The effect of divalent and trivalent cations on aggregation and surface hydrophobicity of selected microorganism

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ABSTRACT

This study investigated the effect of various cations (Ca^{2+} , Mg^{2+} , Al^{3+} , Mn^{2+} , Zn^{2+}) on the autoaggregation (AAG) and surface hydrophobicity (SHb) of three different bacteria (*Brevibacillus panacihumi* strain (ZB1), *Lysinibacillus fusiformis* strain (ZB2) and *Enterococcus faecalis* strain (ZL)) using a 2-level factorial design. The AAG ratio was measured from the changes in the absorbance of the media. Results show that ZB2 had maximum AAG for the three bacteria investigated. A microscopic clustering of cells was observed when Ca^{2+} was added to ZB2. The AAG was in the range of 62%, 58% and 34% for ZB2, ZB1 and ZL, respectively and correlated to the SHb. The aggregation and SHb of the microbial cells increased with increasing ionic strength due to the repulsive steric or overlap forces between the polymer covered surfaces. Ca^{2+} demonstrated a more significant effect on aggregation and SHb of microbial cells due to an attractive binding force.

Keywords: Aggregation, Biogranules, Cations, Surface hydrophobicity

1. Introduction

Granules are formed by the interaction of microbial cells of different or similar strains through basic mechanism of autoaggregation (AAG) or coaggregation. AAG refers to the physical cell-to-cell interaction between genetically identical cells, whereas coaggregation refers to the interaction between genetically distinct bacterial cells. Microbial aggregation is a part of initial cell interaction which results in granule formation. Several factors such as type of seed sludge, type and concentration of substrate, presence of extracellular polymeric substrate (EPS), composition of the media, pH, temperature and operational set-up of the reactor can affect the initial cell interactions. Additionally, surface hydrophobicity (SHb) can also influence granule formation. Cell aggregation and SHb reportedly have a good correlation to the adhesion capacity of bacteria [1-3]. The factors that influence the adhesion ability of microbials cells have been widely investigated and could be measured through microbial aggregation and SHb [2, 4-10].

Granular sludge formed by cell immobilization consist of biofilm, entrapped microorganisms and microbial aggregates [11]. However,

it differs from biofilm formation due to the absence of carrier materials [5, 12-15]. EPS is one of the biofilm components found in granular sludge [8, 16-17]. EPS can cause cell SHb and act as a driving force during biogranulation [5, 8-9, 18-19]. Many studies have reported that EPS and SHb are correlated. They facilitate the aggregation of bacteria and maintenance of the granular structure [8, 18, 20-21]. Several efforts have been made to enhance granulation with additives and change the start up or operating conditions either in aerobic or anaerobic systems [12]. Some of the notable additives are cations [22-26] and synthetic polymers [27-29]. Cations can facilitate the bridging of the negatively charged groups on the cell surface with the secreted EPS [23, 30-31]. This enables the initial cell interaction stage of granulation process. Nomura *et al.* 2009 reported that the aggregation of washed cells (*Lactococcus lactis* JCM 5805) slightly increased at high concentration of cations. In contrast, the aggregation of microbial cell in unwashed cell decreased with increasing cationic strength. Additionally, when EPS was present in suspensions of unwashed cells, a low concentration of cations were found during the aggregation of the microbial cells due to an attractive bridging force. From the application viewpoint, this technology could be useful in flocculation of dispersed

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sludge, and binding of negatively charged cells to promote the granulation process using cations (Ca^{2+} , Mg^{2+} , Fe^{2+} and Fe^{3+}). It is also applicable in bacterial adhesion in wastewater treatment [32].

Granular formation require a long development time depending on the experimental conditions and set-up. In some cases, granules are formed in weeks or months, but require sufficient time to grow and increase in size. Previous studies have reported that bacterial behavior or characteristic is independent of the changes in the environment [33-34].

The aim of this study was to investigate the reaction of a few selected bacteria to any change or modifications in its environment. The effect of various cations (Ca^{2+} , Mg^{2+} , Al^{3+} , Mn^{2+} and Zn^{2+}) on microbial aggregation and SHb of three bacteria (*Brevibacillus panacihumi* strain (ZB1), *Lysinibacillus fusiformis* strain (ZB2) and *Enterococcus faecalis* strain (ZL)) were examined in order to improve the aerobic granulation start-up. Statistical experimental design was used to provide a quantitative understanding of the main interaction effects of the input variables and to develop the correlation between the input variables and responses.

2. Materials and Methods

2.1. Wastewater Composition

The modified synthetic wastewater used in this study is described elsewhere [35]. Glucose (0.5 g/L), ethanol (0.125 g/L), and sodium acetate (0.5 g/L) were used as the mixed carbon sources. The trace elements used in this study were based on the composition recommended by Zhang *et al.* [24].

2.2. Preparation of Bacterial Inoculum

The microorganisms used in this study were ZB1, ZB2 and ZL. Each bacteria was grown overnight in nutrient broth. The cultures were agitated in a rotary shaker at room temperature until the exponential growth phase was reached.

2.3. Reactor Set-up

The experiment was performed using a 1 L schott bottle as reactor. Oxygen was supplied to the reactor at a fixed aeration rate of 2 L/min, in a manner similar to that of Sequential batch reactor (SBR) system in order to mimick the SBR granulation technique and process. The working volume of the experiment was 250 mL (10%v/v of inoculum was used). The bacteria was incubated until the stationary phase was attained, thereafter, analysis was carried out.

2.4. Divalent and Trivalent Cation Solutions

The effect of divalent and trivalent cations on microbial AAg and SHb of three bacteria were investigated using aluminum chloride (AlCl_3), calcium chloride (CaCl_2), magnesium sulfate (MgSO_4), manganese chloride (MnCl_2) and zinc chloride (ZnCl_2). A stock solution of 100 g/L was prepared for each cation and the working concentrations were prepared daily by means of dilution. The experimental conditions for cationic concentration and SHb during the initial cell interaction of the biogranulation pocess was based on previous study [25, 31, 36-37]. All cation solutions were sterilized at 121°C for 5 min [36] as shown in Table 1.

Table 1. Composition of Divalent/Trivalent Cation Solutions

Composition	Concentration(mg/L)
$\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (Al^{3+})	20
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (Ca^{2+})	100
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Mg^{2+})	40
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (Mn^{2+})	10
ZnCl_2 (Zn^{2+})	5

2.5. Aggregation Test

Aggregation tests were conducted to represent the adhesion ability of bacteria under the influence of divalent and trivalent cations based on the procedure used by Chen *et al.* [1] and Rahman *et al.* [2]. Each culture was incubated in the bioreactor at an aeration rate of 2 L/min. From each sample, about 10 mL of the culture was taken from the bioreactor and centrifuged at a slow centrifugation speed of 650 g for 2 min as described by Malik *et al.* [38]. Turbidity measurement was used as an indicator of the optical density (OD) of each sample at the initial and final stationary phase of bacteria growth.

The aggregation ability was expressed as AAg percentage (AAg%) and was calculated using Eq. (1):

$$\text{AAg\%} = [(\text{A}_0 - \text{A}) / \text{A}_0] \times 100 \quad (1)$$

Where

AAg% is the percentage of aggregation,

A_0 is the absorbance of cultured media at 0 h,

A is the absorbance of cultured media after centrifugation

For the AAg%, the culture can be classified into three groups: high coaggregation (HAG: > 70% Ag), medium aggregation (MAG: 20-70% Ag) and low aggregation (LAG: < 20% Ag) cultures. A high aggregation index denotes a strong tendency of the cells to agglomerate into an aggregate [7].

2.6. SHb Test

The SHb of the bacterial strains were based on the microbial adhesion to hydrocarbon assay. The SHb of bacteria was determined according to the methods described by Rahman *et al.* [2] and was modified to suit the sample used in this study.

Each bacteria culture was incubated in the aerated bioreactor. 10 mL of each sample was taken from the glass bottle (bioreactor) after the growth of bacteria reached the stationary phase. The bacterial cells were harvested by centrifugation at 4,000 rpm for 10 min. The pellets obtained were washed twice with 50 mM of K_2HPO_4 (pH 7.0) and then resuspended in the same buffer to obtain an absorbance of about 0.5 at 660 nm. Five (5) mL of bacterial suspension was mixed with 1 mL of xylene ($\text{C}_6\text{H}_4(\text{CH}_3)_2$) by vortexing for 120 s and then allowed to stand for 1 h at room temperature. The absorbance of the bacterial suspension in the aqueous phase after mixing (S) was compared to the absorbance taken at the initial stage of the experiment (S_0). Changes in absorbance due to the bacterial adhesion to the hydrocarbons was measured at 660 nm by using an OD Meter (DR5000 HACH Spectrophotometer). All the samples were analysed in duplicates. The SHb was expressed as SHb% and calculated by Eq (2):

$$\text{SHb\%} = [(\text{S}_0 - \text{S}) / \text{S}_0] \times 100 \quad (2)$$

Where

SHb% is the Percentage of SHb,

S_0 is the absorbance of sample before mixing with xylene,

S is the absorbance of sample after extraction with xylene.

2.7. Experimental Procedure

All three bacteria were cultured separately in nutrient broth until the OD was close to 1. Thereafter, about 25 mL (10% v/v) of the cultured sample was inoculated in a separate 250 mL Schott bottle containing synthetic wastewater. The inoculated synthetic sample was allowed to incubate under aerobic condition until it reached the stationary phase. The aggregation and SHb assay were then carried out. Instead of using physical shaking as commonly reported in previous aggregation test [2, 10, 39-40], aeration was used to provide a close resemblance to the techniques used in typical granulation process. 10 mL of each sample was withdrawn after the stationary phase was reached for the aggregation and SHb tests. To determine the effect of Ca^{2+} , Mg^{2+} , Al^{3+} , Mn^{2+} and Zn^{2+} on aggregation and SHb of ZL, ZB1 and ZB2 cultures, an experimental design was employed using Minitab® (Version 16.3.2). This approach showed the effect of each factor or input variable separately (termed as the main effect) and the effect of the interaction between the variables (termed as the interaction effect) on the responses (i.e., aggregation and SHb). Thus, the outcome was quantitatively determined. The experimental design followed a 2-level factorial design (coded as -1 and +1) comprising of 64 runs. The Ca^{2+} , Mg^{2+} , Al^{3+} , Mn^{2+} and Zn^{2+} range of values used in the experiments are shown in Table 2. The responses were measured as percentage aggregation (%Ag) and percentage SHb (%SHb).

Table 2. Variables and Their Values Used in the Experiment

Composition	Unit	Low value (-1)	High value (+1)
A: Ca^{2+}	mg/L	0	100
B: Mg^{2+}	mg/L	0	40
C: Al^{3+}	mg/L	0	20
D: Mn^{2+}	mg/L	0	10
E: Zn^{2+}	mg/L	0	5

3. Results and Discussion

3.1. AAg and SHb of ZL, ZB1 and ZB2 Strains in Synthetic Wastewater

ZL, ZB1 and ZB2 were obtained from the decolourising bacteria isolated from textile wastewater and palm oil mill effluent [41-43].

3.1.1. Growth profile of ZL, ZB1 and ZB2 in synthetic wastewater

Each bacteria was grown in synthetic wastewater with the addition

of 10% (v/v) nutrient broth under aerobic condition for a 24 h period. Individual bacterial growth was measured using spectrophotometer at a wavelength of 600 nm. The growth profiles were determined by plotting the value of OD_{600} vs time. The growth profile of the various bacteria (ZL, ZB1 and ZB2) were obtained as shown in Fig. 1. The bacteria reached the stationary phase in 12 h.

The AAg and SHb of the strains ZB1, ZB2 and ZL are shown in Table 3. These bacteria can be classified under the medium rank bacteria aggregation.

The plot of the measured AAg% vs the SHb% of the various strains indicates the extent of microbial adhesion to hydrocarbons. Correlation between AAg and SHb for each strain (ZB1, ZB2 and ZL)

Fig. 1. Growth profile of the various bacteria in synthetic wastewater.

Fig. 2. Correlation between the two response variables (AAg% and SHb%).

Table 3. AAg Ability and Shb of Bacterial Strains Used in the Present Study

Species	Strains	AAg (%)	AAg Rank	SHb (%)
<i>Enterococcus faecalis</i>	ZL	30.0 ± 0.04	Medium	33.0 ± 0.40
<i>Brevibacillus panacihumi</i>	ZB1	33.0 ± 0.01	Medium	33.5 ± 3.40
<i>Lysinibacillus fusiformis</i>	ZB2	47.7 ± 3.03	Medium	44.3 ± 10.20

AAg: Autoaggregation; SHb: Surface hydrophobicity; AAg rank: Autoaggregation percentages >70% ranked as high, 20-70% ranked as medium, and <20% ranked as low autoaggregation strains.

Fig. 3. Time course of AAg of ZB1, ZB2 and ZL in synthetic wastewater medium.

was also calculated. A correlation (r^2) of 0.89 was obtained between AAg and SHb for the various strains. This result indicates that a good correlation exist between AAg and SHb. Thus, SHb could be one of the determinants of AAg. The result obtained is in agreement with that of Rahman *et al.* [2].

Fig. 2 and Table 3 show the interdependence of AAg% and SHb% on each other. Thus, SHb% can be referred to AAg% for the various bacteria investigated. However, this hypothesis does not prove that it is applicable to other bacteria. SHb of bacteria can also be caused by the presence of EPS which notably causes adhesion between bacteria and floc [9]. Current understanding is based on a polymer-bridging model, where microbial aggregation is interpreted from the result of interactions of naturally produced EPS and microbial cells. EPS excreted by microbial cells play a key role in adsorbing and bridging cell surfaces. A direct correlation exist between EPS accumulation and microbial aggregation as well as bacteria SHb.

Fig. 3 shows the changes in AAg% with time for ZB1, ZB2 and ZL cultures under aerobic conditions. AAg% in ZB1, ZB2 and ZL

Fig. 4. Phase-contrast micrograph showing the morphology of (a) ZL, (b) ZB1 and (c) ZB2 after growth of 24 h (Mag. 400 x).

bacteria showed an increasing trend with time at the exponential growth phase prior to the stationary growth phase. During the stationary growth phase, there was not much increase in the AAg%. A similar observation was made by Chen *et al.* [1] who noted that the autoaggregation of *Aeromonas punctata* strain T394 and *Ochrobactrum anthropi* strain WZR occurred in the activated sludge system.

Based on the categories [2], ZL, ZB1 and ZB2 were found in the medium AAg group. Although the various bacteria fall in the same rank, AAg% for each bacteria was different. The sequence of AAg% for the three bacteria were ZB2 > ZB1 > ZL. ZB2 also ranked highest during the incubation.

The AAg of the various bacteria could further be compared through their morphology as shown in Fig. 4. Fig. 4(a) - Fig. 4(c) represent the morphological structure of ZL, ZB1 and ZB2, respectively. The results show that the lowest AAg% occurred in ZL due to small number of flocs whereas ZB1 had a cell aggregate formation with irregular-shaped flocs similar to ZB2. However, ZB2 appeared to have a dense clump of cells. Evidently, ZB2 had the most cell aggregate formation with irregular-shaped floc.

3.2. Factorial Design Analysis

3.2.1. Effect of cations on AAg and SHb of ZL, ZB1 and ZB2 bacteria

Previous studies utilized physical shaking to generate shear force [2, 7, 38]. In order to mimic the real situation of the aerobic granulation process, physical shaking method was replaced with aeration in this study. This was to ensure consistent results comparable to conventional methods. Aeration was used to introduce the shear effect onto the microorganisms in the bioreactor. Furthermore, it helped to increase the contact angle of collision between microorganisms and cations which accelerated the aggregation process. The effect of the cations on these variables (AAg and SHb) were presented by the responses produced. When the effect of one input variable affects the responses, there is an obvious interaction effect between the input variables.

The results from the factorial design were analyzed by the analysis of variance (ANOVA) to provide information on the estimated effects with their corresponding coefficients. ANOVA gave a summary of the significance of the main and interaction effects meas-

Table 4. Experimental Results of 2-Level Factorial Design Analysis (in coded levels)

Run No.	Factors					AAg (%)			SHb (%)		
	Ca ²⁺	Mg ²⁺	Al ³⁺	Mn ²⁺	Zn ²⁺	ZL	ZB1	ZB2	ZL	ZB1	ZB2
1	-1	-1	-1	-1	-1	35.6	38.5	33.9	25.7	35.9	46.3
2	+1	-1	-1	-1	-1	34.6	56.5	65.7	19.6	47.4	59.5
3	-1	+1	-1	-1	-1	17.3	39.3	45.7	24.9	43.0	37.8
4	+1	+1	-1	-1	-1	24.1	55.0	31.1	28.7	30.2	70.2
5	-1	-1	+1	-1	-1	19.3	53.7	12.7	17.3	21.9	46.7
6	+1	-1	+1	-1	-1	25.7	59.8	51.7	19.0	32.5	69.3
7	-1	+1	+1	-1	-1	16.6	45.1	16.4	12.8	29.9	46.3
8	+1	+1	+1	-1	-1	35.0	51.7	28.7	34.2	39.0	70.3
9	-1	-1	-1	+1	-1	18.6	44.8	49.8	19.0	41.8	51.4
10	+1	-1	-1	+1	-1	32.6	50.1	32.6	23.6	32.2	58.3
11	-1	+1	-1	+1	-1	15.7	46.2	28.3	17.9	38.4	43.8
12	+1	+1	-1	+1	-1	34.7	53.6	44.5	26.6	31.9	46.3
13	-1	-1	+1	+1	-1	22.3	42.9	49.1	5.3	39.1	45.4
15	-1	+1	+1	+1	-1	22.7	30.3	39.5	19.4	18.6	50.1
16	+1	+1	+1	+1	-1	31.9	62.9	63.7	28.7	45.5	72.9
17	-1	-1	-1	-1	+1	16.9	47.4	59.1	9.3	31.1	39.1
18	+1	-1	-1	-1	+1	32.1	51.7	58.8	27.5	41.6	58.8
19	-1	+1	-1	-1	+1	11.2	47.2	47.1	26.7	18.4	42.7
20	+1	+1	-1	-1	+1	30.5	56.4	53.4	11.7	40.2	61.5
21	-1	-1	+1	-1	+1	18.0	47.0	45.9	14.6	31.3	44.5
22	+1	-1	+1	-1	+1	38.1	59.3	63.4	22.1	39.1	66.3
23	-1	+1	+1	-1	+1	15.9	33.1	37.9	12.4	25.2	59.0
24	+1	+1	+1	-1	+1	30.2	58.9	45.7	19.4	46.3	58.7
25	-1	-1	-1	+1	+1	10.9	22.3	60.1	8.1	37.3	26.6
26	+1	-1	-1	+1	+1	25.2	45.6	28.1	27.3	28.2	78.5
27	-1	+1	-1	+1	+1	20.6	39.4	27.3	12.1	16.0	52.9
28	+1	+1	-1	+1	+1	32.0	47.4	70.8	25.4	31.6	70.9
29	-1	-1	+1	+1	+1	21.2	61.2	63.0	11.1	30.3	67.2
30	+1	-1	+1	+1	+1	25.2	64.9	60.2	18.3	39.6	61.8
31	-1	+1	+1	+1	+1	15.8	45.8	59.8	16.9	24.7	60.2

Table 4. (Cont'd) Experimental Results for 2-Level Factorial Design Analysis (in coded levels)

Run No.	Factors					AAg (%)			SHb (%)		
	Ca ²⁺	Mg ²⁺	Al ³⁺	Mn ²⁺	Zn ²⁺	ZL	ZB1	ZB2	ZL	ZB1	ZB2
32	+1	+1	+1	+1	+1	41.7	59.9	74.0	37.7	29.5	42.1
33	-1	-1	-1	-1	-1	29.6	33.4	54.6	33.4	34.7	42.8
34	+1	-1	-1	-1	-1	31.9	27.2	52.4	27.2	52.2	45.7
35	-1	+1	-1	-1	-1	16.4	19.6	42.3	19.6	40.9	34.3
36	+1	+1	-1	-1	-1	28.0	15.7	52.1	15.7	57.5	27.9
37	-1	-1	+1	-1	-1	13.1	20.0	21.5	20.0	55.7	27.9
38	+1	-1	+1	-1	-1	33.2	31.1	74.5	31.1	59.8	43.7
39	-1	+1	+1	-1	-1	13.7	17.1	30.6	17.1	39.1	24.4
40	+1	+1	+1	-1	-1	26.6	36.8	31.0	36.8	57.7	41.1
41	-1	-1	-1	+1	-1	17.9	13.7	63.9	13.7	36.6	35.1
42	+1	-1	-1	+1	-1	31.5	14.8	42.2	14.8	46.3	31.6
43	-1	+1	-1	+1	-1	19.4	20.6	38.8	20.6	41.3	35.7
44	+1	+1	-1	+1	-1	33.8	22.1	50.6	22.1	56.0	37.3
45	-1	-1	+1	+1	-1	13.9	6.9	50.1	6.9	30.0	42.5
46	+1	-1	+1	+1	-1	36.6	13.6	39.2	13.6	66.5	36.7
47	-1	+1	+1	+1	-1	25.6	21.8	68.1	21.8	33.5	23.2
48	+1	+1	+1	+1	-1	34.8	22.2	68.5	22.2	59.1	45.3
49	-1	-1	-1	-1	+1	15.2	6.3	35.4	6.3	41.0	21.9
50	+1	-1	-1	-1	+1	32.2	21.8	73.0	21.8	51.8	42.9
51	-1	+1	-1	-1	+1	16.8	18.9	74.7	18.9	54.8	27.3
52	+1	+1	-1	-1	+1	34.4	11.8	24.8	11.8	55.8	37.9
53	-1	-1	+1	-1	+1	13.4	10.4	45.7	10.4	37.1	36.3
54	+1	-1	+1	-1	+1	32.8	20.1	69.8	20.1	60.7	38.7
55	-1	+1	+1	-1	+1	14.7	19.6	57.0	19.6	41.6	27.7
56	+1	+1	+1	-1	+1	24.7	20.6	25.8	20.6	62.2	43.7
57	-1	-1	-1	+1	+1	16.8	6.7	72.2	6.7	21.9	39.7
58	+1	-1	-1	+1	+1	29.1	20.2	47.0	20.2	57.5	44.8
59	-1	+1	-1	+1	+1	18.2	19.7	29.3	19.7	37.7	21.0
60	+1	+1	-1	+1	+1	33.7	29.5	40.2	29.5	50.3	45.6
61	-1	-1	+1	+1	+1	29.3	19.6	49.4	19.6	64.1	32.5
62	+1	-1	+1	+1	+1	35.8	21.6	38.9	21.6	77.2	37.2
63	-1	+1	+1	+1	+1	20.9	21.5	45.2	21.5	51.0	36.6
64	+1	+1	+1	+1	+1	38.6	30.1	75.6	30.1	60.6	24.9

ured by the *P*-value. The responses obtained were statistically evaluated at 90% confidence level (*P*-value < 0.1). Table 4 shows the experimental result of the factorial design of the study. Each of the independent variable was investigated at a high (+1) and low (-1) level.

3.2.2. The main effect of cations on AAg of ZL, ZB1 and ZB2

Table 5 shows the five main effects of cations on AAg for ZL, ZB1 and ZB2 (ANOVA summary).

Calcium cation (Ca²⁺) showed a significant effect with *P*-values less than 0.10 in the ZL, ZB1 and ZB2 cultures. Thus, an increase of Ca²⁺ concentration increased the AAg%. It is well known that Ca²⁺ is an important cation for the aggregation process and can increase the bioflocculation of most bacteria [25, 31, 44]. McKinney and Horwood [45] and Tezuka *et al.* [44] proposed the rule of

divalent cationic bridging function (DCB theory) which involves the interaction between divalent cations and negatively charged functional group of the EPS. In most biogranulation development; Ca²⁺ promotes the formation of cell-to-cell bridging, improve aggregation and stabilization of the biopolymer, enhance bio-flocculation and the overall granulation process [30, 44-46].

The effect of magnesium cation (Mg²⁺) on the AAg was not significant in each of the experimented bacteria. This result is in contrast with that of Li *et al.* [47], which stated that Mg²⁺ had similar effect as Ca²⁺ on microbial aggregation which helps in the cell-to-cell bridging of microorganisms. Additionally, it has been reported that Mg²⁺ accelerated the formation of aerobic granules [23]. In this study, Mg²⁺ had a weak effect on the structure of granules and could not play a bridging and core-induced role. This may imply that magnesium enhanced the sludge granulation

Table 5. The *P*-values of the Estimated Effects of Divalent and Trivalent Cations on ZL, ZB1 and ZB2 AAg% after 24 h Aeration

Factors	ZL		ZB1		ZB2	
	Estimated effect	AAg%	Estimated effect	AAg%	Estimated effect	AAg%
Ca ²⁺	13.345	0.000	15.196	0.000	13.444	0.000
Mg ²⁺	-0.679	0.446	-1.070	0.297	0.375	0.857
Al ³⁺	0.597	0.503	6.921	0.000	3.891	0.069
Mn ²⁺	1.843	0.000	-0.932	0.363	-1.185	0.571
Zn ²⁺	-0.938	0.478	1.540	0.137	-1.497	0.475

Fig. 5. Main effects plot for ZL AAg. **Fig. 7.** Main effects plot for ZB2 AAg. **Fig. 6.** Main effects plot for ZB1 AAg.

process through biochemical function. Although Ca²⁺ and Mg²⁺ cations have similar properties, their respective interaction with biopolymers or EPS differ [48]. The bound water content of sludge was not affected by Mg²⁺ addition, but was remarkably reduced with Ca²⁺ was addition [23]. Although the effect of Mg²⁺ on AAg in the various bacteria was not statistically significant, Mg²⁺ is an important trace element and a source of nutrient for metabolic enzymes during the growth of microorganisms [15, 23].

The effect of aluminum cation (Al³⁺) on the AAg of ZB1 and ZB2 was significant, but was non-significant for ZL. The estimated main effects for ZB1 and ZB2 were 6.921 and 3.891, respectively. Fig. 5-7 show that the AAg of ZB1 and ZB2 increased with increasing Al³⁺. It has been reported that Al³⁺ can be used to reduce the required time for granule formation and increase aggregation rate [49].

Unlike most metal cations, Al³⁺ is not required either in the macro or even micro-organisms as nutrients in biological growth [50].

The effect of manganese cation (Mn²⁺) on the AAg of ZL culture was significant, but non-significant for ZB1 and ZB2 (Fig. 5). Mn²⁺ is among the essential nutrient required for microbial growth [50]. Huang *et al.* [51] utilized Mn²⁺ for augmentation during aerobic granule development. The authors found that Mn²⁺ augmentation could enhance the biogranulation process by stimulating the microbial diversity such as cell bridging and EPS secretion. Although there was a slight increase in AAg%, Mn²⁺ demonstrated the ability to cause microbial aggregation in the ZL culture. However the effect was largely different with that of Ca²⁺.

The effect of Zinc cation (Zn²⁺) on the AAg of the various bacteria was not significant. This could be attributed to inappropriate concentration of Zn²⁺ which can inhibit micro-organisms or bacteria activity. In addition, excess concentration of Zn²⁺ resulted to a decrease in EPS production and loosening of aggregated cells [52].

3.2.3. The main effect of cations on SHb of ZL, ZB1 and ZB2

The effect of calcium cations (Ca²⁺) on the SHb for the three bacteria (ZL, ZB1 and ZB2) was significant with estimated main effect of 6.556, 6.882 and 5.515, respectively (Table 6).

From the statistical analysis, it could be explained that addition of Ca²⁺ caused an increase in ZL, ZB1 and ZB2 SHb% (Fig. 8-10).

Ca²⁺ cations acted as a binder between EPS and cells by increasing the cell aggregation. At higher bacteria aggregation, higher hydrophobicity occurs which strengthens cell to cell interaction within the microbial structure [9, 35]. It serves as a protective mechanism for the cells against unfavorable environmental

Table 6. The *P*-values of the Estimated Main Effects of Divalent and Trivalent Cations on ZL, ZB1 and ZB2 SHb% after 24 h Aeration

Factors	ZL		ZB1		ZB2	
	Estimated effect	SHb%	Estimated effect	SHb%	Estimated effect	SHb%
Ca ²⁺	6.556	0.000	6.882	0.000	5.515	0.069
Mg ²⁺	4.338	0.000	-3.725	0.003	-4.624	0.124
Al ³⁺	-0.126	0.906	-0.920	0.429	0.812	0.783
Mn ²⁺	-1.387	0.201	-0.958	0.410	5.042	0.095
Zn ²⁺	-2.206	0.046	-2.058	0.082	7.262	0.019

Fig. 8. Main effects plot for ZL SHb. **Fig. 10.** Main effects plot for ZB2 SHb. **Fig. 9.** Main effects plot for ZB1 SHb.

conditions. Higgins and Novak [53] reported that the addition of Ca²⁺ to activated sludge caused a decrease of the bound water content which increased aggregation.

It is well known that the presence of EPS can enhance cell SHb [18]. EPS is produced when the bacteria is under stress conditions. Although Ca²⁺ does not produce EPS, the increase in Ca²⁺ may create stress condition on bacteria. This could increase the production of the EPS due to the changing environmental conditions [54]. The three bacteria strains used in this study could have been exposed to stress condition due to the presence of excess Ca²⁺. This was evident by the production of EPS and the subsequent SHb occurrence. Fig. 10 shows that the bonding between EPS and the aggregate cells increased with increasing Ca²⁺ concentration, suggesting that Ca²⁺ may create a stronger bond.

The result of the factorial design for the effect of calcium (Ca²⁺) on SHb was significant for all three bacteria experimented. The cell- to-cell bridging with the negatively charged sites on the EPS and bacteria surfaces increased with increasing Ca²⁺ concentration [51, 55]. Additionally, Ca²⁺ served as a cofactor or enzyme to induce the bacterial growth. Li et al. [48] reported that high EPS level was detected in anaerobic and aerobic granulation process in the presence of excess Ca²⁺ because the carbon utilization shifted towards EPS production. Increase in the cell-to-cell bridging between EPS and bacteria caused an increase in the SHb and the bacteria became more hydrophobic which facilitated adhesion or aggregation process [9]. The effect of Ca²⁺ had the same pattern with regards to the responses (AAG% and SHb%) in the presence of EPS. It is well known that EPS can capably mediate both the cohesion and adhesion of cells and play a fundamental role in sustaining the structural integrity in the development of biofilm, anaerobic and aerobic granules [38, 56].

Table 6 shows that the effect of Mg²⁺ on the SHb of ZL and ZB1 was significant, but non-significant for ZB2. The estimated main effect for ZL and ZB1 were 4.338 and 3.725, respectively. From the statistical analysis, addition of Mg²⁺ did not increase the SHb% of ZB2. Although the estimated effect of Mg²⁺ on SHb of ZB1 was negative, the effect was significant because it probably yielded a good interaction with other cations (Fig. 9). The Mg²⁺ cations promoted microbial aggregation and acted as a bridge for other bacteria surfaces and EPS. Additionally, the presence of Mg²⁺ produced higher amounts of EPS. However, further increase of Mg²⁺ did not yield an increase in the SHb% of ZB2. This result is in agreement with that of Nguyen *et al.* [49]. It is also reported that the addition of Mg²⁺ to sludge had no effect on the bound water content [57].

The effect of Al^{3+} on the SHb of ZB2 was significant, indicating that the estimated main effect of Al^{3+} on SHb was positive. Thus, AAg of ZB2 increased with increasing Al^{3+} (Fig. 10). Park *et al.* [57] reported that the concentrations of soluble and colloidal biopolymer are low when Al^{3+} are present in activated sludge, but improves the sludge dewatering property. This indicates that Al^{3+} could reduce the bound water on the surface of aggregate cells. These results suggest that Al^{3+} could improve the ZB2 SHb properties by increasing the cell aggregate bond.

3.2.4. The effect of cations on AAg for ZL, ZB1 and ZB2

Significant 2-way interactions were observed between Al^{3+} and Mn^{2+} ($\text{Al} \times \text{Mn}$), Al^{3+} and Zn^{2+} ($\text{Al} \times \text{Zn}$), Mg^{2+} and Mn^{2+} ($\text{Mg} \times \text{Mn}$) and Ca^{2+} and Zn^{2+} ($\text{Ca} \times \text{Zn}$). The interaction between Al^{3+} and Mn^{2+} was monitored at zero Mn^{2+} concentration. The AAg% slightly decreased from 25% to 22% with increasing Al^{3+} concentration from 0-20 mg/L. The effect of cations on AAg of ZL is presented in Table 7.

Fig.11 shows that the AAg increased in the range of 24-28% at a fixed Mn^{2+} and Al^{3+} concentrations of 10 mg/L and 20 mg/L, respectively. The combined addition of Al^{3+} and Zn^{2+} to the ZL culture at concentrations of 0 mg/L and 20 mg/L caused a slight decrease of AAg from 26% to 22%, but was in the range of 25% to 26% when Zn^{2+} concentration was reduced to 5 mg/L. At Al^{3+} and Zn^{2+} concentrations of 20 mg/L and 5 mg/L, AAg was about 26%.

At a Mn^{2+} concentration of 10 mg/L, the AAg of the ZL culture slightly decreased from 26% to 24%. AAg further increased to 29% with the addition of Mg^{2+} upto a concentration of 40 mg/L.

The AAg of the ZL culture decreased from 20% to 17% when Zn^{2+} concentration of 5 mg/L was added. However, the combined addition of Ca^{2+} and Zn^{2+} at a fixed concentrations of 100 mg/L and 5 mg/L caused an increase of AAg from 30% to 34% as shown in Fig. 11.

The interaction between Mg^{2+} , Al^{3+} and Zn^{2+} ($\text{Mg} \times \text{Al} \times \text{Zn}$) was the only significant 3-way interactions. In the 4-way interactions,

Table 7. The *P*-values of the Estimated Interaction Effects of Divalent and Trivalent Cations on ZL, ZB1 and ZB2 AAg% after 24 h Aeration

Factors	ZL		ZB1		ZB2	
	Estimated effect	AAg %	Estimated effect	AAg %	Estimated effect	AAg %
2-way interaction						
Ca $\times$ Al	0.635	0.476	2.083	0.047	-5.140	0.018
Mg $\times$ Al	0.812	0.363	-6.054	0.000	-2.746	0.194
Al $\times$ Mn	2.837	0.003	4.296	0.000	0.234	0.911
Al $\times$ Zn	1.942	0.035	2.865	0.008	0.420	0.841
Ca $\times$ Mg	1.228	0.173	-0.266	0.794	-2.553	0.226
Ca $\times$ Mn	0.197	0.824	2.027	0.053	-1.571	0.454
Ca $\times$ Zn	1.671	0.067	-0.960	0.349	-1.882	0.370
Mg $\times$ Mn	3.403	0.001	-0.392	0.700	-0.299	0.886
Mg $\times$ Zn	1.164	0.196	0.965	0.609	-0.176	0.933
Mn $\times$ Zn	0.528	0.553	0.522	0.346	0.748	0.720
3-way interaction						
Ca $\times$ Mg $\times$ Al	-0.528	0.557	2.183	0.038	-1.818	0.386
Ca $\times$ Al $\times$ Mn	-1.402	0.121	0.555	0.587	-1.741	0.407
Ca $\times$ Al $\times$ Zn	-0.943	0.292	-0.951	0.353	-4.594	0.034
Mg $\times$ Al $\times$ Mn	-2.698	0.159	-1.250	0.225	0.936	0.654
Mg $\times$ Al $\times$ Zn	-0.943	0.004	-0.951	0.488	-4.594	0.039
Al $\times$ Mn $\times$ Zn	-0.115	0.897	6.252	0.000	3.111	0.143
Ca $\times$ Mg $\times$ Mn	0.501	0.573	-1.371	0.184	1.137	0.587
Ca $\times$ Mg $\times$ Zn	0.199	0.573	-1.333	0.196	-4.964	0.022
Ca $\times$ Mn $\times$ Zn	-1.783	0.823	-1.241	0.228	2.346	0.266
Mg $\times$ Mn $\times$ Zn	-0.408	0.897	-1.887	0.071	-0.366	0.861
4-way interaction						
Ca $\times$ Mg $\times$ Al $\times$ Mn	1.490	0.100	0.085	0.933	5.525	0.012
Ca $\times$ Mg $\times$ Al $\times$ Zn	1.327	0.141	1.588	0.126	0.658	0.753
Ca $\times$ Al $\times$ Mn $\times$ Zn	1.769	0.053	-6.546	0.351	-2.478	0.240
Ca $\times$ Mg $\times$ Mn $\times$ Zn	2.237	0.016	-0.956	0.000	0.712	0.733
Mg $\times$ Al $\times$ Mn $\times$ Zn	1.029	0.251	-1.679	0.106	-2.48	0.242
5-way interaction						
Ca $\times$ Mg $\times$ Al $\times$ Mn $\times$ Zn	1.789	0.051	1.790	0.086	1.654	0.430

Significant Level = 90% (0.1)

Fig. 11. Interaction plot of AAg% of ZL.

Fig. 12. Interaction plot for ZB1 AAg.

the three significant groups were Ca^{2+} , Mg^{2+} , Al^{3+} and Mn^{2+} ($\text{Ca}^{2+} \times \text{Mg}^{2+} \times \text{Al}^{3+} \times \text{Mn}^{2+}$), Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Mn} \times \text{Zn}$) and Ca^{2+} , Al^{3+} , Mn^{2+} and Zn^{2+} ($\text{Ca} \times \text{Al} \times \text{Mn} \times \text{Zn}$). In the 5-way interactions, $\text{Ca} \times \text{Mg} \times \text{Al} \times \text{Mn} \times \text{Zn}$ were significant. The results of the factorial analysis for the individual and combined cationic effect on the AAg of the ZL culture varied inconsistently. The 2-way interaction effects between Ca^{2+} and Al^{3+} ($\text{Ca} \times \text{Al}$), Ca^{2+} and Mn^{2+} ($\text{Ca} \times \text{Mn}$), Mg^{2+} and Al^{3+} ($\text{Mg} \times \text{Al}$), Al^{3+} and Mn^{2+} ($\text{Al} \times \text{Mn}$) and Al^{3+} and Zn^{2+} ($\text{Al} \times \text{Zn}$) were significant as shown in Fig. 12. The AAg of ZB1 increased from 39-43% at Al^{3+} concentration of 20 mg/L. An interaction effect was observed when Ca^{2+} concentration of 100 mg/L was added to the ZB1 culture, resulting in an increase of AAg from 50-65% (Fig. 12). In contrast, the AAg of ZB1 culture decreased from 43-39% when exposed to Mn^{2+} concentration of 10 mg/L, but increased from 55% to 58% when Ca^{2+} concentration of 100 mg/L was added to the culture (Fig. 12).

The AAg of ZB1 culture also showed an increasing trend from 41% to 59% when exposed to Al^{3+} concentration of 20 mg/L but slightly increased from 49% to 50% when Mg^{2+} concentration of 40 mg/L was added to the culture (Fig. 12). However, AAg

Fig. 13. Interaction plot for ZB2 AAg.

of ZB1 culture decreased from 49% to 43% when exposed to Mn^{2+} concentration of 10 mg/L, but increased in the range of 50-55% when Al^{3+} concentration of 20 mg/L was added to the culture.

The AAg of ZB1 culture remained almost constant in the range of 46-45% when exposed to Al^{3+} concentration of 5 mg/L, but increased in the range of 50-55% when exposed to Al^{3+} and Zn^{2+} concentrations of 20 mg/L and 5 mg/L, respectively. In the 3-way interactions, the five significant effects observed were Ca^{2+} , Mg^{2+} and Al^{3+} ($\text{Ca} \times \text{Mg} \times \text{Al}$), Ca^{2+} , Mg^{2+} and Mn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Mn}$), Ca^{2+} , Mg^{2+} and Zn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Zn}$), Mg^{2+} , Al^{3+} and Zn^{2+} ($\text{Mg} \times \text{Al} \times \text{Zn}$) and Al^{3+} , Mn^{2+} and Zn^{2+} ($\text{Al} \times \text{Mn} \times \text{Zn}$). In the 4-way interactions, the only significant interaction was between Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} whereas the 5-way interactions were also significant.

In the ZB2 culture, the 2-way interaction effects between Ca^{2+} and Al^{3+} ($\text{Ca} \times \text{Al}$), Mg^{2+} and Al^{3+} ($\text{Mg} \times \text{Al}$) and Al^{3+} and Mn^{2+} ($\text{Al} \times \text{Mn}$) were significant. The AAg of ZB2 increased from 41-55% when exposed to Al^{3+} concentration of 20 mg/L, but decreased from 62-50% when Ca^{2+} concentration of 100 mg/L was added (Fig. 13). In contrast, AAg of ZB2 culture increased from 49-50% when exposed to Al^{3+} and Mg^{2+} concentrations of 20 mg/L and 40 mg/L, respectively and further increased to 59% when Mg^{2+} was excluded from the culture as shown in Table 6.

For the interaction effect between Mg^{2+} and Al^{3+} , AAg of ZB1 culture slightly increased from 55-56% when exposed to Mg^{2+} and Al^{3+} concentrations of 40 mg/L and 20 mg/L, respectively but was in the range of 50-60% when Mg^{2+} was excluded. In the ZB2 culture, AAg decreased from 52-50% when exposed to Zn^{2+} concentration of 5 mg/L and further decreased from 56-54% when Al^{3+} concentration of 20 mg/L was added to the culture.

In the 3-way interactions, the two significant interactions were between Ca^{2+} , Mg^{2+} and Mn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Mn}^{2+}$) and Ca^{2+} , Mg^{2+} and Zn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Zn}$) (figure not shown).

3.2.5. Interaction effect of cations on SHb for ZL, ZB1 and ZB2

The significant 2-way interaction effect on ZL SHb was observed between Ca^{2+} and Al^{3+} ($\text{Ca} \times \text{Al}$), Mg^{2+} and Al^{3+} ($\text{Mg} \times \text{Al}$), Mn^{2+} and Zn^{2+} ($\text{Mn} \times \text{Zn}$) and Mg^{2+} and Mn^{2+} ($\text{Mg} \times \text{Mn}$) as shown in Table 8.

The SHb of ZL culture decreased from 18-15% when exposed to Al^{3+} concentration of 20 mg/L, but increased from 21-24% when Ca^{2+} concentration of 100 mg/L was added to the culture (Fig. 14). Exposure of the ZL culture to Mn^{2+} concentration of 10 mg/L caused a decrease of SHb from 20-14%, but showed an increasing trend from 20-24% when Mg^{2+} concentration of 40 mg/L was added to the culture Fig. 14.

The SHb of ZL culture also decreased from 18-14% when exposed to Zn^{2+} concentration of 5 mg/L and further decreased from 23-21% when Zn^{2+} and Mg^{2+} concentrations of 10 mg/L and 40 mg/L was added to the culture. The SHb further decreased from 19-14% when exposed to Al^{3+} concentration of 20 mg/L and further decreased from 62-50% when Ca^{2+} concentration of 100 mg/L was added to the culture. In the 3-way interactions of the ZL culture, the five significant interactions was between Ca^{2+} , Mg^{2+} and Al^{3+} ($\text{Ca} \times \text{Mg} \times \text{Al}$), Ca^{2+} , Al^{3+} and Mn^{2+} ($\text{Ca} \times \text{Al} \times \text{Mn}$), Ca^{2+} , Al^{3+} and Zn^{2+} ($\text{Ca} \times \text{Al} \times \text{Zn}$), Mg^{2+} , Al^{3+} and Zn^{2+} ($\text{Mg}^{2+} \times \text{Al}^{3+} \times \text{Zn}^{2+}$), Ca^{2+} , Mg^{2+} and Mn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Mn}$), and

Fig. 14. Interaction plot for ZL SHb.

Table 8. The *P*-values of the Estimated Interaction Effects of Divalent and Trivalent Cations on the SHb% of ZL, ZB1 and ZB2

Factors	ZL		ZB1		ZB2	
	Estimated effect	SHb%	Estimated effect	SHb%	Estimated effect	SHb %
<i>2-way interaction</i>						
$\text{Ca} \times \text{Al}$	2.125	0.054	2.043	0.024	5.208	0.085
$\text{Mg} \times \text{Al}$	2.577	0.021	1.483	0.205	3.367	0.259
$\text{Al} \times \text{Mn}$	-0.044	0.967	0.310	0.788	9.900	0.002
$\text{Al} \times \text{Zn}$	2.179	0.049	2.046	0.084	2.691	0.365
$\text{Ca} \times \text{Mg}$	-0.353	0.742	2.724	0.024	0.275	0.926
$\text{Ca} \times \text{Mn}$	1.664	0.127	-3.022	0.013	-3.326	0.264
$\text{Ca} \times \text{Zn}$	1.654	0.130	2.757	0.022	-2.982	0.316
$\text{Mg} \times \text{Mn}$	3.971	0.001	-0.976	0.401	6.219	0.041
$\text{Mg} \times \text{Zn}$	0.001	0.999	-1.021	0.380	-0.455	0.877
$\text{Mn} \times \text{Zn}$	4.675	0.000	-0.939	0.419	-3.552	0.234
<i>3-way interaction</i>						
$\text{Ca} \times \text{Mg} \times \text{Al}$	2.697	0.016	1.484	0.205	-3.685	0.217
$\text{Ca} \times \text{Al} \times \text{Mn}$	-2.874	0.011	-0.464	0.689	-1.314	0.000
$\text{Ca} \times \text{Al} \times \text{Zn}$	-2.355	0.034	-4.902	0.000	-1.559	0.598
$\text{Mg} \times \text{Al} \times \text{Mn}$	0.639	0.552	-2.016	0.088	6.493	0.034
$\text{Mg} \times \text{Al} \times \text{Zn}$	-1.847	0.092	-0.035	0.976	-0.185	0.950
$\text{Al} \times \text{Mn} \times \text{Zn}$	1.453	0.181	-2.552	0.033	-2.016	0.496
$\text{Ca} \times \text{Mg} \times \text{Mn}$	1.191	0.271	3.118	0.011	16.488	0.000
$\text{Ca} \times \text{Mg} \times \text{Zn}$	-3.049	0.007	0.478	0.680	1.209	0.682
$\text{Ca} \times \text{Mn} \times \text{Zn}$	1.937	0.078	-1.214	0.298	4.357	0.146
$\text{Mg} \times \text{Mn} \times \text{Zn}$	-0.780	0.181	-1.758	0.135	-0.729	0.806
<i>4-way interaction</i>						
$\text{Ca} \times \text{Mg} \times \text{Al} \times \text{Mn}$	-1.216	0.261	-2.196	0.065	-1.877	0.526
$\text{Ca} \times \text{Mg} \times \text{Al} \times \text{Zn}$	2.078	0.059	-3.921	0.002	1.320	0.655
$\text{Ca} \times \text{Al} \times \text{Mn} \times \text{Zn}$	0.942	0.382	-0.304	0.006	1.903	0.520
$\text{Ca} \times \text{Mg} \times \text{Mn} \times \text{Zn}$	3.555	0.002	-3.382	0.793	3.236	0.277
$\text{Mg} \times \text{Al} \times \text{Mn} \times \text{Zn}$	0.018	0.986	2.058	0.082	0.694	0.814
<i>5-way interaction</i>						
$\text{Ca} \times \text{Mg} \times \text{Al} \times \text{Mn} \times \text{Zn}$	0.173	0.872	-3.487	0.005	-2.475	0.404

Significant Level = 90% (0.1)

Fig. 15. Interaction plot for ZB1 SHb.

Ca^{2+} , Mg^{2+} and Zn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Zn}$). In the 4-way interactions, two groups were significant including Ca^{2+} , Mg^{2+} , Al^{3+} and Zn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Al} \times \text{Zn}$) and Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Mn} \times \text{Zn}$). The 5-way interactions had significant effect on SHb (figure not shown).

In the ZB1 culture, the SHb for the 2-way interactions between Ca^{2+} and Mg^{2+} ($\text{Ca} \times \text{Mg}$), Ca^{2+} and Al^{3+} ($\text{Ca} \times \text{Al}$), Ca^{2+} and Mn^{2+} ($\text{Ca} \times \text{Mn}$), Ca^{2+} and Zn^{2+} ($\text{Ca} \times \text{Zn}$) and Mn^{2+} and Zn^{2+} ($\text{Mn} \times \text{Zn}$) were significant. The interaction effect of the cations on the SHb of ZB1 is shown in Fig. 15. The SHb decreased from 35-18% when ZB1 culture was exposed to Mg^{2+} concentration of 40 mg/L and further decreased from 39-37% when Ca^{2+} concentration of 100 mg/L was added. This showed that only Ca^{2+} effect influenced the SHb since addition of Mg^{2+} decreased the SHb. A similar decrease of SHb from 33-28% was observed when the ZB1 culture was exposed to Al^{3+} concentration of 20 mg/L, but increased from 37-39% when Ca^{2+} concentration of 100 mg/L was added (Fig. 15).

In contrast, the SHb of ZB1 culture increased from 28-32% when exposed to Mn^{2+} concentration of 10 mg/L, but decreased from 40-35% when Ca^{2+} concentration of 100 mg/L was added to the culture. The SHb slightly decreased from 36-35% when exposed to Zn^{2+} concentration of 5 mg/L and further decreased from 36-32% when Ca^{2+} concentration of 100 mg/L was added to the culture (Fig. 16). However, SHb slightly increased from 37-38% when exposed to Zn^{2+} and Mn^{2+} concentrations of 5 mg/L and 10 mg/L, respectively. In the 2-way interactions, the three significant interaction groups for SHb were Ca^{2+} and Al^{3+} ($\text{Ca} \times \text{Al}$), Al^{3+} and Mn^{2+} ($\text{Al} \times \text{Mn}$) and Mg^{2+} and Mn^{2+} ($\text{Mg} \times \text{Mn}$) (Table 8). The SHb decreased from 47-42% when exposed to Al^{3+} concentration of 20 mg/L, but increased from 47-55% when Ca^{2+} concentration of 100 mg/L was added to the culture.

In the ZB2 culture, the SHb decreased from 50-45% when exposed to Mn^{2+} concentration of 10 mg/L, but increased from 40-56% when Al^{3+} concentration of 20 mg/L was added to the culture for the three way interaction (Fig. 16). SHb also decreased from 52-50% when exposed to Mn^{2+} concentration of 10 mg/L, but increased from 40-53% when Mg^{2+} concentration of 40 mg/L was added to the culture (Fig. 16).

Fig. 16. Interaction plot for ZB2 SHb.

The five significant interaction groups in the 3-way interaction effects on SHb of ZB2 were between Ca^{2+} , Mg^{2+} and Al^{3+} ($\text{Ca} \times \text{Mg} \times \text{Al}$), Mg^{2+} , Al^{3+} and Mn^{2+} ($\text{Mg} \times \text{Al} \times \text{Mn}$), Ca^{2+} , Mg^{2+} and Mn^{2+} ($\text{Ca} \times \text{Mg} \times \text{Mn}$), Mg^{2+} , Al^{3+} and Mn^{2+} ($\text{Mg} \times \text{Al} \times \text{Mn}$) and Al^{3+} , Mn^{2+} and Zn^{2+} ($\text{Al} \times \text{Mn} \times \text{Zn}$) (Table 8). In the 4-way interactions, the two significant groups were calcium, magnesium, aluminium, and manganese ($\text{Ca}^{2+} \times \text{Mg}^{2+} \times \text{Al}^{3+} \times \text{Mn}^{2+}$) and calcium, magnesium, manganese and zinc ($\text{Ca}^{2+} \times \text{Mg}^{2+} \times \text{Mn}^{2+} \times \text{Zn}^{2+}$). The 5-way interactions also had significant effect on ZB2 SHb.

High interactions amongst the cations did not influence the AAg and SHb of microbial organisms. From the experiment and analysis, most of the cations had effects that could stimulate the microbial AAg and SHb. Literally, Ca^{2+} and Mg^{2+} were the most influential cation binder and played a vital role in microbial aggregation [23]. In biological treatment, Al^{3+} is the second most influential ion binder and it is known to enhance AAg of micro-organisms [29, 58].

The significant effect of the combination of cations on AAg and SHb for each species of bacteria tested differed. Some of the cations such as Ca^{2+} had similar effects in all three bacteria. The presence of single or a mixture of cations caused different effects towards each of the species of bacteria. The result obtained also showed that the presence of cations influenced the bacterial diversity for AAg and SHb. The relationship between AAg and SHb for the three bacteria was correlative. However the selection of the most performing cations could not rely on the significant effects obtained from ANOVA.

Rahman *et al.* [2] reported that heterogeneity of bacteria play a vital role in SHb. Heterogeneity in SHb of different bacterial strains was also reported by Del Re *et al.* [40]. According to Perez *et al.* [59], heterogeneity in SHb was due to the involvement of several factors interfering with adhesion of cells to hydrocarbons. Thus cations could be the factors that influence the heterogeneity of bacteria. Thus, different effects were observed for the various bacteria strains or species. There was good correlation between AAg and SHb in the three bacteria. This indicates that SHb and AAg results are similar and SHb could be one of the determinants of AAg. Similar to AAg, the statistical result on SHb to some extent

provides an explanation with respect to the presence of cations towards SHb of cells structure and granule formation [18, 60].

4. Conclusions

The investigated input variables i.e. divalent and trivalent cations imposed significant linear effect on the selected bacterial aggregation and SHb. The ANOVA showed an overall result. Thus, Ca^{2+} gave the most significant effect on AAg and SHb of the selected bacteria experimented. Overall, the factorial design was purposely used in this study to test the effects of cations on the selected bacteria through the estimation of the equivalent AAg% and SHb%. In the presence of the cations especially Ca^{2+} , ZB2 had an aggregation up to 62% which was the highest AAg amongst all of them. This was followed by ZB1 and ZL at 58% and 34%, respectively. The AAg of each bacteria correlated to the SHb.

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