Desalination and Water Treatment
www.deswater.com

doi: 10.1080/19443994.2015.1074116

57 (2016) 16086–16095
July

Taylor & Francis
Taylor & Francis Group

Effects of cationization hybridized biopolymer from *Bacillus subtilis* on flocculating properties

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Received 15 November 2014; Accepted 13 July 2015

ABSTRACT

Bacillus subtilis isolated from palm oil mill wastewater was able to produce biopolymer with high flocculating activity in treating kaolin clay suspension ($Al_2Si_2O_5(OH)_4$). Hybridization of the biopolymer with monovalent, divalent, and trivalent metal ions could enhance the flocculating activity. Hybridized biopolymer with divalent metal ion (Ca^{2+} or Mg^{2+}) obtained the highest flocculating activity compared to that of monovalent (K^+) and trivalent (Al^{3+}) metal ions. Biopolymer hybridized with Ca^{2+} and Mg^{2+} ions achieved 71.61 and 62.72% flocculation efficiency, respectively, at dosage of 600 mg/L. Electron configuration of the metal ions is the prime factor that affects the formation of bonding between biopolymer and kaolin clay suspension. Under optimum growth condition, *B. subtilis* was able to produce 4.15 g/L of purified biopolymer with multivalent metal ion hybridization. CHNS/O analyzer has been used to analyze the ratio of C:H:N:S:O elements contained in the biopolymer. Fourier transform infrared spectrum has been used to analyze the functional group of biopolymer and reaction between hybridized biopolymer and kaolin clay suspension.

Keywords: Biopolymers; *Bacillus subtilis*; DNA; Microbial growth; Water pollution; Kaolin clay suspension

1. Introduction

Biopolymer is a type of natural polymer that can be used for suspended solids agglomeration through formation of flocs or flakes in flocculation process. Unlike inorganic (aluminum chlorohydrate, aluminum sulfate, calcium oxide, and ferrous sulfate) and organic (chitosan, gelatin, and alginates) synthetic polymers which tend to transfer contaminants from one phase to another [1] and subsequently causing secondary environmental pollution [2]. For instance, residual aluminum is a neurotoxicant which plays a role in the etiology and pathogenesis of Alzheimer's disease [3,4]. Moreover, carbon nanotubes are widely used for filtration and separation in water and wastewater treatments, have also detrimental health and environmental impacts through interference and damage of deoxyribonucleic acid (DNA) [5]. Recently,

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biopolymer as a naturally occurring polymer has caught a great interest by many researchers due to its advantages: harmless to human and environmental, biodegradable characteristic, and lack of secondary pollution [6,7]. Commonly, soil [8–10] and activated sludge from wastewater treatment plant [11–13] are sources used to produce biopolymer. Hitherto, only two bacteria were discovered and identified from palm oil mill (POM) wastewater which are able to produce biopolymer. They were *Chryseomonas luteola* [14] and *Staphylococcus cohnii* [15] with flocculationg efficiency of 96.15 and 70.3%, respectively. Other than the above two, wastewater from POM still contains many micro-organisms that have not been isolated and identified for their potential in producing biopolymer.

In this study, *Bacillus subtilis* is one of the most striking species found in the POM wastewater. *B. subtilis* is usually found in soil samples [16–18], but it has never been reported in the presence of POM wastewater. This is the first finding of *B. subtilis* isolation from the POM wastewater to produce biopolymers. Time course of biopolymer production, effect of biopolymer with and without metal ion hybridization, and effect of metal ion dosage were studied to investigate the optimum performance of the biopolymer in treating kaolin clay suspension. The characteristics of the biopolymer were analyzed through Fourier transform infrared spectroscopy (FT-IR) and CHNS/O analysis. Morphological characteristic of the bacteria was viewed by scanning electron microscope (SEM).

2. Material and methods

2.1. Sampling

POM wastewater was collected from the anaerobic wastewater pond of MALPOM Industries Sdn. Bhd. located at Sungai Bakap, Penang, Malaysia. The collected POM wastewater sample was transferred to the laboratory and stored at −40°C in a freezer until further used.

2.2. Medium and culture condition

The culture medium contained (in gram per liter (g/L)): meat extract 10.0; peptone 10.0; yeast extract 5.0; sodium chloride 5.0; sodium thioglycollate 2.0; sodium formaldehyde sulfoxylate 1.0; and bacteriological agar 15.0. The broth medium contained (in gram per liter (g/L)): meat extract 10.0; peptone 10.0; yeast extract 5.0; sodium chloride 5.0; sodium thioglycollate 2.0; sodium formaldehyde sulfoxylate 1.0; and methylene blue 0.0002. Bacteriological agar was added as solidifying agent for the making of agar plate. Sodium thioglycollate (98% purity, Acros Organics) and sodium formaldehyde sulfoxylate (98% purity, Acros Organics) were added to the culture medium for the purpose of oxygen reduction (Laboratorios Conda 2010). This was to ensure the anaerobic condition for the broth medium. Methylene blue was added to the culture medium as an indicator of anaerobic condition. The pH of the broth medium was adjusted to pH 7.0 ± 0.2 with 1 M sodium hydroxide (NaOH) or 0.1 M hydrochloric acid (HCl) and measured using a pH meter (Metrohm, 826 pH Mobile). Subsequently, the medium was sterilized at 121°C for 15 min in the high-pressure steam sterilizer (Tomy ES-315, High-pressure Steam Sterilizer). All cultivations were done in the anaerobic chamber (Fisher Scientific, Forma Anaerobic System) at 35°C.

2.3. Isolation, characterization, and identification

Isolation of the bacteria was started with serial dilution. The serial dilution was prepared by taking out 1 mL of the raw POM wastewater and diluted with 9 mL of sterile saline water. The dilution was continued until colonies on the agar plate were countable where single inoculum micro-organism can be obtained through streaking. The single colony of the micro-organism was characterized with morphology and gram straining. The micro-organism was then identified using 16S rDNA sequences. One mL of isolated strain cultivation liquid was inoculated and centrifuged at 17,900 × g for 1 min. The bacteria genomic DNA was extracted via i-genomic DNA extraction kit (QIAGEN) with addition of 200 μL of lysis buffer, 10 μL of proteinase K, and 3 μL of RNAase A. The sample was incubated at 65°C for 20 min with the tube inverted every 2 min. After incubation, 250 μL of binding buffer, 700 μL of washing buffer, and 100 μL of elution buffer were added separately to the sample. The sample was incubated at 25°C and centrifuged at 13,000 revolutions per minute (rpm) for 1 min to elute the DNA. Lastly, the extracted DNA was stored at −80°C.

Polymerase chain reaction (PCR) amplification of 16S rDNA was performed in 50 μL of reaction mixture which contained 10 μL of 5 × colorless GoTaq® buffer, 1 mM $MgCl_2$, 0.1 mM dNTP, 0.02 U/μL of Taq DNA polymerase, 0.5 μM forward primer, 0.5 μM reverse primer, 6 ng of DNA template, and 29.3 μL of sterile deionized distilled water. Two primer pairs were used to detect and amplify the 16S rDNA sequences: 27F (5′ AGA GTT TGA TCM TGG CTC AG 3′) and 1492R (5′ TAC GGY TAC CTT GTT ACG ACT T 3′). Table 1 shows the PCR program used to amplify 16S rDNA

Table 1
PCR program used to amplify 16S rDNA with primers 27F and 1492R

Program	Temperature (°C)	Time
Initial denaturation	95	2 min
Denaturation	94	50 s
Annealing	58	45 s
Elongation	72	45 s
Final elongation	72	8 min

with primers 27F and 1492R. Amplified product was gel purified using Qiagen gel purification kit and the PCR-amplified 16S rDNA was sequenced using the ABI Prism model 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were blasted with currently available micro-organism sequences in GenBank.

2.4. Biopolymer-producing bacteria analysis

A bacteria strain was grown in culture broth by incubating in incubator shaker at 130 rpm in the anaerobic chamber at 35°C for 7 d. A small amount of sample was taken daily for standard plate counting and flocculating activity measurement. Standard plate count method was used to study growth rate for each of the bacteria strain by colony-forming unit (CFU). One mL of the sample was transferred and spread onto nutrient plate for bacteria growth. The result was recorded in the unit of CFU/mL. The best strain with the highest flocculating activity was further analyzed in various parameters such as effect of biopolymer-producing bacteria with and without metal ion hybridization and effect of metal ion dosage.

2.5. Flocculating activity determination

Flocculating activity was a measurement used to determine flocculation efficiency of biopolymer. Synthetic kaolin clay suspension (5 g/L) was used for the selectivity of the best biopolymer production. During the determination of flocculating activity, 100 mL of synthetic kaolin clay suspension was mixed with 10 mL of bacteria strain and 600 mg/L of $CaCl_2$. The mixture underwent rapid mixing at 300 rpm for 3 min, slow mixing at 100 rpm for 10 minutes using laboratory stirrer (WiseStir, MSH-20D) and was left to settle for 5 min. Twenty mL of the sample was withdrawn using automatic pipette (Eppendorf Research plus) from a depth of 2 cm below the surface of kaolin clay suspension for optical density (OD) measurement. The OD of the clarified solution (A) and control (B) were measured with spectrophotometer at 550 nm. The flocculating activity of biopolymers was calculated by applying the following equation:

$$\text{Flocculation Efficiency } (\%) = \frac{dB - dA}{dB} \times 100 \quad (1)$$

where dA and dB are the optical densities of the sample and control, respectively.

2.6. Effect of biopolymer with and without metal ion hybridization

In this study, biopolymer without metal ions hybridization was referred to biopolymer in the culture broth with no metal ion addition. On the other hand, biopolymer with metal ion hybridization was referred to biopolymer with a known dosage of metal ion (potassium ion (K^+), calcium ion (Ca^{2+}), magnesium ion (Mg^{2+}), and aluminum ion (Al^{3+})) being added into the culture broth. The culture broths were incubated together with the individual metal ions in the incubator shaker at 130 rpm and 35°C for several days until the bacteria reached its optimal growth.

2.7. Effect of multivalent metal ion dosage

Different metal ions (K^+, Ca^{2+}, Mg^{2+}, and Al^{3+}) dosages from 0.1 to 1.0 g/L were added separately to the culture broth using the same incubating method as stated in Section 2.6. Subsequently, the flocculating activity in treating kaolin clay suspension at various dosages was determined.

2.8. Biopolymer purification and characterization

The culture broth containing biopolymer-producing bacteria strain was incubated in incubator shaker at 130 rpm and 35°C for several days at the highest bacteria growth. The culture broth was then centrifuged at 6,000 rpm for 15 min at 4°C to remove cell pellets. The supernatant was mixed with two volumes of absolute chilled ethanol, stirred and left to stand at −20°C in the freezer for 2 h. The precipitate was collected after centrifugation at 6,000 rpm for 15 min at −4°C. After three such steps, the crude biopolymer was obtained and dried in vacuum freeze drier (Labconco, Freezone6). The functional groups of the crude biopolymer were analyzed and identified using FT-IR. The ratio of C:H:N:S:O elements was analyzed using CHNS/O analyzer (Perkin Elmer 2400, Series II).

2.9. Microscopic techniques

Sample preparation for SEM was performed using hexamethyldisilazane (HMDS) method. The bacteria was fixated with McDowell–Trump fixative reagent and stored at 5°C for at least 2 h. Then, it was rinsed with 0.1 M phosphate buffer and post-fixated with 1% osmium tetroxide for 1 h. The osmium tetroxide was discarded and the residue was rinsed twice with distilled water. Dehydration of the bacteria was performed by sequential immersion in serial dilution of absolute ethanol solution of 50, 75, 95, and 100% (×2) for 10 min in each dehydration process. Lastly, the sample was suspended in HMDS and left for air-dry in room temperature before put in the desiccator to maintain its low humidity level for further sputtered process. The sample was then sputtered with gold for SEM analysis (Zeiss EVO, Germany).

3. Results and discussion

3.1. Identification of biopolymer-production bacteria

A new biopolymer-producing bacteria strain was screened and isolated from POM wastewater. Base on the morphological test (Fig. 1) shows that the bacterium is in rod-shaped, gram-positive with flagellum and motile, facultative anaerobes, mesophilic temperature range and endospore formation. According to the 16S rDNA which was sequenced after PCR amplification and compared with sequences deposited in database, the similarity of the 16S rDNA sequences of the strain with *B. subtilis* (GenBank nucleotide sequence accession number is EF488090) reached up to 99%.

Normally, *B. subtilis* is referred to soil-dwelling bacterium which is easily found in soil to decay organic materials [19]. Using *B. subtilis* from POM wastewater for organic waste degradation in fermentation process with nitrate instead of oxygen as an electron acceptor was reported by Inatsu et al. [20]. According to Kunst et al. [21], a putative respiratory nitrate reductase gene was found in *B. subtilis*. An anaerobic growth of *B. subtilis* was demonstrated experimentally by Nakano et al. [22]. Hence, it has been proven that *B. subtilis* is a facultative anaerobe. Fig. 1 shows the SEM image of *B. subtilis* and the corresponding histograms of cell length and width. With the magnification of 15,000× and extra high tension of 20,000 V, *B. subtilis* is shown to be rod shaped with cell length of 1.121 μm and cell width of 565.7 nm.

3.2. Time course of the biopolymer production

B. subtilis was further analyzed for its optimal growth and biopolymer production. Fig. 2 shows the growth rate of *B. subtilis* and flocculation efficiency in treating kaolin clay suspension within the duration of 168 h. The graph displays three major phases of *B. subtilis* growth pattern: logarithmic phase (0–48 h), stationary phase (48–72 h), and death phase (72–168 h). In the logarithmic phase, flocculation efficiency increased drastically from 62.94 to 71.26%, which is directly proportional to the cell growth of *B. subtilis* (21,000–71,500 CFU/mL). The cell growth of *B. subtilis* is correlated with the production of biopolymer. As the *B. subtilis* cell growth rate increased, the biopolymer yield also increased, hence flocculation efficiency in treating kaolin clay suspension increased. This situation proved that the biopolymer was produced during the growth of *B. subtilis*, instead of cell autolysis [13,23]. Cell autolysis is referring to the

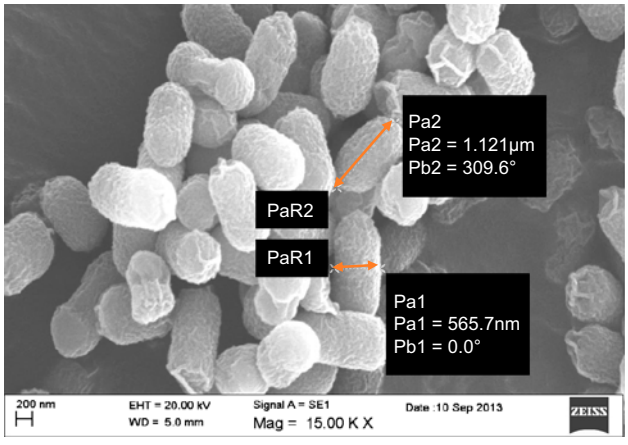

Fig. 1. Scanning electron microscopy image and cell length and width of *B. Subtilis.*

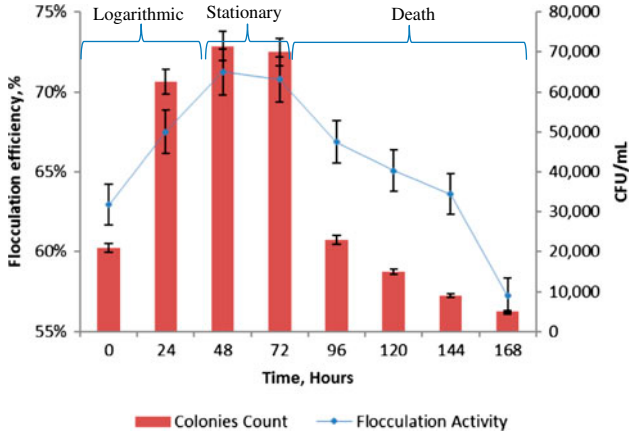

Fig. 2. Time course of *B. Subtilis* growth pattern and flocculation efficiency.

destruction of cell through the action of its own enzymes with cessation of active processes in the cell. *B. subtilis* cell growth became steadily constant from 48 to 72 h with 71,500 and 70,000 CFU/mL in the stationary stage. This phenomenon happened because growth rate and death rate of the *B. subtilis* cell are homogeneous. This equilibrium condition was resulted from nutrients' limitation in the culture broth. In conjunction with the results of bacteria growth rate, flocculation efficiency in treating kaolin clay suspension showed the same trend with an almost constant performance (70.78–71.26%). After 72 h, *B. subtilis* cell growth decreased drastically and it is known as death phase, resulting from 70,000 (72 h) to 5,000 CFU/mL (168 h). The flocculation efficiency also shows a reducing trend due to insufficient nutrients to support cell division.

3.3. The effect of biopolymer with and without metal ion hybridization

Fig. 3 demonstrates the effect of biopolymer with and without metal ion hybridization in treating kaolin clay suspension. Biopolymer without metal ion shows lower flocculation efficiency compared with biopolymer hybridized with multivalent metal ions (K^+, Ca^{2+}, Mg^{2+}, and Al^{3+}). For biopolymer without metal ion hybridization, it shows only 1.22% of flocculation efficiency. In order to improve the flocculation efficiency of biopolymer in treating kaolin clay suspension, multivalent metal ions were introduced and hybridized with the biopolymer. According to Lu et al. [23], the addition of metal ions greatly affects the flocculation of kaolin clay suspension. The biopolymer hybridized

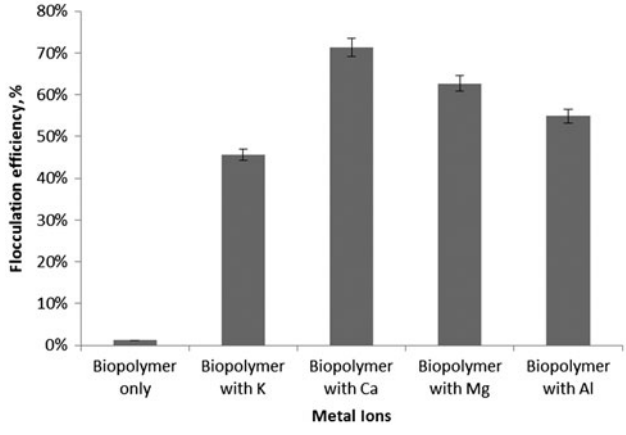

Fig. 3. The effect of biopolymer with and without metal ions hybridization on the flocculating activity (Metal ion concentration was constant at 600 mg/L and the optimal incubation period was 48 h).

with multivalent metal ions increased the flocculation efficiency by 44.34% for K^+, 70.10% for Ca^{2+}, 61.45% for Mg^{2+}, and 53.66% for Al^{3+}. This is because the biopolymer hybridized with multivalent metal ions had highly increased the initial adsorption of biopolymers on kaolin particles by increasing the positive charge of the polymer surface. The carboxylate groups in the biopolymer structure served as bonding sites for the metal ions [24]. Hence, addition of multivalent metal ions to hybrid with biopolymer improved the efficiency of treating kaolin clay suspension through the formation of complex flocs mediated by metal ions.

The effects of using biopolymers hybridized with multivalent metal ions (K^+, Ca^{2+}, Mg^{2+}, and Al^{3+}) on the flocculation efficiency in treating kaolin clay suspension are compared. Divalent metal ions in the hybrids (71.61% for Ca^{2+} and 62.72% for Mg^{2+}) show higher flocculation efficiency compared to trivalent (54.88% for Al^{3+}) and monovalent (45.56% for K^+) metal ions. The monovalent K^+ shows the lowest flocculation efficiency because it has bound stably with carboxylate group in the biopolymer. Monovalent K^+ has a single valence on its outer electron configuration to form a single ionic bond with biopolymer. Thus, K^+ possesses limitation for further complex formation since it requires very high ionization energy for second oxidation [25]. On the other hand, the presence of trivalent metal ion was also less effective than divalent metal ions in terms of flocculation efficiency. This is because the presence of trivalent Al^{3+} not only increased the positive charge density of the biopolymer, but also increased the positive charge density over the kaolin particle surface by its extra valence on its outer electron configuration, causing instability of the system. Therefore, trivalent Al^{3+} inhibits stable flocs formation between hybridized biopolymer and kaolin clay suspension. However, residual diffused ion which moves freely in the solution will cause displacement of H^+ ions in kaolin clay suspension. Adsorption of aluminum ion on kaolin could create swelling, internal stress in the molecules, and a decrease in shear stress [26]. Lastly, divalent metal ion (Ca^{2+} and Mg^{2+}) with a valence of 2+ on its outer electron configuration is able to form two ionic bonds with biopolymers and kaolin clay suspension. It holds the biopolymers and kaolin clay suspension closer and stronger together [27].

Besides the influence of the multivalent metal ions, electron configuration of the divalent ions is also a factor affecting flocculation efficiency. In Fig. 3, Ca^{2+} and Mg^{2+} have the same covalent ions of M^{2+} but they show different flocculation efficiencies in treating kaolin clay suspension, 71.61% for Ca^{2+} and 62.72% Mg^{2+}. This is because Ca^{2+} is less stable than Mg^{2+}.

Table 2
Comparison of flocculation efficiency with different type of biopolymer-producing bacteria and metal ions on suspended solid

Micro-organism	Metal ion in biopolymer	Flocculation efficiency (%)	Refs.
Bacillus sp. F19	No metal ion	97	[10]
Serratia ficaria	Ca^{2+}	94.75	[8]
Staphylococcus cohnii ssp.	Al^{3+}	88.9	[15]
Staphylococcus cohnii ssp.	Ca^{2+}	70.3	[15]
Bacillus sp. DYU1	Ca^{2+}	97	[18]
Bacillus mojavensis strain 32A	Ca^{2+}	89.7	[24]
Halobacillus sp. Mvuyo	Ca^{2+}	75.7	[29]
Cobetia sp.	Mn^{2+}	95.02	[30]
Bacillus licheniformis X14	Ca^{2+}	98.1	[31]
Bacillus subtilis	Ca^{2+}	71.32	Present study

Ca^{2+} has an electron configuration of $4s^2$ and atomic radius of 197 pm (trillionths of a meter) compared to Mg^{2+} which only has an electron configuration of $3s^2$ and atomic radius of 160 pm. Hence, Ca^{2+} can easily bind with carboxylate group in the biopolymer structure [28]. As a result, the biopolymer hybridized with Ca^{2+} significantly improved the flocculation behavior of the biopolymer by neutralizing and stabilizing the charges of kaolin clay suspension. The type of biopolymer-producing bacteria and the presence of metal ions in the biopolymers toward the flocculation efficiency are summarized in Table 2. It can be observed that Ca^{2+} is the most commonly used metal ion biopolymer hybrid for the treatment of suspension in wastewater.

3.4. The effect of multivalent metal ion dosage on production of hybridized biopolymer

This section is attempted to determine the most suitable dosage of multivalent metal ions to be added for hybridization which gave the highest flocculation efficiency. Generally, insufficient or excessive metal ions inhibit flocculation efficiency. If insufficient metal ion is added, it may cause weak strength of bond between the flocs. Subsequently, a loose structure of flocs would be formed between biopolymer and kaolin clay suspension. However, if the addition of metal ions is excessive, it may cause a drop in the flocculation efficiency. This is because excessive metal ions will increase the diffused metal ions in the broth solution. The diffused metal ion will act as a competitor for the metal ion-hybridized biopolymer by forming a bridge between the diffused metal ion and the charged colloid surface. Therefore, the diffused metal ion inhibits flocs formation between hybridized biopolymer and kaolin clay suspension. Details of the explanation are shown in the Fig. 4. With the

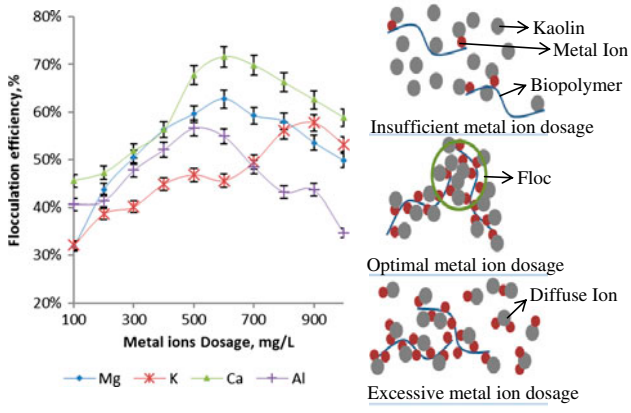

Fig. 4. The effect of multivalent metal ions dosage on flocculation efficiency.

corresponding optimum metal ion dosage used, the flocculation efficiency of hybridized biopolymer from *B. subtilis* are: 56.62% at a dosage of 500 mg/L for Al^{3+}, 71.61% at a dosage of 600 mg/L for Ca^{2+}, 62.72% at a dosage of 600 mg/L for Mg^{2+}, and 57.73% at a dosage of 900 mg/L for K^{+}. These results are supported by Wong et al. [15] who reported that metal ions play an important role in neutralization and stabilization of the negative charge for kaolin clay suspension flocculation [15]. Hence, dosage of metal ion to be added will influence the bridging effect between biopolymer and particles. It leads to the formation of stable flocs in terms of higher flocs density, bigger flocs size, and flocs resistance to shear [18].

3.5. Characteristics of biopolymer hybridized with Ca^{2+}

The biopolymer hybridized with Ca^{2+} after 48 h incubation could extract up to 4.15 g of crude biopolymer compared to unhybridized biopolymer whereby

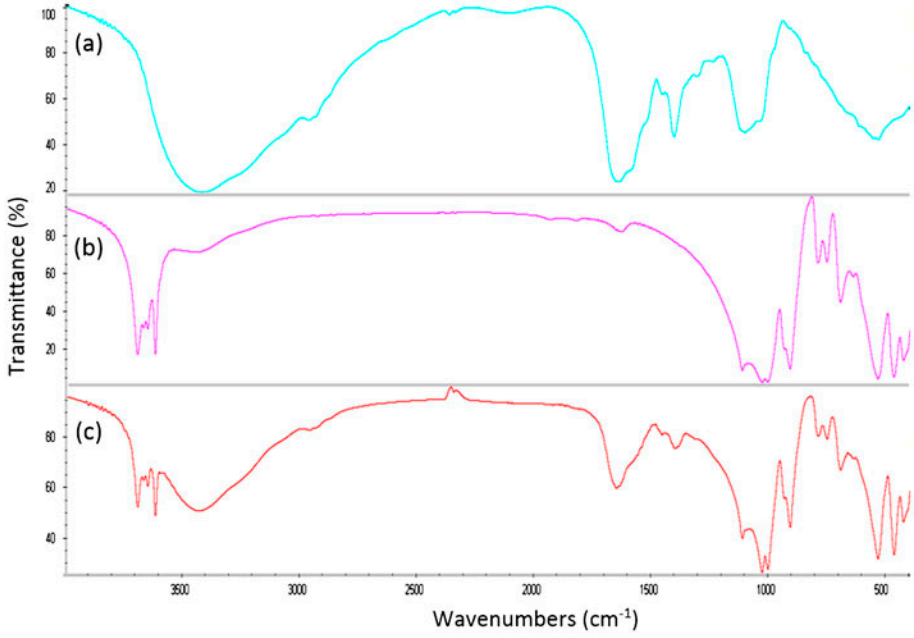

Fig. 5. The infrared spectrum of (a) purified biopolymer hybrid with Ca^{2+}, (b) kaolin clay suspension, and (c) sludge of kaolin clay suspension treated by hybridized biopolymer.

only 1.89 g of crude biopolymer was extracted from 1 L of culture broth. The yield is obviously much higher than the crude biopolymer which only 0.4 g/L obtained from *Aspergillus flavus* by Aljuboori et al. [32]. Elemental analysis of the biopolymer revealed that the weight fraction of the element C:H:O:N:S was 30.18:5.64:35.75:7.51:1.61 (w/w). Furthermore, the Fourier transform infrared spectrum of biopolymer (Fig. 5) displays a broad stretching intense peak in the range from 3,400 to 3,500 cm^{-1}, which is assigned to hydroxyl group and amine [11,33]. The peak at 3,421.29 cm^{-1} is characteristic of –OH stretching from hydroxyl group and R_2NH from secondary amine group. The peak at 2,936.89 cm^{-1} is characteristic of C–H stretching vibration. The absorption peaks at 1,639.51 and 1,580.28 cm^{-1} are C=O group and COO– stretching absorption bond, respectively [18]. The absorption peak at 1,407.54 cm^{-1} is a bending vibration of CH_3 and scissor vibration of CH_2 [24]. The strong peaks in the range from 1,000 to 1,200 cm^{-1} generally indicate as sugar derivatives. In summary, the infrared spectrum and elemental analysis show that the biopolymer is polyglutamic acid.

3.6. Final product of hybridized biopolymer in flocculation with kaolin clay suspension

The final product of kaolin clay suspension after flocculation was never reported in any literature. Fig. 6 shows the final product of hybridized biopolymer with kaolin clay suspension which is first reported in this article. The chemical composition of kaolin is $Al_2Si_2O_5(OH)_4$, with one silicon oxygen (SiO_4) tetrahedral layer and one alumina [$Al(OH)_6$] octahedral layer. In Fig. 5, the silicon oxygen (SiO_4) tetrahedral layer demonstrates three strong infrared bands in the region of 1,130–1,000 cm^{-1} [34]. The wavenumbers 912.28 cm^{-1} from FT-IR shows a strong band of Si–O–Al compounds. On the other hand, the stretching vibration of alumina octahedral is present

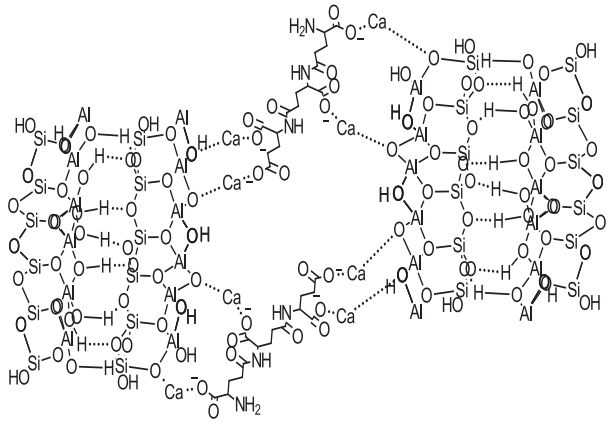

Fig. 6. The structure of the final product of calcium-hybridized biopolymer with kaolin clay suspension.

at the peaks 536.42 and 697.01 cm^{-1} [35]. The peak at 643.00 cm^{-1} is characteristic of the symmetric stretching band of Al–O bond [36]. The biopolymer produced by *B. subtilis* is glutamic acid with the chemical composition $C_5H_9NO_4$. It holds carboxylic acid at both ending sites of the organic chain. Glutamic acid is easily deprotonated to glutamate where carboxylic acid will lose a hydrogen ion (H^+) to form carboxylate anion. The FT-IR wave number of 1,580.28 cm^{-1} is the evidence of the COO– stretching absorption bond. This is because the carboxylic acid is not stable at pH values greater than 4.1. Hence, Ca^{2+} takes the opportunity to form a hybrid with carboxylate anion through ionic bonding [28]. On the other hand, the negatively charged kaolin clay suspension surface creates counter-anion for the attachment of biopolymer hybridized with Ca^{2+} [27]. It forms high-density flocs with larger floc size and high settling rate [18]. These are shown in the FT-IR spectra where distinct peaks were obtained for biopolymer hybridized with Ca^{2+}, kaolin clay suspension, and sludge of kaolin–calcium biopolymer. The appearance of wavenumbers at 3,424.99, 1,653.57, and 1,401.28 cm^{-1} in the sludge proves that the bonding between biopolymer hybridized with Ca^{2+} and kaolin clay suspension has been established.

4. Conclusion

B. subtilis—a biopolymer-producing bacterium, is first time isolated from POM wastewater and it is identified using 16S rDNA sequencing. Biopolymer hybridized with multivalent metal ions gave better flocculation performance compared to biopolymer alone in treating kaolin clay suspension. For multivalent metal ions, divalent metal ions gave the best flocculation efficiency compared to monovalent and trivalent metal ions. The optimum metal ion dosage for biopolymer hybridization was 600 mg/L for Ca^{2+} with 71.61% flocculation efficiency. In the optimal culture condition, biopolymer hybridized with Ca^{2+} after 48 h incubation could extract up to 4.15 g of crude biopolymer from 1 L of culture broth. Elemental analysis and FT-IR spectrum of the biopolymer show that the novel biopolymer is polyglutamic acid.

Acknowledgments

The authors would like to express their thanks for the support by Universiti Sains Malaysia (USM) Research University (RU) grant (1001/PTEKIND/814160) for financial support and Universiti Malaysia Perlis (UniMAP).

Nomenclature

CFU	—	Colony-forming unit
DNA	—	Deoxyribonucleic acid
FT-IR	—	Fourier transform infrared spectroscopy
HMDS	—	Hexamethyldisilazane
M	—	Molarity
PCR	—	Polymerase chain reaction
POM	—	Palm oil mill
RPM	—	Revolution per minute
SEM	—	Scanning electron microscopy

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