Effect of Anion and Cation on Ecotoxicity of Ionic Liquids towards Different Microbes

by

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Dissertation submitted in partial fulfilment of
the requirements for the
Bachelor of Engineering (Hons)
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Universiti Teknologi PETRONAS,
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Perak Darul Ridzuan

CERTIFICATION OF APPROVAL

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Approved by,

_____
(Dr. Muhammad Moniruzzaman)

UNIVERSITI TEKNOLOGI PETRONAS
BANDAR SERI ISKANDAR, PERAK
May 2015

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

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WAN NUR HAZIQAH BINTI WAN ABDULLAH

ABSTRACT

Ionic liquids (ILs) have been used in industrial application including electrochemical, chemical engineering, chemistry and others. ILs can be easily tuned by combining selected cation and anion in order to achieve desired characteristics. Nowadays, all industries are trying to use green application. However, the information regarding the toxicity of ionic liquids are still limited. The objective of this study is to evaluate the toxicity of ionic liquids towards selected microbes. The effect of anion and cation on the toxicity of ionic liquids also have been discussed. Toxicity of ionic liquids are determined by conducting Minimum Inhibitory Concentration (MIC) test. MIC test is conducted and the results obtain used to determine EC50 value for each microorganism towards different ionic liquids determine by plotting dose-response curve graph. The findings from this research are hydroxide anion and phosphonium cation found to be toxic towards microorganisms. While ammonium cation and acetate anion is found to be non –toxic towards microorganisms.

ACKNOWLEDGEMENT

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CHAPTER 1
INTRODUCTION

1.1 Background of Study

Ionic liquids (ILs) are salt from the combination of cation and anion which have melting points below 100ºC (Markiewicz et al., 2013). Nowadays, ILs are an alternative solvent to replace conventional solvents. The usage of conventional solvents give impact to environment due to their toxicity, flammability and volatility (Ventura et al., 2012) . ILs can be easily tuned by selecting different combination of cation and anion to achieve desired characteristics. The characteristics of ILs are negligible vapor pressure, non-flammable, high conductivity, high chemical, thermal and electrochemical stability (Petkovic et al., 2010).

Usage of ILs in industrial applications including electrochemistry, biological uses, analytics, solvent and catalysis, engineering and physical chemistry has been increasing due to its characteristics. Figure 1.1 shows some of application of ILs in some industries.

Majority of industries are trying to use green application, however the information regarding the toxicity, biodegradability and recyclability are lacking compared to the conventional solvent (Rajathi and Rajendran, 2013). According to Pretti et al. (2009) the toxicity of different ILs towards aquatic organisms are different. The toxicity of ILs depends on the cationic head. However, the toxicity level decreases from aromatic heterocyclic nitrogen-containing compounds (pyridinium and imidazolium) to non-aromatic cyclic and acyclic compounds (pyrrolidinium, ammonium, and morpholinium). Further research need to be conducted in order to determine the toxicity of ILs.

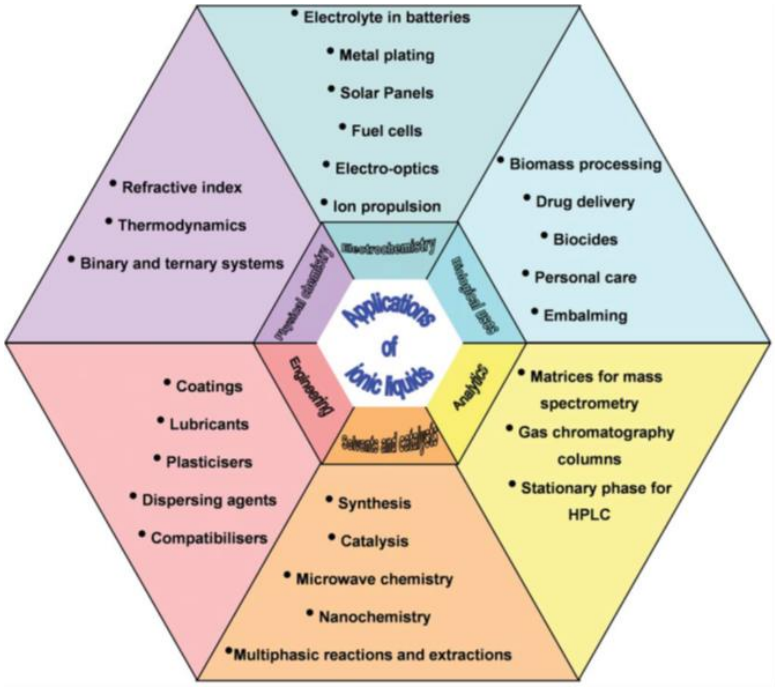


FIGURE 1.1 Application of ILs (adapted from Plechkova and Seddon, 2008)

1.2 Problem Statement

Ionic liquid has gained attention in industries using green technology. Green technology is technology used not giving impact to environment or can be described as environmental friendly. However the information regarding the toxicity of ionic liquid still limited. The usage of tetrabutylammonium hydroxide, tetrabutylphosphonium hydroxide, tetrabutylammonium acetate, and tetrabutylphosphonium acetate toxicity are still questionable. It is important to have knowledge regarding the toxicity in order to know what will be the impact if ILs being released to environment. The ionic liquid which found to be toxic can be used as anti-microbial in pharmaceutical industry while non-toxic ionic liquids can be used in bioprocess. There are still limited information regarding the toxicity of ionic liquid towards microorganism. This study will investigate the effect of anion and cation on ecotoxicity of ionic liquid towards microorganisms.

1.3 Objectives

1. To determine EC50 towards selected microorganisms (*Aeromonas Hydrophilia, Listeria Monocytogenes, Escherichia Coli and Staphylococcus Aureus*) using different ILs; tetrabutylammonium hydroxide, tetrabutylphosphonium hydroxide, tetrabutylammonium acetate, and tetrabutylphosphonium acetate.
2. To investigate the effect of using anion towards toxicity level of ILs.
3. To investigate the effect of using cation towards toxicity level of ILs.

1.4 Scope of Study

The toxicity of selected ionic liquids (ILs) will be evaluated using Minimum Inhibitory Concentration (MIC) test using selected microorganisms. The study will focus on few types of ILs; tetrabutylammonium hydroxide, tetrabutylphosphonium hydroxide, tetrabutylammonium acetate, and tetrabutylphosphonium acetate with different concentration. The microorganisms that will be used are *Aeromonas Hydrophilia*, *Listeria Monocytogenes*, *Escherichia Coli* and *Staphylococcus Aureus.* The results will be evaluated after 24 hours depending on the nature of microorganisms.

CHAPTER 2
LITERATURE REVIEW

2.1 Ionic Liquid (ILs)

2.1.1 Properties of ILs

The combination of cation and anion will form ionic liquids (ILs). They are molten salt that usually have melting point below 100ºC (Thuy Pham et al., 2010). ILs are also non–flammable as their vapor pressure are negligible under ambient conditions (Welton, 2004). They are also called as 'green solvent' as when released to the environment they will give less impact compare to conventional solvent (Plechkova and Seddon, 2008).

ILs have high thermal stability and also high chemical stability where they are stable towards organic and inorganic substances (Gilmore, 2011). The viscosity, hydrophobicity, density and solubility of ILs can be varied by selecting different combination of cation and anion according to specific characteristics. ILs can be divided into two groups; water miscible and water immiscible depending on their solubility in water. Miscibility of ILs in water depending on anion of ILs (Moniruzzaman and Goto, 2011).

Not only that, ILs have good conductivity compared to organic solvent or electrolyte systems. They are also more viscous than common molecular solvents. Van der Waals forces and hydrogen bonding used to determine the viscosity of ILs. The effect of alkyl chain length also increase the viscosity due to stronger of van der Waals force between cations. The conductivity of ILs inversely linked with viscosity which mean higher viscosity exhibit low conductivity. The conductivity of ILs increase when the temperature increases thus lowering the viscosity. ILs are more denser than water and the lengthening of the alkyl chain will decrease the density of ILs (Endres and El Abedin, 2006).

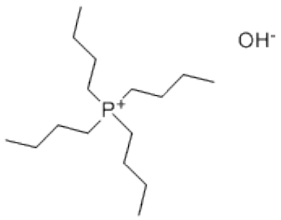
2.1.2 Composition of ILs

ILs specific applications can be formed by selecting the cation and anion. This is because ILs are designer solvents and their properties can be tuned. (Tokuda et al., 2004).

TABLE 2.1 Commonly used cation and anion (adapted from Tokuda et al., 2004)

Cation	Anion
• Imidazolium (IM)	• Chloride (Cl)
• Pyridinium (Py)	• Bromide (Br)
• pyrrolidinium(Pyr)	• Tetrafluoroborate (BF_4)
• Morpholinium (Mor),	• Hexafluorophosphate (PF_6)
• Piperidinium (Pip)	• Bis(trifluoromethylsulfonyl)imide $[(CF_3SO_2)N]^-$
• Quinolinium (Quin),	• Dicyanamide $[(CN)_2N]^-$
• Quaternary ammonium (N)	
• Quaternary phosphonium (P)	

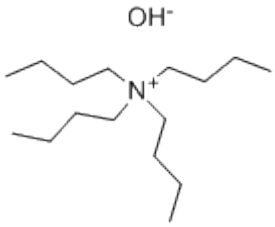
a)



Tetrabutylphosphonium hydroxide

(source: http://www.chemicalbook.com)

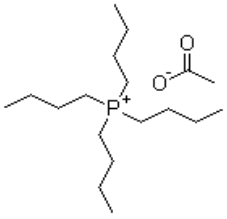
b)



Tetrabutylammonium hydroxide

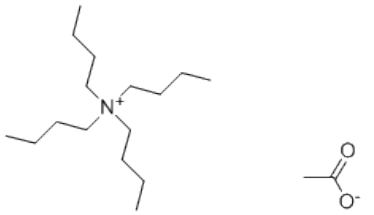
(source: http://www.chemicalbook.com)

FIGURE 2.1 (a) Tetrabutylphosphonium hydroxide (b) Tetrabutylammonium hydroxide

c) 

Tetrabutylphosphonium acetate

source :(http://www.chemspider.com/)

d) 

Tetrabutylammonium acetate

(source: (http://www.chemspider.com/)

FIGURE 2.2 (c) Tetrabutylphosphonium acetate (d) Tetrabutylammonium acetate

2.2 Application of ILs

Conventional organic solvents are hazardous and have high toxicity properties which may affected the environment. The ILs are suggested to replace the conventional organic solvents. Application of ILs in industries including those of biotechnology, chemistry, chemical engineering, coating and energy (Thuy Pham et al., 2010).

In pharmaceutical applications, ILs are gaining more attention from drug designers and researchers in finding new medical treatments and also delivery options. According to Moniruzzaman et al. (2010) usage of ILs in microemulsions increase the solubility of sparingly soluble drug. Not only that, ILs that have phosphonium and ammonium can be used to treat cancer (Kumar and Malhotra, 2009).

Also, by dissolving lithium in ILs it can be used as electrolytes in lithium batteries (Galiński, et al., 2006).ILs can also be used as performance additives for lubrication oil, sensors (Wei and Ivaska, 2008) and dye-sensitived solar cell (Grätzel, 2003) as their physicochemical properties are tunable.

2.3 Toxicity of ILs

It is important to know the toxicity of different ILs towards living organisms and be aware of the impact of the solvent to the environment (Wood, 2011). It is crucial to recognize the toxicity of different ILs in order to be aware the impact if they are released to environment (Gilmore, 2011). By selecting biocompatible organic cation and inorganic anions; non-toxic ILs could be produced.

Recently ionic liquids that contained imidazolium, pyridinium, piperidenium and quaternary ammonium cations has been studied in order to determine the toxicity and biodegradability. Mori et al. (2015) had conducted a research on the toxicity of tetramethylammonium hydroxide (TMAH) and test it on aquatic organisms. It can be seen tetramethylammonium hydroxide (TMAH) is toxic towards *Daphnia Magna*. *Daphnia Magna* can be found in ponds and lakes and also called as water fleas. It has single large compound eyes and slightly large antennae (Clare, 2002).

Researches had been conducted using cholinium (quaternary ammonium cation) with alkanoates anions to investigate the impact of anions on ionic liquid toxicities and tested it using filamentous fungi. From the research, anion toxicity depends on its liphophilicity. This types of ILs have the potential to be used as biotechnological applications due to their biodegradability, harmlessness to environment and also as a good solvent (Petkovic et al., 2010).It would be more safer to use bulkyl cholinium, phosphonium or ammonium based ILs with shorter side of chain for pharmaceutical uses (Moniruzzaman and Goto, 2011). Hydrophobic phosphonium with long alkyl chain are observed to be less toxic where it may have the potential for chemical and biocatalytic processes (Ventura et al., 2012).

It is widely reported that the toxicity of ILs are depends on the increase of alkyl chain length associated (C1-C12) with cations (Couling et al., 2006).In regards to the anion effects, toxicity increases when using trifluoromethanesulfonate due to the liphophilicity of the anion (Latała et al., 2009).

2.4 Types of Microorganisms

The bacteria will be divided into two classes which are gram positive and gram negative. The methods to determine whether the bacteria is gram-positive or gram – negative by undergo gram stain test. The purple –coloured stain show that it is gram-positive bacteria while pinkish or red is gram-negative bacteria (Antimicrobial Drug Resistance, 2012). Gram-negative bacteria not all can pass through it as it have thick bilayer on the outside. Gram- positive bacteria more disposed to antibiotics compared to gram-negative since everything can pass through it easily (Enger and Ross, 2003).

Aeromonas Hydrophilia lives in aquatic environment is gram-negative bacteria and also aerobics and anaerobics. Gelatin, haemoglobin and and also elastin can be digested by this bacteria. It can resist cold temperature and chlorine. *Listeria Monocytogenes* is gram- positive, facultative anaerobic and having size for about 0.4-0.2 µm × 0.5-2 µm.it will be growth well at temperature 4° C (Beverly, 2004) *Staphylococcus Aureus* is also gram- positive, facultative anaerobes and a human pathogen (Foster ,n.d).

2.5 Microorganisms and Its Toxicity

The usage of different microorganisms are tested in order to observe the effect of ILs. The growth inhibition zone obtained will be used to test the biocompatibility of ILs which are biocompatible will have lower inhibition halo. Research had been conducted using *Vibrio fischeri* to test toxicity of quaternary ammonium ILs. The liphophilicity of cation related to antimicrobial effects when tested using quaternary ammonium chloride. Quaternary ammonium ILs are found to be less toxic compare to pyridinium and imidazolium compounds (Couling et al., 2006).

CHAPTER 3
METHODOLOGY

3.1 Project Flowchart

FIGURE 3.1 Project Flowchart

3.2 Equipment/Tools Used

3.2.1 Autoclave

Autoclave is used to sterilize equipment and apparatus with temperature at 121°C and 15 psi for about 15 minutes. It is used to prevent any bacteria contaminated the equipment or apparatus that may affect the viability of bacteria.

3.2.2 96 well-plate reader

In the laboratory microplate reader is used to do analysis. The biological chemical or physical events of sample in microtiter plates can be identified using this equipment. This plate reader will be used to analyze the sample of different types of ILs with different type of microorganisms. The data will be transported to Microsoft Excel and graph are constructed using GraphPad Prism 6 based on the results.

3.2.3 GraphPad Prism 6

The EC50 for each microorganisms on different ILs will be calculated based on the result obtained. GraphPad Prism 6 is used in order to construct dose-response curve. This graph will shows the relationship between the increase of concentration of the dose of the drug and the response from increasing concentration. Based on this project the dose response curve will be showing the effect of increasing concentration of ILs and the viability of the microorganisms.

3.3 Synthesis of Ionic Liquids

FIGURE 3.2 Synthesis of Ionic Liquids

3.4 Methods for Sub culturing Microorganisms

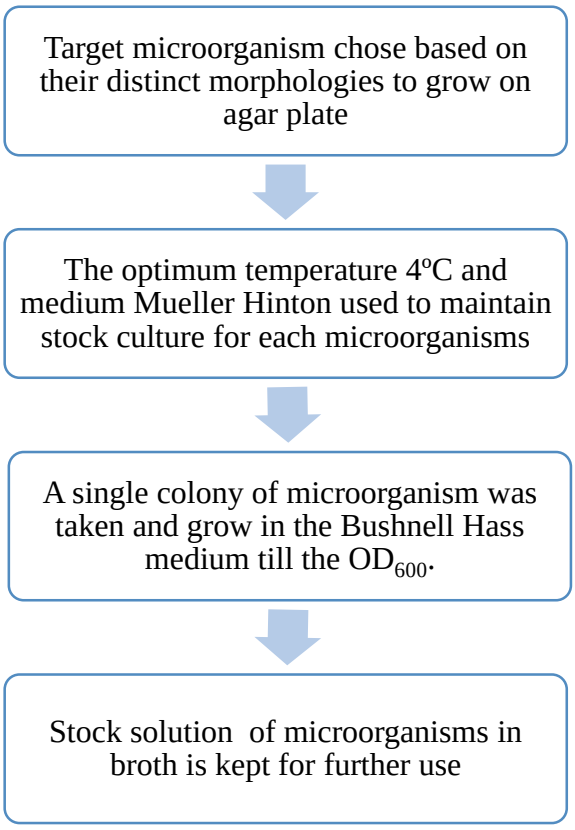


FIGURE 3.3 Flowchart of Sub-culturing Microorganisms (adapted from Ventura et al.2012)

3.5 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is used to determine the lowest concentration of ILs that will inhibit the growth of microorganisms. EC50 is the half maximal effective concentration. EC50 is the concentration where exposed organisms is killed or immobilized 50%. MIC test can be conducted after the microorganisms are subculture. MIC can be done using 96-well plates by inoculate the organisms into well which contain broth and serial dilution of ILs. The sample will be incubated for about 24 hours and the plate will be analyzed to determine the growth of microorganisms.

3.5.1 Serial Dilution

Serial dilution is method to identify the viability of microorganism in amount of liquid. ILs is mix with the broth in 96 wells plate. The dilution of ILs will be started from 10000 ppm.

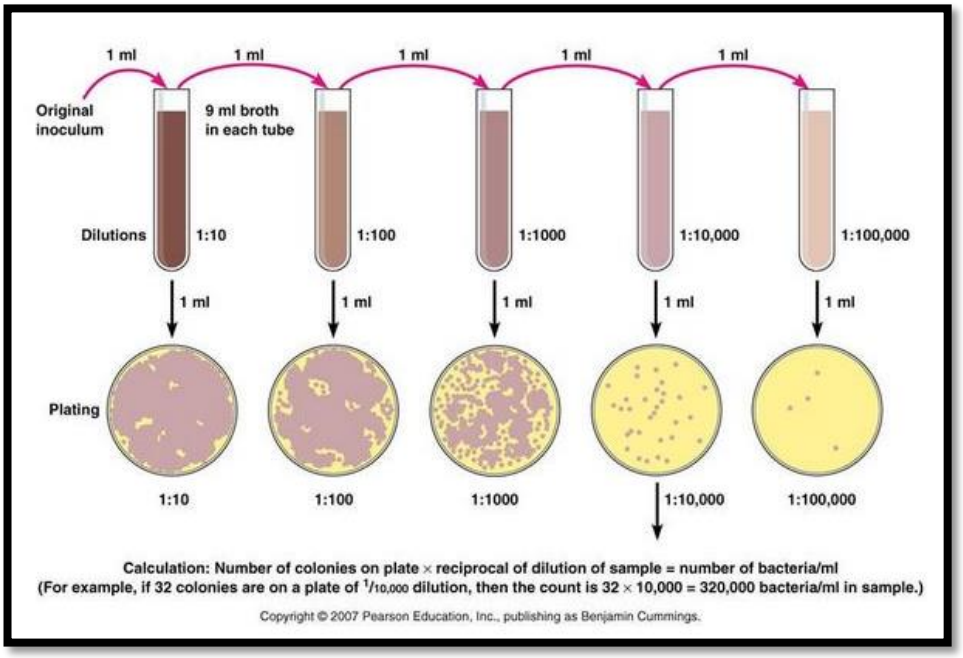


FIGURE 3.4 Serial Dilutions (source: http://classes.midlandstech.com)

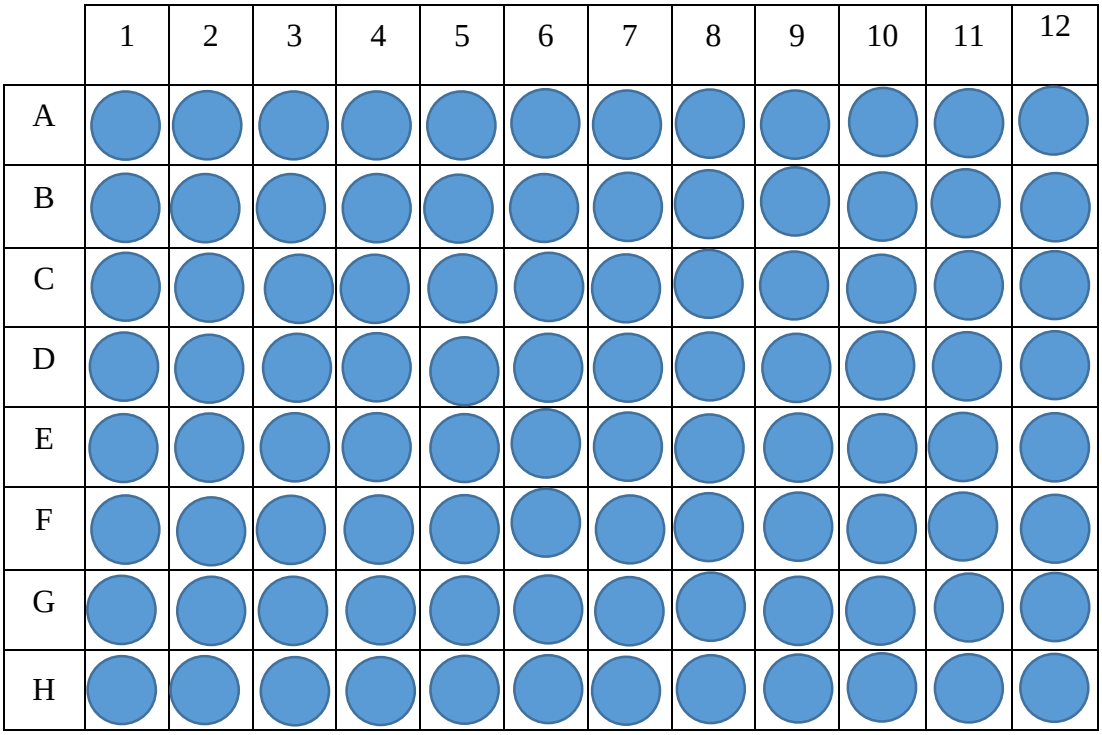


FIGURE 3.5 Division of ILs in 96-wells plate

Tetrabutylphosphonium hydroxide can be placed at (A-B), Tetrabutylphosphonium acetate at (C-D) and Tetrabutylammonium hydroxide at (E-F) and Tetrabutylammonium acetate at (G-H). Column 12 will be filled with ILs and bacteria while for column 11-2 the serial dilution method will be used and column 1 filled with microorganism and the broth which acts as positive control. For example one 96 wells plate it will be testing with *Aeromonas Hydrophilia* but with different ILs. Then, the experiment will be repeated by using the other types of bacteria.

Table 3.1 Division of ILs in 96 wells plate

Matrix	Ionic Liquids (ILs)
A-B	Tetrabutylphosphonium hydroxide
C-D	Tetrabutylphosphonium acetate
E-F	Tetrabutylammonium hydroxide
G-H	Tetrabutylammonium acetate

3.5.2 Method to Conduct MIC

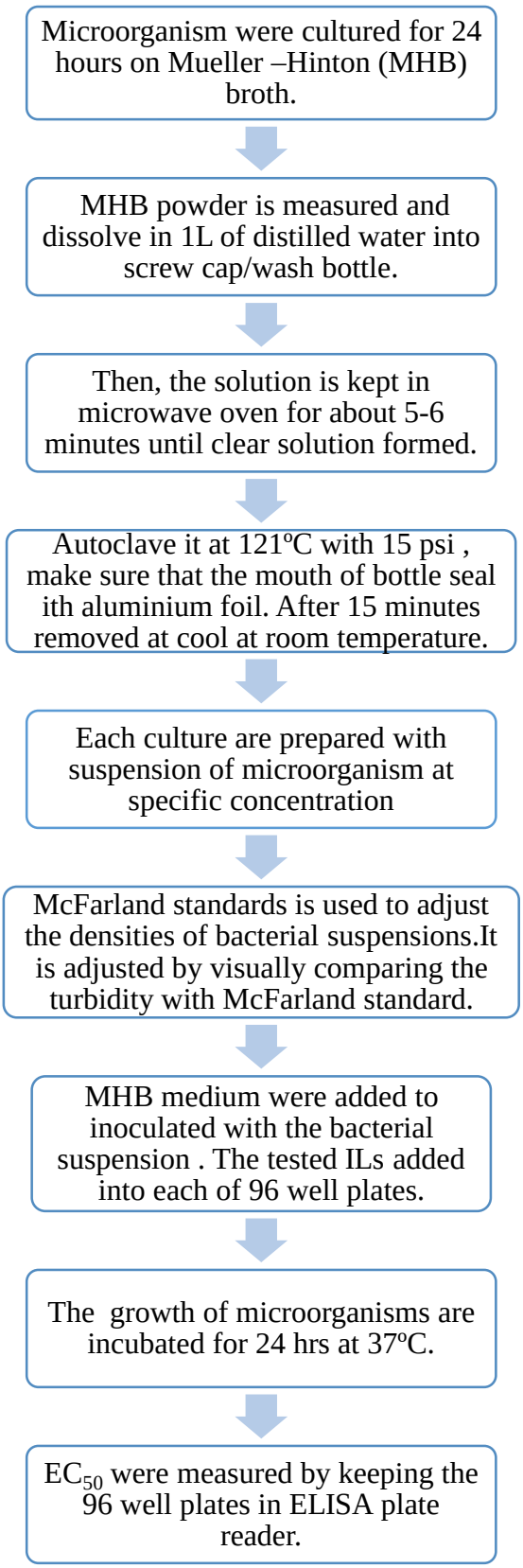


FIGURE 3.6 Flowchart of Conducting Minimum Inhibitory Concentration (MIC) (adapted from Ataee et al., 2012)

3.5.3 Steps to Construct the Dose-Response Graph

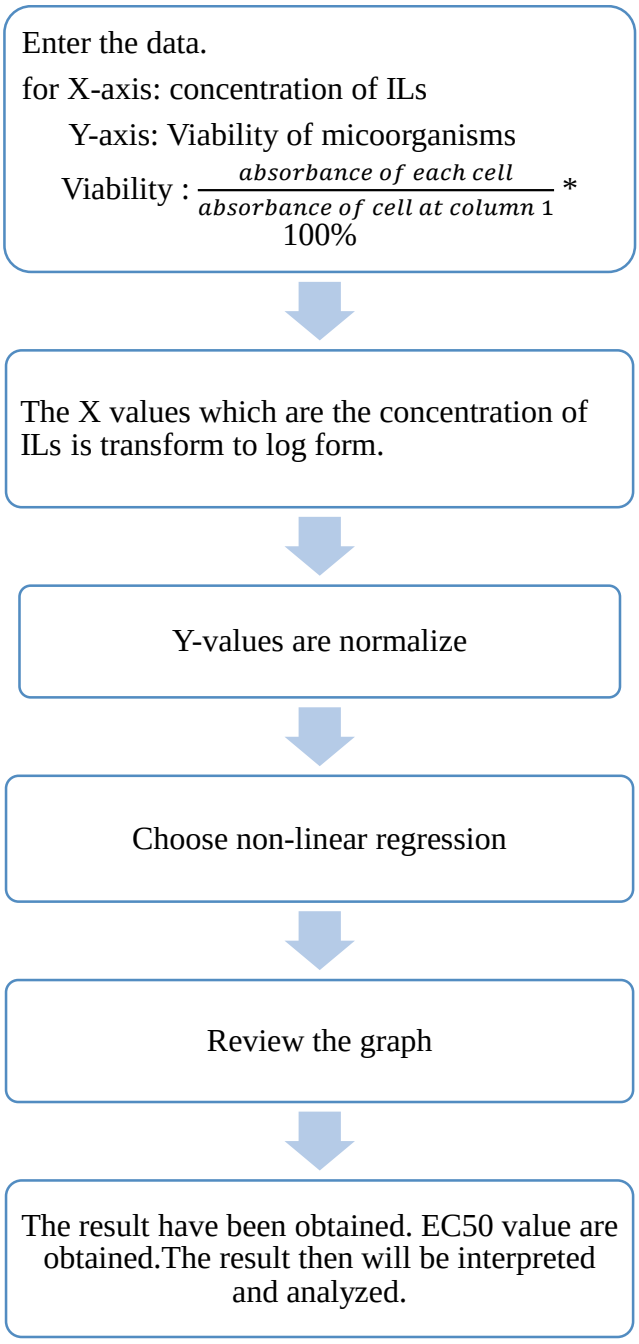


FIGURE 3.7 Flowchart of Constructing Dose-Response Graph (source: http://www.graphpad.com)

3.6 Gantt Chart

3.6.1 FYP I

TABLE 3.2 Gantt chart for FYP I

NO	GANTT CHART	PERIOD OF PLANNING													
	DESCRIPTION OF PLANNING	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	Tittle selection														
2	First meeting with supervisor. Discuss about the project tittle.														
3.	Literature review														
4.	Preparing extended proposal														
5.	Chemical Selection														
6.	Submission of extended proposal to supervisor														
7.	Preparation for proposal defense														
8.	Proposal defense														
9.	Experimental Work														
10.	Submission of Interim Draft Report														
11	Submission of Interim Final Report														

3.6.2 FYP II

TABLE 3.3 Gantt chart for FYP II

NO	GANTT CHART	PERIOD OF PLANNING													
	DESCRIPTION OF PLANNING	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	Project work continues.														
2	Preparation of ionic liquids and microbes														
3.	Submission of progress report														
4.	Project work continues.														
5.	Pre -SEDEX														
6.	Submission of draft final report														
7.	Submission of Dissertation (soft bound)														
8.	Submission Technical Paper														
9.	Viva														
10.	Submission of Project Dissertation (hard bound)														

3.7 Key Milestone

TABLE 3.4 Key Milestone FYP I

Week	Activities
Week 1-2	▪ Received project title from coordinator ▪ Understanding the overall project idea ▪ Identifying the scope of study and objectives
Week 3-4	▪ Find literature review of the project ▪ Meet with supervisor for further understanding of the project
Week 5-6	▪ Read the research paper ▪ Find out what are the ionic liquid that will be tested
Week 7-8	▪ Selecting the ionic liquid for the toxicity test ▪ Preparation and submission of extended proposal ▪ Find out the method to test toxicity of ionic liquid
Week 9-12	▪ Proposal defence ▪ Start the lab work ▪ Preparation of Interim Report
Week 13-14	▪ Submission of Interim Report ▪ Continuation of lab work.

Table 3.5 Key Milestone FYP II

Week	Activities
Week 1-6	▪ Project works continues from previous progress. ▪ Preparation of ionic liquids and microbes
Week 7	▪ Submission of progress report
Week 8-12	▪ Project work continues ▪ Analyzing the data
Week 11	▪ Submission of Draft Final Report ▪ Pre -SEDEX
Week 12	▪ Submission of Dissertation (soft bound) ▪ Submission Technical Paper
Week 13-14	▪ Viva ▪ Submission of Project Dissertation (hard bound)

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Synthesis of Ionic Liquids

The toxicity test is conducted to four selected ILs which are:

1. Tetrabutylphosphonium hydroxide
2. Tetrabutylammonium hydroxide
3. Tetrabutylphosphonium acetate
4. Tetrabutylammonium acetate

Tetrabutylphosphonium hydroxide and Tetrabutylammonium hydroxide are commercially available. However for Tetrabutylphosphonium acetate Tetrabutylammonium acetate, these two ILs need to be synthesized. These two ILs are synthesized by used acid base neutralization method. Once the acid base neutralization reaction completed ILs needed to undergo purification.

4.1.1 Nuclear magnetic resonance (NMR)

NMR test is conducted in order to ensure the purity of ILs formed. Based on the major peak shown in Figure 4.1 and 4.2 it is clearly shown that the ILs are successfully formed.

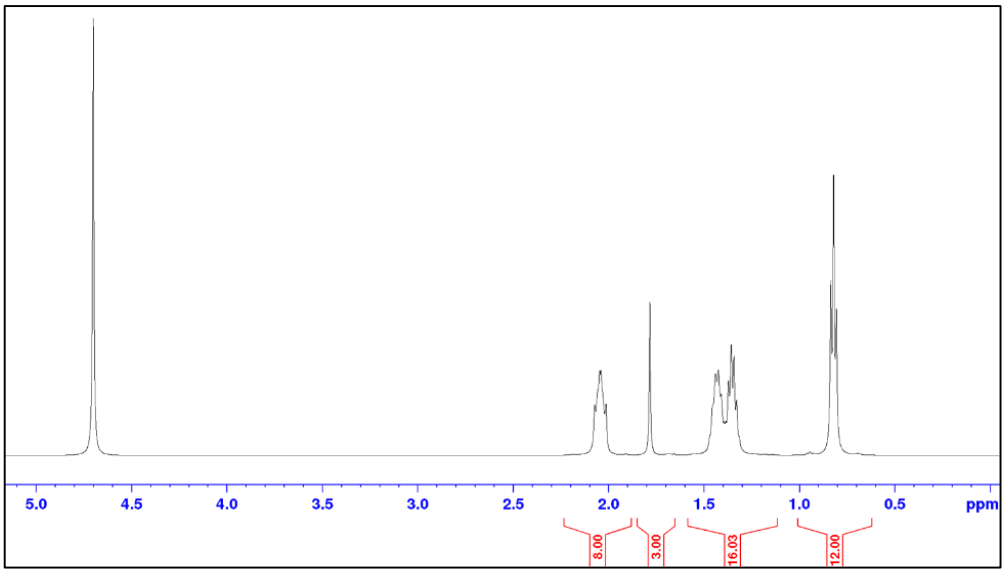


FIGURE 4.1 NMR Tetrabutylphosphonium Acetate

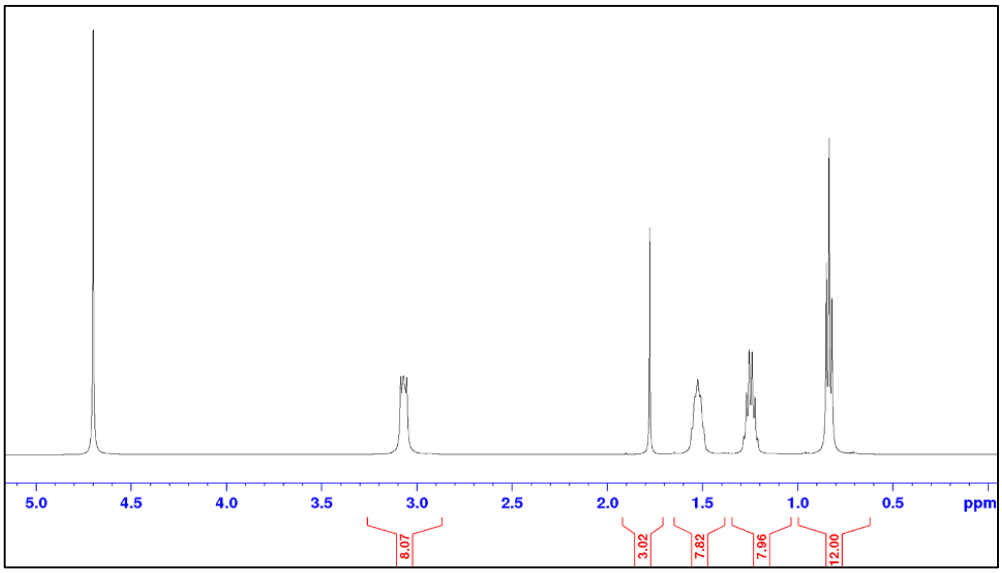


FIGURE 4.2 NMR Tetrabutylammonium Acetate

4.1.2 Thermogravimetric analysis (TGA)

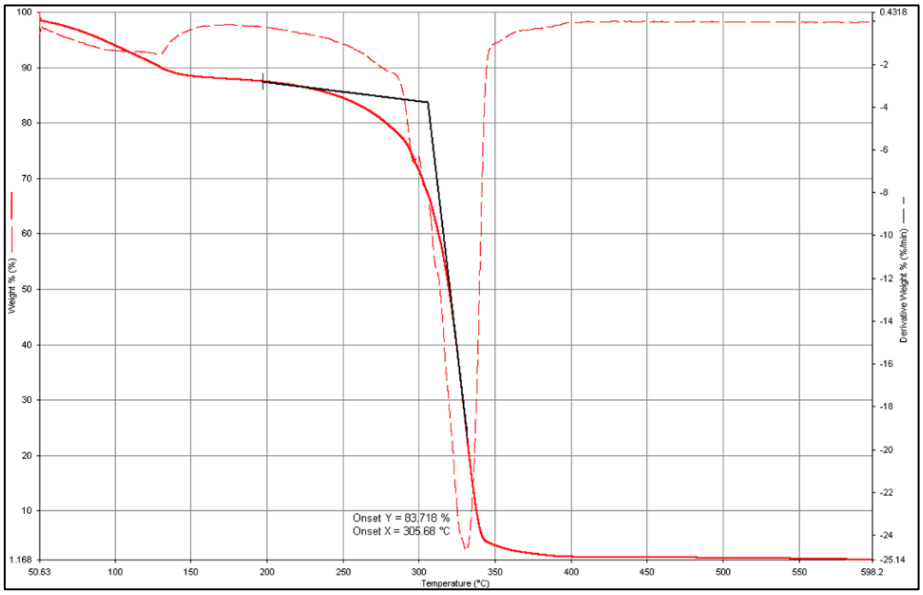


FIGURE 4.3 TGA Tetrabutylphosphonium Acetate

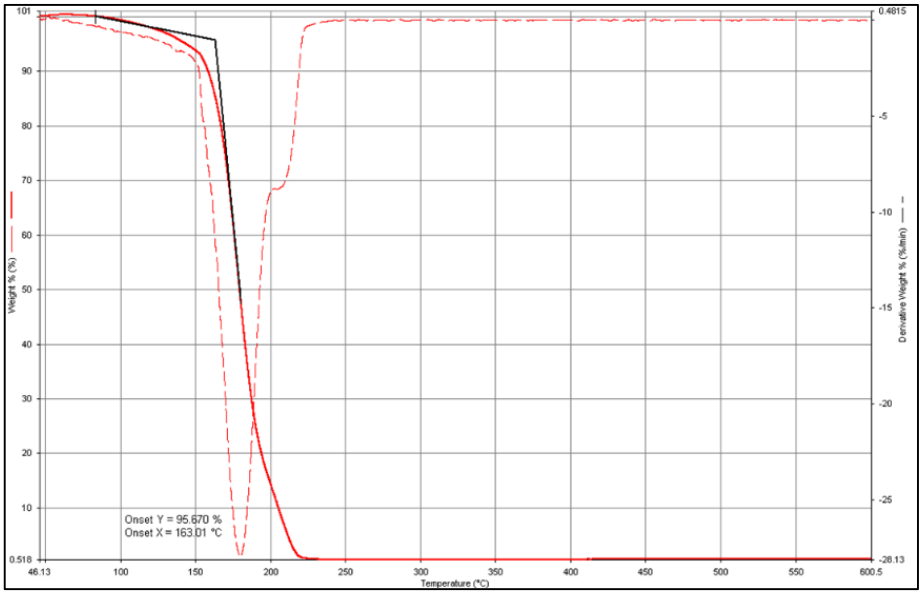


FIGURE 4.4 TGA Tetrabutylammonium Acetate

Thermogravimetric analysis or thermal gravimetric analysis (TGA) is conducted in order to determine the decomposition temperature of ILs that have been synthesized. Based on the analysis the temperature where Tetrabutylphosphonium acetate started to decompose is from 305.68°C while for Tetrabutylammonium acetate it started to decompose at 163.01°C. Starting from these temperature the ILs will started to decompose and cannot be used anymore.

4.2 Types of Microorganisms Used

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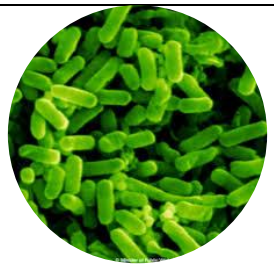
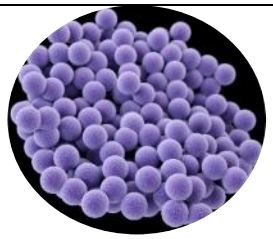
<td> 
Source:
http://pigeonracingpigeons.files.wordpress.com

Escherichia Coli (EC)
• Gram- negative bacteria
• Facultative anaerobic</td>
<td> 
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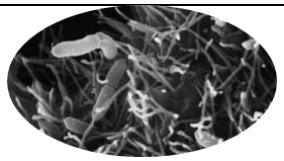
http://www.bacteriainphotos.com/
MRSA_images.html

Staphylococcus Aureus (SA)
• Gram- positive bacteria
• Facultative anaerobic</td>
</tr>
<tr>
<td> 
Source:
http://microbewiki.kenyon.edu/index.php/
Aeromonas_Hydrophila
Aeromonas Hydrophilia(AH)

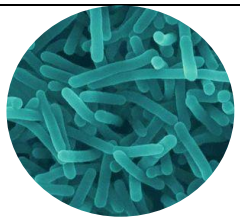
• Gram-negative
• Aerobic and anaerobic</td>
<td> 
Source :
http://www.foodsafety.asn.au/resources/
Listeria-monocytogenes/
Listeria Monocytogenes (LM)
• Gram- positive bacteria
• Facultative anaerobic</td>
</tr>
</table>

FIGURE 4.5 Microorganisms used to test toxicity of different ILs

Figure 4.5 shows the four bacteria that were used for to test the toxicity of selected ILs. There are widely research on *E. coli* bacteria in the field of biotechnology and microbiology. However for the other three bacteria which are *Staphylococcus Aureus, Aeromonas Hydrophilia* and *Listeria Monocytogenes* there is less research on them. These bacteria are chosen because of they are available in environment. Not only that, these bacteria can grow easily and the cost of growing it is cheaper.

4.3 Bacteria Cultivation

The four types of selected microorganism have been sub-cultured and keep in temperature 4 °C for further use.

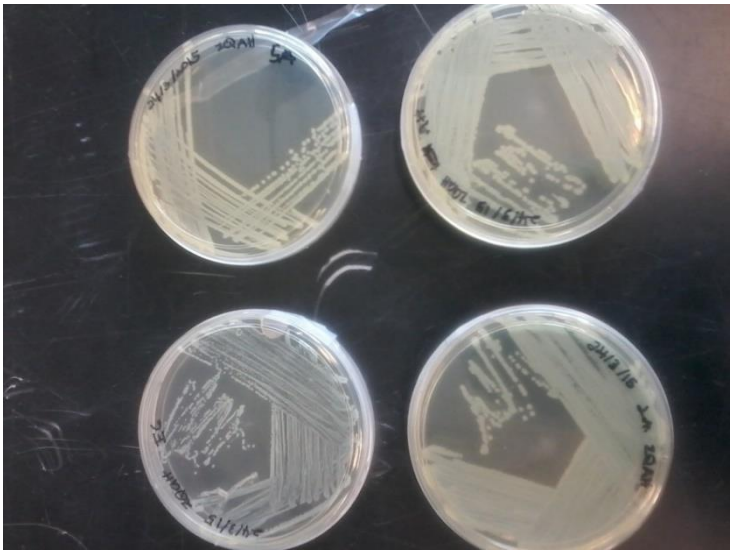


FIGURE 4.6 Agar plate that contain the selected microorganisms

The bacteria cultivation process also have been done according to the following procedure:

A single colony of each microorganisms was taken and put into the Luria Bertani (LB) media to grow the bacteria and put under condition of 37°C, 175 rpm for about 18-20 hours in a shaking incubator.

FIGURE 4.7 Single colony of each microorganism in LB media are put in the incubator shaker

After that 10 ml of LB media is put into the bottle that contain the 10 ml Mueller Hinton Broth. Then, the turbidity of the bacterial suspension is compared with the McFarland standard.

4.3.1 Turbidity against McFarland Standard

McFarland Standard are used as a standard to adjust the densities of bacterial suspensions according to the following procedure:

1. The McFarland Equivalence Turbidity Standard is inverted to fully suspend the polystyrene microparticles.
2. The turbidity of active grow broth culture or bacterial suspension is compared visually using McFarland Standard.
3. White card with contrasting black line is used to compare.

TABLE 4.1 McFarland Standard

Standard No	0.5	1.0	2.0	3.0	4.0	5.0
Approximate Cell Density($x10^8$/ml)	1.5	3.0	6.0	9.0	12.0	15.0

The turbidity of the bacterial suspension is compared with the standard no 1.0 which the density is approximately to 3.0 $x10^8$/ml. The bacteria then can be used to test the toxicity of ILs.

4.4 Minimum Inhibitory Concentration (MIC) Test

MIC test conducted by following the procedure above. When MIC test is conducted the result will be obtain by using 96-wells plate reader. The viability for the different microorganisms towards different ILs will be calculated. Viability is calculated in order to identify the ability of microorganisms to maintain its potentialities. Viability can be calculated by using the formula below:

$$\text{Viability: } \frac{absorbance\ at\ each\ cell}{absorbance\ of\ cell\ at\ column\ 1} \times 100\% \qquad (1)$$

Thus, as the viability have been calculated EC50 value can be determined by constructing the graph. EC50 values refer to the concentration at which 50% of the exposed organisms are immobilized or killed.

4.5 Dose Response Graph

The value of EC50 of each microorganism on different ILs can be determined by constructing dose-response graph. The x-axis of dose response graph is the log concentration of ILs (ppm) where y-axis is the viability of microorganisms (%). In this project MIC test is done three time for all bacteria against one type of ILs. The lowest EC50 value indicate that the IL is toxic towards that microorganisms.

4.6 Discussion

4.6.1 Dose Response Curve for Tetrabutylphosphonium Hydroxide

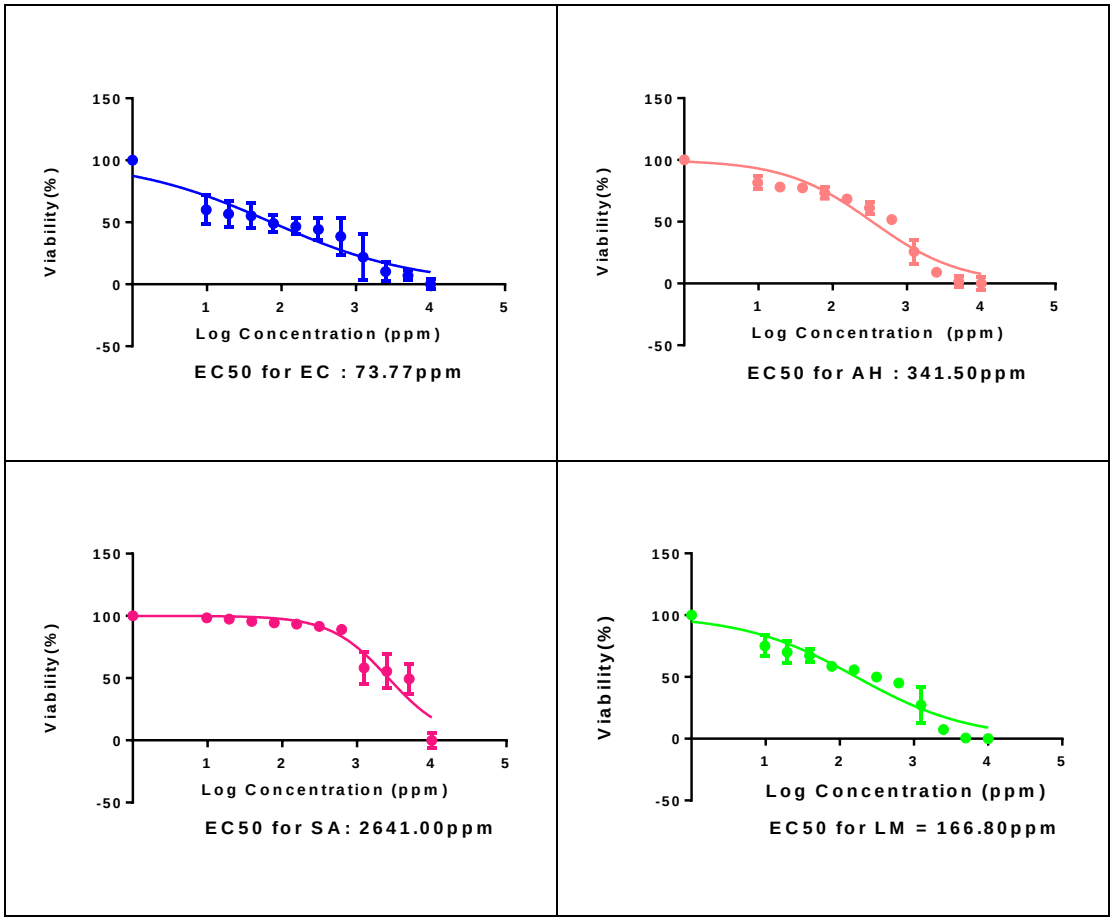


FIGURE 4.8 Graph of Viability vs Log Concentration for Tetrabutylphosphonium Hydroxide for different microorganisms

Figure 4.8 shows the graph of viability vs. log concentration of Tetrabutylphosphonium Hydroxide for 4 different microorganisms which are *Escherichia Coli* (EC**)**, *Staphylococcus Aereus* (SA**)**, *Aeromonas Hydrophilia* (AH) and *Listeria Monocytogens* (LM). Based on the graph we can see that the trend of the graphs are decreasing. The viability of the microorganisms are decreasing as the concentration of Tetrabutylphosphonium Hydroxide increasing. Based on the results, EC has the lowest EC50 value which is 73.77ppm. This shows tetrabutylphosphonium hydroxide is toxic towards EC. SA has the highest EC50 value which is 2641.00ppm.

4.6.2 Dose Response Curve for Tetrabutylammonium Hydroxide

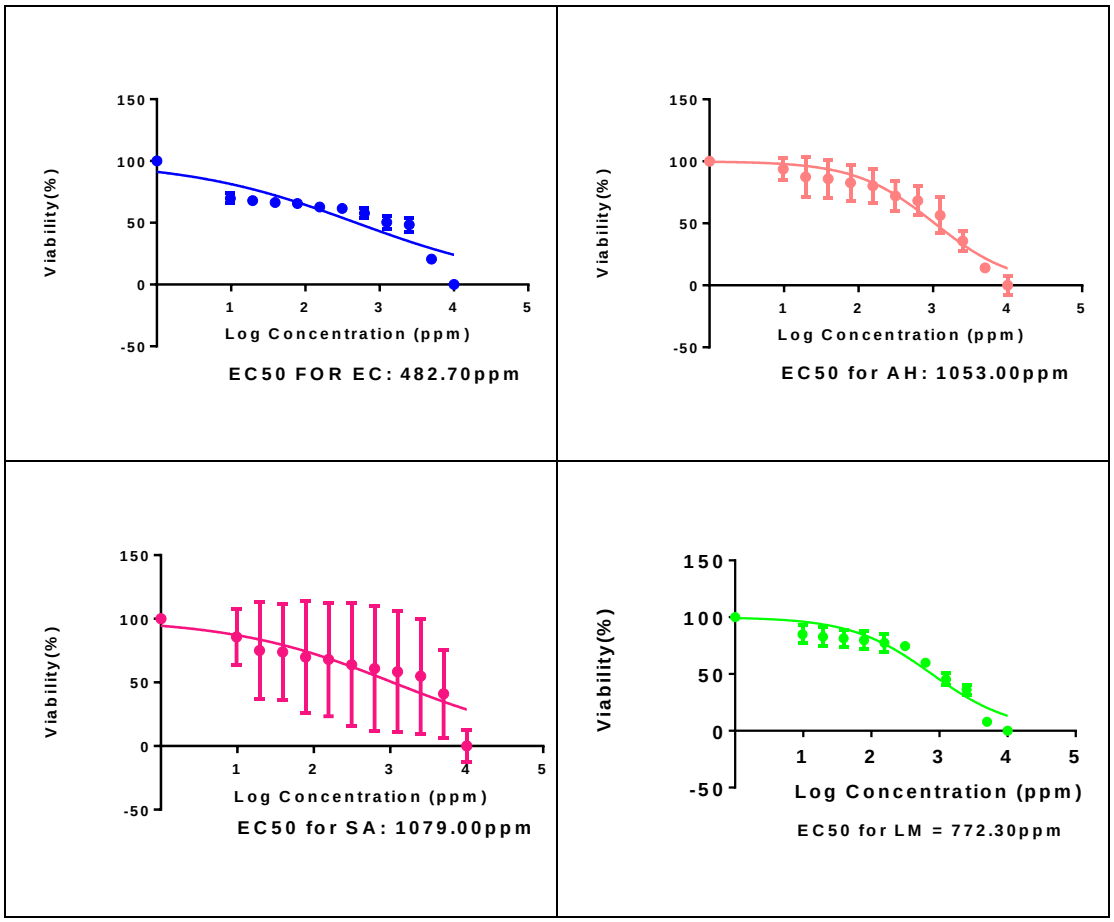


FIGURE 4.9 Graph of Viability vs Log Concentration for Tetrabutylammonium Hydroxide for different microorganisms

Based on Figure 4.9, all the grapg are showing decreasing trend. EC has the least EC50 value which is 482.70ppm. This shows tetrabutylammonium hydroxide is toxic towards EC. SA has the highest EC50 value which is 1079.00ppm.

4.6.3 Dose Response Curve for Tetrabutylphosphonium Acetate

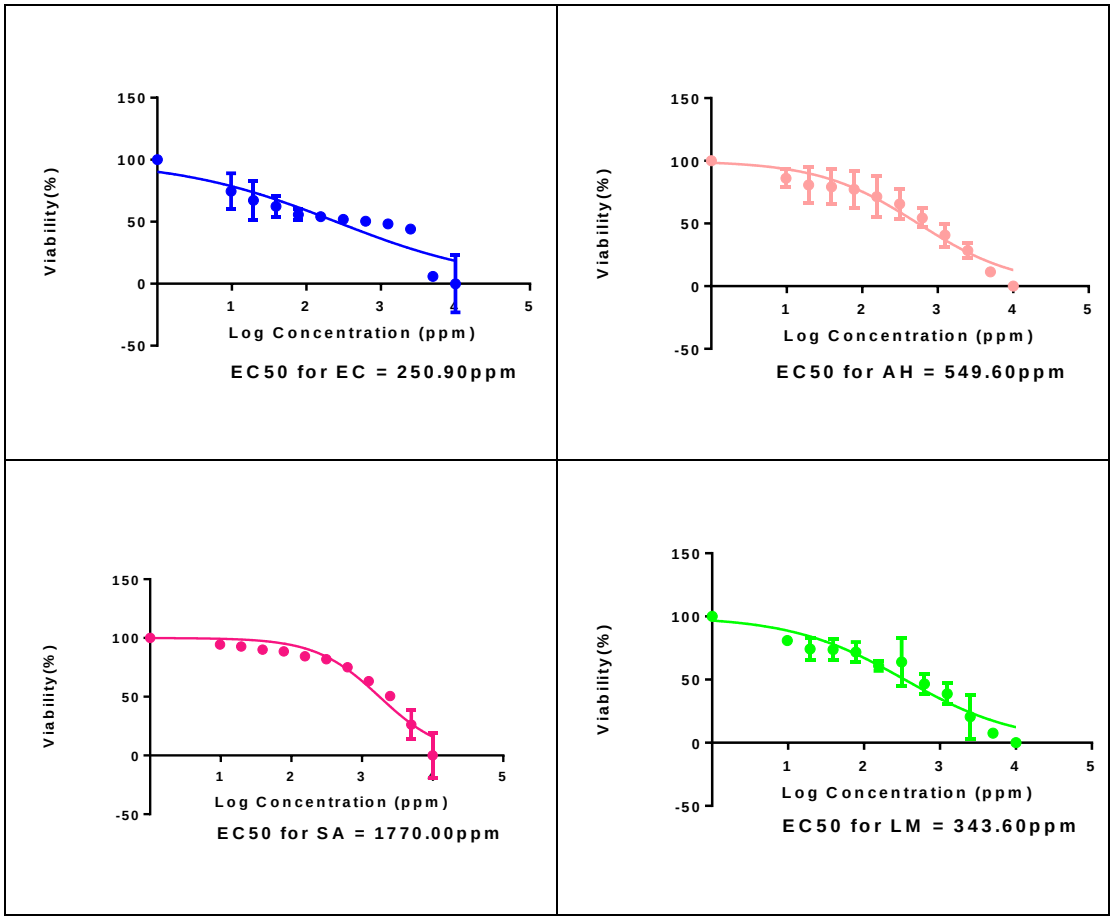


FIGURE 4.10 Graph of Viability vs Log Concentration for Tetrabutylphosphonium Acetate for different microorganisms

All graphs show decreasing trending where the viability (%) decreasing as the concentration increasing. EC has the lowest EC50 value which is 250.90ppm compare to other types of microorganisms. This shows Tetrabutylphosphonium Acetate is toxic towards EC. SA has the highest EC50 value which is 1770.00ppm.

4.6.4 Dose Response Curve for Tetrabutylammonium Acetate

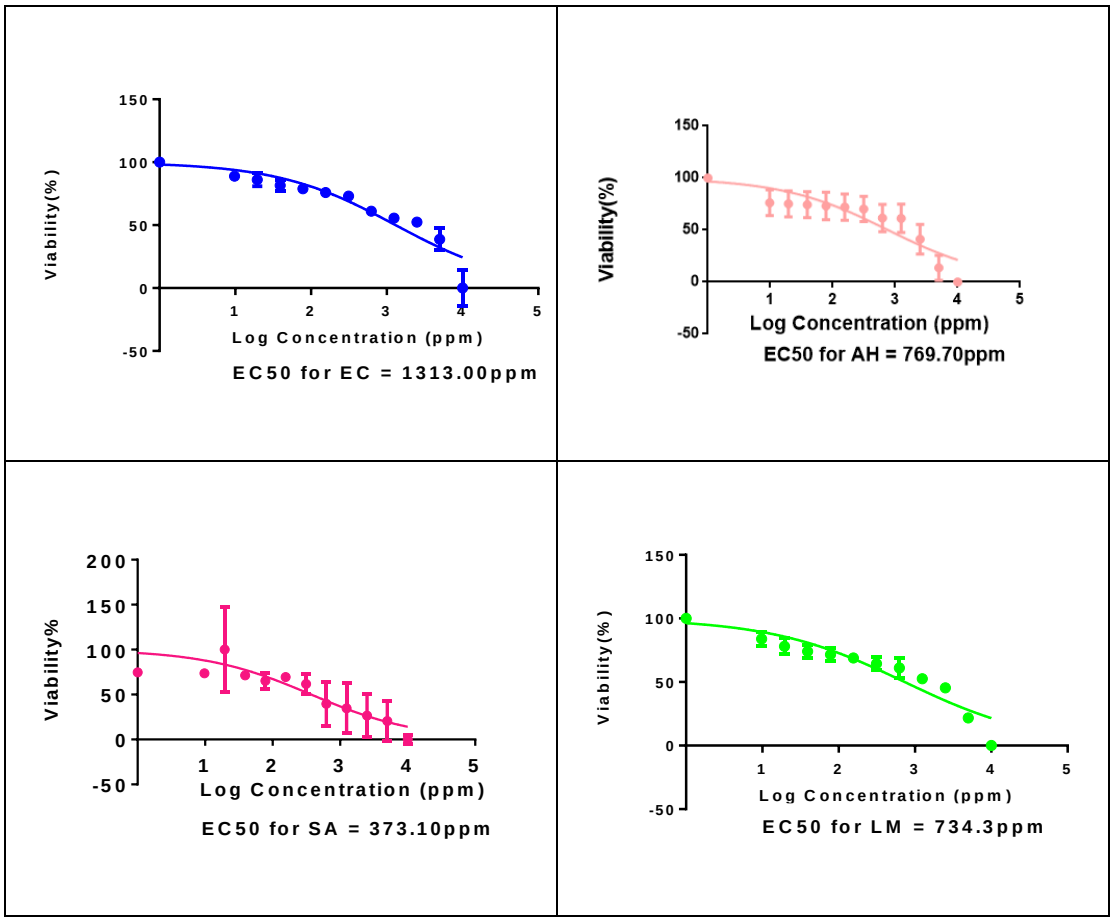


FIGURE 4.11 Graph of Viability vs Log Concentration for Tetrabutylammonium Acetate for different microorganisms

The trend of the graph is decreasing. Based on the results, the lowest EC50 value is 373.10ppm which is from SA. This shows Tetrabutylammonium Acetate is toxic towards SA. EC has the highest EC50 value which is 1313.00ppm.

TABLE 4.2 Summary of EC50 for all microorganisms

	EC50(ppm) (lower limit ;upper limit)			
Ionic Liquid	***Escherichia Coli (EC)***	***Aeromonas Hydrophilia (AH)***	***Staphylococcus Aereus (SA)***	***Listeria Monocytogens (LM)***
Tetrabutylphosphonium Hydroxide	**73.77 (46.22;117.70)**	**341.50 (262.10;444.90)**	**2641.00 (2078.00;3356.00)**	**166.80 (121.40;229.20)**
Tetrabutylammonium Hydroxide	**482.70 (291.70;798.80)**	**1053.00 (758.80;1461.00)**	**1079.00 (240.90 ;4828.00)**	**772.30 (583.10;1023.0)**
Tetrabutylphosphonium Acetate	**250.90 (142.70;441.20)**	**549.60 (396.30;762.40)**	**1770.00 (1308.00;2397.00)**	**343.60 (240.70;490.50)**
Tetrabutylammonium Acetate	**1313.00 (918.90;1875.00)**	**769.70 (443.30;1336.00)**	**373.10 (189.2;736.0)**	**734.30 (509.40;1058.0)**

The lowest EC50 value show that the ILs is higher towards the microorganisms. The Based on the table, the results shows that Tetrabutylphosphonium Hydroxide is toxic towards *Escherichia Coli*. The EC50 value is 73.77ppm. The lower limit and upper limit of EC50 value for Escherichia *Coli* are 46.22ppm and 117.70ppm.

The increasing toxicity level is represented by:

- **EC**

  Tetrabutylammonium Acetate ($C_{18}H_{39}O_2N$) < Tetrabutylammonium Hydroxide ($C_{16}H_{37}ON$) < Tetrabutylphosphonium Acetate; ($C_{18}H_{39}O_2P$) < Tetrabutylphosphonium Hydroxide; ($C_{16}H_{37}OP$)

- **AH**

  $C_{16}H_{37}ON$< $C_{18}H_{39}O_2N$<$C_{18}H_{39}O_2P$< $C_{16}H_{37}OP$

- **SA**

  $C_{16}H_{37}OP$< $C_{18}H_{39}O_2P$< $C_{16}H_{37}ON$< $C_{18}H_{39}O_2N$

- **LM**

  $C_{16}H_{37}ON$< $C_{18}H_{39}O_2N$< $C_{18}H_{39}O_2P$< $C_{16}H_{37}OP$

The effect of cation was studied by using phosphonium and ammonium based ILs. While the effect of anion was studied based on hydroxide and acetate. The effects observed are depending on the morphologic aspect of the microorganisms tested. According to Wood (2011), the respond of different organisms towards different ILs will be different. This can be explained by the difference between gram negative bacteria and gram positive bacteria. Both of the type of bacteria shared the same internal but they have different external structure. Gram positive bacteria has a thick and multilayered cell wall while gram negative bacteria have thin layer. According to Ventura et al., (2012) the sensitivity level of microorganisms is represented by:

Yeast<mold<gram-negative bacteria<Gram- positive bacteria

4.6.5 Effect of Cation

The cation will give a significance impact on toxicity of ILs. Based on the EC50 value it is observed that phosphonium cation is more toxic towards all microorganisms except *S. Aereus (SA).* This results are similar to the finding of Carvalho et al., (2014) who found phosphonium is more toxic compare to ammonium when tested on bacterium *Vibrio fischeri*. According to several research it is believe that the toxicity of ILs is affected most by the cation compared to anion (Matzke et al., 2007). According to Kumar (2009) a research has been conducted in order to study phosphonium and ammonium cation based ILs on the anti-cancer activities by using NCI 60 of human tumor cell lines. It is found that phosphonium cation is more active compared to ammonium cation. The phosphonium based ILs shows that they are sensitive against all 60 tumor cell lines compared to ammonium.

4.6.6 Effect of Anion

Anion component are also observed in order to compare the toxicity level. For this project hydroxide and acetate anion is compared. Based on the results, hydroxide anion is found to be toxic towards microorganisms. Nevertheless there is not much findings in regards to these four bacteria tested. Researches should be done more on this matter to support this findings. According to the research conducted by Saadeh et al., (2009) all the terabutylammonium salts; acetate affected gram-positive bacteria. This is similar with the result obtain which show terabutylammonium acetate is toxic towards *Staphylococcus Aereus* by having EC50 value 373.10ppm.

4.7 Possible Error

Based on the results, it is observed that hydroxide anion is more toxic compared to acetate anion. The results obtained may be due to some possible errors which are:

1. Experimental error while doing the ½ fold dilution which causing error on the concentration of ILs.
2. The growth of bacteria are affected by some external constraints including temperature, concentration, pH and many more.

CHAPTER 5
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

It is important to know the toxicity of those ILs to society in order for the society to be aware regarding the effect of the toxicity of ILs. As a conclusion, the project investigate the toxicity of ionic liquids towards microorganism. The toxicity of the ILs are different depending on the types of ILs. The objectives of this project have been achieved which is to determine the EC50 for each microorganisms towards selected ILs. Not only that the effect of cation and anion towards toxicity level of different ILs are also determined. The toxicity of ionic liquid can be determine by observing the lowest number of EC50. Based on the results obtained phosphonium cation is the most toxic ILs towards the microorganisms. While, hydroxide anion is the most toxic towards microorganisms.

5.2 Recommendations

It is recommended to test the ILs with different organisms in order to know the impact of the ILs towards environment. Not only that, the test should be repeated in many times in order to get the accurate reading. The bacteria growth rate should be take into consideration as there is no significant if the ILs are tested on the bacteria that are already dead.

Thus, the procedures to growth the bacteria should be followed carefully or should be repeated in order to ensure the bacteria is growth. The ½ fold dilution

should be done carefully as it will impact the results of the toxicity. Researchers should ensure that they are put the ILs into the well plate correctly.

Not only that, this project can be continue by conducting biodegradability test. These experiment can be conducted by using the other ILs since there is lack of information regarding the toxicity.

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APPENDICES

Appendix 1: Table of Viability for *Staphylococcus Aereus (*SA)

Concentration (ppm)	0	9.766	19.531	39.0625	78.125	156.25	312.5	625	1250	2500	5000	10000
	1	2	3	4	5	6	7	8	9	10	11	12
TBPOH	100.000	99.558	99.293	98.851	98.408	98.143	97.524	96.905	87.445	86.737	82.935	51.724
	100.000	98.948	98.247	97.283	96.670	96.407	95.355	93.602	77.651	76.074	74.321	56.968
	100.000	99.299	98.861	97.721	97.283	96.319	95.618	94.391	77.213	75.723	72.831	53.287
Average	100.000	99.268	98.800	97.952	97.454	96.956	96.166	94.966	80.770	79.511	76.696	53.993
TBPAce	100.000	98.939	98.320	97.524	97.171	95.579	94.518	93.722	91.512	85.588	76.481	68.258
	100.000	98.320	98.143	97.613	97.347	96.375	95.579	94.872	90.716	87.887	79.752	70.822
	100.000	98.422	97.984	97.195	96.757	96.056	96.056	92.200	89.483	88.519	87.029	84.049
Average	100.000	98.560	98.149	97.444	97.092	96.003	95.384	93.598	90.570	87.331	81.087	74.377
TBAOH	100.000	99.293	98.497	98.232	97.878	96.375	95.314	94.695	92.573	88.329	76.923	37.489
	100.000	98.773	97.984	96.757	96.319	96.056	94.917	92.200	90.184	88.519	75.635	47.327
	100.000	75.986	58.019	57.406	50.657	49.080	43.909	41.630	41.192	40.578	39.702	32.252
Average	100.000	91.351	84.833	84.132	81.618	80.504	78.046	76.175	74.650	72.475	64.087	39.023
TBAAce	100.000	99.299	98.598	97.721	97.371	96.845	96.231	95.793	95.443	87.905	83.348	55.478
	100.000	99.735	98.939	98.408	97.878	97.171	96.640	74.713	70.999	68.347	66.667	61.450
	100.000	99.211	145.136	98.335	88.782	97.195	85.276	70.202	66.082	62.489	58.545	56.880
Average	100.000	99.415	114.224	98.155	94.677	97.070	92.716	80.236	77.508	72.914	69.520	57.936

Appendix 2: Table of Viability for *Escherichia Coli (EC)*

Concentration (ppm)	0	9.766	19.531	39.0625	78.125	156.25	312.5	625	1250	2500	5000	10000
	1	2	3	4	5	6	7	8	9	10	11	12
TBPOH	100.000	64.351	63.041	62.254	60.682	58.978	55.570	47.051	40.760	34.862	34.731	29.489
	100.000	74.967	72.608	71.560	69.201	66.710	66.317	65.007	61.861	45.085	40.236	33.028
	100.000	79.920	76.862	75.532	66.888	66.223	65.293	63.564	39.362	38.431	36.968	34.973
Average	100.000	73.080	70.837	69.782	65.590	63.970	62.393	58.540	47.328	39.459	37.312	32.497
TBPAce	100.000	83.879	80.341	79.948	77.982	77.457	76.409	75.754	74.050	69.594	50.983	61.992
	100.000	81.520	76.802	76.802	74.705	74.443	73.788	73.001	72.477	71.035	52.294	39.318
	100.000	95.479	92.287	85.372	78.989	77.394	75.798	74.734	73.670	73.138	51.596	44.548
Average	100.000	86.959	83.143	80.707	77.225	76.431	75.331	74.496	73.399	71.256	51.624	48.619
TBAOH	100.000	82.447	80.718	79.920	79.388	73.936	73.404	68.218	62.234	60.505	48.138	34.309
	100.000	77.064	76.802	76.016	75.360	75.098	74.443	72.477	68.676	67.235	43.644	30.013
	100.000	78.768	77.064	75.623	75.098	74.836	73.919	73.132	67.890	66.972	46.265	31.717
Average	100.000	79.426	78.195	77.186	76.616	74.624	73.922	71.276	66.267	64.904	46.016	32.013
TBAAce	100.000	91.622	88.963	86.968	86.569	86.303	84.973	76.463	75.399	71.676	71.410	35.239
	100.000	94.626	93.709	91.481	88.991	86.632	84.535	78.768	74.836	73.788	64.089	49.279
	100.000	95.151	94.233	90.826	89.253	86.894	85.452	79.817	75.754	75.098	62.385	48.624
Average	100.000	93.800	92.302	89.758	88.271	86.610	84.987	78.349	75.330	73.521	65.961	44.381

Appendix 3: Table of Viability for *Aeromonas Hydrophilia (*AH)

Concentration (ppm)	0	9.766	19.531	39.0625	78.125	156.25	312.5	625	1250	2500	5000	10000
	1	2	3	4	5	6	7	8	9	10	11	12
TBPOH	100.000	86.237	85.101	84.343	82.955	77.904	73.864	65.530	43.939	33.965	33.333	32.449
	100.000	91.069	85.912	85.535	82.642	79.371	68.428	67.799	42.138	34.340	28.302	27.799
	100.000	83.396	82.138	81.887	77.107	75.094	74.591	63.145	54.843	36.855	27.421	25.409
Average	100.000	86.901	84.384	83.922	80.901	77.456	72.294	65.491	46.974	35.053	29.686	28.552
TBPAce	100.000	91.540	90.783	89.899	85.354	75.758	73.232	72.854	59.217	49.369	41.667	35.732
	100.000	95.220	94.465	93.333	94.465	93.585	86.289	73.208	67.925	56.855	43.774	32.327
	100.000	86.038	76.855	76.101	75.346	74.591	72.830	64.403	56.226	53.082	40.503	35.346
Average	100.000	90.933	87.368	86.444	85.055	81.311	77.451	70.155	61.123	53.102	41.981	34.468
TBAOH	100.000	98.737	97.601	96.086	93.308	89.394	76.263	73.990	72.348	51.515	42.424	35.101
	100.000	99.497	97.736	96.604	94.591	94.214	90.566	87.421	79.119	62.264	40.126	34.340
	100.000	88.931	78.868	78.491	76.855	75.975	76.101	73.585	59.497	54.969	41.887	26.038
Average	100.000	95.722	91.402	90.393	88.252	86.528	80.976	78.332	70.322	56.249	41.479	31.826
TBAAce	100.000	93.813	93.182	92.677	92.298	91.162	89.520	83.333	82.576	69.318	52.399	33.333
	100.000	81.384	80.629	79.874	78.994	78.113	77.862	75.094	76.604	64.403	41.384	35.220
	100.000	78.491	77.862	77.484	76.226	75.975	74.340	66.289	65.031	51.321	37.358	35.849
Average	100.000	84.562	83.891	83.345	82.506	81.750	80.574	74.906	74.737	61.680	43.714	34.801

Appendix 4: Table of Viability for *Listeria Monocytogens (*LM)

Concentration (ppm)	0	9.766	19.531	39.0625	78.125	156.25	312.5	625	1250	2500	5000	10000
	1	2	3	4	5	6	7	8	9	10	11	12
TBPOH	100.000	84.982	80.403	73.626	58.791	54.762	54.212	47.985	37.363	7.509	1.465	1.099
	100.000	71.313	65.316	64.992	58.509	56.888	50.081	44.733	35.656	12.480	1.621	0.810
	100.000	69.854	65.316	64.344	59.643	57.212	46.840	44.084	11.021	5.024	1.783	1.297
Average	100.000	75.383	70.345	67.654	58.981	56.287	50.378	45.601	28.013	8.338	1.623	1.069
TBPAce	100.000	85.714	84.799	84.066	81.868	67.033	58.608	57.692	50.549	43.223	13.370	2.930
	100.000	79.254	70.340	70.016	68.720	59.643	51.702	45.057	37.439	16.694	10.373	3.079
	100.000	80.227	71.151	70.502	68.395	61.264	86.386	44.084	36.143	12.480	11.021	7.131
Average	100.000	81.732	75.430	74.862	72.994	62.647	65.565	48.944	41.377	24.132	11.588	4.380
TBAOH	100.000	94.139	92.674	90.476	89.011	86.813	78.938	64.286	51.465	42.125	9.341	1.648
	100.000	80.065	77.958	76.823	75.851	73.906	73.258	58.023	45.867	35.981	8.590	0.972
	100.000	81.199	78.282	77.634	75.041	72.771	72.609	59.157	41.005	33.387	9.724	1.783
Average	100.000	85.134	82.971	81.644	79.967	77.830	74.935	60.489	46.112	37.164	9.218	1.468
TBAAce	100.000	90.659	85.531	80.769	78.022	73.810	70.147	69.963	56.960	50.916	23.810	3.114
	100.000	81.686	75.527	72.123	69.854	67.747	66.451	62.237	51.053	43.760	26.094	2.107
	100.000	80.713	75.041	71.313	69.368	67.585	59.806	54.457	53.809	45.867	22.042	3.079
Average	100.000	84.353	78.699	74.735	72.415	69.714	65.468	62.219	53.941	46.848	23.982	2.767