{--}50 \text{ mg L}^{-1}$), probably due to the fact that the specific binding sites for Zn for EPS may already be saturated by Zn cations present in the broth during cultivation [23]. It is possible that EPS produced using Zn metal stress would not be suitable for Zn removal in wastewater, although Zn reportedly triggers metal stress in *Bacillus* spp. [23], and improved properties of EPS of *Xylella fastidiosa* and *Rhizobium leguminosarum* [40, 41]. Other than Zn, several other metals (Cu, Pb, Sb, Cd, Ni) have also been studied to induce metal stress in different microbes (*Bacillus* sp. S3, *Vibrio*

fluvialis, *Enterobacter cloacae*, *Cyanothece* sp.), with Cu and Pb identified as more effective compared to Sb, Cd and Ni in inducing metal stress and improving EPS quality [36, 42, 43]. The improved quality of EPS in removing metals is attributed to the fact that bacteria cells will produce more specialised EPS when cultivated under the influence of metal stress. During cultivation, additional glycoproteins and extracellular transport proteins are synthesised in the EPS matrix to act as barriers against metal pollutants and to transport excess metal out of the cell [36]. Although higher metal stress (Zn) may produce better quality EPS, it is noted that the EPS yield decreased (Section 3.1.2). As such, while Zn metal stress is observed to improve the quality of EPS, the yield was significantly decreased, thus it is not an ideal improvement strategy. Other metals such as Cu and Pb may be considered in future studies.

3.2.3 Supplementation with sugarcane molasses

It is evident that low levels of molasses significantly improved the quality of EPS for metal removal (Fig. 7). When low concentrations (1, 2, 3%) of sugarcane molasses was supplemented, the quality of EPS was significantly enhanced, with 1% molasses (Cu: 58%, Pb: 98%, Zn: 83%, Cd: 73%, Cr: 96%) identified as the concentration enabling the highest quality of EPS for metal removal (Fig. 7). However, at higher concentrations of





molasses, the quality of EPS decreased, with EPS from 4% molasses (Cu: 21%, Pb: 82%, Zn: 23%, Cd: 19%, Cr: 38%) and 5% molasses (Cu: 22%, Pb: 84%, Zn: 28%, Cd: 9%, Cr: 49%) reporting significantly lower metal removal efficacies compared to the control (Cu: 28%, Pb: 34%, Zn: 23%, Cd: 15%, Cr: 26%) (Fig. 7). These results were similar to other studies conducted using EPS from *B. sphaericus* and *B. subtilis*, whereby the ideal concentration of sugarcane molasses used were 1–3% [37, 44]. This phenomenon is due to the fact that at low concentrations of molasses, bacteria cells are able to utilise the additional carbon source and vitamins from molasses to synthesise additional polysaccharide chains in the EPS matrix, thus producing higher quality EPS [18]. In contrast, at high concentrations, catabolite repression of oxidative pathways in bacteria cells may occur, which hinders the formation of the EPS matrix and slows the growth rate of bacteria cells [18, 37].

Comparing the three approaches tested (co-cultivation, inducing metal stress during cultivation, supplementing molasses during cultivation), the most feasible approach would be through supplementation of molasses. This approach improved both quality and yield of EPS. Sugarcane molasses at 1% concentration produces EPS of the best quality as its metal removal is the highest across all metals and its EPS yield was double of yield of EPS from control. In addition, the quality of EPS from all three

approaches tested was acceptable. No peaks were detected at 260–280 nm when scanned from 190 to 500 nm wavelength using a UV-Vis spectrophotometer (data not shown). This indicated that the EPS was not contaminated by DNA, a common EPS contaminant. Thus, EPS supplemented with 1% of molasses is considered potentially possible for utilisation in wastewater treatment. It is also not expected that the purity of EPS would influence the metal removal efficacy as most EPS are extracted and obtained in a similar manner, and would have achieved a certain level of acceptable quality.

4 Conclusions

Of the three strategies tested, the EPS produced from supplementation with molasses was the most feasible and effective. Cultivation of EPS at low concentrations of molasses (1–3%) showed significant improvement in EPS quality and yield, with 1% molasses concentration producing EPS of the highest metal removal efficacy and a high yield. This study concludes that among the strategies of co-cultivation, metal stress, and supplementation with molasses, supplementing molasses during cultivation was the most effective as it improved both EPS yield and quality of EPS significantly. There is potential application of modified EPS via supplementation of molasses for up-scaled and applied wastewater treatment.

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Authors' contributions

Caleb Cheah, Yuen Lin Chew and Adeline Su Yien Ting contributed to the conception and design of the study. Material preparation, data collection and analysis were performed by Caleb Cheah and Adeline Su Yien Ting. The first draft of the manuscript was written by Caleb Cheah and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare that they have no conflict of interest with any parties.

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