Manila Journal of Science ***13*** *(2020), pp. 40–52*

Effect of Bacteria Consortium, Carbon, and Cation Sources on Bioflocculant Production and Flocculating Rate

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ABSTRACT

The cohabitation of organisms within similar environment or culture conditions has been reported to enhance efficiency and production of bioflocculants due to synergistic activity. This study aimed to harvest crude bioflocculants produced from such consortium at varying culture conditions to evaluate the flocculating activity and adaptation in treatment of water. Confirmed isolates from the institute of molecular science and biotechnology were obtained and separated in to three groups comprising four isolates in each group. The isolates and the respective groups are T1 (*Providencia alcalifaciens MTK03),* T2 (*Pseudomonas otitidis MTK01*), T3 (*Aeromonas caviae MTK12*), T4 (*Aeromonas rivopollensis MTK15*), K1 (*Aeromonas veronii MTK18*), K2 (*Pseudomonas aeruginosa MTK10*), K3 (*Aeromonas caviae MTK12*), K4 (*Lysinibacillus sphaericus MTK16*), and L1 (*Aeromonas* sp. *MTK19*), L2 (*Pseudomonas* sp. *MTK17*), L3 (*Lysinibacillus sphaericus MTK20*), and L4 (*Aeromonas* sp. *MTK11*). Scanning electron microscopy with energy dispersive spectroscopy was employed to display the respective structure and elements in the bioflocculants and treatment reaction. The result revealed glucose and trivalent $FeSO_4$ as the best supportive culture condition for effective flocculating activity over sucrose and divalent $CaCl_2$. Highest and lowest flocculating activities were achieved with isolates within Group 1 but of different consortium at T1,2,4 (98%) and T3,4 (0.4%), which depicts synergistic and antagonistic activity while the least calculated overall activity across each group was 8.7% (Group 3) with glucose and $CaCl_2$ culture condition. This study has revealed that the flocculating activity of crude bioflocculant can be enhanced through the synergistic activity from consortium of bacteria isolates.

Keywords: bioflocculants, consortium, bacteria, carbon source, trivalent cation

INTRODUCTION

Bioflocculants have been neither associated with any medical problems nor reported as toxic or carcinogenic (Luvuyo, 2013). Bioflocculant does not lead to secondary pollution; it is biodegradable and does not consume reagents when compared with inorganic salts (Gao et al., 2009) and thus its potential usage in wastewater treatment plants, drinking water treatment, fermentation, and downstream processes. Synthetic flocculants are widely used because of their low cost and flocculating efficiency; nonetheless, some of their degraded monomers have been discovered to be carcinogenic and neurotoxic (Okaiyeto et al., 2016; Shih et al., 2001). Due to these demerits, biopolymers from microorganisms have been gaining attention as an alternative. Amongst other factors, the environment where the flocculant-producing organisms are isolated contributes to the stability and efficiency of the bioflocculants (Kolawole et al., 2019). Fresh water, marine water, soil, and activated sludge are some of the environments that have been documented (Cosa et al., 2013).

Coexistence of isolate in the same environment has been suggested to form a protocooperation relationship, which in turn could improve efficiency and production of bioflocculants (Kurane et al., 1994; Zhang et al., 2007). Flocculating efficiency is one of the limiting factors affecting the application of bioflocculants (Zhang et al., 2007), and to overcome this quandary, previously confirmed bioflocculants producing bacteria isolated from an open river in Ilorin, Kwara State, Nigeria (Kolawole et al., 2019), were combined to enhance production and flocculating activity as a consortium.

MATERIALS AND METHODS

Collection of Isolates

Stock culture of the isolates, namely, *Providencia alcalifaciens MTK03, Pseudomonas otitidis MTK01, Aeromonas caviae MTK12, Aeromonas rivopollensis MTK15*, *Aeromonas veronii MTK18*, *Pseudomonas aeruginosa MTK10*, *Aeromonas caviae MTK12*, *Lysinibacillus sphaericus MTK16*, *Aeromonas* sp. *MTK19*, *Pseudomonas* sp. *MTK17*, *Lysinibacillus sphaericus MTK20,* and *Aeromonas* sp. *MTK11*, were collected from the Institute of Molecular Science and Biotechnology Laboratory (IMSB), University of Ilorin, for subculturing on freshly prepared nutrient agar on a petri dish. This was further randomly distributed in to three groups (T, K, and L i.e. 1, 2 and 3 respectively) to attain the respective consortium with a maximum of four isolates per group (Table 1).

Table 1. Grouping of Isolates

Group T	Group K	Group L
Providencia alcalifaciens MTK03, Pseudomonas otitidis MTK01, Aeromonas caviae MTK12, Aeromonas rivopollensis MTK15	*Aeromonas veronii MTK18, Pseudomonas aeruginosa MTK10, Aeromonas caviae MTK12, Lysinibacillus sphaericus MTK16*	*Aeromonas* sp. *MTK19*, *Pseudomonas* sp. *MTK17*, *Lysinibacillus sphaericus MTK20*, *Aeromonas* sp. *MTK11*

Culture Media Preparation

The culture media used were nutrient agar (NA) and bioflocculant production broth medium (BPB). The nutrient agar was prepared by weighing 7 g of nutrient agar powder into a sterile conical flask and dissolving in 250 mL of distilled water, after which it was mixed thoroughly to obtain a homogeneous mixture. It was thereafter boiled with frequent agitation to aid dissolution, subsequently sterilized by autoclaving at 121°C for 15 min and then allowed to cool before use (Sagar, 2015). While the growth medium for bioflocculants production was composed of glucose (10 g) or sucrose (10 g) when varying, $MgSO_4.7H_2O$ (0.3 g), K_2HPO_4 (5 g), peptone (1 g), and KH_2PO_4 (2 g) in a liter of distilled water at pH 7 and sterilized by autoclaving (Zhang et al., 2007).

Bioflocculant Production and Determination of Flocculating Activity

Each bacterial isolate was inoculated into 5 mL of sterile growth medium contained in a plain bottle and incubated at 25°C with shaking at 120 rpm for 7 days. The fermentation broth was centrifuged at 4000 × g for 30 min at 4°C to sediment the cells. The cell free culture supernatant was then used to assay for the flocculating activity (Zhang et al., 2007).

Kaolin clay was used as the test material, and flocculating activity of the bioflocculants was determined according to the method described by Kurane et al. (1994) and Zhang et al. (2007) with minor modifications. A concentration (4 g/L) of kaolin suspension was made, from which 100 mL of the kaolin suspension was measured into a 250-mL flask; 3 mL of 1% $FeSO_4$ and also $CaCl_2$ when varying and 2 mL of culture supernatant were then added. The mixture was agitated vigorously for 60 s in the conical flask and then poured into a 100-mL measuring cylinder and allowed to sediment for 5 min at room temperature. Five milliliters (5 mL) of the clarified supernatant was withdrawn, and then the optical density (OD) of the clarified supernatant was measured at 550 nm with a UV spectrophotometer in duplicate and the mean was recorded. A control experiment was prepared following the same process, but the bioflocculants were replaced with distilled water, and the flocculating activity was also determined.

Flocculating activity was calculated with the following formula:

Flocculating activity

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

A = Optical density at 550 nm (OD550) of the control

B = Optical density at 550 nm (OD550) of a sample

Optimization of Culture Conditions for Bioflocculant Production

Use of Carbon Sources

Using the description of Lachhwani (2005), the effects of different carbon sources on bioflocculant production by the test bacteria of the mixed cultures were evaluated. Carbon sources, namely, glucose and sucrose, were used. The test bacteria were inoculated into the various culture broths containing these carbon sources and then incubated for 7 days at 25°C in an incubator shaker, shaking at 120 rpm; their flocculating activity was then measured according to the method described by Zhang et al. (2007).

Use of Cations

The effects of cations on flocculating activity of the bioflocculant produced by the mixed culture was done in a similar way as described above for flocculating activity using solutions of 1% (w/v) of different salts like $CaCl_2$ and $FeSO_4$ as cation sources according to He et al. (2010).

Scanning Electron Microscopy (SEM) With Energy Dispersive Spectroscopy (EDS) Analysis

The surface morphology structure of the crude bioflocculants was attained with a scanning electron microscope. Images of crude bioflocculants and kaolin clay before and after flocculation were scanned. The system was left to automatically detect the elements via energy dispersive spectroscopy (EDS) (Goldstein et al., 2003).

RESULTS AND DISCUSSION

The result in Tables 2, 3, and 4 displays the respective calculated flocculating activities of the bioflocculants from the isolates of each group, that is, T1 to T4 (*Providencia alcalifaciens MTK03, Pseudomonas otitidis MTK01, Aeromonas caviae MTK12, Aeromonas rivopollensis MTK15*), K1 to K4 (*Aeromonas veronii MTK18*, *Pseudomonas aeruginosa MTK10*, *Aeromonas caviae MTK12*, *Lysinibacillus sphaericus MTK16*), and L1 to L4 (*Aeromonas* sp. *MTK19*, *Pseudomonas* sp. *MTK17*, *Lysinibacillus sphaericus MTK20*, *Aeromonas* sp. *MTK11*). The tables also reveal the culture condition with the overall highest flocculating activity. Amongst the consortium with two isolates, T3,4, T2,3, K2,3, L1,2, L1,3, and L2,3 are the few notable ones that appeared to have the least record of activity while T123, K234, and L124 for consortium with three isolates had the least record of activity less than 10%. Figures 1 and 2 show the overall activity of the respective consortium under the respective culture conditions for the highest and lowest flocculating activities.

Table 2. Flocculating Rate of Microbial Consortium at Different Carbon and Cation Sources (Grp1)

	Glucose and $FeSO_4$	**Glucose and $CaCl_2$**	**Sucrose and $FeSO_4$**	**Sucrose and $CaCl_2$**
T1,2	84.0	22.0	17.0	65.0
T1,3	64.0	17.0	42.0	29.0
T1,4	80.0	23.0	50.0	46.0
T2,3	68.0	4.0	74.0	43.0
T2,4	78.0	16.0	44.0	68.0
T3,4	8.0	0.4	81.0	62.0
T1,2,3	49.0	8.0	83.0	34.0
T1,2,4	98.0	10.0	75.0	37.0
T1,3,4	73.0	12.0	81.0	21.0
T2,3,4	86.0	15.0	76.0	13.0
T1,2,3,4	81.0	11.0	91.0	54.0
Overall FA	**69.9%**	**12.6%**	**64.9%**	**42.9%**

Key: T1 (*Providencia alcalifaciens MTK03),* T2 (*Pseudomonas otitidis MTK01*), T3 (*Aeromonas caviae MTK12*), and T4 (*Aeromonas rivopollensis MTK15*). The recorded activities are in percentage.

Table 3. Flocculating Rate of Microbial Consortium at Different Carbon and Cation Sources (Grp2)

	Glucose and $FeSO_4$	Glucose and $CaCl_2$	Sucrose and $FeSO_4$	Sucrose and $CaCl_2$
K1,2	44.0	8.0	46.0	49.0
K1,3	87.0	15.0	53.0	65.0
K1,4	91.0	19.0	51.0	53.0
K2,3	63.0	4.0	28.0	37.0
K2,4	50.0	6.0	54.0	58.0
K3,4	82.0	13.0	45.0	57.0
K1,2,3	78.0	17.0	66.0	26.0
K1,2,4	40.0	13.0	75.0	40.0
K1,3,4	79.0	13.0	65.0	49.0
K2,3,4	61.0	9.0	42.0	44.0
K1,2,3,4	60.0	10.0	74.0	56.0
Overall FA	**66.8%**	**11.5%**	**54.5%**	**48.5%**

Key: K1 (*Aeromonas veronii MTK18*), K2 (*Pseudomonas aeruginosa MTK10*), K3 (*Aeromonas caviae MTK12*), and K4 (*Lysinibacillus sphaericus MTK16*). The recorded activities are in percentage.

Table 4. Flocculating Rate of Microbial Consortium at Different Carbon and Cation Sources (Grp3)

	Glucose and $FeSO_4$	Glucose and $CaCl_2$	Sucrose and $FeSO_4$	Sucrose and $CaCl_2$
L1,2	2.08	1.78	35.86	91.76
L1,3	68.78	9.58	85.42	54.54
L1,4	71.66	11.83	64.51	69.88
L2,3	92.66	8.14	74.01	67.99
L2,4	75.42	8.41	57.79	57.19
L3,4	73.34	15.86	57.92	81.43
L1,2,3	93.46	10.74	74.01	79.16
L1,2,4	79.88	1.90	48.92	70.26
L1,3,4	74.23	11.22	75.91	21.40
L2,3,4	35.38	15.11	53.23	85.13
L1,2,3,4	75.62	1.57	56.52	55.49
Overall FA	**67.5%**	**8.7%**	**62.2%**	**66.8%**

Key: L1 (*Aeromonas* sp. *MTK19*), L2 (*Pseudomonas* sp. *MTK17*), L3 (*Lysinibacillus sphaericus MTK20*), and L4 (*Aeromonas* sp. *MTK11*). The recorded activities are in percentage.

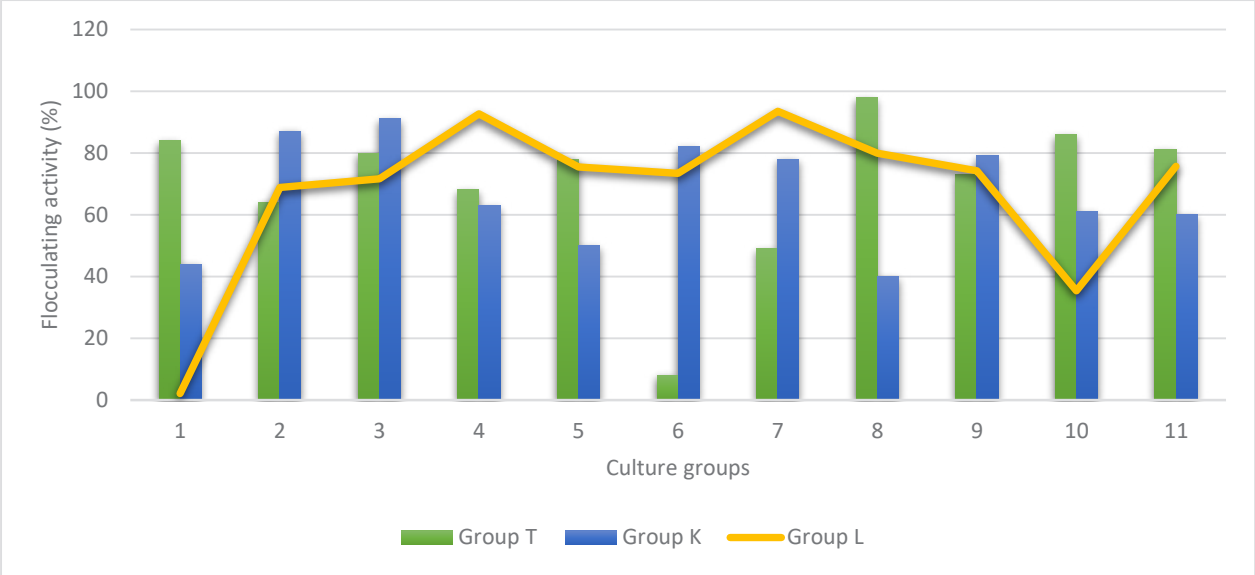

Figure 1. Flocculating activity across the groups with glucose and $FeSO_4$ (highest).

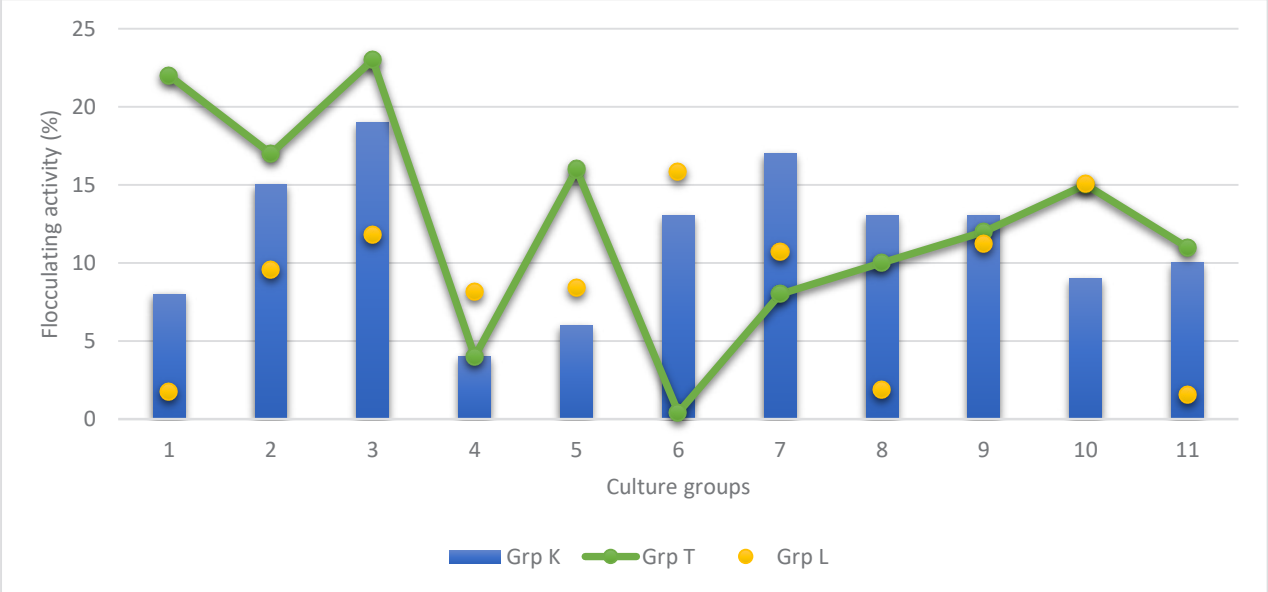

Figure 2. Flocculating activity across the groups with glucose and $CaCl_2$ (lowest).

Plates 1 and 2 show the images from the scanning electron microscopy (SEM) at 10,000 and 15,000 magnification, respectively, while Figure 3 shows the energy dispersive spectroscopy (EDS). The displayed plates include the image for kaolin clay; crude bioflocculants of T1,2,4, K1,4, and L1,2,3; and the outcome of treatment after addition of the respective crude bioflocculants to the kaolin clay suspension. The elements from the dispersal graph included sodium, sulfur, iron, aluminum, potassium, silicon, and oxygen at different concentration as displayed in Figure 3.

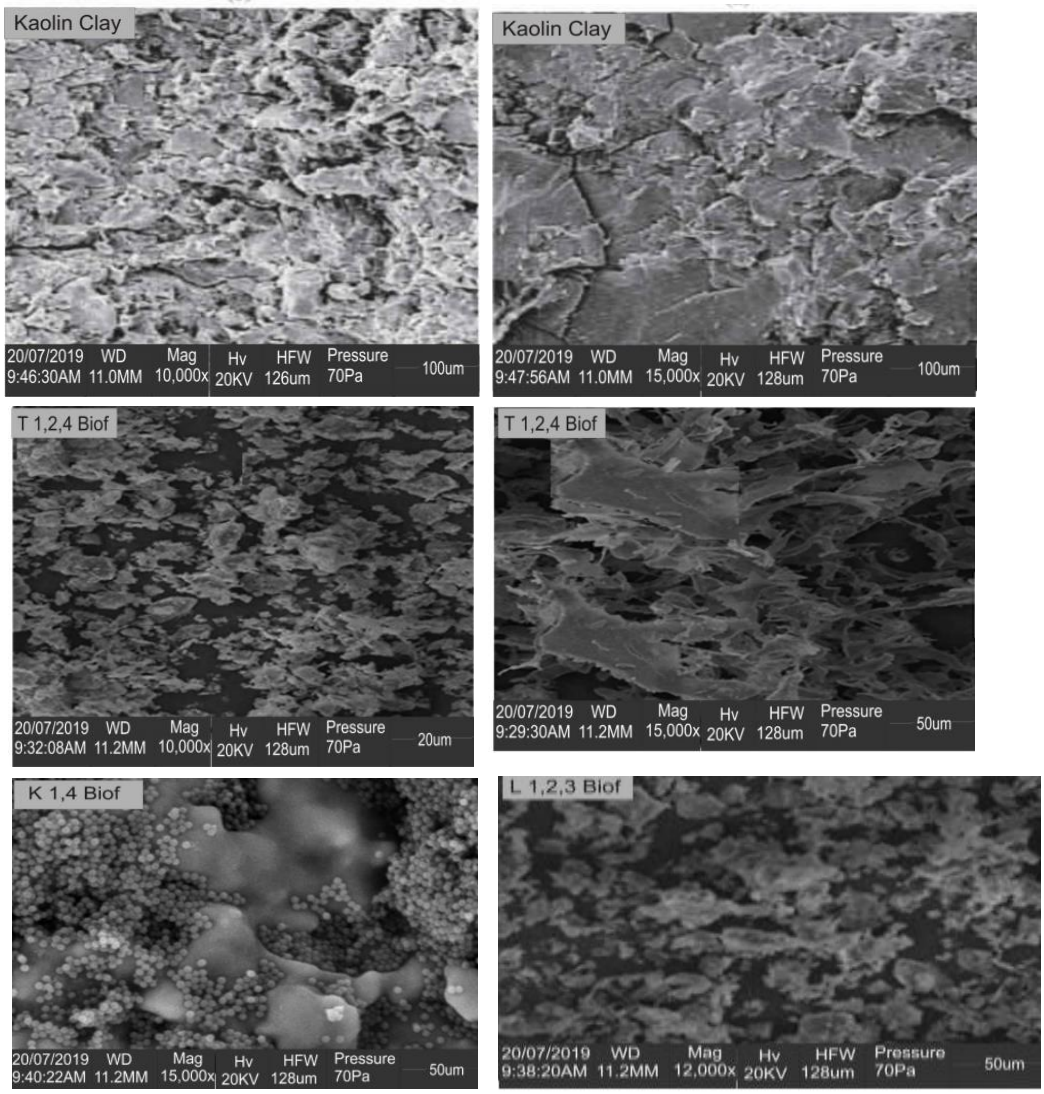

Plate 1. Scanning electron micrographs of the kaolin clay suspension and the bioflocculants from T124, K14, and L123 at different magnifications.

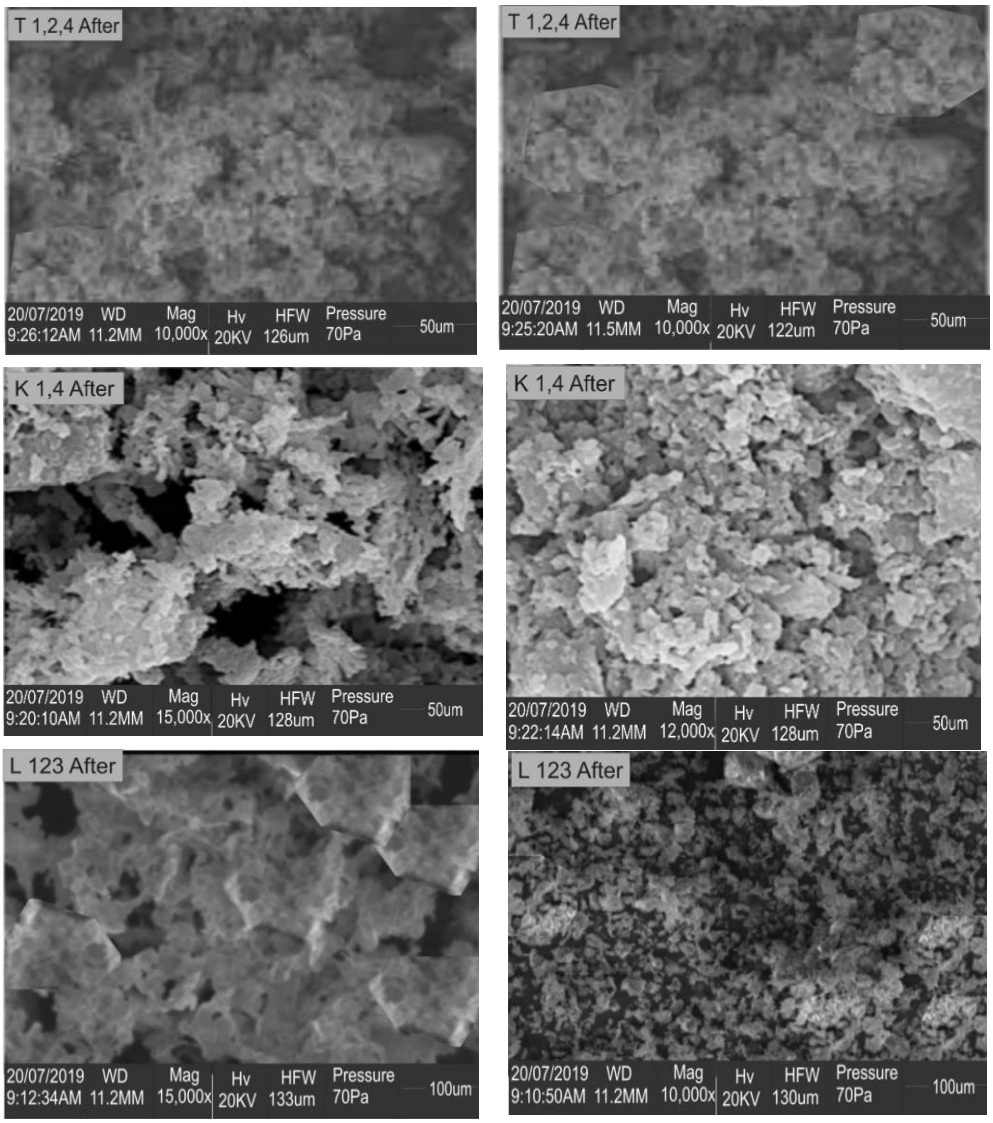

Plate 2. Scanning electron micrographs after addition of bioflocculants from T124, K14, and L123 to kaolin clay suspension at different magnifications.

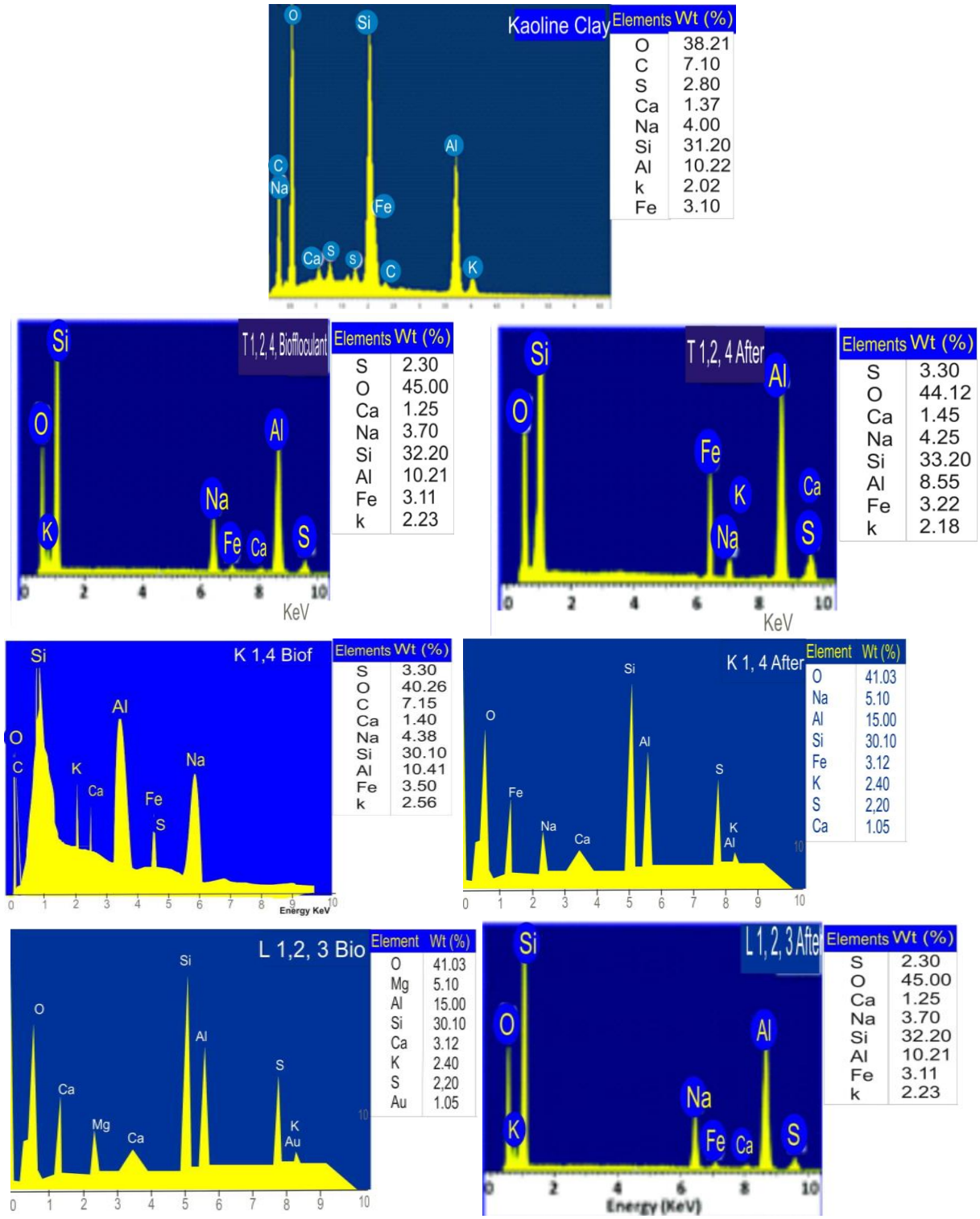

Figure 3. EDS micrographs of the kaolin clay suspension and the respective bioflocculants, after addition of the bioflocculants to the kaolin suspension.

The result across the isolates showed glucose and $FeSO_4$ as the most supportive carbon and cation source due to the higher yield that was recorded. This was followed by sucrose and $FeSO_4$ for Groups 1 and 2, but for the third group, the use of $CaCl_2$ as replacement for $FeSO_4$ was revealed to have enhanced the flocculating activity better (Table 4). This was evaluated by the cumulative percentage of the activities across the respective consortium. This report is contrary to that of Li, Chen, et al. (2009) and Li, Zhong, et al. (2009) of an enhancement of flocculating activity (FA) of bioflocculants produced by *Bacillus licheniformis* and *Bacillus circulans* in the presence of Al^{3+}, Fe^{3+}, and Ca^{2+}. A report by Luvuyo et al. (2013) revealed the activity of $CaCl_2$ to be the best divalent cation that enhanced the FA of the bioflocculants at 90% compared to other cations. This compares favorably with the report of this study in Table 4. The recorded overall enhancement of flocculating activity with glucose and $FeSO_4$ conforms to a study that reported trivalent cations such as Fe^{3+} as the most supportive (Zufarzaana et al., 2012). Kaolin clay was used as test material because its surface is negatively charged and when cations are added it has the ability to neutralize the negatively charged functional groups of the bioflocculant molecules and suspended particles thereby increasing the adsorption of the bioflocculants onto the suspended particle and resulting in effective flocculation (Li, Zhong, et al., 2009). By reducing the negative charge on kaolin particles, the distance between the molecules are shortened, and thus, floc can be formed by the binding of the bioflocculants and cation to form a colloid (Kolawole et al., 2019; Okaiyeto et al., 2014).

Furthermore, the relation between the isolates proved to be more of synergistic across the three groups with a record of 50% and above activity using a combination of glucose, sucrose, and $FeSO_4$, while the antagonistic activity was more evident in others (Tables 2 and 3). This may be due to the ability of the bacteria consortium to break down and utilize the carbon sources effectively. Similar preferences for carbon were reported by Gong et al. (2008), who stated that *Serratia ficaria* had flocculating activities of 96.69% and 97.15% using glucose and lactose as the carbon source. However, reports of carbon sources such as maltose, fructose, lactose, and starch have also been shown to enhance flocculating activity (Kolawole et al., 2019; Tsolanku et al., 2019). The highest activity was recorded amongst the culture mix of T124, which is *P. alcalifaciens MTK03*, *P. otitidis MTK01*, and *A. rivopollensis MTK15*, while the lowest (0.4%) was with the T3,4, which is *A. caviae MTK12* and *A. rivopollensis MTK15* (Table 2). Although the activity was not as high as that of T124, which had 98% FA, the highest activity for Group 2 as presented in Table 2 was recorded amongst *A. veronii MTK18* and *L. sphaericus*, that is, K1,4 with 91.0% FA, which shows a lesser synergistic relation than the former. The highest antagonistic activity interpreted as lower FA was noticed amongst the K2,3, which is *P. aeruginosa* and *A. caviae*.

Likewise, synergistic activity with the consortium of *Aeromonas* sp. *MTK19*, *Pseudomonas* sp. *MTK17*, and *L. sphaericus MTK20* yielded the highest FA while the least was recorded with *Aeromonas* sp. *MTK19* and *Pseudomonas* sp. *MTK17* (L1, 2). The activity of the produced flocculant from L1 and L2 was noticed to be generally low across the different culture conditions except with sucrose and $CaCl_2$ as the respective carbon and ion sources. This portrays that the activity of organisms can be greatly influenced by the environment. Furthermore, antagonistic activity was noticed amongst the consortium of *Aeromonas* sp. *MTK19*, *Pseudomonas* sp.

MTK17, *L. sphaericus MTK20*, and *Aeromonas* sp. *MTK11* (L1,2,3,4), L1,2,4, L2,3, L2,4, and L1,3 as examined in the evidently lower flocculating activity in comparison to other data (Table 3). However, this consortium also had the highest values of overall flocculating activity of 67.5%, 62.2%, and 66.8% compared to the other groups. Previous studies have reported flocculating activity of bioflocculants from mixed culture of genera of *Oerskovia, Acinetobacter*, *Agrobacterium*, and *Enterobacter* termed APR-3 (Kurane & Matsuyama, 1994). Although in the fermentation industries decomposition of substances by pure microbes is important, the relationship between mixed microbials has been considered to be more efficient in decomposition and removal of substances.

From the cumulative percentages across each group, the highest and lowest flocculating activities of the consortium and the respective culture were determined. The result revealed glucose as the best supportive culture condition for effective flocculating activity over sucrose but also depicted that the activity of glucose or the ability of the microorganisms to access glucose constituent for their metabolism can be limited by the choice of ion. This is evident in the use of $FeSO_4$ and $CaCl_2$, which yielded the highest and the lowest activities, respectively (Figures 1 and 2).

Surface morphology plays a vital role in the effectiveness of flocculation (Feng et al., 2009). The image (Plates 1 and 2) shows the kaolin clay sample as fine particles crowdedly dispersed all over while the spectroscopy analysis revealed the presence of the following elements and the respective percentage weights: oxygen (38.21), carbon (7.10), sulfur (2.80), calcium (1.37), sodium (4.00), silicon (31.20), aluminum (10.22), potassium (2.02), and iron (3.10) (Figure 3). The result from the SEM image is contrary to a study by Ayat et al. (2017), where separate particles were obtained using ordinary clay. The elemental analysis for the respective bioflocculants is lower in some elements such as oxygen and calcium when compared to other reports from different bacteria. Furthermore, analysis of a purified bioflocculant by Tsolanku et al. (2019) reported more elemental composition. All the bioflocculants from this study are polymers of glycoprotein as confirmed by the presence of carbon, nitrogen, and oxygen at varying levels. This conforms to studies by Pathak et al. (2015) and Cosa et al. (2013). The peaks from the spectrum of the bioflocculants reveal the presence of eight elements except for crude bioflocculants from the consortium of *Aeromonas veronii MTK18* and *Lysinibacillus sphaericus MTK16*, which had nine. This SEM analysis conforms partly with Agunbiade et al. (2016) on the structural morphology of kaolin and bioflocculants; however, more elements were recorded in this study.

Conclusively, this study has revealed the enhanced flocculating activity of crude bioflocculant from consortium of bacteria isolates and thus suggests more of synergistic than antagonistic activity amongst the isolates.

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