EFFECT OF CATIONS ON MICROBIAL AGGREGATION USING *BREVIBACILLUS PANACIHUMI* STRAIN ZB1, *LYSINIBACILLUS FUSIFORMIS* STRAIN ZB2 AND *ENTEROCOCCUS FAECALIS* STRAIN ZL

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Dedicated to my beloved mak, Zaliah Binti Kamdi,
and to my brothers and sisters, Rohana Alias, Ahmad Nazri Alias, Khairani Alias,
Jamiah Alias, Ahmad Nizam Alias, Azhar Alias and Azlina Alias…

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ABSTRACT

Microbial aggregation and surface hydrophobicity are two important variables often used to evaluate the initial stage of granules development. Most studies only focused on the development of granules but have not studied the ability of microbial aggregation and surface hydrophobicity (SHb) of bacteria in the initial stage of biogranulation process. This study investigated the effect of metal cations in improving granules development based on microbial aggregation and surface hydrophobicity (SHb). Autoaggregation (AAg) and SHb of *Brevibacillus panacihumi* strain ZB1, *Lysinibacillus fusiformis* strain ZB2 and *Enterococcus faecalis* strain ZL cells were studied using batch culture. Synthetic wastewater under aerobic condition with the addition of Ca^{2+}, Mg^{2+}, Al^{3+}, Mn^{2+} and Zn^{2+} was applied. Initial screening for AAg and SHb using 2-level factorial design showed that Ca^{2+} caused a significant increase in these two parameters for all the bacteria. Based on the AAg ratio measured from changes in absorbance of the culture medium, all of the three bacteria were classified as medium AAg. *L. fusiformis* strain ZB2 had the highest value of AAg by having a compact and large microscopic clustering of cells, followed by *B. panacihumi* strain ZB1 and *E. faecalis* strain ZL. The AAg ability of each bacterium was well correlated with the SHb. Addition of selected mixed cations (Ca^{2+}, Mg^{2+}, $Al^{3+}$and Mn^{2+}) increased the AAg ability of the bacterial strains from 35% to 41% for *E. faecalis* strain ZL, 43% to 56% for *B. panacihumi* strain ZB1, and 49% to 57%, for *L. fusiformis* strain ZB2. The SHb of the investigated bacteria had also increased from 32% to 37% for *E. faecalis* strain ZL, 45% to 55% for *B. panacihumi* strain ZB1, and 51% to 57%, for *L. fusiformis* strain ZB2. Addition of mixed cations has also caused a significant increase in the microbial aggregation and SHb of the mixed bacterial culture. The mixed culture consisting of all bacteria had the highest microbial aggregation (44.7%). On the contrary, the mixed culture consisting of *B. panacihumi* strain ZB1 and *E. faecalis* strain ZL had the highest SHb (28.8%). As a conclusion, addition of different cations resulted in an increase of AAg and SHb in individual and consortium of the tested bacteria.

ABSTRAK

Agregasi mikrob dan kehidrofobikan permukaan adalah dua pembolehubah penting yang selalu digunakan untuk menilai peringkat awal pembentukan granul. Kebanyakan kajian hanya tertumpu kepada pembentukan granul tetapi tidak mengkaji tentang keupayaan agregasi mikrob dan kehidrofobikan permukaan (SHB) daripada bakteria dalam peringkat awal proses pembentukan granul. Kajian ini dilakukan untuk menyelidik kesan kation logam dalam meningkatkan proses pembentukan granul berdasarkan agregasi mikrob dan kehidrofobikan permukaan (SHb). Agregasiauto (AAg) dan SHb bagi sel-sel *Brevibacillus panacihumi* strain ZB1, *Lysinibacillus fusiformis* strain ZB2 dan *Enterococcus faecalis* strain ZL telah dijalankan secara kultur berkelompok. Kajian dijalankan menggunakan airsisa sintetik dalam keadaan aerobik dengan tambahan Ca^{2+}, Mg^{2+}, Al^{3+}, Mn^{2+} dan Zn^{2+}. Penyaringan awal bagi AAg dan SHb menggunakan reka bentuk 2-tahap faktorial menunjukkan bahawa Ca^{2+} memberikan kesan peningkatan besar dua parameter tersebut kesemua bakteria yang dikaji. Berdasarkan nisbah AAg yang diukur menerusi perubahan penyerapan media kultur, ketiga-tiga bakteria ini diklasifikasikan sebagai jenis bakteria yang memiliki AAg sederhana. *L. fusiformis* strain ZB2 mempunyai nilai AAg tertinggi dengan sel mikroskopik yang padat dan gumpalan yang besar, diikuti oleh *B. panacihumi* strain ZB1 dan *E. faecalis* strain ZL. AAg untuk setiap bakteria yang dikaji mempunyai korelasi yang baik dengan SHb. Penambahan campuran kation terpilih (Ca^{2+}, Mg^{2+}, Al^{3+}dan Mn^{2+}) telah meningkatkan keupayaan AAg untuk setiap strain bakteria daripada 35% hingga 47% bagi *E. faecalis* strain ZL, 42% hingga 57% bagi *B. panacihumi* strain ZB1 dan 42% hingga 56% bagi *L. fusiformis* strain ZB2. SHb bagi bakteria yang dikaji juga menunjukkan peningkatan daripada 32% hingga 37% bagi *E. faecalis* strain ZL, 45% hingga 55% bagi *B. panacihumi* strain ZB1 dan 51% hingga 57% bagi *L. fusiformis* strain ZB2. Penambahan kation campuran juga memberikan kesan terhadap peningkatan agregasi mikrob dan SHb bagi kultur bakteria campuran. Kultur campuran yang terdiri daripada kesemua bakteria mempunyai agregasi mikrob tertinggi (44.7%). Kultur campuran yang terdiri daripada *B. panacihumi* strain ZB1 dan *E. faecalis* strain ZL memberikan peratusan kenaikan SHb tertinggi (28.8%). Sebagai kesimpulan, penambahan kation yang berlainan menghasilkan peningkatan AAg, dan SHb secara individu dan gabungan bakteria konsortia yang diuji.

TABLE OF CONTENTS

LIST OF TABLE

TABLE NO. | **TITLE** | **PAGE**

LIST OF FIGURES

LIST OF ABBREVIATION

ABBREVATION		DESCRIPTION
cfu	-	Colony Forming Unit
cm	-	Centimeter
µL	-	Microliter
mg	-	Milligram
mg/L	-	Miligram per liter
mL	-	Mililiter
mm	-	Milimeter
mM	-	Milimolar
AAg	-	Autoaggregation
$AlCl_3$	-	Aluminium Chloride
$CaCl_2 \cdot 2H_2O$	-	Calcium Chloride dihydrate
CAg	-	Coaggregation
$CoCl_2 \cdot 6H_2O$	-	Cobalt Chloride
$CuCl_2 \cdot 2H_2O$	-	Copper Chloride
Na_2EDTA	-	Disodium Ethylenediaminetetraacetic Acid
EPS	-	Extracellular Polymeric Substance
$FeCl_3 \cdot 4H_2O$	-	Iron (III) Chloride
H_3BO_3	-	Boric Acid
KH_2PO_4	-	Monopotassium Phosphate
K_2HPO_4	-	Dipotassium Hydrogen Phosphate
KI	-	Potassium Iodide
$MgSO_4 \cdot 7H_2O$	-	Magnesium Sulfate Heptahydrate
$MnCl_2 \cdot 4H_2O$	-	Manganese Chloride
$NaMoO_4 \cdot 2H_2O$	-	Sodium Molybdate Dihydrate
NH_4Cl	-	Ammonium Chloride
OD	-	Optical Density

rpm	-	Rotation per Minute
SHb	-	Surface Hydrophobicity
v/v	-	Volume over Volume
$ZnCl_2$	-	Zinc Chloride

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of study

Biogranulation is a branch of biotechnology for wastewater treatment. Biogranulation can be divided into anaerobic and aerobic processes. Although it is different in operational processes, the fundamental method of granules formation in both systems are the same. The development of granules requires the microbial cell to aggregate to one another either among the same or different microorganisms. This can be achieved autoaggregation or coaggregation among the bacterial strains. Auto-aggregation is referred to physical cell-to-cell interaction between genetically identical cells, while coaggregation refers to the interaction between genetically distinct bacterial cells. Granules are formed by cell immobilization and consists of biofilm, entrapped microorganisms and microbial aggregates (Liu and Tay, 2002). However it is different from the formation of biofilm because no carriers or supporting materials is needed to develop granules (Tay *et al.*, 2006; Di Iaconi *et al.*, 2007; Adav *et al.*, 2008a; Liu *et al.*, 2009).

In general, biogranulation is a process that involves the transformation of the seed sludge to sludge aggregates and then the formation of compact clumps. After which is the formation of granular sludge before forming mature and stable granular sludge. Mature and stable granular sludge is compact and forms nearly spherical shape (Tay *et al.*, 2006; Sondhi *et al.*, 2010; Liu *et al.*, 2010; Zheng *et al.*, 2011). Compared to conventional activated sludge floc, anaerobic and aerobic granules have a regular, dense and strong physical structure, good settling ability, high sludge retention and able to withstand shock-loading rate. Application of compact granules based technology ease the conventional associated problem such as sludge bulking, large treatment plant space, and high production of sludge waste (De Kreuk *et al.*, 2005).

There are two types of reactor system used for biogranulation development; upflow anaerobic sludge blanket (UASB) and sequencing batch reactors (SBRs). UASB reactor systems have been one of the important anaerobic wastewater treatment system for over two decades. There have been successfully operated to treat various types of wastewater such as domestic wastewater (Kalogo *et al.*, 2001), latex wastewater (Boonsawang *et al.*, 2008) and industrial wastewater such as beverage, brewery, food and tannery industries (Karthikeyan and Kandasamy, 2009), agro-industrial wastewater (Rajagopal *et al.*, 2013), grey water (Elmitwalli *et al.*, 2007) and leachate (Torres *et al.*, 2009). The UASB reactors system were commonly used by researchers for anaerobic biogranulation development.

Basically, SBR was used for activated sludge treatment in which all of the treatment processes take place in the reactor tank and clarifiers are not required. It consists of five stages which involves fill, react, settle, decant and idle conditions. Research in this area has led to the development of granules in laboratory scale SBRs on a wide variety of easily degradable carbon sources such as glucose, acetate and ethanol. SBRs have been used for nutrient removal (Keller *et al.*, 2001) such as to treat municipal and agricultural wastewater (Abdullah *et al.*, 2011). It has also been used to treat industrial and other hazardous wastewater. Research on aerobic granulation has concentrated mainly in sequencing batch reactors (SBR) because the reactor operation conditions (cyclic feeding and starvation, high shear stress and short settling time) promote development of granules (Liu *et al.*, 2002a)

1.2 Problem Statement

The anaerobic and aerobic biogranulation wastewater treatment demonstrates effective and reliable removal performance, together with outstanding settleability sludge profile. However, both systems require relatively long start-up period (Yu *et al.*, 2009). Development of granules will take weeks to several months depending on the condition of the experimental set-up. Sometimes granules could be formed and be seen in the reactor in just a week, however it woud take a long period of time to make the whole reactor filled with granules (De Kreuk and Van Loosdrecht, 2004; Di Iaconi *et al.*, 2007).

Many research have been conducted to reduce the start-up period for biogranulation development by adjusting the configuration of reactors. Optimizing certain conditions in the reactor system such as increasing aeration rates, reducing the settling time, extending the aeration period and varying the organic loading rate have been reported to reduce the biogranulation time and enhance the performance (Qin *et al.*, 2004; Ivanov *et al.*, 2006; Li *et al.*, 2009; Gao *et al.*, 2011). Apart from the optimization of certain conditions for the biogranulation development, many researchers have also used other alternatives such as adding bioaugmentation bacteria, substances, co-substrates, polymers and divalent cations to enhance the microbial aggregation during the initial start-up (Tay *et al.*, 2006).

These studies show that addition of any material as previously mentioned was basically to improve the microbial aggregation hence to speed up the biogranulation in the initial stage. According to Guo *et al.* (2011), the changes in bacterial population and extracellular polymeric substance (EPS) production could influence the cell surface hydrophobicity (SHb), which could also have an effects on microbial aggregation. In this case, any factor that can increase the EPS production and surface hydrophobicity, may help to improve the microbial aggregation. According to Liu *et al.* (2004a), cell SHb is one of the important factor that trigger the biogranulation. However there is still lack of understanding on the effects of foreign materials or enhancers on the bacterial cell. Furthermore much research have proven that different types of bacteria are non-identical and may act differently towards the materials that are used as enhancers, either as in individual or mixed culture (Adav and Lee, 2009; Lamprecht, 2009).

Previous research in biogranulation development explains that bacterial behavior or characteristic is not necessarily similar to the changes of the environment. The bacteria or microorganisms would react to any changes or modifications that would probably give a similar or different result. The aim of this study was to examine the effect of cations on microbial aggregation and SHb, and find a way to improve the aerobic granulation start-up from the perspective of cells aggregation. Hence, in order to invstigate the microbial aggregation and cell SHb on the addition of cations, three dye-degrading bacteria, *Enterococcus faecalis* strain ZL, *Brevibacillus panacihumi* strain ZB1 and *Lysinibacillus fusiformis* strain ZB2 were used in this study. These bacteria were kindly donated from Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia. *E. faecalis* strain ZL was indigenously isolated from a palm oil mill effluent (POME). While *B. panacihumi* strain ZB1 and *L. fusiformis* strain ZB2 were isolated from local textile effluent. From here onwards, *Enterococcus faecalis* strain ZL will be referred to as ZL, *Brevibacillus panacihumi* strain ZB1 will be referred to as ZB1 and *Lysinibacillus fusiformis* strain ZB2 will be referred to as ZB2.

1.3 Objectives of the study

a) To determine the classification of the autoaggregation (AAg) and surface hydrophobicity (SHb) for *Brevibacillus panacihumi* strain ZB1, *Lysinibacillus fusiformis* strain ZB2 and *Enterococcus faecalis* strain ZL.

b) To investigate the effect of selected cations that influence microbial aggregation (AAg) and surface hydrophobicity (SHb) of the bacteria using 2 level factorial design on individual bacteria.

c) To investigate the effect of combined cations on microbial aggregation and surface hydrophobicity on the mixed culture.

1.4 Scope of study

In this study, three dye-degrading bacteria, *Brevibacillus panacihumi* ZB1, *Lysinibacillus fusiform* ZB2 and *Enterococcus faecalis* ZL were used to represent mixed culture in real wastewater. This research involve the investigation of cation on microbial aggregation and cell surface hydrophobicity (SHb) of the bacterial strain either in the form of single and mixed cultures under aerobic condition using modified synthetic textile wastewater. This study was divided into three parts; firstly the classification of aggregation and SHb of each bacteria used in this study. Secondly experimental design; using a 2-level factorial design, the effects of AAg and SHb on the three bacteria were studied individually. Finally the best cations were selected and effects of the cations on AAg and SHb of the mixed culture was studied.

1.5 Significance of Study

This study investigated the effect of cations on the biogranulation initial start-up. This is because biogranulation development significantly depends on the mechanism during the initial stage of the granules formation. This includes the process of microbial aggregation and SHb. The significance of this study is to improve the initial stage of aerobic biogranulation development by observing the microbial aggregation and surface hydrophobicity of bacterial cells. In this study, the effect of single cation and interaction between cationic affinity to the change of microbial aggregation and SHb of tested bacterial culture were also been observed. Nevertheless, the method used in this study will provide an alternative that could be used to overcome the long start-up period of aerobic biogranulation development.

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