MERCURY REMOVAL FROM PETROLEUM BASED INDUSTRIES WASTEWATER BY *Pseudomonas putida* ATCC 49128 IN MEMBRANE BIOREACTOR

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ABSTRACT

Mercury is an extremely toxic pollutant that currently being emitted and distributed globally. Microbes have been used to solve environmental wastewater problems for many years. The objective of the study is to remove mercury from petrochemical wastewater using *Pseudomonas putida* (*P. putida*), ATCC 49128 in membrane bioreactor to enhance water quality to meet EQA – Industries Effluent 2011 requirements. The approach of this study is focused on the removal or reduction of mercury concentration including development studies of pure culture of *P. putida* and growth related parameters involved in designing the membrane bioreactor system. Furthermore, this research also provide an understanding of *P. putida* behavior in rehydrating freeze-dried growing method, growth parameters and optimum operating condition in membrane bioreactor. A field survey on mercury concentrations at several petroleum based industrial plants in Peninsular Malaysia revealed a maximum reading of 2318 ppb which is higher than the Department of Environmental (DOE), Malaysia requirement for Standard B (50 ppb). Hence, treatment study of *P. putida* on mercury contaminated wastewater was conducted at mercury concentration in the range of 1000 – 4000 ppb. Optimum operating conditions of 24 hours acclimatization time, 180 rpm of orbital shaker speed, temperature of 37^{o}C, pH 7 and nutrient concentration of 8 g/L were identified for *P. putida* growth in a shaked flask. The overall percentage of mercury removal for sample with 4000 ppb mercury in bioreactor is 99.60% for the first 6 hours and 99.80% removal after the microfiltration membrane system. Microfiltration membrane enhanced further the treatment of the wastewater by retaining the *P. putida* from escaping during the release of treated wastewater, reducing the turbidity by 94.2% (5.32 NTU) and concentration of suspended solids up to 60.4%. (0.09 mg/L). It was identified that the mechanism of mercury detoxification in the membrane bioreactor was based on reduction of Hg^{2+} to non-toxic Hg^{0} by mercury reductase enzyme produced by *P. putida*. The findings from this study can be used as references for future application of petroleum based industries wastewater treatment as well as other industries related to mercury contamination in their wastewater treatment plant such as gold mining, chemical industries, agriculture etc.

ABSTRAK

Merkuri merupakan bahan pencemar amat toksik yang kini telah tersebar dan teragih secara global. Mikrob telah digunakan untuk menyelesaikan masalah air sisa buangan alam sekitar selama bertahun-tahun. Objektif kajian ini ialah untuk menyingkir merkuri daripada air sisa buangan industri berasaskan petroleum menggunakan *Pseudomonas putida* (*P. putida*) ATCC 49128 di dalam Bioreaktor Membran untuk meningkatkan kualiti air bagi mematuhi keperluan EQA – Efluen Industri 2011. Pendekatan kajian baru ini memfokuskan kepada membuang atau mengurangkan kepekatan merkuri termasuk kajian pembangunan kultur tulen *P. putida* dan parameter-parameter yang berkaitan dengan pertumbuhannya dalam mereka sistem Bioreactor Membran. Seterusnya, penyelidikan ini menyediakan kefahaman terhadap kelakuan *P. putida* dalam metod pertumbuhannya secara pengeringan sejuk beku hidratan semula, parameter-parameter pertumbuhan dan kondisi operasi optima dalam Bioreaktor Membran. Tinjauan lapangan di beberapa loji industri berasaskan petroleum di Semanjung Malaysia mendapati bacaan kepekatan maksima merkuri ialah 2318 ppb yang mana bacaan ini melebihi daripada tahap yang dibenarkan oleh Jabatan Alam Sekitar (DOE) Malaysia bagi Piawaian B (50 ppb). Oleh yang demikian, kajian rawatan air sisa buangan yang tercemar dengan merkuri dalam julat 1000 – 4000 ppb menggunakan *P. putida* telah dijalankan. Didapati kondisi operasi optima ialah 24 jam untuk masa sesuaian iklim,180 rpm kelajuan penggoncang orbital, suhu 37^{o}C, pH 7 dan kepekatan nutrisi sebanyak 8 g/L adalah untuk pertumbuhan *P. putida* dalam kelalang penggoncang. Peratus keseluruhan pembuangan merkuri untuk sampel berkepekatan 4000.00 ppb dalam bioreaktor ialah 99.60% untuk 6 jam pertama dan 99.8% pembuangan selepas sistem membran penapisan mikro. Membran penapisan mikro mampu meningkatkan rawatan air sisa buangan dengan menahan *P. putida* daripada terlepas semasa pelepasan air yang telah dirawat, mengurangkan kekeruhan sebanyak 94.2% (5.32 NTU) dan mengurangkan kepekatan pepejal terampai sehingga 60.40% (0.09 mg/L). Di dalam bioreaktor, didapati mekanisma penyahtoksikan dilakukan oleh enzim merkuri "reductase" yang dihasilkan oleh *P. putida* dengan melakukan penurunan Hg^{2+} kepada Hg^{0} yang tidak toksik. Penemuan daripada kajian ini boleh digunakan sebagai rujukan untuk aplikasi akan datang bagi rawatan air sisa buangan berasaskan petroleum dan industri yang berkaitan dengan pencemaran merkuri dalam loji rawatan air sisa buangan seperti perlombongan emas, industri kimia, pertanian dan lain-lain lagi.

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LIST OF SYMBOLS

kPa	-	Kilopascal
P_f	-	Inlet pressure of feed stream, kPa
P_c	-	Pressure of concentrate stream, kPa
P_p	-	Pressure of permeate stream, kPa
[S]	-	The concentration of the reactant
P	-	Pressure, Pa
k	-	Growth rate constant
n	-	Stoichiometric yield constant
[P]	-	The concentration of the product
X	-	The concentration of biomass in the bioreactor
μ	-	The specific growth rate
Y_{XS}	-	Biomass yields
Y_{PS}	-	Product yields
t	-	Time
μ_m	-	Maximum specific growth rate
K_s	-	Monod constant
n	-	Number of generation
g	-	Generation time
A	-	Initial mercury concentration
B	-	Final mercury concentration

LIST OF ABBREVIATIONS

ml	-	Milliliter
min	-	Minute
rpm	-	Rev- per- minute
ppm	-	Parts-per-million
ppb	-	Parts-per-billion
hr	-	Hour
NTU	-	Nephelometric Turbidity Unit
Hg^{2+}	-	Inorganic Mercury (+2)
Hg^{0}	-	Gaseous Elemental Mercury
Hg	-	Mercury
HgS	-	Mercury Sulfide
MeHg	-	Methyl Mercury
THg	-	Total mercury
P. putida	-	*Pseudomonas putida*
MBR	-	Membrane Bioreactor
MF	-	Microfiltration
UF	-	Ultrafiltration
TMP	-	Transmembrane pressure gradient, kPa
$Hg(NO_3)_2$	-	Mercury(II) Nitrate
KH_2PO_4	-	Monopotassium Phosphate
$MgSO_4.{}_7H_2O$	-	Magnesium Sulfate Heptahydrate
NaCl	-	Natrium chloride
HNO_3	-	Nitric Acid
HCl	-	Hydrochloric Acid
NaOH	-	Sodium Hydroxide
NaOCl	-	Sodium Hypochlorite
H_2SO_4	-	Sulfuric Acid
NA	-	Nutrient Agar
NB	-	Nutrient Broth
DOE	-	Department of Environmental
EQA	-	Environmental Quality Act
RO	-	Reverse Osmosis

OD	-	Optical Density
EPA	-	Environmental Protection Agency
WHO	-	World Health Organization
MAHL	-	Maximum Allowable Headworks Loading
COD	-	Chemical Oxygen Demand
BOD	-	Biological Oxygen Demand
OSHA	-	Occupational Safety and Heath Administration
NIOSH	-	National Institute for Occupational Safety and Health
REL	-	Recommended Exposure Limit
TWA	-	Time Weighted Average
ACGIH	-	American Conference of Government Industrial Hygienists
ATSDR	-	Agency for Toxic Substances & Disease Registry
MRL	-	Minimal Risk Level
TLV	-	Threshold Limit Value
FDA	-	Food and Drug Administration
RCRA	-	Resource Conservation and Recovery Act
EOR	-	Enhance Oil Recovery
GAC	-	Granular Activated Carbon
PAC	-	Poly Aluminium Chloride
EDTA	-	Ethylene Diamine Triacetic Acid
AAGR	-	Average Annual Growth Rate
OSM	-	Outer Skin Membrane
TSS	-	Total Suspended Solids
TCE	-	Trichloroethylene
BTEX	-	Benzene, Toluene, Ethylbenzene, Xylene
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate
CGTase	-	Cyclodextrin Glycosyltransferase
vvm	-	Volume (air) per volume (media) per minute
ATCC	-	American Type Culture Collection
UV-Vis	-	Ultra-violet Visible
TDS	-	Total Dissolved Solid
PVC	-	Polyvinyl Chloride
P1L1	-	Plant 1 and Location 1
P1L2	-	Plant 1and Location 2

OTR - Oxygen Transfer Rate

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF RESEARCH

Mercury (Hg) pollution of the environment by mining activities and petroleum based industries wastewater has resulted in worldwide contamination of large areas of soils and sediments (Miller et al.,1996; Degetto et al.,1997; Suchanek et al., 1998 and Horvat et al., 1999) and led to elevated atmospheric mercury levels (Ebinghaus and Slemr, 2000). The amount of Hg in wastes only in the EU has been estimated at about 990 metric tonnes (Busto et al., 2010). Because of lack of suitable and effective cleanup technologies for low concentration of mercury in the petroleum based industries, efforts to deal with polluted sites are directed toward the mechanical removal of contaminated material and its deposition elsewhere (Hosokawa, 1993; Miserocchi et al., 1993 and Busto et al., 2010). Such processes are costly and often result in remobilization of toxic mercury compounds during the dredging process.

Mercury is one of the most toxic elements found on earth. It binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions. After being discharged into the environment, mercury enters the sediments where it remains for many decades. It is taken up by aquatic organisms in the form of highly toxic methylmercury and is subsequently biomagnified through the food chain, hence threatening the health of top predators, such as birds, fish, seals, and man, is thereby threatened (Braune et al., 1999 and Muir et al., 1999). At high concentrations, mercury vapor inhalation produces acute necrotizing bronchitis and pneumonitis which could lead to death from respiratory failure. Long term exposure to mercury vapor primarily affects the central nervous system.

Mercury also accumulates in kidney tissues, directly causing renal toxicity, including proteinuria or nephritic syndrome (Chang et al., 1998). High concentration of Hg^{2+} causes impairment of pulmonary function and kidney, chest pain and dyspnousea (Manohar et al., 2002). Therefore, the discharge of mercury into the environment by mercury emitting industries must be prevented and this could be achieved through the use of efficient and cost effective treatment technologies.

Purification of areas polluted by heavy metals such as mercury is difficult, because the metals cannot be transformed into harmless elements (Al-Malack and Anderson, 1996a). Over decades, communities have made concerted efforts to face this problem through treating and removing the heavy metals (Chandra et al., 2003). Various types of technology are available for removing mercury from water and wastewater, which include chemical precipitation, conventional coagulation, reverse osmosis, ultrafiltration, magnetic filtration, ion exchange and activated carbon adsorption and chemical reduction (Derek and Coates, 1997).

Mercury remediation through common physico-chemical technologies are not only expensive but not environmental friendly and used only to treat high mercury concentration in wastewater treatment plant. Stringent legislation requires expensive and efficient method of treatment of wastewater in order to fulfill the discharge limit requirement (Demirbas, 2008).

There are three principle advantages of biological technologies for the removal of pollutants: firstly, biological processes can be carried out *in-situ* at the contaminated site, secondly, bioprocess technologies are usually environmentally benign (no secondary pollution) and thirdly, they are cost effective (Vijayaraghavan and Yun, 2008).

Biological systems have been thought to be adopted for the removal of toxic heavy metals such as mercury from petroleum based industries wastewater (Zeroul et al., 2001 and Malakahmad et al., 2011). Bioremoval is a biological system for removing metal ions from polluted water has the potential to achieve greater performance at lower cost than non-

biological wastewater treatment (Kondoh et al., 1998). Developments in the field of environmental biotechnology indicate that bacteria, fungi, yeasts and algae can remove heavy metals from aqueous solution by adsorption (Saglam et al., 1999).

Bacterial resistance to mercury is related to enzymatic reduction of Hg^{2+} to volatile Hg^{0} (Devars et al., 2000). Mercury detoxification process originates from proteins of the microbial mercury resistance (mer) microbial mercury resistance, mer operon located in either plasmids or transposable elements in the mercury resistant microorganisms. Specific transport of bulk mercury across the cell membrane is achieved by two mer operon genes merP and merT, which express cystein-rich protein to deliver ambient mercuric toward intracellular mercuric reductase for subsequent reduction of mercuric ions to volatile Hg^{0} (Weon and Ashok, 2001).

1.2 PROBLEM STATEMENT

Mercury is one of the heavy metals of concern, found in wastewaters coming from oil refinery, chloralkali manufacturing industry, paint, pharmaceutical, paper and battery manufacturing industries. Mercury and mercurial compounds are highly toxic contaminants in the aquatic systems and soils. They are dangerous pollutants because they can disperse widely into environment due to their high mobility and potentially dangerous concentration throughout the food chain (Carpi, 2001).

Mercury contamination of hydrocarbon production and processing systems can be more than a mere nuisance (Li et al., 2009). Early detection and accurate quantification of mercury is necessary to assure equipment integrity, to comply with regulations and to ensure safety of workers. High concentrations of mercury are found in several regions of the world and operators have developed measures to cope with the major ramifications but all such measures benefit from early recognition of potential problems. Routine maintenance and inspection activities become non-routine when mercury is present in fluids above a few ppb and become problematic when mercury concentrations reach approximately 100 ppb.

Mercury in crude oil or gas affects quality and price of salable products and raises equipment integrity concerns in proportion to concentration that may be present. In rare cases of South East Asia, mercury can be present in sufficient quantities to interfere with the normal function of heat exchangers, separators and conditioning systems (Camanella et al., 1986).

Mercury in crude oil above certain limits can be problematic to refinery operations as mercury would deactivate catalysts and reduces the quality of refined products. The Environmental Protection Agency (EPA) of the United State has specified that waste with mercury below 260000 ppb concentrations must be stabilized before disposal. Environmental impacts are also important because running mercury-laden crude's can produce wastewater and solid waste streams having mercury concentrations that exceed regulatory limits. Mercury originating in crude feeds could deposit in equipment and thus can become an important health and safety issue during inspection and maintenance operations (Campanella et al., 1986).

Methods for removing mercury ion (Hg^{2+}) from aqueous solution mainly consist of physical, chemical and biological technologies. Among conventional methods suggested by researchers for removing mercury ions from aqueous solution are chemical precipitation and electrochemistry (Pascal et al., 2007), ion exchange (Chen et al., 2008), membrane technologies (Broom et al., 1994), adsorption on activated carbon and etc (Demirbas, 2008).

Most of mercury remediation through common physico-chemical technologies are expensive, not environmental friendly and suitable only for the treatment of higher mercury concentration in wastewater treatment plants. For example, chemical precipitation and conventional coagulation treatment are ineffective, especially when metal ion concentration in aqueous solution in the range 10000 to 100000 ppb, and it also produce large quantity of sludge which would subsequently require extensive treatment. Ion exchange, membrane technologies and activated carbon adsorption process are extremely expensive when treating large amount of water and wastewater containing heavy metal in low concentration

and they are impractical for large scale treatment (Volesky, 2001). In addition, treatment of mercury at low concentration using physico-chemical technique is not recommended.

Today, stringent legislation in Malaysia requires expensive and efficient methods of wastewater treatment in order to fulfill the discharge limit requirements, where the maximum allowable concentration of mercury is below or equivalent to 5 ppb for Standard A and 50 ppb for Standard B. In recent years, the application of biotechnology in controlling and removing mercury pollution has gained much attention, gradually becoming a hot topic in the field of heavy metals pollution control because of its potential application in wastewater treatment plant especially in petroleum based industries that are facing mercury ions contamination in their processing systems (Ahmadun et al, 2009).

One potential process in biotechnology that can be implemented in treatment of mercury contaminated wastewater is by introducing *Pseudomonas putida* (*P. putida*) as a biosorption approach which utilizes natural materials of biological origin. This method was pioneered by Nakamura el al., (1986) for the treatment of mercury contaminated wastewater discharged near Minamata Bay. The biosorption process possesses metal-sequestering properties and can be used to decrease the concentration of mercury ions in solution. It can effectively sequester dissolved mercury ions out of dilute complex solutions efficiently and quickly, thus making it an ideal candidate for treating high volume and low concentration complex wastewaters (Wang and Chen, 2006).

Even though much has been discussed in literatures regarding the potential usefulness of *P. putida* in treating mercury contaminated wastewater, no specific strains were mentioned. Established research findings showed that specific types of *P. putida* strain are suitable for specific application in treating contaminated wastewater, which include *P. putida S12* for phenol treatment (Wierckx et al., 2009), *P. putida KT2440* for biodegradable plastics (Wang and Nomura, 2010) and etc. Obviously the findings indicated that there is no specific strain that can naturally and effectively treat mercury contaminated wastewater unless the strain has been subjected to acclimatization and

conditioning. Hence, there is a need to conduct research on a chosen *P. putida* strain to understand its behavior in treating mercury contaminated wastewater under various conditions.

P. putida are free-living bacteria found in soil or water with a single polar flagellum and non-pathogenic as compared to other *pseudomonads* that can cause disease (Auerbach et al., 2000). *P. putida* plays an important role in the decomposition and biodegradation of wastewater undergoing treatment. In addition, *P. putida* have been used in applied studies by chemists and environmental engineers for green chemistry application and bioremediation for its enzymes (Felske et al., 2003). This strain is also grows well with benzene, toluene, ethylbenzene and p-cymene (Mazzeo et al., 2010).

These characteristics are most viable and capable for treating wastewater treatment especially in petroleum based industries. Toxic compound pollution from these industries and the accumulation of toxins in the food chain can lead to serious ecological and health hazards as a result of their solubility and mobility.

1.3 OBJECTIVE AND SCOPES OF THE RESEARCH

The objective of the proposed study is to remove mercury from petroleum based industries wastewater using *P. putida* ATCC 49128 in Membrane Bioreactor process to enhance water quality to meet the Environmental Quality Act (EQA), under Environmental Quality (Industries Effluent) Regulations 2011.

With the intention of achieving this objective, the following series of scopes are outlined:

i) To evaluate mercury contamination levels of two petroleum based industries wastewater treatment plants in Malaysia

ii) To study the method of *P. putida* growth (freeze dried culture), acclimatization time and the required nutrient

iii) To determine the optimum parameters for growth of *P. putida* in shake flasks culture

iv) To study the effects of the operation condition (temperature, pH, nutrient, shaking time) in the reduction of Hg using model/synthetic wastewater sample

v) To investigate reduction of Hg concentration in actual petroleum based industries wastewater using *P. putida* at optimum condition

vi) To evaluate the performance of membrane bioreactor (MBR) for Hg removal in petroleum based industries wastewater application

1.4 SIGNIFICANCE OF THE RESEARCH

This study focuses on removal or reduction of mercury concentration, development studies of pure culture (*P. putida*), operating parameters involved and the application of membrane bioreactor (MBR) process in wastewater treatment. The biological method and MBR design are emerging as a potential alternative that have been demonstrated to be robust, highly selective, efficient and environmental friendly (green technology) in minimizing mercury in wastewater with low mercury concentration. This approach is also suitable for treating wastewater with high total suspended solids (TSS), high total dissolved solid (TDS) and turbidity. This research will also provide an understanding of *P. putida* behavior using the rehydrating freeze-dried method, growth parameters, optimum operating condition and design of membrane bioreactor for mercury ions removal from wastewater.

The bioremediation method in treating low concentration mercury contaminated wastewater is expected to be able to overcome the disadvantages faced by the present

physico-chemical treatment method, such as high energy consumption, generation of toxic sludge through conversion of mercury into another form of complex, high capital and operation cost, and not environmental friendly.

Bioremediation is often considered a cost effective and environmental friendly method and is gradually making inroads in environmental clean-up application. In this study *P. putida* naturally possess the ability to degrade and transform toxic mercury (Hg^{2+}) to less toxic elemental mercury (Hg^{0}) through enzymatic transformation.

The findings from this study can be used as references for future application for petroleum based industry wastewater treatment as well as other industries and activities such as such as gold mining, chemical industries and agriculture, which are related to mercury contamination in their wastewater treatment plant.

1.5 OUTLINE OF THE THESIS

The thesis is arranged into five chapters in order to describe and report the research being carried out. Chapter 1 provides an introduction of mercury contamination in industrial wastewater, biological treatment, objectives, scopes and outline of approach to the research in order to achieve the target.

Chapter 2 titled Literature Review covers the review on mercury exposure and impact on health and environmental, and mercury exposure limit. This chapter discusses on mercury contamination at two wastewater treatment plants, and current treatment methods available today for mercury removal in wastewater plant especially in petroleum based industries. This chapter also presents work of previous researchers on mercury contamination, *P. putida* characteristic and mechanism of mercury removal, the ground work of *P. putida* culturing and growth related parameters. This chapter also states and elaborates on bioreactor operation, membrane bioreactor theories and processes and fermentation kinetics models, which are important in understanding the processes themselves.

Chapter 3 explains the methodology of the experiment. A research methodology flow chart is attached in order to simplify and facilitate better understanding of the research undertaken. The methods or the experimental steps taken to carry out the research are elaborated. Operating condition parameters investigated include optical density (OD) for the growth of *P. putida*, orbital shaker temperature, nutrient concentration, growth pH, growth time/period, agitator/orbital shaker speed, mercury concentration and water quality which is measured by total dissolved solid (TDS), total suspended solids (TSS) and turbidity.

Chapter 4 presents the results and discussions of case studies of mercury contamination at two petroleum based industries wastewater treatment plants. For experimental purposes the maximum mercury concentration in wastewater is identified as a benchmark for synthetic wastewater. This chapter also reports on *P. putida* seeding method for determining acclimatization time for the optimum and healthy growth in order to culture more *P. putida*. Operating conditions such as orbital shaker speed, orbital shaker temperature, pH, nutrient concentration and agitator speed in the shake flask and bioreactor are elaborated and discussed in this chapter. The performance of mercury removal in shake flask, bioreactor and membrane bioreactor using synthetic wastewater and actual wastewater are also discussed. Actual wastewater quality determined by total dissolved solid (TDS), total suspended solids (TSS) and turbidity before and after the treatment by *P. putida* in membrane bioreactor are reported and discussed.

Chapter 5 concludes the research work and summaries all the relevant findings generated from the study. Some suggestions and recommendations to improve the study and to conduct further investigation in the future are also given.

CHAPTER 2

LITERATURE REVIEW

2.1 MERCURY OVERVIEW

Mercury was known to the ancient Chinese and Hindus, and was found in Egyptian tombs that date from 1500 BC. In China, India and Tibet, mercury use was thought to prolong life, heal fractures, and maintain generally good health. China's first emperor, Qin Shi Huang Di said to have been buried in a tomb that contained rivers of flowing mercury, representative of the rivers of China was driven insane and killed by mercury pills intended to give him eternal life. The ancient Greeks used mercury in ointments and the Romans used it in cosmetics. By 500 BC mercury was used to make amalgams with other metals. The Indian word for alchemy is *Rasavatam*which means 'the way of mercury' (Eisler, 2006).

Alchemists often thought of mercury as the first matter from which all metals were formed. Different metals could be produced by varying the quality and quantity of sulfur contained within the mercury. An ability to transform mercury into any metal resulted from the essentially mercurial quality of all metals. The purest of these was gold, and mercury was required for the transmutation of base (or impure) metals into gold as was the goal of many alchemists (Farrar and Williams, 1977).

Hg is the modern chemical symbol for mercury. It comes from *hydrargyrum*, a Latinized form of the Greek word *`Υδραργυρος* (*hydrargyros*), which is a compound word meaning 'water' and 'silver', since it is liquid, like water, and yet has a silvery metallic

sheen. The element was named after the Roman God Mercury, known for speed and mobility. It is associated with the planet Mercury. The astrological symbol for the planet is also one of the alchemical symbols for the metal. Mercury is the only metal for which the alchemical planetary name became the common name (Carpi, 2001).

2.1.1 Chemistry of Mercury

Mercury is the only common metal liquid at ordinary temperatures. Mercury is sometimes called quicksilver. It rarely occurs free in nature and is found mainly in cinnabar ore (HgS) in Spain and Italy (De and Ramaiah, 2006). It is a heavy, silvery-white liquid metal (Srivastava and Majumder, 2007). It is a rather poor conductor of heat as compared with other metals but is a fair conductor of electricity. It alloys easily with many metals, such as gold, silver, and tin. These alloys are called amalgams. Its ease in amalgamating with gold is made use of in the recovery of gold from its ores. Appendix 2.1 – 2.5 illustrate detail of mercury properties such as general properties, physical properties, atomic properties, miscellaneous of mercury and selected isotopes of mercury.

There are three important types of mercury which is the pure element, inorganic compounds such as mercuric nitrate and organic mercury compounds such as phenyl mercuric propionate. Mercury generally exists as elemental mercury (Hg(0) or Hg^0), monovalentmercury (Hg(I)), divalent mercury (Hg(II) or Hg^{2+}) and monomethylmercury (CH_3-Hg^+), commonly called methylmercury, MeHg (Sarkar, 2002).

Elemental mercury is the most volatile form of mercury. It has a vapour pressure of 0.3 Pa at 25°C and transforms into the vapour phase at room temperatures. In particularly, if elemental mercury is not enclosed, elemental mercury evaporates and forms mercury vapours which dissolve only slightly in water (56 µg/L at 25°C). Mercury vapours are colourless and odourless. The higher temperature, the more vapours are released from liquid elemental mercury. Elemental mercury is used to extract gold from ore at the amalgamation process of artisanal and small scale gold mining in a lot of countries, and mercury vapour is released to the atmosphere when amalgam is burned.

Monovalent and divalent mercury is compound form between mercury and other compounds. Many inorganic and organic compounds of mercury can be formed from Hg(II). Monovalent mercury (Hg(I)) includes mercury (I) oxide (mercurous oxide or dimercury monoxide) and mercury (I) chloride (mercurous chloride). Mercury (I) oxide is Hg_2O and unstable and easily decomposes into metallic mercury and divalent mercury (Japan Public Health Association, 2001). Chemical formula of mercury (I) chloride is Hg_2Cl_2. Mercury (I) chloride is known as calomel or mercurous chloride and is an odourless solid which is the principal example of mercury (I) compound (ILO, 2000).

Divalent mercury (Hg(II) or Hg^{2+}) includes mercury (II) chloride (mercuric chloride), mercury (II) oxide (mercuric oxide, mercuric oxide red and mercuric oxide yellow) (Japan Public Health Association, 2001). Mercury (II) chloride (Hg_2Cl_2) or sublimate is a poisonous white soluble crystalline salt of mercury (ILO, 2000). In some countries, it was used in insecticides, batteries and as antiseptic, disinfectant, etc. Mercury (II) oxide is HgO and exists as an irregularly shaped, orange-yellow powder (yellow precipitate) or/and orange-red powder (red precipitate) with high lustre. It is still used as a material for anodes for mercury batteries (ILO, 2000).

Mercury also forms organometallic compounds which are a covalently-bonded compound and does not include mercury bound to proteins nor salts formed with organic acids. Methylmercury (MeHg) is CH_3Hg^+ and one of organometallic forms. It can bioaccumulate up the food chain and is recognised as a bioaccumulative environment toxicant. Due to a bioaccumulative environmental toxicant, methylmercury can lead to high concentrations of methylmercury in predatory fish which is a very important source of protein and other nutrients for human. These organometallic compounds are stable, though some are readily broken down by living organisms, while others are not readily biodegraded (Japan Public Health Association, 2001). In the past, methylmercury was produced directly and indirectly as part of several industrial processes such as the manufacture of acetaldehyde (Tajima, 1970).

2.1.2 Exposure Risks to Mercury

Three important types of mercury are considered among the most harmful materials in the environmental because of its toxicity, high volatility and potential for bioaccumulation even at low concentration (Byun et al., 2011). These include metallic mercury, organic mercury and inorganic mercury.

2.1.2.1 Impact of Mercury to Health

i. Metallic Mercury Exposure

Inhalation of mercury vapor poses the greatest risk to health and safety because mercury is absorbed more rapidly through the lungs than through the digestive tract or skin (Mortazavi et al., 2005). Metallic mercury is highly lipophilic (has a high affinity for body fat) and is absorbed almost completely by the lungs upon inhalation. A few drops of mercury can raise the vapor concentration in surrounding indoor air to a dangerous level. Air saturated with mercury vapor at 20˚C contains a concentration that greatly exceeds toxic limits for humans. Inhaled mercury enters the bloodstream, where it can accumulate and stay in the kidney and brain for weeks to months. One study of Japanese workers exposed to metallic mercury indicated high mercury levels in the brain ten years after their last exposure to metallic mercury (Wilhelm and Bloom, 2000).

Dermal absorption is much slower than inhalation, but mercury exposure may produce skin irritations and allergic reactions. Absorption by ingestion is much slower than for inhalation. Ingested mercury does not enter the blood stream easily, and is mostly expelled in the feces. Mercury is also expelled from the body via exhalation, saliva, bile, and sweat. The half-life of metallic mercury in the human body is approximately one to two months in the body as a whole. The half-life in blood ranges from two days to approximately one month (Wilhelm and Bloom, 2000).

ii. Organic mercury exposure

Renal toxicity from exposure to organic forms of mercury has only been reported in cases of severe poisoning accompanied by symptoms of neurologic toxicity (Ledakowicz et al., 1995). Cinca et al., (1979) report that two children who died of complications from severe poisoning after consuming pork contaminated by ethylmercury were found to have high urinary protein, urinary sediment, and blood urea at the onset of their symptoms, and severe nephritis on autopsy. In addition, 62 out of 86 cases of ethyl mercury poisoning from Iraqi grain seed exhibited clinical symptoms of kidney damage such as, oliguria, polydipsia, polyuria, and albuminuria (Jalili and Abbassi, 1961).

iii. Inorganic mercury exposure

Inorganic mercury can damage the stomach and intestines, producing symptoms of nausea, diarrhea, or severe ulcers if swallowed in large amounts. Effects on the heart have also been observed in children after they accidentally swallowed mercuric chloride. Symptoms included rapid heart rate and increased blood pressure. There is little information on the effects in humans from long-term, low-level exposure to inorganic mercury (US EPA, 1997).

iv. Physical Damage from Mercury

Chronic and acute mercury poisoning can produce irreversible physical damage to the kidneys, lungs, spinal cord, and central nervous system (Green-Ruiz, 2006). Developing fetuses may also be damaged if the mother is exposed to mercury. Mercury poisoning has not been shown to cause cancer in animals, but it produces a variety of other types of damage, often irreversible. Victims may experience digestive disturbances, skin irritation, eye damage, leg cramps, loss of sensation around the lips, ataxia (inability to control voluntary muscle movements), or tunnel vision (US EPA, 1997).

Chronic mercury poisoning may develop gradually without conspicuous warning signs because mercury accumulates in body tissues (Hussein et al., 2005). Symptoms of repeated exposure include gray skin color, gum problems, tremors, memory and concentration problems, mood changes, and visual disturbances including clouding of the eyes. Rescuers are not directly at risk from individuals exposed to mercury vapor, although contaminated clothing can expose rescuers through direct contact or off gassing of mercury vapor (Horn et al., 1994).

2.1.2.2 Impact of Mercury to Environmental

Mercury in the air would settle into water bodies and affect aquatic environment (Wagner-Dobler, 2003 and Hassan et al., 2008). This airborne mercury can fall to the ground in raindrops, in dust, or simply due to gravity (known as "air deposition"). Mercury density higher than 5 g/cm^3 (Hussein et al., 2005). After the mercury falls, it can end up in streams, lakes, or estuaries as inorganic mercury, where it can be transferred to organic mercury (methylmercury) through microbial activity. Mercury is a persistent, mobile and bioaccumulative element in the environment and retained in organisms. Most of the mercury found in the environment is inorganic since mercury is never broken down into other chemical and harmless form. Once mercury enters into the environment, mercury permanently exists in the environment by changing its chemical forms depending on the environment (Mortazavi et al., 2005 and Li et al., 2009). Figure 2.1 shows the mercury species and transformation in the environment (Wagner-Dobler, 2003).

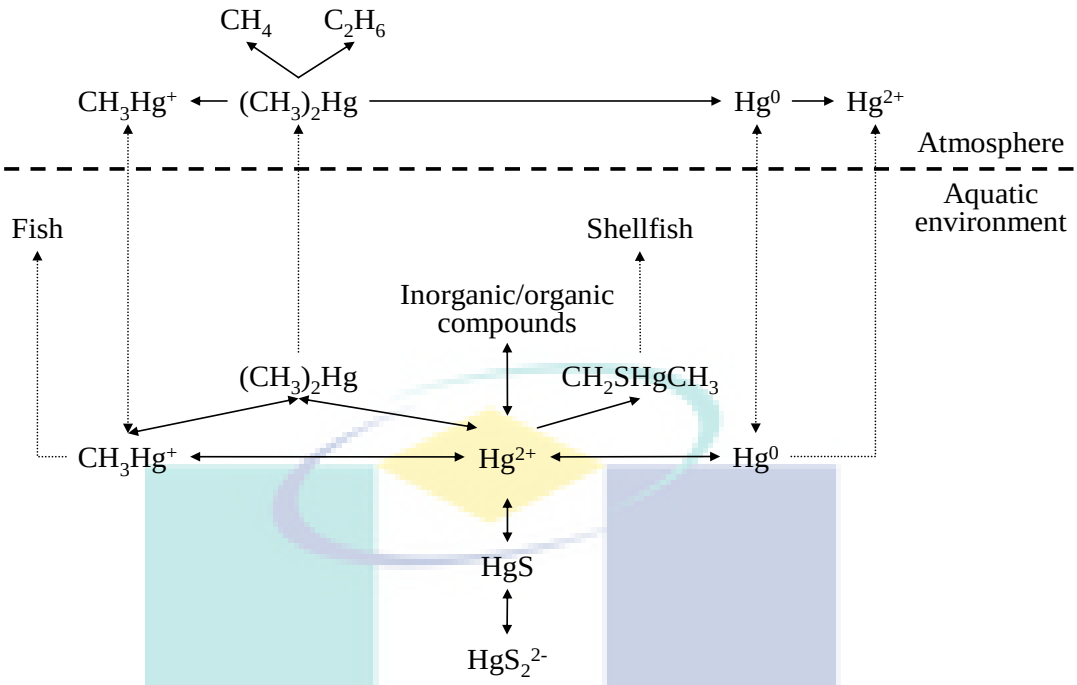


Figure 2.1: Mercury species and transformation in the environment

Mercury in the atmosphere is broadly divided into gas form and particulate form. Most of mercury in the general atmosphere is in gas form (95 % or more) Gaseous mercury includes mercury vapour, inorganic compounds (chlorides and oxides), and alkyl mercury (primarily methylmercury). However, 90 – 95 % or more of the gaseous mercury is mercury vapour (Japan Public Health Association, 2001).

In the aquatic environment under the suitable conditions, mercury is bioconverted to methylmercury, called methylation (Wood, 1974). Methylmercury is bioaccumulated within organisms from both biotic (other organisms) and abiotic (soil and water) sources and biomagnified in the food chains (Wagner-Dobler et al., 2000 and Uslu and Tanyol, 2006). Therefore, methylation is the source of mercury exposure to human and its mercury exposure is chronic exposure to human health through consuming fish and seafood (ShamsiJazeyi and Kaghachi, 2010).

Methylmercury accumulates in fish at levels that may harm the fish and the other animals that eat them. Mercury deposition in a given area depends on mercury emitted from

local, regional, national, and international sources. The amount of methylmercury in fish in different water bodies is a function of a number of factors, including the amount of mercury deposited from the atmosphere, local non-air releases of mercury, naturally occurring mercury in soils, the physical, biological, and chemical properties of different waterbodies and the age, size and types of food the fish eats (Malakahmad et al, 2011).

Birds and mammals that eat fish are more exposed to methylmercury than any other animals in water ecosystems. Similarly, predators that eat fish-eating animals are at risk. Methylmercury has been found in eagles, otters, and endangered species. Some highly-exposed wildlife species are being harmed by methylmercury. Effects of methylmercury exposure on wildlife can include mortality (death), reduced fertility, slower growth and development and abnormal behaviour that affect survival, depending on the level of exposure. In addition, research indicates that the endocrine system of fish, which plays an important role in fish development and reproduction, may be altered by the levels of methylmercury found in the environment (Kannan and Krishnamoorthy, 2005).

2.1.2.3 Exposure Limits

i. Mercury Limit in Wastewater

The Environmental Protection Agency (EPA) is the regulating body for Springs Utilities' IPP in the USA. In 1999, the EPA approved a maximum limit 25 parts per trillion (ppt) or 0.01 pounds per day for mercury, (known as the Maximum Allowable Headworks Loading or MAHL) based on acceptable levels entering the influent of the wastewater treatment plant, and limits on mercury in the Springs Utilities' Colorado Discharge Permit System.

Environmental legislation has been enacted in most countries and the standards have been made more stringent than ever including Malaysia. DOE (Department of Environment) of Malaysia is empowered under the Environmental Quality Act 1974 (Act 127), Regulations, Rules & Orders, under Environmental

Quality (Industries Effluent) Regulations 2011, to control and prevent pollution, as well as to protect and enhance the quality of the environment in Malaysia. Appendix 2.6 shows parameter limits of effluent of Standard A and Standard B under Environmental Act 1974, Amendment 2011, (EQA, 2011).

Malaysia government has initiated programs to control scheduled waste management to safeguard the environment and the safety of the people. In the Environmental quality (Scheduled Wastes) Regulations 2011, prescribed a listing of 107 categories of toxic and hazardous wastes defined as "scheduled wastes." In 1993 the government introduced the legal control on import/export of scheduled wastes. DOE, Ministry of Science, Technology and Environment, Kuala Lumpur reported that in 1998, the generation of hazardous wastes was metal finishing, chemical, electronics and electrical, printing and packaging. The wastes were solids, liquids and semi-solids with difference chemical compositions. For mercury, the limit of concentration mercury in wastewater is 50 ppb for Standard B and 5 ppb for Standard A (EQA, 2011).

ii. Mercury Vapor Limit

Several government agencies have established limits for various types of mercury exposure. Many of these limits deal with the chronic exposure of workers in industries that use mercury or mercury-containing devices. Other limits deal with the effects of acute exposure, such as might result from a mercury spill.

Occupational Safety and Health Administration's (OSHA) legally enforceable ceiling limit for workplace exposure is set at 100 micrograms per cubic meter ($\mu g/m^3$). At no time should the mercury concentration exceed this level. The National Institute of Occupational Safety & Health (NIOSH) Recommended Exposure Limit (REL) for mercury vapor is set at 50 $\mu g/m^3$.

The American Conference of Governmental Industrial Hygienists (ACGIH) set their Threshold Limit Value (TLV) at 25 µg/m^3 of mercury vapor (as averaged during 8 hours workday). The U.S Environmental Protection Agency (USEPA) has set a reference concentration of 0.30 µg/m^3 for inhalation exposure. The Agency for Toxic Substances & Disease Registry's (ATSDR) Minimal Risk Level (MRL) is 0.20 µg/m^3 with an action level of 1 µg/m^3 that triggers remediation if exceeded in air as shown at Appendix 2.7.

iii. Mercury Limit in Drinking Water

The EPA and Food and Drug Administration (FDA) limit for mercury in drinking water is 1 ppb (Ledakowicz et al., 1995 and Oehman et al., 2006). Ingesting 0.3 g of mercury can be fatal to humans, and 75 mg/day in drinking water is fatal. The Resource Conservation and Recovery Act (RCRA) limit for mercury in leachate is 200 ppb.

There is a significant potential contribution to the overall exposure from contact with the skin, eyes, and mucous membranes. Dermal exposure can result in over exposure even though air levels are less than the specified limits.

2.2 MERCURY SOURCE IN PETROLEUM INDUSTRIES

Mercury is a trace component of all fossil fuels including natural gas, gas condensates, crude oil, coal, tar sands and other bitumen. The use of fossil hydrocarbons as fuels provides the main opportunity for emissions of the mercury they contain to the atmospheric environment but other avenues also exist in production, transportation and in processing systems (Wilhelm and Bloom, 2000). These other avenues may provide mercury directly to air, water or solid waste streams. In addition, the distribution and transformation of mercury in production, transportation and processing are considered relative to the determination of mercury in air emissions, wastewater, and products from oil and gas processing facilities (Rebhun and Galil, 1994).

2.2.1 Mercury Emissions from Oil and Natural Gas Production and Processing

Wastewaters originate from production operations in the form of produced and in refining and gas processing as wastewater. Solid waste streams are generated in production, transportation and in refining. Air emissions originate from fugitive emissions (process equipment) and combustion. Combustion to be vastly dominant as a possible avenue by which mercury in oil and gas may be transferred from produced hydrocarbons to the environment as wastewater (US EPA, 1971).

Oil and gas industry distinguishes between upstream and downstream operations. The upstream category refers to primary production and whatever processing is necessary to place the produced fluids in the transportation system. The term downstream operations refer to refining and gas processing to produce salable products (Wilhelm and Bloom, 2000).

2.2.2 Mercury Emissions to Water

The main wastewater streams that derive from petroleum production and processing are produced water from both oil and gas production and refinery wastewaters. Very minor amounts of water (relative to produced water and refinery wastewater) derive from gas processing and these are mainly water from separators at gas plants (essentially produced waters) and condensed water from dehydration. No wastewater streams originate from transportation systems other than the very small amounts that come from pipeline pigging operations and tanker ballast (Wilhelm and Bloom, 2000).

2.2.3 Produced Water

Normal production operations of both crude oil and natural gas involve primary separation of water, gas and oil. Separated water (referred to as produced water when separated close to the well) is either discharged (to an ocean, lake or stream or evaporation pond) or reinjected (usually to the formation it came from). Reinjection is utilized to

enhance oil recovery (EOR) or to comply with regulatory requirements stemming from environmental concerns. Produced water normally containing high (>100.00ppb) concentration of mercury dissolved (Wilhelm and Bloom, 2000).

Produced water is the largest waste stream in the oil and gas industry. Produced water varies greatly in composition and salinity, depending on the geologic source of the water, type of production, and the treatment of the water once brought to the surface. The salinity of produced water ranges from essentially fresh water to brines that are several times more saline than seawater. Some states allow surface discharge of produced water, but many do not allow (Bloom and Falke, 1996).

Produced water originating on offshore platforms can be discharged to the ocean unless the platforms are located in sensitive areas or the water is unusually hazardous due to a particular characteristic (salinity, hydrocarbon content, toxicity). In sensitive coastal areas, produced water is closely regulated with permit requirements that severely limit options for discharge thus necessitating treatment or re-injection. Appendix 2.8 shows mercury in produced water at different location (U.S. EPA, 2001).

About 70 percent of the offshore produced water is discharged to the ocean (approximately 0.3 trillion liters annually). Currently it is not possible to assign either a mean or range to mercury concentrations in produced and discharged water. The mercury species present in produced waters are unknown but likely include higher percentages of suspended forms (HgS) and ionic forms than the produced crude oil. Applying an estimated mean mercury concentration in produced water of 1 ppb to 0.5 trillion liters (0.2 onshore and 0.3 offshore yearly), one obtains the result that on the order of 250 kg mercury may enter the aqueous environment annually from waters associated with U.S. oil and gas production (U.S. EPA, 2001).

2.2.4 Refinery Wastewater

The chemical compositions of refinery wastewater vary widely, as do the volumes of water (per barrel of oil processed) produced by refineries (Campanella et al., 1986). Gill and Fitzgerald (1984), reported range of mercury pollution in ocean waters is broad i.e. 0 – 1000 ppb. Major of water compositional differences stem from process configuration (products produced) and from the type of crude oil that is processed (high sulfur crude, sweet crude). The production of petroleum based industry wastewater generally consists of polycyclic and aromatic hydrocarbons, cyanide, oil, phenols, metal derivatives, sulphides and other chemicals (Campanella et al. 1986).

The wastewater that enters water treatment systems at refineries is a composite of water discharges from individual processing units that differ in type and function. Water streams from process units are differentiated and categorized as waters that contact hydrocarbons (including condensed steam from stripping) and cooling waters that typically do not contact hydrocarbons directly but may contain some hydrocarbon contamination from leakage (Wilhelm and Bloom, 2000).

The mean and range of mercury concentration in refinery wastewater cannot be stated with certainty. Very little information is available in the published literature that speaks directly to this issue. The EPA study of refinery effluents from the early 80's (Ruddy, 1982) provides a mean close to 1 ppb but the methodology to arrive at this number is poorly documented. The amount of refinery wastewater discharged to the environment (rivers, lakes and oceans) is approximately 1.50 billion barrels yearly (U.S. EPA 2001 and U.S. EPA 1997). Applying the 1982 EPA mean value of 1 ppb as maximum to this amount yields approximately 250 kg as an upper limit to the total amount of mercury discharged in refinery wastewater. Pollutant concentration for a typical refinery wastewater can be seen in Appendix 2.9.

2.3 TECHNOLOGIES OF MERCURY TREATMENT IN WASTEWATER

The literature, however, provides only limited information on actual full-scale treatment technology performance and almost no full-scale economic data or information on mercury recovery (Chen et al., 1998). The effectiveness of treatment provided by each type of technology depends on the chemical nature and initial concentration of mercury as well as the presence of other constituents in the wastewater that may interfere with the process. Other factors, however, such as residuals management and costs, weigh heavily in selecting the appropriate treatment approach (Camanella et al., 1986 and Merzouk et al., 2009).

There are several conventional methods can be adopted for the removal mercury from wastewater i.e. precipitation, coagulation/flocculation, ion exchange, membrane system and chemical reduction. However, most of these methods suffer from drawbacks such as high capital costs, and problems concerning the disposal of the residual metal sludge (Wang and Chen, 2006 and Agarwal et al., 2010).

2.3.1 Precipitation Treatment Process

Precipitation and coagulation/co-precipitation technologies are among the most established approaches for removing mercury from wastewater (Trivunac and Stevanovic, 2006). Sulfide precipitation is one of the more commonly precipitation methods for removal of inorganic mercury from wastewater. In this process, sulfide (sodium sulfide or another sulfide salt) was added to the waste stream and mixed in a stirred reaction vessel to convert the soluble mercury to relatively insoluble mercury sulfide form (HgS), which is then precipitated.

The precipitated solids can then be removed by gravity settling in a clarifier. Flocculation, with or without a chemical coagulant or settling aid, can be used to enhance the removal of precipitated solids. Precipitation efficiency declines significantly at pH above 9 (Patterson, 1985, Fu and Wang, 2010).

Removal of both inorganic and organic mercury by coagulation/ co-precipitation for a variety of mercury-containing wastewater can be achieved via addition of coagulant is employed such as aluminum sulfate (alum), iron salts, and lime. For alum and iron, the dominant mercury removal mechanism is most likely by adsorptive co-precipitation. Nevertheless, there are no commercial precipitants available that can irreversibly and permanently bind the mercury at low concentration (Patterson, 1985 and Blue et al., 2007).

2.3.2 Adsorption Processes

Adsorption processes actually are most applied methods in the industries, and the most extensively used methods for up-taking metal ions (Chiarle et al., 2000 and ShamsiJazeyi and Kaghachi, 2010). The predominant adsorption process utilizes activated carbon, but the uses of other adsorbents also are reported in the literature (Iyer et al., 2005). Metal hydroxides are also used as adsorbents. When metal hydroxides are employed for adsorptive treatment, the process is commonly termed as coagulation or co-precipitation. Variables other than adsorbent type and dosage can also affect adsorption efficiency. Common variables include wastewater pH and pollutant speciation (Agarwal et al., 2010).

Granular activated carbon (GAC) is the most commonly used adsorbent system for treating industrial waste (U.S. DOE, 1994 and Cyr et al. 2002). This process is used in a variety of configurations, GAC systems may be either pressure or gravity type. The polyaluminium chloride (PAC) is normally not regenerated for reuse due to unfavorable economics including poor recovery of the PAC (Wu et al., 2005 and Zhao et al., 2010). The study also demonstrated that the addition of mercury chelating agents, such as ethylene diamine triacetic acid (EDTA) or tannic acid, prior to contact with the PAC increased mercury removal efficiency. The other activated carbons studied displayed maximum total mercury (II) removal at pH 4 to 5, and the percent mercury (II) removal dropped markedly at pH values greater than and less than 4 to 5. Pretreatment or modification of activated carbon with carbon disulfide solution before used has been shown to enhance mercury removal (Fu and Wang, 2010).

2.3.3 Ion Exchange Treatment

Ion exchange processes are typically operated as packed columns. Usually four operations are carried a complete ion exchange cycle: service, backwash, regeneration, and rinse. In the service step, the ion exchange resin in the packed column is contacted with the water containing the mercury to be removed. After a target concentration of mercury in the column effluent is reached, the resin is said to be spent. A backwash step is then initiated to expand the bed and to remove fines that may be clogging the packed bed. The spent resin is then regenerated by exposing it to a concentrated solution of the original exchange ion, so that a reverse exchange process occurs. The rinse step removes excess regeneration solution before the column is brought back online for the next service cycle (Agarwal et al., 2010).

Ion exchange technology for mercury removal has historically been limited to the use of anion resins to treat industrial wastewater that contains inorganic mercury (Ritter and Bibler, 1992 and Chiarle et al. 2000). If the chloride content of the wastewater is low, either chlorine or chloride salt could be added to improve removal process efficiency. Cation exchange of mercury may be effective if the anion content of the wastewater is low (Sorg, 1979). The disadvantages include high cost and partial removal of certain ions (Clifford et al., 1986 and Dabrowski et al., 2004).

2.3.4 Chemical Reduction

The standard electrode potential of metals determines their placement in the electromotive series, which is a series of elements in descending order of their standard potential (Merzouk et al., 2009). Ionic mercury can be displaced from solution via reduction by another metal higher in the electromotive series, and then separated by filtration or other solids separation technique. Reducing agents include aluminum, zinc, iron, hydrazine, stannous chloride, and sodium borohydride. Although the literature includes much discussion of reduction processes, only limited actual treatment data are presented. The main advantage claimed for reduction is that mercury can be recovered in the metallic state (Patterson, 1985). However, most of reduction processes cannot

effectively achieve mercury levels below 100 ppb, and their use would likely require second-stage polishing.

Table 2.1 gives a comparative description of different methods available. According to Green-Ruiz (2006), conventional techniques for removing mercury may not be effective, especially when the metal concentration is less than 100 000 ppb. Such processes are costly and often result in remobilization of toxic mercury compound during the dredging process (Wagner-Dobler et al., 2000 and Hussein et al., 2005).

Table 2.1: Summaries of various techniques adopted for mercury removal from wastewater

No	Method	Technique	Advantages	Disadvantages
1	Precipitation	-sulfide -reagents include sodium dimethyldithiocarbamate (SDTC) and sodium thiocarbonate (STC),(Matlock et al., 2001)	-neutral pH, water can reused, fast	-the costs of the absorption process is expensive (Vaart et al., 2001) -monitoring and generate toxic residue (Tarangini, 2009 and Agarwal, 2010)
2	Adsorption	-activated carbon -metal hydroxide -natural product (Natale et al., 2006) -silica, polyacrylamide, and hybrid silica–polyacrylamide aerogels (Ramadan et al., 2010) - iron oxide (Wu et al, 2006)	-the costs for regeneration of the Natale et al., 2006	-slow process and generate toxic residue (Agarwal, 2010)
3	Ion exchange	-packed column -aluminium and iron electrodes (Njiki et al., 2009)	-efficiency close to 100% (Njiki et al., 2009)	-regenerated brine must be disposed, cannot typically used for water with high total dissolve content, high setup cost and generate toxic residue (Agarwal, 2010)
4	Chemical reduction	reducing agent (aluminum, zinc, Iron, hydracine, stannous chloride and sodium borohydride.)	- can be applied at higher mercury concentration, fast process	- the costs of the process is expensive (Lyold and Lovly, 2001)

2.4 MEMBRANE BIOREACTOR (MBR)

Research into combining membranes with biological processes for wastewater treatment began over 30 years ago, and membrane bioreactor has been used commercially for the past 20 years. A recent review shows an exponential growth of number of plants and their installed capacity in the past decade with the estimate of market growth rate above

10% per year (Kraumer and Drews, 2010). At the same time, Cornel and Krause (2006) reported a significant decline in annualized costs from about USD0.90/m^3 ten years ago to USD0.08/m^3 in 2005 primarily due to lower membrane cost but also due to improved energy efficiency to below than 0.4 kWh/m^3.

2.4.1 Definition of Membrane Bioreactor

Membrane bioreactors (MBR) can be defined as the combination of two basic processes that is biological degradation process and membrane separation into a single process where suspended solid and microorganisms responsible for the biodegradation are separated from the treated water by a membrane filtration unit (Gao et al., 2009). The entire biomass is confined within the systems, providing both perfect control of residence time for the microorganisms in the reactors (sludge age) and the disinfection of the effluent (Judd, 2006).

According to this definition, MBR process should be distinguished from other treatment processes where membrane process is installed downstream of the biological processes, such as activated sludge or fixed film process, as a refining stage or tertiary treatment (Melin et al., 2005 and Wang and Chen, 2008).

The general operation of the MBR is illustrated in the Figure 2.2. The influent enters the bioreactor, where it is brought into contact with the biomass. The mixture then is pumped from the bioreactor and at under pressure condition, it was filtered through the membrane. Permeate is discharged from the system while the entire biomass is returned to the bioreactor. Excess sludge is pumped out in order to maintain a constant sludge age and the membrane is regularly cleaned by back washing, chemical washing, or both of the techniques (Stephenson et al., 2000).

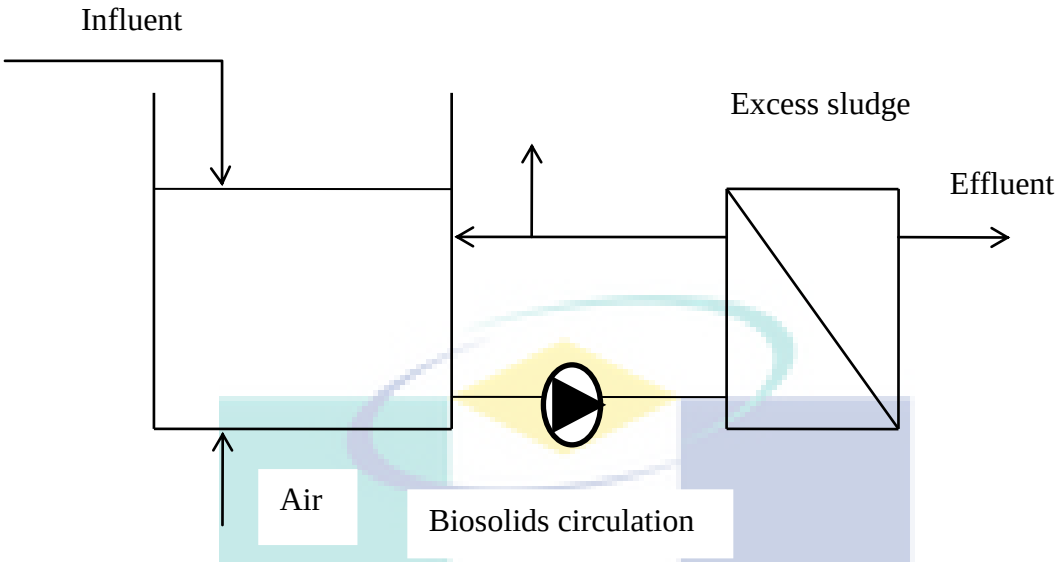


Figure 2.2: The general aeration Membrane Bioreactor (MBR)

The biological reactor and membrane units can be combined in two ways (Melin et al., 2005):

1. Internally

An internal MBR is typically used for medium to high amounts of waste streams and forms an option against conventional wastewater treatment. It requires outer skin membranes (OSM) and the most widely common MBR. The diagram of internal membrane bioreactor is shown at Figure 2.3.

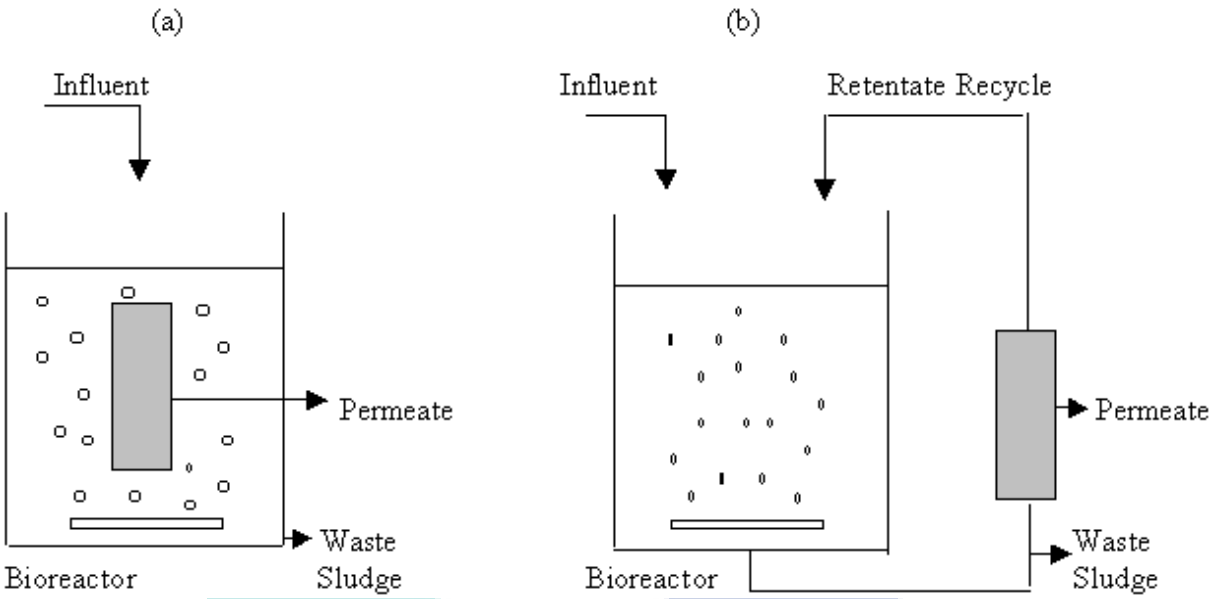


Figure 2.3: (a) Internal Membrane Bioreactor and (b) External Membrane Bioreactor Reactor (Recirculated)

2. Externally (recirculated)

In this case the biomass must circulate between the bioreactor and the membrane, as illustrated on the Figure 2.3b. An external MBR is typically used for smaller waste streams which are highly concentrated (Stephenson et al., 2000).

Apart from the differences in the types of membranes, these two configurations can be distinguished by the technology used to create the pressure gradient between the two sides of the membrane (driving force). The pressure across the membrane in the integrated MBR can be applied only by suction through the membrane or by pressurizing the bioreactor in a recirculated MBR, pressure across the membrane can also be created by recirculating flow through the membrane (Radjenovic et al., 2007).

The list of the principle advantages and disadvantages of the biomass separations MBR are (Melin et al., 2005; Judd, 2006; Qin, et al., 2007 and Hermanowics, 2011):

- Small footprint
- Complete solids removal from effluent
- Effluent disinfection
- Combined COD, solids and nutrient
- High loading rate capability
- Low /Zero sludge production
- Rapid start up
- Sludge bulking not a problem
- Modular/retrofit

The disadvantages are (Qin, et al., 2007 and Kim et al., 2008):

- Aeration limitations
- Membrane fouling
- Membrane costs

2.4.2 Membrane Structure and Materials

The principle objective in membrane manufacture is to produce a material of reasonable mechanical strength, and which can maintain a high throughput of a desired permeate with a high degree of selectivity. The efficiency of a membrane system will depend on its structure which essentially determines the mechanism of separation and thus the application (Shamel, 1998).

The range of available membrane materials is very diverse. They vary widely both in chemical composition and physical structure. On this basis, two types of structures may be categorized as either symmetric or asymmetric membrane (Mulder, 1991).

The choice of a particular material can be considered critical in order to achieve excellent performance. There are a number of criteria which must be satisfied on a particular choice of membrane material (Shamel, 1998):

- The material must be chemically stable over a range of conditions.
- The material must be formable.
- The material may need to be approved for food or water contact for certain markets.

The commonly used materials for the manufacture of membrane together with their characteristics and typical applications are summarized in Appendix Table 2.10.

2.4.3 Membrane Module and Membrane Processes

The development or selection of membrane materials and configurations for MBR has been governed by the need to suppress membrane fouling (Kim et al., 2008). The nature and extent of fouling depend both upon the feed water quality and specific facets of the membrane separation system (Tay et al., 2007). As with most other membrane applications, the preferred membrane materials for MBRs are invariably polymeric on the simple basis cost. Appendix 2.11 is shown the membrane configurations (Metcalf and Eddy, 2003).

Membrane processes may be classified according to the types of membranes used. Appendix 2.12 summarizes the different membrane processes, their characteristics and typical applications (Metcalf and Eddy, 2003).

2.4.4 The Cross-flow Filtration

The cross-flow filtration is a process design where a tangential flow is forced along the filter surface (Al-Malack and Anderson, 1996a). During filtration, material retained at the membrane. The accumulation is visualized as concentration polarization which increased the total filtration resistance, reduces the flux and may alter the membrane separation properties due to pore plugging, pore narrowing or the growth of a fouling layer (Stephenson al., 2000 and Dizge et al., 2011). Not only diffusive but also convective back-transport can, in optimal situations, keep the boundary layer above the filter surface free

from accumulated foulants (Melin et al., 2005). Generally, however, the cross-flow is insufficient and some material gathers in the vicity of the membrane or adsorbs to the membrane. Some adsorption is even inevitable.

Cross-flow membrane filtration has been widely used for cell separation and processing as well as for immobilizing or containing cells and enzymes (Alberti et al., 2006). Microorganisms immobilized by cross-flow system for heavy metal removal have been studied using hollow fiber or spiral-wound membrane cartridge (Al-Malack and Anderson, 1995 and Wagner-Dobler, 2003). Compared with gel or solid support-immobilization system, cross-flow systems may have the following advantages: in terms of removing and recovering heavy metals by immobilized microorganisms (Chen et al. 1998; Katayon et al., 2004 and Trivunac and Stevanovic, 2006):

i. Cell leakage, which was observed with gel or solid support-immobilization systems, could be prevented. This is particularly significant when genetically engineered organisms are used for metal remediation since their release into the environment would be subjected to government regulation.

ii. The membrane system would allow maximum microbe-metal interaction and reduce the diffusional barrier for the metal observed with gel or solid support-immobilization systems.

iii. High cell densities can be produced.

iv. Metal-laden cells could be easily recovered after treatment. In addition, cell harvesting and metal clean up could be processed in membrane system.

One of the disadvantages of using the hollow fiber bioreactor with cell recycle permits only a single equilibrium stage (Chung et al., 2005). However, its performance in terms of lowering effluents Hg^{2+} levels can be improved by using two reactors in series. In

this study, effective removal and recovery of Hg^{2+} at low concentrations was achieved even though only one reactor was used (Wagner-Dobler et al., 2000b).

2.4.5 Microfiltration Membrane

Microfiltration (MF) membranes are used for the separation of cells, cell debris and particles from a solution (Porter et al., 2005). Polymeric MF membranes are much porous than UF membranes, commonly are symmetric structure with rougher surface and tortuous paths rather than defined pores. MF membranes are in general characterized by their (nominal) pore size, ranging from 0.1 to 5.0 microns (Broom et al., 1994 and Al-Malack and Anderson, 1996a). In this study, MF membranes have been used in the MBR. The membrane functions to separate the treated wastewater from the active biomass in an aerobic bioreactor (Brady et al., 1994 and Chung et al., 2005).

In advanced treatment applications, MF has been used, most commonly as a replacement for depth filtration to reduce turbidity, remove residual suspended solids, and reduce bacteria to condition the water for effective disinfection (Stephenson et al., 2000). The characteristics and typical operating information for MF membrane technologies used for wastewater including operating pressures and flux rates is stated in Appendix 2.13.

Porous MF membranes are capable of removing only suspended materials, generally down to around 0.05 μm in size. It is the porous membranes that are used in MBRs to retain the suspended solid materials, mainly biomass, within the reactor treatment which requires no further purification due to the operational cost (Meng et al., 2005). MF is also several disadvantages to be known is using MF membranes. The advantages and disadvantages are listed in Appendix 2.14 (Stephenson et al., 2000).

2.4.6 Operating Conditions Factors

Most studies reported on industrial wastewater treatment in MBRs have used side-stream systems with pore size ranging between 0.001 μm and 0.10 μm (Vera et al., 1997

and Chen et al., 1998). Consequently, is most schemes, the membranes are operated at realatively high TMPs (150 – 300 kPa) and crossflow velocities (1.6 – 4.5 m/s). From this studies, high actual flux up to 0.15 m^3/m^2h but low specific flux rates (<0.001 m^3/m^2h kPa) observed.

i. Transmembrane Pressure (TMP)

Transmembrane pressure (TMP) is the pressure gradient, which is the driving force for almost all the pressure-driven membrane processes applied to wastewater treatment the desired permeate is water, such that the retained or rejected material (retentate) is concentrated (Al-Malack and Anderson, 1996b). Studies were shown that TMP as a function of time at different fluxes (Qin et al., 2007). However, membrane filtrations TMP need to be increased from time to time in order to maintain the constant flux value (Tay et al., 2007). Defrance and Jaffrin (1999) mentioned that the flux stabilizes itself at the same value, independent of TMP exceeds 85 kPa. Thus, TMP can be considered among the indicator for the limit of MBR operation, especially for studies.

$$TMP = \frac{(P_f + P_c)}{2} - P_p \qquad (2.1)$$

Where TMP = transmembrane pressure gradient, kPa

P_f = inlet pressure of feed stream, kPa

P_c = pressure of concentrate stream, kPa

P_p = pressure of permeate stream, kPa

ii. Flux

Membrane flux, defined as the mass or volume rate of transfer through the membrane surface, in terms of m^3/m^3h, is an important design and operating parameter that affects the process economics (Meytcalf and Eddy, 2003). Efficiency of membrane performance is determined selectivity and the flow through the membrane which often

denoted as the flux or permeation rate (Mulder, 1991). SI unit used in this system is $l/m^2/hr$ or LMH

$$\text{Flux (LMH)} = \frac{\text{Flowrate (L/hr) x 0.06}}{\text{Membrane area } (m^2)} \quad (2.2)$$

Flux selection provides the most significant factor in determining membrane performance (Gao et al., 2009). At high convection towards the membrane (i.e. at high flux), colloidal aggregation and heterogeneous deposits are observed. Internal and/or irreversible will also take place more rapidly at higher fluxes, more or less in agreement with theoretical predictions.

At low flux, visual observation of the membrane with a microscope confirms the absence of floc deposition. However, it has been shown that the composition of the adsorbed material on the membrane was very close to that of the material on the membrane was very close to that of the supernatant of the mixed liquor (Trivunac and Stevanovic, 2006 and Defrance et al., 2000). The small but linear TMP increase observed at low fluxes thus appears to arise from deposition of the solute and colloidal fraction of the sludge, which are likely to interact with the membrane in the incipient stages of filtration (Kim et al., 2008).

2.5 *Pseudomonas putida* REVIEW

2.5.1 Introduction

In Minamata Bay, mercury-resistant *Pseudomonas spp*. were isolated from sediments near the drainage outlet to the Bay. *Pseudomonas spp*. dominated the bacteria with the highest resistance to mercury (Eisler, 2006). The mercury-resistant *Pseudomonas* strains were more resistant to inorganic mercury, methylmercury and phenylmercury (Nakamura et al., 1986). Previous studies showed that mercury causes an increased relative abundance of mercury resistant bacteria isolates has rarely been carried out Moreover,

studies showed mercury did not affect the number of culturable *Pseudomonas spp*. even though the number of bacteria growing on general medium was affected (Holtze et al., 2006).

Pseudomonas putida (P. putida) was isolated from a polluted creek in Urbana, IL by enrichment culture with ethylbenzene as the sole source of carbon and energy. *P. putida* is one of the most well-studied aromatic hydrocarbon degrading bacterial strains. Well over 200 articles have been written about various aspects of *P. putida* physiology, enzymology, and genetics by microbiologists and biochemists, in addition to more applied studies by chemists and environmental engineers utilizing *P. putida* and its enzymes for green chemistry applications and bioremediation (Eisler, 2006).

Strain grows well with benzene, toluene, ethylbenzene, and *p*-cymene. Mutants of strain that are capable of growth with *n*-propylbenzene, *n*-butylbenzene, isopropylbenzene and biphenyl are easily obtained (Ouyang et al., 2006). In addition to aromatic hydrocarbons, the broad substrate toluene dioxygenase in strain can oxidize trichloroethylene (TCE), indole, nitrotoluenes, chlorobenzenes, chlorophenols and many other aromatic substrates. Although *P. putida* cannot use TCE as a source of carbon and energy, it is capable of degrading and detoxifying TCE in the presence of an additional carbon source (Walia et al.,2003).

2.5.2 Characteristic of *Pseudomonas putida*

"*Pseudomonad*" is a general term for diverse group of bacteria which morphologically and physiologically resemble members of the genus *Pseudomonas*. In addition to the genus Pseudomonas, a variety of other bacteria are sometimes lumped under this title if they share the following characteristics (Loh and Cao, 2008):

- gram-negative
- rod-shaped
- non-spore forming
- typically motile with one or more polar flagella

- aerobic metabolisme
- able to grow on wide variety of organic substrates

Most *pseudomonads* are free-living saprophytic organisms in soil, water and plant rhizosphere where they play an important role in decomposition, biodegradation, and the carbon and nitrogen cycles (Boopathi and Rao, 1999). They are able to colonize various environments and play important roles in metabolic activities (Vijayaraghavan and Yun, 2008). Because of this lifestyle, *pseudomonads* are characterized by great metabolic diversity and are able to utilize a wide range of carbon source, including molecules which few other organisms can break down. Consequently, they have great potential for different biotechnological applications, particularly in bioremediation and biocatalyst.

Most *pseudomonads* are motile of a single polar flagellum as shown at Figure 2.4 and characteristic of *P. putida* at Appendix 2.15. The most important factor is the non-pathogenic properties of *P. putida* than other *pseudomonads* which can cause disease (pathogenic). Pathogenic is the microbe that will bring disease and harm to human, plant and animal while non-pathogenic is vice versa (Boopathi and Rao, 1999).

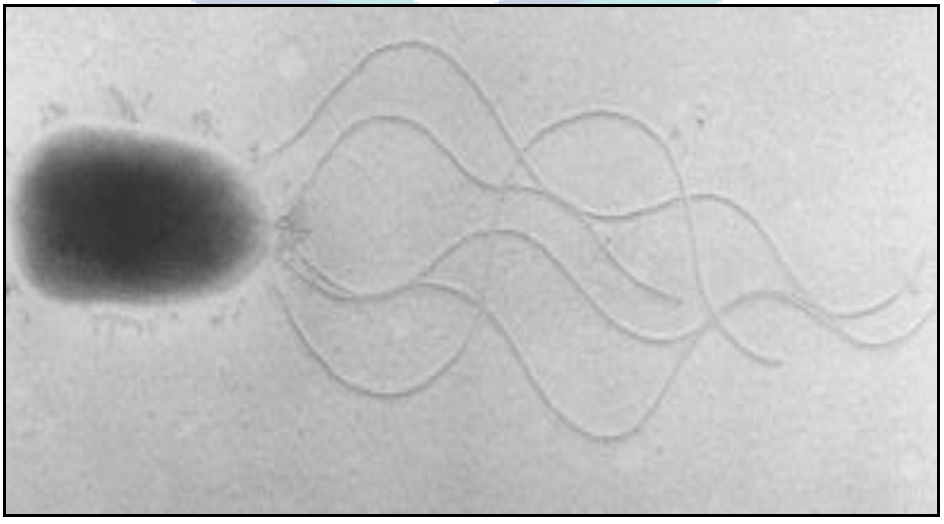


Figure 2.4: *Pseudomonas putida* , 1.0µm.

P. putida is a gram negative rod shaped saprophytic soil bacterium. It is a versatile environmental isolate that is capable of growth on mercury and several aromatic hydrocarbons, including benzene, toluene and ethylbenzene. *P. putida* is known to be chemotactic to aromatic hydrocarbons and chlorinated aliphatic compounds and has the potential for use in bioremediation applications (Loh and Cao, 2008).

Members of the genus *Pseudomonas* are characterized by their ability to grow in simple media at the expense of the great variety of organic compound, ability to grow under controlled conditions and resilience to wide range of environmental situations. They have strict respiratory metabolism and are motile by one several polar flagella. *P. putida* strains shows optimal growth 30˚C - 37˚C and easily isolated from soil and water by enrichment culture in mineral media with various carbon sources (Wang and Chen, 2008 and Loh and Cao, 2008)

For many years, the ability of *P. putida* to degrade hydrocarbon in wastewater such benzene, toluene, and o-xylene has a direct bearing on the development of strategies for dealing with environmental pollution (Robledo-Ortiz et al., 2011). Therefore, these characteristics make *P. putida* a valuable microorganism in bioremediation process. For example, many underground gasoline storage tanks are leaking and contaminating groundwater supplies with benzene, toluene, ethylbenzene and xylenes (BTEX). *P. putida* can oxidize all of these compounds and thus a detailed knowledge of the physiology and biodegradation capabilities of this organism will essential for providing the scientific foundations for the emerging bioremediation industry (Mazzero et al., 2010). Table 2.2 shows *P. putida* with different strain to treat contaminated hydrocarbon in petroleum based industries since they are able to biodegrade target pollutants.

Table 2.2: *P. putida* with different strain and their application in petroleum based industries

	***P. putida* Strain**	**Application**
1	*P. putida* S12	-Solvent-tolerant that converts glucose into phenol (Wierckx et al., 2009) -Bioproduction of p-hydroxybenzoate from renewable feedstock (Verhoef et al., 2007) -Transposon mutations in the flagella biosynthetic pathway (Kieboom et al., 2001)
2	*P. putida* CCRC 14365	-Biodegradation of single phenol and sodium salicylate, SA (Tsai and Juang, 2006)
3	*P. putida* KT2440	-Model bacterial strain for genetic, physiological, and applied studies (Yun et al., 2011) -Bioremediation and biosynthesis of biodegradable plastics (Wang and Nomura, 2010)
4	*P. putida* ND6	-Mineralization of naphthalene--degrading genes -Salicylaldehyde dehydrogenase gene *nahV* and salicylate hydroxylase gene *nahU* (Li et al., 2010)
5	*P. putida* KT2442	-Biotransformation of aromatic compounds to chiral cis-diols (Ouyang et al., 2006) -Produces medium-chain-length (MCL) polyhydroxyalkanoates (PHA) from fatty acids (Ma et al., 2009) -Degradation of benzene, toluene, and phenol (Reardon et al., 2000)
6	*P. putida* F1	- Production of catechol, 3-methyl-catechol, and 3, 6-dimethyl-catechol from benzene, toluene, and xylene; and second the mineralization of those intermediaries to produce CO_2 and water (except in the case of 3, 6-dimethyl-catechol, which is not mineralized by *P. putida* F1 (Yu et al., 2001) -Degradation of Benzene, toluene and o-xylene (Robledo-Ortiz et al., 2011)
7	*P. putida* OCNB-1	Degradation of o-chloronitrobenzene as the sole carbon and nitrogen sources (Wu et al., 2005)

8	*P. putida* BCRC14349	Phenol degradation sodium from tripolyphosphate-crosslinked chitosan beads entrapped (Hsieh et al., 2008)
9	*P. putida* OU83	Degrading 3-nitrotoluene (3-NT), 2,4-dinitrotoluene (DNT), 2,6-DNT, and mono and polychlorinated biphenyls (Walia et al., 2003)
10	*P. putida* CCRC14365	Phenol degradation (Chung et al., 2003)
11	*P. putida* CSV86	Utilizes aromatics over glucose and co-metabolizes them with organic acids (Basu et al., 2006)
12	*P. putida* CP1	Degradation of 2-chlorophenol (Farrel and Qualty, 2002)

2.5.3 Microbial Transformations of Mercury

Microbial transformation of mercury can be defined as a process that involves microorganisms through their enzymes to return the natural environment altered by contaminant to its original condition (Tarangini, 2009). Although micro-organism cannot destroy metals but they can alter their chemical properties via a surprising array of mechanism that can be used to treat toxic metal contamination by involved highly specific biochemical pathways that have evolved for their protection (Lloyd and Lovly 2001). In this context, mercury is unique because of the combination of the extreme toxicity, no biological function and low vapor pressure of elemental mercury (Wagner-Dobler, 2003). Microorganism that highly resistant to mercury are extremely important in detoxifying the mercury compounds by NADPH-linked enzyme called mercuric reductase and organomercurial lyase (Brock et al., 2006, De and Ramaiah, 2006). Microorganisms are known to mediate four typed of enzymatic transformations of mercury (Barkay and Wagner-Dobler, 2005):

1. reduction of Hg^{2+} to Hg^{0}
2. breakdown of organomercury compounds (including $MeHg^{+}$), resulting in formation of Hg^{0}

3. methylation of Hg^{2+}
4. oxidation of Hg^{0} to Hg^{2+}

Reduction Hg^{2+} reaction in (1) and (2) are performed by mercury reductase enzyme and proteins of the microbial mercury resistance (mer). Mercury metal (Hg^{0}) can volatilize out the system and subsequently recovered, either mercury vapor sorption onto various materials or through sorption to packed column (Oehman et al., 2009). Thus, the resistant *P. putida* can contribute to mercury removal. Moreover, researchers have used mercury-resistant bacteria in bioremediation (Xiao-xi et al., 2010).

The detoxification (resistance) mechanism for mercury is based on the unique peculiarities of this metal: the electrochemical potential of Hg^{2+}/ Hg^{0} at pH 7 is +430mV, which means living cells are able reduce Hg^{2+} to elemental form Hg^{0}, which is non-toxic to human and microorganism (Singh et al., 2008). Secondly, the melting point of mercury is extraordinary low (melting point -39^{o}C, boiling point 357^{o}C), so that elemental mercury does not remain inside the cell but leave it by passive diffusion and is then either evaporated into the air or precipitated due to its low solubility in water. In either case, the bacterial cell is effectively freed of toxic Hg^{2+} or organomercury compounds.

2.5.4 Mechanism of Hg^{2+} Reduction to Hg^{0} by *P. putida*

The complex structure of microorganisms implies the mechanism for the metal to be taken up by the microbial cell. The cell surfaces of all microorganisms are negatively charge owing to the presence of various anionic structures (Barkay and Wagner-Dobler, 2005). Hussein et al., (2005) and Vijayaraghavan and Yun (2008) reported that carboxyl groups also actively participate in binding process. This gives bacteria the ability to bind metal cations. Detoxification for mercury removal by *P. putida* involved mechanisms of redox transformation where the enzyme-catalysed reduction of the toxic mercury ion (Hg^{2+}) to non-toxic elemental mercury, Hg^{0} (Llyold and Lovly, 2001 and Wagner-Dobler, 2003). Under aerobic growth conditions, Hg^{0} is removed from the immediate environment of resistant microbes because of its low water solubility (6 x 10^{-6} g/100 mL water at 25^{o}C and

high vapor pressure (Henry's constant, *H*= 0.3), allowing for the commencement of cellular growth (Barkay and Wagner-Dobler, 2005).

The biochemical basis of resistance to inorganic mercury compounds such as Hg $(NO_3)_2$ appears to be quite similar in several different species. This enzyme has been characterized in plasmid-carrying strains of *P. putida*. This reductase is flavoprotein, which catalyzes the NADPH dependent on reduction of Hg^{2+} to Hg^0 then this allows for more CH_3Hg^{2+} to be converted to Hg^{2+} for compound of organic mercury.

Genetically, these genes involved in mercury reduction mechanism called mer genes, are arranged in an operon (mer operon) which consists of series of genes (MerR, MeO, MerP, MerT, MerA, MerD) and which are under control of the regulatory protein MerR which function as either a repressor (absence of Hg^{2+}) or transcriptional activater (presence of Hg^{2+}). MerO still as unknown function, MerP as a periplasmic binding protein, MerT as transport gene, MerA is is responsile for the production of mercury reductase enzyme and MerD as gen for regulation (Horn et al., 1994, Bitton, 2005 and Brock et al., 2006).

When Hg^{2+} is present, the cellular machinery encoded by the microbial mer operon provides specific uptake proteins (merT, merP) that transport Hg^{2+} into cytoplasm. Hg^{2+} forms a complex with MerR, which then functions as an activator to transcription of mer operon. The protein MerP is a perplasmic Hg^{2+}which binds to amino acid cystein in protein in an operon. MerP binds Hg^{2+} and transfers it to a membrane protein MerT, which transport Hg^{2+} into the cell for reduction by intracellular mercuric reductase. As a result, this mechanism as prevention from cell damaging (Weon and Ashok, 2001 and Brock et al., 2006).

Inside the cell, Hg^{2+} is reduced by NADPH to Hg^0 by mercury reductase (merA), which is related to glutathione reductase (Schiering et al., 1991 and Felske et al., 2003). The reaction takes place within minutes. The final result is reduction of Hg^{2+} to Hg^0, which is volatile and is released from the cell but is non-toxic to human and microbes (Holtze et

al., 2006). The microbial reduction of mercury is a detoxification reaction that requires energy rather than producing it. Thus, in a bioreactor, the bacteria have to be supplied by nutrients. Figure 2.5 shows schematic representative of a sample model for mercuric ion reduction in *P. putida* (Schiering et al., 1991).

Mercury reduction is an inducible trait; the transcription of the mer operon is suppressed if no mercury is present due to binding of merR protein to the operator/ promoter region of mer operon. If mercury is present, it binds to the merR protein and cause it to leave the operator/promoter region, where transcription can then start. Truncation of merR gene from the mer operon resulted in recombinant strains with constitutive expression of mercury resistance (Horn et al., 1994).

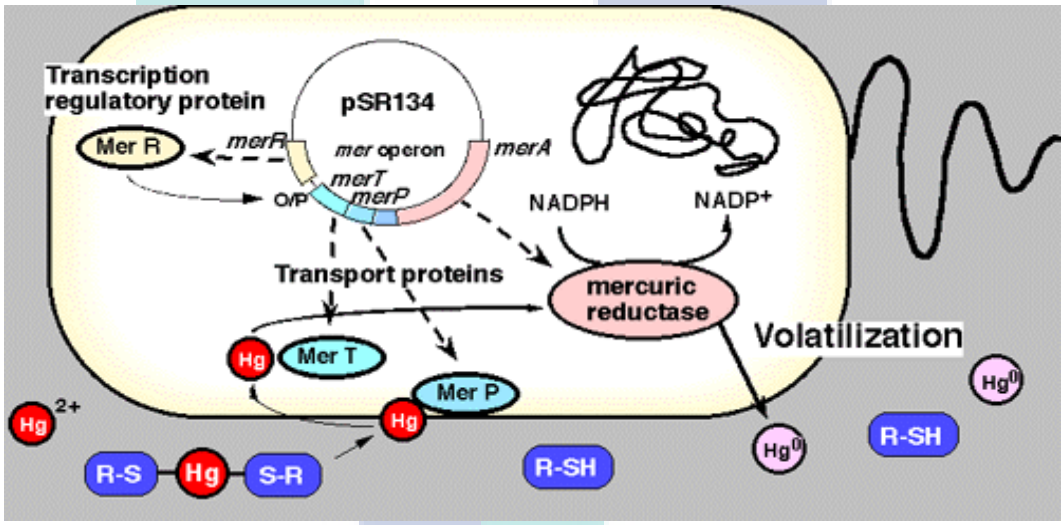


Figure 2.5: Schematic representation of a simple model for mercuric ion reduction in *P. putida*

2.5.5 Retention of Elemental Mercury in Hollow Fiber Membrane

Elemental mercury produced by microbial reduction diffuse out the cells and can easily be volatilized into atmosphere. Remediation technologies based on mercury volatilization have been explored (Fry et al. 1992; Saouter et al. 1994 and Gadd 2000), but

have never proceeded beyond laboratory scale, because collecting the volatilized mercury is tedious and expensive on a technical scale (Srivastava and Majumder, 2007).

There are two main types of continuous systems which can be used for mercury removal: fixed beds or fluidized beds. Column containing fixed beds have been used as the conventional method for ion exchange resins and have been extensively used to evaluate biosorption/bioaccumulation by natural occurring strains. Work performed at the National Research Institute for Biotechnology showed that the elemental mercury formed could also retain in packed bed bioreactor (Brunke et al. 1993). Mercury accumulated in the carrier material and, microdroplets of mercury were visible by light microscopy and scanning microscopy as shown at Figure 2.6 (Wagner-Dobler, 2003).

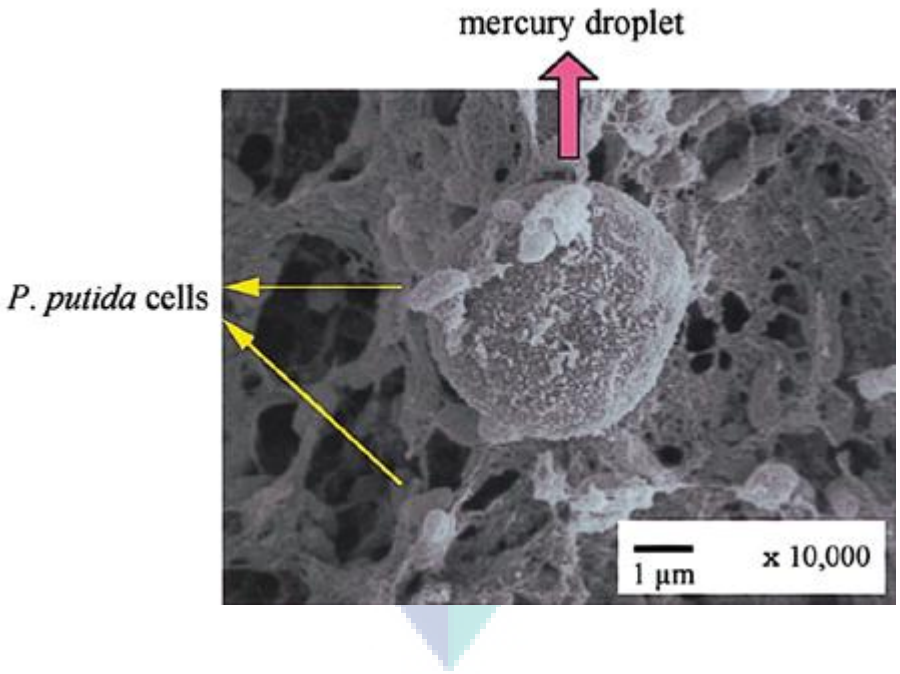


Figure 2.6: Scanning electron micrograph of a Ca-alginate bead used to immobilize bacterial cells in the mercury removal

In this study, hollow fiber cartridge was used to retain cells for Hg^{2+} bioaccumulation (Chen et al., 1998 and Wagner-Dobler, 2003). Juang and Tsai, 2006b) reported that microporous hollow fiber can be used as the reactor for biotechnology

applications such as cell cultures and biodegradation (Loh and Yu, 2000 and Chung et al., 2003). The cell immobilized by polymeric membranes, particularly in the form of hollow fibers, to form an immobilized culture can be generically employed as the membrane bioreactor for biotechnology application (Juang and Tsai, 2006b). The membrane physically separates the wastewater from biology medium where degradation takes place, making it a useful technique for the treatment petroleum based industries wastewater (Chung et al., 2005). At first, a backflush operation mode was adopted to immobilize the cells for bioaccumulation. Cells growth was monitored by measuring the optical density (OD) in bioreactor reservoir. The microporous membrane bioreactor is promising for practical application if the substrates were not so toxic even at high levels and the solution conditions were relatively mild (Juang and Tsai, 2006c and Juang et al., 2007).

2.6 GROWTH OF *P. putida* POPULATIONS

2.6.1 Introduction

Growth is defined as an increase in the number of microbial cells in a population, which can be measured as an increase in microbial mass. For microbes, growth is their most essential response to their physiochemical environment (Volesky, 2001). Growth is a result of both replication and change in cell size (Bitton, 2005). *P. putida* can grow and adapt under a variety of physical, chemical, and nutritional conditions (Kulkarni and Chaudri, 2006). In suitable nutrient medium, organisms extract nutrients from the medium and convert them into biological compounds. Parts of these nutrients are used for energy production and parts are used for biosynthesis and product formation. As a result of nutrient utilization, microbial mass increase with time (Volesky, 2001).

2.6.2 Growth Patterns of *P. putida*

When a liquid nutrient medium is inoculated with a seed culture, the organism selectively take up dissolved nutrients from the medium and convert them into biomass (Chen et al., 2008). A typical growth curve includes the following phase; (1) lag phase, (2)

logarithm or exponential growth phase, (3) deceleration phase, (4) stationary phase, and (5) death phase. Figure 2.7 describes the microbial growth kinetics (Shuler and Kargi, 2002).

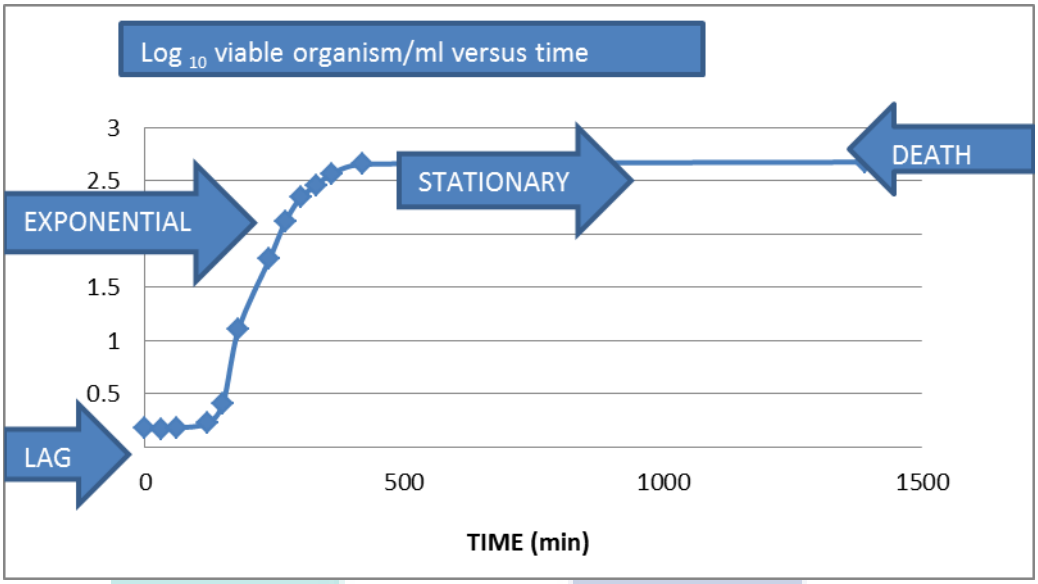


Figure 2.7: Typical growth curve for *P. putida* population

The lag phase occurs immediately after inoculation and is a period of adaption of cells to a new environment (Lee, 2003). Microorganisms reorganize their molecular constituents when they are transferred to a new medium. During this phase, cell mass may increase a little, without increasing in cell number density (Bitton, 2005). In commercial production of enzyme, lag phase should be as short as possible through the right choice of inoculum (Ee, 2004).

The exponential growth phase is also known as the logarithm growth phase. In this phase the cells have adjusted to their new environment. After this adaption period, cells can multiply rapidly, and cell mass and cell number density may increase. This is a period of balanced growth in which all the components of a cell grow with the same rate (Xiao et al., 1999). That is, the average composition of a single cell remains approximately constant during this phase of the growth. During balanced growth, the specific growth rate determined from either cell number or cell mass would be the same. Since the nutrient

concentration is large in this phase, the growth rate is dependent of nutrient concentration in this phase (Shuler and Kargi, 2002 and Bitton, 2005).

The deceleration phase follows the exponential phase. In this phase, growth decelerates due to either depletion of one more essential nutrients or the accumulation of toxic by-products of growth. For a typical bacterial culture, these changes occur over a very short period of time. During the exponential growth phase, the specific growth rate, μ is approximately constant (Monteiro et al., 2000). The rapidly changing environment results in unbalanced growth. In the exponential phase, the cellular metabolic control system is set to achieve maximum rates reproduction (Bitton, 2005 and Brock et al., 2006).

The stationary phase starts at the end of deceleration phase, when the net growth rate is zero or when the growth rate is equal to the death phase. Even though the net growth is zero during the stationary phase, cells are still metabolically active and produce secondary metabolism. Bull and Whitten (1974) pointed out that the stationary phase is a misnomer in terms of physiocology of the organism, as the population is still metabolically active during this phase and may produce products called secondary metabolites, which are not producing during the exponential phase.

During the stationary phase, the cell catabolizes cellular reserves for new building blocks and for energy. The reason for termination of growth may be either exhaustion of an essential nutrients or accumulation of toxic products. If an inhibitory product is produced accumulation in the medium, the growth rate will slow depending on inhibitor production and at certain level of inhibitor concentration, the cell growth will stop (Brock et al., 2006).

The death phase or decline phase follows the stationary phase. However, some cell death may start in the stationary phase, and a clear demarcation between these two phases is not always possible. Often, dead cell lyse, and the living organism will use intracellular nutrient released into medium during stationary phase. At the end of the stationary phase, either because of nutrient depletion or toxic product accumulation, the death phase begins (Shuler and Kargi, 2002).

Besides meeting requirements for growth and product formation, the metal uptake process, however, is complex and the length of time required for the removal of these compounds in environment depends on various factors that affect the growth kinetics of the participating microorganisms, such as techniques of culture, acclimatization, substrate concentration, pH variation, temperature, chemistry of metal ions, metal concentration, aeration, agitation and presence of toxic intermediates. Any one of these factors or a combination of them can play a role in limiting the rate of biomass growth and substrate biodegradation (Brock et al., 2006).

2.6.3 Culture Techniques for *P. putida*

The success of the cell culture and their survival depends on technique used (Lebeau et al., 2002). In case of *P. putida* is stored in freeze-drying ampoules before conducting culturing steps. Freeze-drying is popular method of preservation microorganisms because the long-term viability is excellent in most cases and the storage and distribution requirements are simple (Shinohara et al., 2000).

In the batch biodegradation study conducted by Abuhamed et al., (2003a), acclimatization of *P. putida* for adaption was more rapid than non-adapted cells. For the growth of *P. putida* as aerobes, it is necessary to provide extensive aeration. This is because the oxygen that is consumed by the organisms during growth is not replaced fast enough by diffusion from air. Forced aeration of cultures is frequently needed and can be achieved either by vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium through a fine glass tube or porous glass disc. Aerobes usually grow much better with forced aeration than when oxygen is provided by simple diffusion. *P. putida* were grown aerobically on a rotary shaker at 120 rpm (Kulkarni andChaudhari, 2006).

2.6.4 pH and Incubation Temperature

Temperature is an important factor affecting cell performance in terms of microbial growth and survival (Bitton, 2005). Temperature also affects product formation but the

optimum temperature for growth and product formation may be different (Shuler and Kargi, 2002 and Vijayaraghavan and Yun, 2008). As temperature rises, chemical and enzymatic reaction in the cell proceed at more rapid rates, and growth becomes faster. However, above a certain temperature, particular proteins may be irreversibly denatured. Thus, as the temperature is increased within the range, growth and metabolic function increase up to point where denaturation reaction is set. Above this point, cell functions fall sharply to zero. It is evident in both the cases that the increase in the maximum specific growth rates with temperature from 15 ^{o}C – 30^{o}C follows an exponential trend as predicted by Arrhenius equation. However, between 30^{o}C – 35^{o}C the increase is clearly not exponential, which suggests that the optimum temperature might fall between 30^{o}C and 35^{o}C. In the temperature range of 30^{o}C to 37^{o}C, good cell growth was observed (Ee, 2004).

Hydrogen ion concentration (pH) affects the activity of enzymes and therefore the microbial growth rate. The optimal pH growth may be different from that for product formation. Most organisms show a growth pH range of 2 - 3 units. Thus, pH control by means of a buffer or an active pH control system is important. The pH was controlled by feeding 1M NaOH solution the bioreactor to prevent acidification (Kim and Kim, 2004). Most natural environments have pH values between 5 and 9, and organisms with optima in this range are most common (Srivastava and Majumde, 2007).

Vijayaraghavan and Yun, (2008) reported that the activity of binding sites can also be altered by adjustment of pH due to the negatively charged of carboxyl groups which are responsible for the binding metal via ion exchange mechanism. *P. putida* was reported to be optimal for biodegradation of phenol at pH 6.8 – 7.0 (Chung et al., T. 2003; Tsai and Juang, 2006 and Srivaastava and Majumde, 2007).

2.6.5 Aeration and Agitation

In fermentation using bioreactor, aeration is to provide microorganisms in submerge culture with sufficient oxygen for metabolic requirements (Ee, 2004). The pump was used to add and re-circulate the solution to the reactor at flow rate of 2.00 L/min and agitation

speed at 100 rpm for the study carried out by Kim and Kim (2004). However, studies on the effect of aeration and agitation on *P. putida* are scarce.

Ishenny, (2006) studied on effect of aeration on for *Candida* cylinddricae, which the aeration was varied from 0.25 vvm to 1.00 vvm and found out by increasing the aeration would increase the growth rate because the rate of oxygen consumption may exceed the rate oxygen supply, leading to oxygen limitation. Rosso et al., (2002) are reported on the effects of aeration an agitation on other microbial products are diversified. Feng et al. (2003) is suggested that maximum b-mannanse production from *Bacillus licheniformis* was attained when aeration rate and agitation speed were controlled at 0.75 vvm and 600 rpm respectively. Walther et al. (2001) discovered that at high aeration rate, *Candida tropicalis* grew vigorously, leading to high xylitol production rate.

2.7 BIOREACTOR OPERATION MODES AND KINETICS MODEL

2.7.1 Introduction

Large scale cultivation is performed in specialized reaction vessels known as bioreactor or fermenters. The term of fermentation is often used to describe the cultivation of cells in fermenters. There are three main mode of bioreactor operation: batch, fed batch and continuous (Ishenny, 2006).

2.7.2 Batch Mode

The batch mode of fermentation is an example of closed-system fermentation where the nutrients are limited (Stanbury et al., 1984). At zero time, the sterile nutrient solution is inoculated with microorganism and incubation is allowed to proceed under optimal physiological conditions. Therefore, in such a bioreactor, the concentration of nutrients, cells and products varies with times as growth proceeds (Ee, 2004 and Sheikh, 2006).

In the batch mode, all nutrients required during one run of cultivation, except for molecular oxygen in an aerobic process or other chemicals for pH adjustment, are added to the medium before cultivation is stated, and the broth containing product is withdrawn only at the end of each batch run (Yamane, 1994 and Ishenny, 2006) as shown in Figure 2.8 (Yamane, 1994).

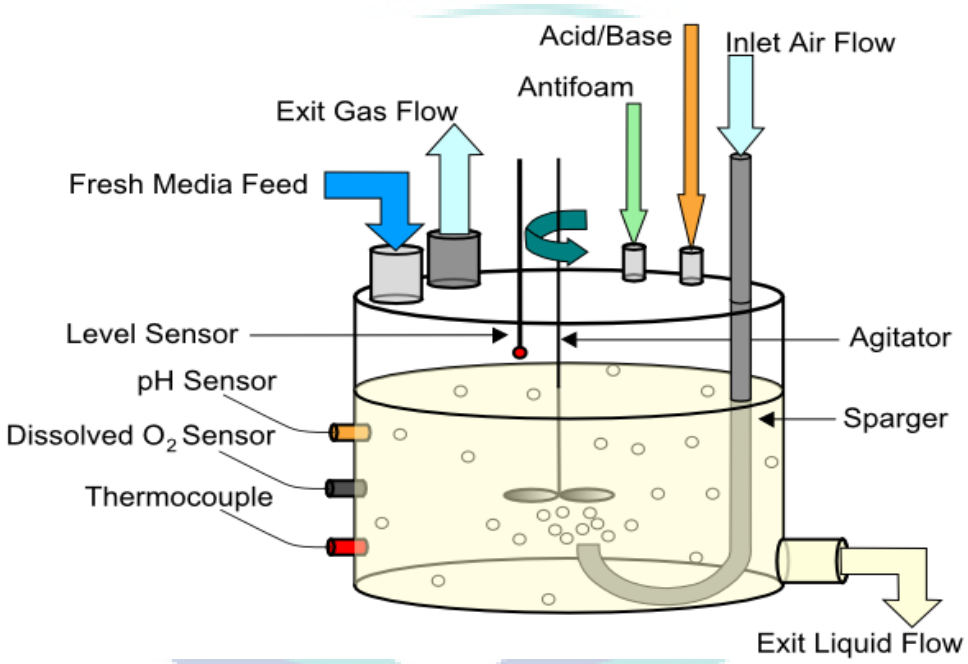


Figure 2.8: Diagram of bioreactor for *P. putida* cultivation

Batch mode operation is the most primitive mode and the least efficient; the most serious drawback is that the environment (nutrient concentration) surrounding the organisms are not controlled at all in a batch culture even though their functions and activities vary greatly depending on change in the environment (Yamane, 1994 and Ee, 2004).

Procedure for any batch culture is basically the same, although some small differences are observed on the kind of microorganism and kind of metabolite desired. Major equipment consists of the main bioreactor, both being preparation, steam sterilization, cooling, air compression, air filtering, stirring, antifoam. The seed bioreactor

is used to cultivate cell mass initially necessary for the main bioreactor, and the desire metabolite is obtained from the main bioreactor (Ee, 2004 and Lee, 2003).

Firstly, organisms on slant in a test tube are inoculated in a shake flask, from which the grown cells are then transferred to seed bioreactor. In some cases more than stages of seed culture are necessary. In practice, a series of the seed cultures work are (Kim and Kim, 2004):

1. The vacant tank is preliminary sterilized with steam followed by sterilization of the medium prepared in the tank.
2. After cooling, the microorganism pre-cultured in the flask is inoculated. The microorganisms grown in the shake flask are transferred to the main bioreactor after sufficient cell concentration has been achieved. Sufficient steam sterilization and air filtration are necessary to kill and remove microbial contaminants during the inoculations and transfers.
3. Once cultivation starts in the bioreactor, temperature and pH are controlled at their optimum values and the culture broth is stirred and air is supplied (aeration) during the whole cultivation.
4. The cultivation is completed when the desired level of cell growth and metabolite accumulation is attained, and the culture broth is subjected to the next downstream treatment for product recovery.

One run of batch operation range from several hours to more than several weeks. The total process time for one run of batch culture is the sum of the times required for input of the sterilized medium in the bioreactor, transfer of innoculum, lag of cell growth, major microbial reaction, harvesting (broth out), inside cleaning, and vacant sterilization (Yamane, 1994 and Alagappan and Cowan, 2003).

2.7.3 Fermentation Kinetics

2.7.3.1 Introduction

The large scale cultivation of cells is central to the production of a large proportion of commercially important biological products. Not surprisingly, the maximization of profits is closely linked to optimizing product formation by cellular catalyst; i.e. producing the maximum amount of product in the shortest time at the lowest cost (Ishenny, 2006).

To achieve this objective, cell culture systems must be described quantitatively. In other words, the kinetics of the process must be known. By determining the kinetics of the system, it is possible to predict yields and reaction times and thus permit the correct sizing of a bioreactor.

Obviously, reaction kinetics must be determined prior to the construction of the full scale system. In practice, kinetic data is obtained in small scale reactors and then used with mass transfer data to scale-up the process (Ee, 2004 and Ishenny, 2006).

2.7.3.2 The Kinetics of Batch Fermentation

Cell culture systems are extremely complex. There are many inputs and many outputs. Unlike most chemical systems, the catalysts themselves are self-propagating. To assist in both understanding quantifying cell culture systems, biotechnologists often use mathematical models (Dunn et al., 1992 and Ishenny, 2006). At Chemical reactions can be simplified. For example, a first order chemical reaction in which 1 mole of reactant (*S*) is converted to a product (*P*):

$$S = nP \tag{2.3}$$

Can be expressed as a differential equation of the form:

$$(d[S])/dt = -k[S] \quad (2.4)$$

where [*S*] is the concentration of the reactant and k is a rate constant.

A differential equation describing product formation is:

$$d[P]/dt = -n(d[S])/dt \quad (2.5)$$

or

$$d\,[P]/dt = -nk[S] \quad (2.6)$$

Where [*P*] is the concentration of the product and n is the stoichiometric yield constant describing the relationship between the removal of *S* and formation of *P*. Note that as the concentration of *S* decrease, the concentration of *P* increase and hence the negative sign in equation 2.3 and 2.4. By solving these two equations, it is possible to predict the values of *S* and *P* at any time (Dunn, et al., 1992 and Ishenny, 2006).

2.7.3.3 The Exponential Growth Curve

At the exponential or log phase, the growth rate of the cells increase in a short time and the cells grow at constant, maximum rate (Abuhamed, 2003a and Ee, 2004). The rate increase cell (or biomass) is depand on the concentration of cells present in the reactor. The number of cells increased exponentially and the exponential growth varies with the type of microorganism and growth conditions. The growth follows a geometric progression (2^0, 2^1, 2^2, 2^n) (Bitton, 2005).

$$X_t = X_0 e^{\mu t} \quad (2.7)$$

Where X_t is the concentration of biomass or the number of cells in the bioreactor after time, *t*. X_0 is the initial number or biomass of cells and μ is the specific growth rate. This model of microbial growth is referred to as the exponential growth model (Park et al.,

2002 and Tsai and Juang, 2006). Biomass concentrations are typically expressed in $g.L^{-1}$ of dry weight or density/turbidity of cell under measurement optical density at 600 nm wavelength is used (Shuler and Kargi, 2002).

Using the natural logarithms on both sides of Equation 2.7, thus can be re-written as:

$$\ln X_t = \ln X_0 + \mu t \tag{2.8}$$

where μ is given by

$$\mu = \frac{\ln X_t - \ln X_0}{t} \tag{2.9}$$

The specific growth rate (μ) describes how fast the cells are reproducing. The higher the value of specific growth rate, then the faster the cells are growing. When cells are not growing, then their specific growth rate is zero. During exponential phase, the specific growth rate is relatively constant (Dunn, et al. 1992, Bitton, 2005, Brock et al., 2006).

A mathematical relationship during the exponential growth involved the following parameters (Shuler and Kargi, 2002; Ee, 2004 and Brock et al., 2006):

- Specific growth rate, μ is defined as the increase in cell mass per unit time, e.g., grams cells (g) per gram cells (g) per hours: ($g.g^{-1}.h^{-1}$). The specific growth rate is commonly units are in reciprocal hours (hr^{-1}), however, it can also be expressed in reciprocal seconds (s^{-1}) or minutes (min^{-1}) or any other units of time.

- Number of generation, $n = 3.3 \ln X_t/X_o = 3.3 \ln OD/OD_0$

- Generation time, g is defined the time required for formation of two cells from one, doubling time, $g = t/n$, where t is duration of exponential growth expressed in days, hours, or minutes depending on the organism and the growth condition.

- Growth rate constant to measure of the number of generation that occur per unit time in an exponential growing culture, $k = \ln 2/g = 0.693/g$

Armed with knowledge of n and t, g and k can be calculated. These parameters will show the different microorganisms growing under the different culture conditions. This is often useful for optimizing for testing the positive or negative effect of some treatment on the bacteria culture (Shuler and Kargi, 2002 and Brock et al., 2006).

2.7.3.4 Monod Model

A typical plot of specific growth rate against the concentration of growth limiting nutrient is shown in Figure 2.9 (Shuler and Kargi, 2002 and Brock et al, 2006).

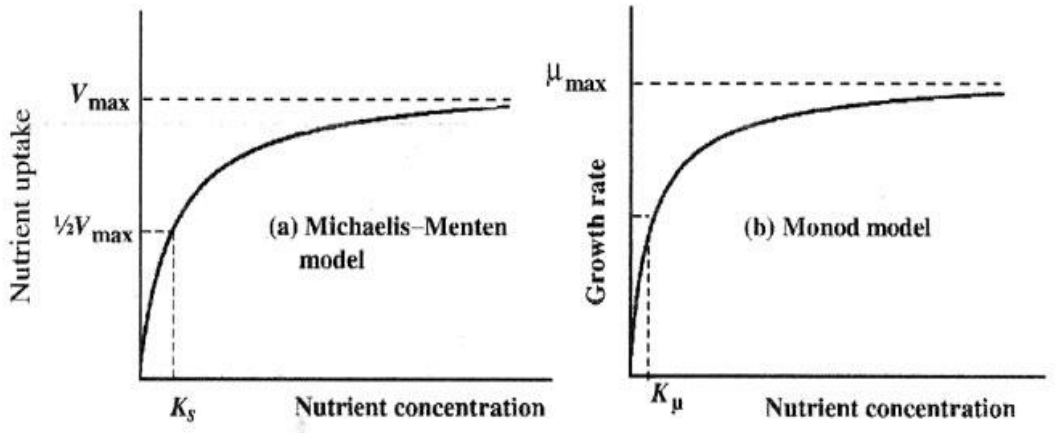


Figure 2.9: Typical plot of specific growth rate against substrate concentration, Monod model and Michaelis-Menten model

In 1942, Jaques Monod proposed that the following mathematical relationship could be used to describe the effect of a growth limiting nutrient on specific growth rate:

$$\mu = \frac{\mu_m S}{K_s + S} \tag{2.10}$$

Where

- μ_m is maximum specific growth rate
- K_S is the saturation or Monod constant
- S is the concentration of the growth limiting substrate

Equation 2.6 can be therefore be re-written as:

$$\frac{dX}{dt} = \frac{\mu_m S \ X}{K_s + S} \tag{2.11}$$

The maximum specific growth rate (μ_m) is the maximum growth rate achievable when the concentration of the growth limiting nutrient is not limiting. The higher the value of μ then the faster the rate at which the organism can grow.

The Monod constant (K_S) is the concentration of the growth limiting nutrient at which the specific growth rate is half the maximum value. It represents an affinity the organism has for the nutrient.

The value of μ_m and K_S are dependent on the organism, the growth limiting nutrient, fermentation medium and environmental factors such as pH and temperature. Value of the μ_m is in the range of 0.01 to 3.00 hr^{-1}. Monod constant values are typically less than 0.10 gL^{-1} (Reardon et al., 2000 and Ishenny, 2006).

The Monod Model and the Michaelis Menten Model

The Monod Model

$$\mu = \frac{\mu_m S}{K_s + S} \tag{2.12}$$

Look similar to Michaelis Menten equation.

$$V = \frac{V_{\max}[S]}{K_m + [S]} \tag{2.13}$$

The parameters μ_m and K_s are analogous to V_{max} and K_m. both models predict that only when the concentration of a rate limiting substrate or nutrient becomes limiting, then the reaction rate will be reduced. There is however one very distinct difference between the two models. The Michaelis Menten equation was derived using the mechanism of enzyme action as a basis. The Monod Model is contrast is used because it fits the typical curve shown in Figure 2.9. The Monod Model is therefore classified as an empirical model, while the Michaelis Menten equation is a mechanistic model (Lee et al., 2003; Bitton, 2005 and Brock et al., 2006).

Monod model parameter estimation – Monod constant

Determine the value of the Monod constant is more difficult when using a batch cultivation system. One method would be to take tangents to plot of X vs t and then obtain a plot of μ vs S as shown in Figure 2.9 and of K_s could then be calculated as the substrate concentration which corresponds to ½ μ_m (Bitton, 2005).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 INTRODUCTION

This chapter describes the details of all research techniques and activities carried out in this study consisting of materials, process equipment, apparatus used for experimental analysis, sterilization techniques, culture preservation and fermentation process. The flowchart provides an overview of the research elements considered and undertaken in achieving the objectives mentioned in Chapter 1. Glassware used in this experiment underwent through autoclaving and proper sterilization to avoid contamination. Safety measures and precautions have been taken into account when designing and preparing the experiments and the procedure related to microorganism were carried out in the laminar flow hood.

3.2 MATERIALS

The materials used in this research consisted of microorganisms and chemicals for the preservation of the microbe in slant, culture medium and for analytical procedures. Various chemicals and materials are also used for sample analysis of wastewater and also as cleaning chemical.

3.2.1 Microorganism

Bacteria *P. putida* used in this study was obtained from Merck (Malaysia) Sdn. Bhd as local agent dealing with the bacteria. The *P. putida* used is ATCC 49128 (freeze dried) sourced from Microbiologics, 217 Osseo Ave. North, St. Cloud, USA. Appendix 3.1 shows freeze-dried of *P. putida* packed in a container. The ingredients of *P. putida* nutrient consist of 5% of pepton meat and 3% meat extract. The growth medium for *P. putida* were prepared by suspending 8g nutrient powder in 1 L of de-ionized (DI) water. The growth media was sterilized in an autoclave at a temperature of 121^{o}C and a pressure of 15 psi for 25 minutes. The culture was kept below 5^{o}C and this culture stock was used for all subsequent works.

Analytical grade ethanol used as cleaning solvent was supplied by Merck (Malaysia) Sdn. Bhd. Tap water was used for washing glassware, cleaning and as cooling water. Distilled water was used to prepare the culture medium and deionized water was used for analytical purposes such as in spectrophotometer and mercury analyzer, and also for the preparation of chemical standard solution to determine standard calibration curve, and for sample dilution.

3.2.2 Chemicals

All chemicals and medium used in the experiments were of analytical grade, including peptone, yeast extract, KH_2PO_4, $MgSO_4.7H_2O$, Urea, NaCl, HNO_3, HCl, NaOH, were purchased from Merck (Malaysia) Sdn. Bhd. Stannous Chloride solution ($SnCl_2$), $Hg(NO_3)_2$ as mercury standard solution (1000 000 ppb) and H_2SO_4 were obtained from Orbiting Scientific & Technology Sdn. Bhd.

3.2.3 Medium

The medium was used for inoculum, growth and wastewater treatment purpose. Medium for nutrient agar in test tubes is prepared similar to that of the growth media. The

main ingredients were combination of 10% of peptone from meat, 5% of meat extract, 15% of agar or broth plus other ingredients. In preparing the nutrient broth (NB) for the stock culture, 8 g of nutrient (Merck: BD 234000) was magnetically suspended in unheated 1L of de-ionized water. For the nutrient agar (NA) 20 g of nutrient (Merck: BD 213020) was suspended in 1L of de-ionized water where the solution was heated using hot plate and stirred. Then, both of nutrients were sterilized in an autoclave at temperature of 121^{o}C at 15 psi for 25 minutes before being kept in a freezer at temperature below 5^{o}C. Appendix 3.2 shows the process of preparing *P. putida* nutrient using hotplate.

3.2.4 Process of Culturing *P. putida* from Freeze-Dried

Freeze-drying is one of the most commonly adopted methods used in the preparation of microbial culture. The method offers the convenience of storing and transportation the culture by mail, as well as the ability to store for long periods. Freeze-drying of different organisms using different methods have been found to have different surviving rate (Shinohara et al., 2005).

The method used in this study was developed by Dagegett and Simione (1987). The appropriate biosafety protocol of cultures are adopted in this experiments and the steps taken are as follows: preparing the culture in biological safety cabinet, wearing of suitable eye protection, holding vials away from face, wearing of gloves and sterilizing all empty vials and fragments before disposal.

The packing skin of the culture must also be removed using a sharp blade or by soaking in water for a few minutes while the ampule briskly scored once with a sharp file about one inch from the tip. After the ampule was disinfected with alcohol-dampened gauze, the gauze is wrapped around the ampule to break the scored area. Care should be taken not to have the gauze too wet to prevent the alcohol from being sucked into the ampule when scored area is broken. The cotton was removed with a sterile forcep, and after properly mixing the culture in 0.50 ml nutrient broth, the suspension is transferred to an oven heated to 30^{o}C to let the culture to incubate for 24 hours incubating. A few drops of

this suspension is then transferred to slanting agar, nutrient broth and plate agar to start the growth of the culture (Dagegett and Simione, 1987).

Figure 3.1 illustrates the overall processes of culturing of *P. putida* from freeze-dried sample, where strict procedures need to be adopted to ensure the growth of the microbes in nutrient broth (NB), agar plate or nutrient agar (NA) and slanting agar as the stock cultures were used in all the subsequent studies. However, note that sampling of *P. putida* from prepared agar slant should not be more than five times to prevent insufficient cell growth (Stanbury, et al. 1984).

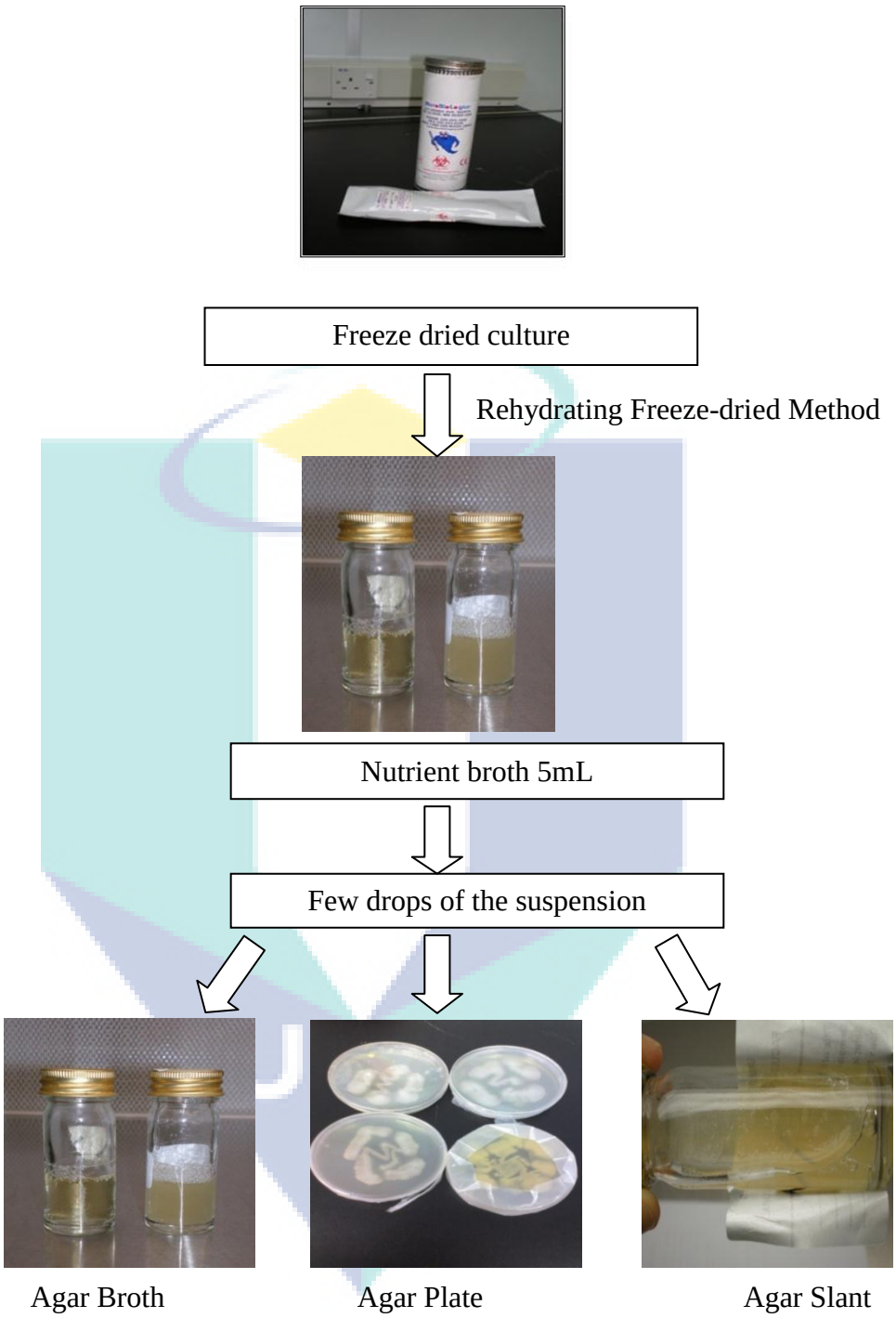


Figure 3.1: The Overall Processes of Culturing *P. putida* from Freeze-Dried Sample

3.2.5 Stock Culture

It is essential to ensure the purity of the stock culture so that the culture can be used for a long period of time without losing or lacking any nutrient. The preserved nutrient is preserved and broth can only last for a few weeks and after that the culture will start to decay due to nutrient depletion and accumulation of toxic by- product. A stock culture can be stored in a refrigerator up to six month (Stanbury, et al. 1984).

There are several ways on how to transfer a culture from broth culture to a slanting agar in a laminar flow cabinet (ESCO) as shown in the Appendix 3.3. The technique used is known as aseptic technique where sterility of the media must be maintained during transfer a pure culture, *P. putida*. First, the inoculating loop is sterilized using a flame from a Bunsen burner until it is red hot. The loop is cooled down before dipping into the broth culture. The test tube containing the pure culture is shaken to disperse the cells. The cap of the test tube is removed and the lip of the test tube is sterilized using the flame from a Bunsen burner. The culture tube is then held in a slanting position while the inoculating loop is inserted into the culture broth.

After that, the culture tube is capped after its lip has been flamed with the Bunsen burner. After leaving the culture tube aside, a test tube containing NA is opened and its lip is also flamed. The inoculating loop that contains a smear of culture is inserted into the second test tube while gently sliding the loop in a continuous streaking motion on the surface of the agar. The loop is again flamed after use it. The test tube containing the pure culture on NA is then incubated at 30°C for 24 hours. It is then kept in a refrigerator to be used as stock culture.

3.3 EQUIPMENTS

In this study, in general the equipment used can be divided into experimental apparatus and analysis apparatus. Both apparatus have their own function in setting up a series of experimental activities for data collection.

3.3.1 Experimental Apparatus

The experimental apparatus are the basic parts of the apparatus that are required in order to carry out the experiments. These experimental apparatus that required in the experiments are auto-clave, H+P Varioklav Stream Sterilizer ESCO, Shaker (B. Braun,German), microbiological incubator (Mermmert-Germany/BE 600), 2L bioreactor (B. Braun Biotech), laminar flow cabinet (ESCO), oven (Haeraeus), Cross-flow Membrane Filtration (Amersham/ Kucklas/ Quixstand Crossflow Membrane).

3.3.2 Analysis Apparatus

The analysis apparatus, which is different from experimental apparatus, are the apparatus that are involved in the analysis and determination of the condition of the compound or substance as well as to identify operating parameters of the process. In this section, the analysis apparatus used are mercury analyzer, (RA-3000 Mercury, NIC), UV-Vis. Spectrophotometer (U-1800, Hitachi), pH Meter (Mettler Toledo), vacuum pump (HACH), Turbidity Meter HACH 2100P and Analytical Balance (Mettler Toledo).

3.4 METHODS

The overall experimental procedure is further detailed in the following sections. Generally, the aim of the experiments are to achieve and establish the objectives previously mentioned that involves a series of research activities as summarized in the experimental flowchart. The data collected are often graphically correlated and calculations for process efficiency are later carried out, and finally, a discussion leading to a conclusion is presented.

3.4.1 Overall Experimental Sequence of the Study

Sterilization, culture preservation, fermentation, membrane bioreactor separation, procedure for analysis of samples and experimental data are covered in this section.

Basically, the experiments carried out in this study are divided into four main stages. The first two stages were carried out in shake flasks and the next two stages were conducted in 2L bioreactor and membrane bioreactor. The first stage is to investigate variables involved in fermentation during *P. putida* growth such as culturing technique, nutrient/substrate concentration, acclimatization time, pH, incubator shaker speed and temperature. The second stage is to determine and study the effects of the variables on the capability of *P. putida* to reduce or remove mercury in wastewater. The third stage is to identify the performance of *P. putida* with optimum growth parameter in a bioreactor to reduce mercury by determining the aeration rate and propeller speed. Finally, studies with membrane bioreactor using actual petroleum based industries wastewater were conducted and analyses of wastewater were carried out before and after the treatment. The summary of the methodology is presented in experimental flowchart Figure 3.2.

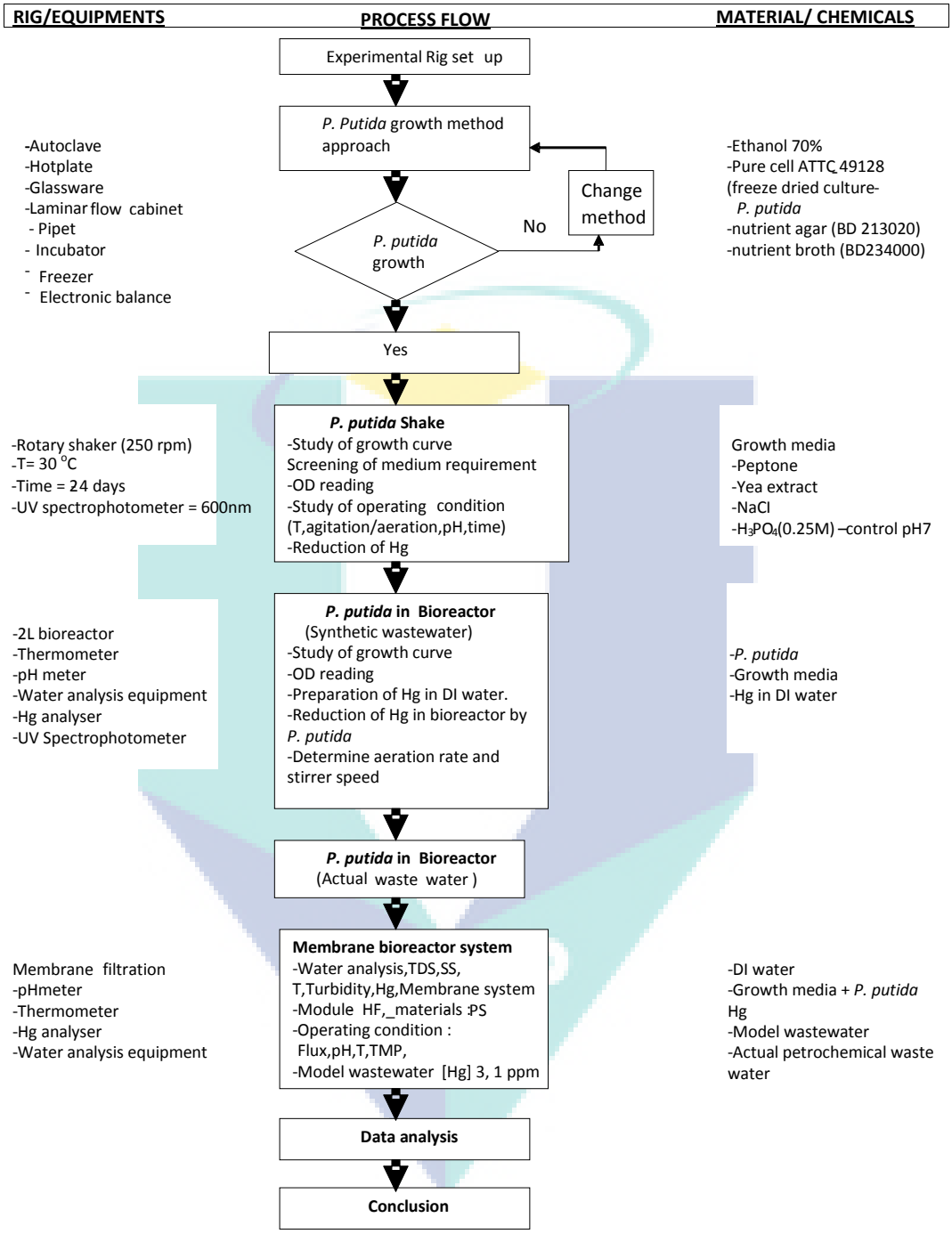


Figure 3.2: The overall processes of the research methodology

3.5 MERCURY CONTAMINATION LEVEL IN PETROLUEM BASED INDUSTRIES WASTEWATER

3.5.1 Introduction

The combustion of hydrocarbons is identified as a major anthropogenic of mercury emissions to the atmosphere and its distribution in the environment contributes to the mercury cycle. The contribution of mercury from petroleum based industries to global mercury cycle is difficult to estimate due to lack of data and information especially in petroleum based industries wastewater especially in Malaysia.

3.5.2 Regional Case Studies

This study summaries the case studies that have been carried out to determine contamination of mercury at two different petroleum based industries in the East Coast and southwest of Peninsular Malaysia. Wilhelm and Bloom, (2000) reported that, water discharge from plants in petroleum based industrial plants have been identified as sources of aquatic mercury contamination. The first industry (P1) was located at the East Coast has started its operation in 1984 and is the first production division that is involved in gas production. It is located approximately 110 km south of Kuala Terengganu in an industrialized area dedicated to oil and gas facilities. This industry has been designed to accommodate gas and condensate. It is built with a facility to receive slugs and separation of two phases of fluid received from the Platform Collector.

Meanwhile the second industry (P2) was located 90 km from Kuala Lumpur in the south west of Peninsular Malaysia is a refinery complex built on a 962 acres of land and the plant is still undergoing expansion. The main processes carried out at the plant are crude distillation unit, naphtha hydro-treating unit, catalytic, reforming unit with continuous catalytic regenerator, saturated gas concentration unit, sour water stripping unit, kerosene and heavy naphtha treating unit and mercury removal unit.

3.5.2.1 Case Study 1

In the first case study, the source of samples are collected from a wastewater treatment plant of a petroleum gas processing industry (P1) situated in the east coast of Peninsular Malaysia. Wastewater was sampled at two different locations of the wastewater treatment plant. The first sampling point (P1L1) is located at the exit point of the treatment plant while the second sampling point (P1L2) is located at the inlet point of the treatment plant. Sampling at P1L1 was conducted daily for period of 14 days (one sample per day), while the sampling at P1L2 was done consecutively for a period of 12 days.

3.5.2.2 Case Study 2

The second case study, the source of samples are collected from a wastewater treatment plant of a petroleum refinery (P2) situated in the south west of Peninsular Malaysia. Wastewater was sampled at six different locations within zone 1 of the treatment plant (P2L1) on a single day. This activity was repeated for a period of 5 days. At zone 2 (P2L2), 34 samples were collected at various points within the zone in a duration of 5 days.

3.5.3 Wastewater Sample Collection

For this investigation, samples from two petroleum based industrial wastewater plants were collected following the guidelines for sampling or handling of Standard Methods as presented in Appendix 3.4.

In general, the wastewater from petroleum based industries are characterized by a diversity of pollutants including hydrocarbons, polycyclic and aromatic hydrocarbons such as phenols, mercaptans, sulfides, oil, ammonia, metal derivatives such as mercury, cyanides and other chemicals (Eisler, 2006).

Wastewater samples were collected around the center of wastewater pond and only clean containers (bottles, beakers) were used for this purpose. The containers were first

rinsed several times first with the water to be sampled. Normally, the sample containers were slowly filled to avoid turbulence and air bubbles. However, it is difficult to obtain the true representative of the water when collecting surface water samples.

Generally, as little time as possible should elapse between collecting the sample and analyzing it. However, depending on the test, special precautions in handling the sample may be necessary to prevent natural interferences such as organic growth or loss and gain of dissolved gases.

3.5.4 Samples Storage and Preservation

Proper sample storage and preservation are required after the samples were taken from both sources of effluent. This is to ensure any undesired interferences present in contaminated are removed. These procedures involved acid-washing bottles where the glassware or plasticware are cleaned with laboratory detergent. After rinsing well with tap water, the glassware are rinsed with a 1:1 of hydrochloric acid (HCl) solution followed by thoroughly rinsed with de-ionized water and finally dried in the oven.

The least expensive containers that can be used for storing the samples are polypropylene or polyethylene and the best and most expensive containers were quartz or TFE (tetrafluoroethylene, Teflon) as stated in (Appendix 3.4). Sample preservation is prepared by adding acid to slow the chemical and biological changes that continue to happen after collection. Normally, the samples are analyzed as soon as possible after collection, especially when the analyzed concentration is expected to be low. This also reduces the probability of error and minimizes labor. Preservation methods include pH control (acid addition), refrigeration and freezing at below 5^{o}C are needed to protect the samples. In this study, samples were immediately refrigerated at a temperature below 5^{o}C.

Normally, based on the experience in the industries, contamination problems associated with the PVC/glass bottles sampler or the handling operations during sampling process in the fields were considered not likely to happen. Hand-held collection tests of

PVC sampler versus glass bottle collections during the sampling experiments indicate that any measurable differences are within the analytical precision of the method, which is 0.20 ppb (Gill and Fitzgerald, 1984).

Adsorptive losses of Hg in the collectors were not considered suspect for several reason:

(1) No detectable losses of Hg on the wall of the collectors (PVC/ glass bottles) are observed over short periods of time.

(2) If adsorptive losses were a serious concern in the collector, then the collections that remain in the sampler for longer periods of time should show lower Hg levels.

3.5.5 Mercury Contamination Study Flowchart

In this experiment, the main element of research is to identify the seriousness of mercury contamination by having surveys in several petroleum based industries. These research surveys would require full involvement and commitment by industries in order to achieve satisfactory findings. The experimental procedures are summarized in experimental flowchart shown in Figure 3.3.

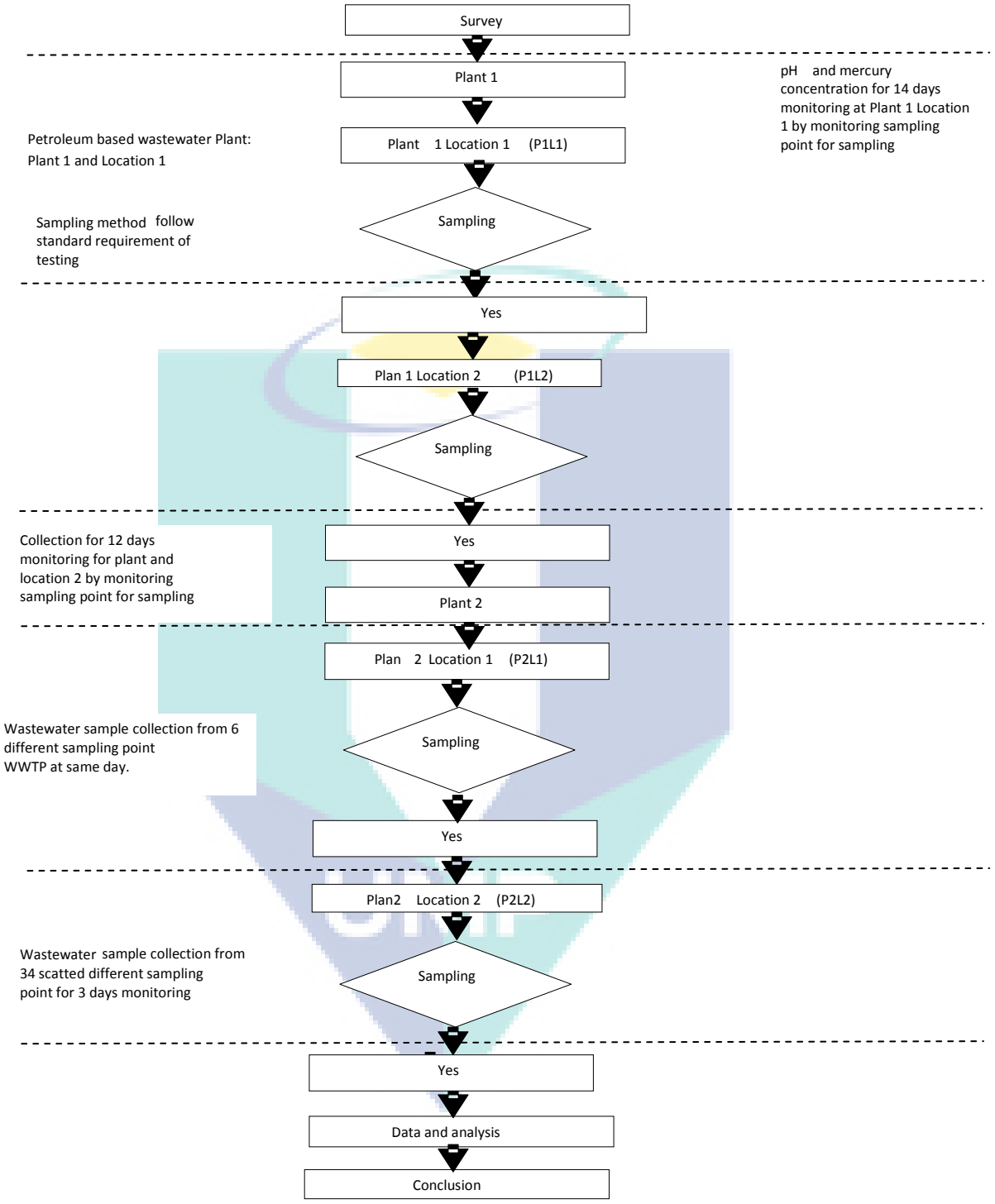


Figure 3.3: The experimental procedure for two mercury contamination case studies at petroleum based industrial wastewater treatment plants

3.6 *P. putida* IN SHAKE FLASK

It is essential to understand the growth characteristics of *P. putida* growth in shake flask before proceeding with the experiments in the bioreactor and membrane bioreactor using bigger scale and with actual wastewater from petroleum based industries. Therefore, a series of experiments were carried out in order to study the optimum operating conditions for the growth of *P. putida*.

3.6.1 Experimental Set-up Flowchart

The experiments were conducted in order to investigate the performance of *P. putida* growth in shake flask. The outcomes from the experiments were then utilized as a guide when the experiments are conducted in bioreactor and membrane bioreactor in determining the optimum operating parameters for the growth of *P. putida*. The flowchart of experimental set-up for investigating the growth of *P. putida* in shake flask is illustrated Figure 3.4.

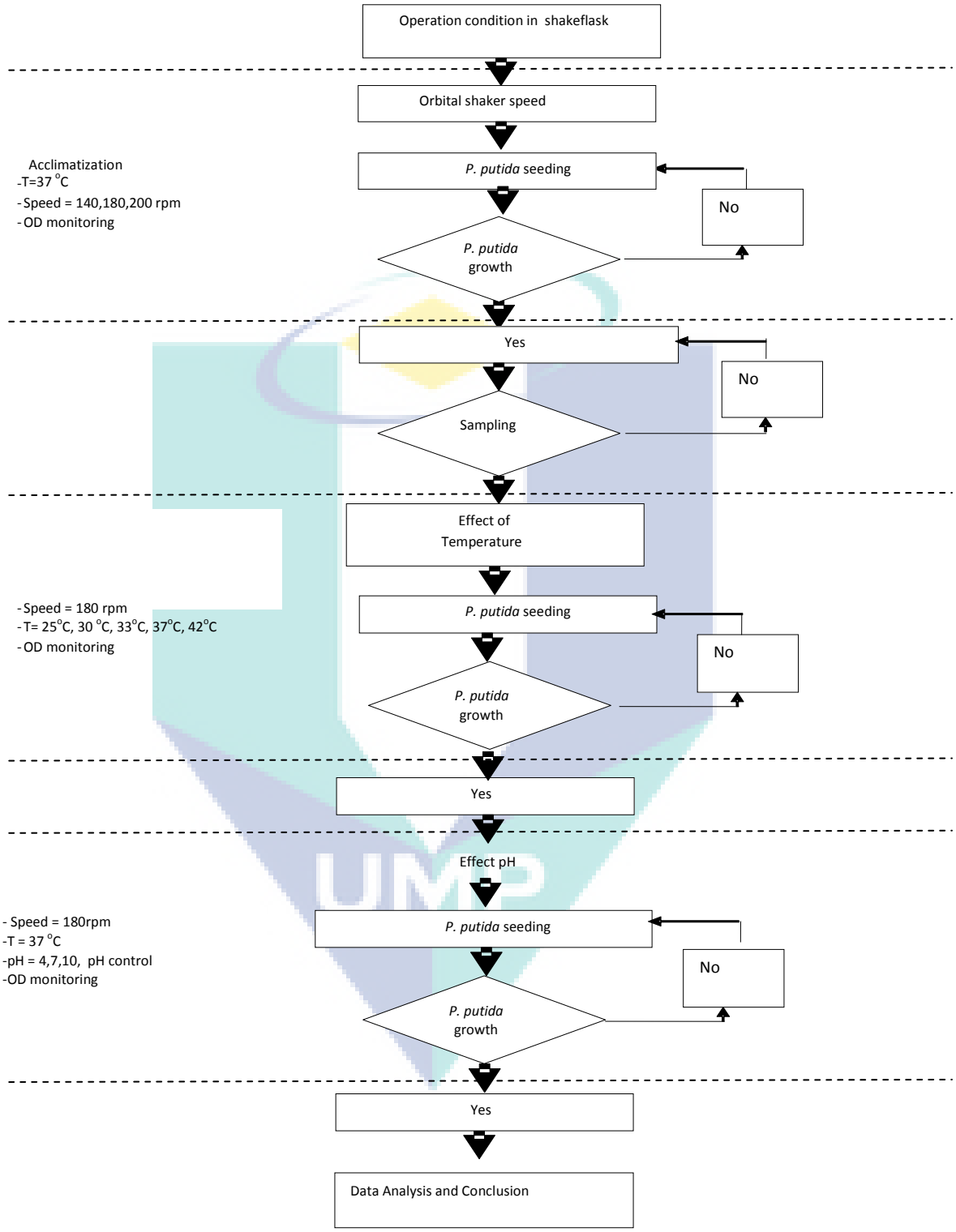


Figure 3.4: The flowchart of the experimental set-up for *P. putida* growth in shake flask

3.6.2 Sterilization of Glassware

All glassware were thoroughly washed with liquid detergent using clean brush, rinsed with running tap water, followed by dipping in 1% HCl to neutralize any remaining detergent before rinsing once again with distilled water and then dried in an oven for 15 minutes. Before sterilization, the test tube and Erlenmeyer flasks were covered either with a cap, a cotton plug or an aluminum foil. Before sterilization, all Petri disk and pipettes were placed inside a metal can or metal pipette box. All glassware was autoclaved at a temperature of 121^{o}C and pressure at 15 psi for 25 minutes. After the sterilization cycle is completed sterilization cycle is over, the autoclave was allowed to cool for 15 minutes before opening it. All glassware was stored in another sterile container (oven) until required for use. The medium that has been fermented in shake flasks were also sterilized with the standard procedure of autoclave sterilization (Sheikh, 2006). Appendix 3.5 shows the autoclave used for sterilization.

3.6.3 Preparation of Bacteria Inoculum

The preparation of inoculum is done by picking a loop-full of *P. putida* colony, ATCC 49128, from a freshly grown culture which has been cultivated on nutrient agar (NA). The culture is then transferred into 10 ml of nutrient broth (NB) which is 10% of the medium volume or with the ratio of 1:9, and is then incubated at 30^{o}C for 24 hours as proposed by the manufacturer (Merck (Malaysia) Sdn. Bhd.). The quantity of inoculum normally used is between 3% – 10% of the medium volume (Stanbury, et al. 1984), but 10% of volume is used in this study. After 24 hours, the colony is transferred to a 25 ml inoculum flask containing 90 ml nutrient broth. Cells are grown at 37^{o}C while being vigorously shaken at 180 rpm. Samples of 10.00 ml are drawn at regular intervals of 30 minutes using an aseptically syringes. The samples are then analyzed by UV spectrophotometer at optical density (OD) of 600nm to monitor the growth of *P. putida* (Shuler. and Kargi, 2002).

3.6.4 Operating Conditions for *P. putida* Growth

From the experiments conducted using set-up above, a summary of the operating conditions and parameter values that has been identified for the growth of *P. putida* in shake flask and bioreactor are shown in Table 3.1 and Table 3.2.

Table 3.1: Effect of operating condition for *P. putida* growth studied in shake flask

Experiment	Operating Conditions Study	Parameter Value
1	Acclimatization Time (hr)	24 48 72
2	Orbital Shaker Speed (rpm)	140 180 200
3	Temperature (^{o}C)	25 30 33 37 42
4	Substrate Concentration (g/L)	4 8 12 16
5	pH	4.0 7.0 10.0 6.7 (control)
6	Mercury at low concentration (ppb)	1 6 18

Table 3.2: Effect of operating conditions for *P. putida* growth studied in bioreactor

Experiment	Operating Conditions Study	Parameter Value
1	Propeller Speed (rpm)	100 180 200
2	Aeration Rate (vvm)	0.25 0.50 0.75
3	Mercury Concentration (ppb)	1000 3000

3.6.5 Determination of *P. putida* Growth

UV spectrophotometer was used to assess the growth of *P. putida* and the method is based on the absorption of light by suspended cells in media of the sample culture. The intensity of the transmitted light is measured using a spectrophotometer. Turbidity or optical density (OD) measurement of the culture medium provides a fast, inexpensive, and simple method of estimating cell density in the absence of other solids or light-absorbing compound. Light transmission in a sample chamber is a function of cell density and the thickness of the chamber as it is modulated and scattered by broth absorption. Proper procedure entails using a wavelength that minimize absorption by medium components (600 nm wavelength are often used), "blanking" against medium, and the use of a calibration curve (Shuler. and Kargi, 2002, Brock et al., 2006).

5 ml of liquid samples are taken every hour to be analyzed until decay phase is observed at all concentrations, and the shake flasks are detached from the orbital shaker

during the sampling. Using a UV Spectrophotometer (UV-VIS, U-1800, Hitachi), the growth of *P. putida* is monitored at 600 nm absorbance. Appendix 3.6 shows the samples of *P. putida* with nutrient and mercury ready to be analyzed and Appendix 3.7 shows the UV Spectrophotometer (UV-VIS, U-1800, Hitachi) for monitoring the growth of *P. putida*.

3.6.6 Determination of Cell Dry Weight

Determination of cellular dry weight is the most commonly used direct method for determining cell mass concentration and is applicable only for cell grown in-solid free medium. The mixture is incubated at 100^{o}C for 20 minutes and centrifuged at 5000 rpm for 3 minutes. The supernatant is then decanted and the cells filtered onto pre-weighted 0.20 μm cellulose nitrate filter (Whatman), washed twice with distilled water and dried in an oven at 95^{o}C for 24 hours. Following this, the dry cell weight is measured. Fresh medium that has been similarly treated is used as blank for reduction impurities in the sample (Shuler and Kargi, 2002 aand Ee, 2004).

After the nutrient is depleted, the biomass is measured by taking a 1 ml sample and centrifuged at 5000 rpm for 5 minutes in a microcentrifuge. The cell pellet is washed twice with saline solution and is first dried in vacuum drier and followed by drying in an oven at 80^{o}C until its weight become constant.

The flowchart of the process used to determine the cell dry weight is shown in Figure 3.5 while Appendix 3.8 shows the refrigerated centrifuge (5810 R) and Appendix 3.9 shows the samples in the oven for cell dry weight test.

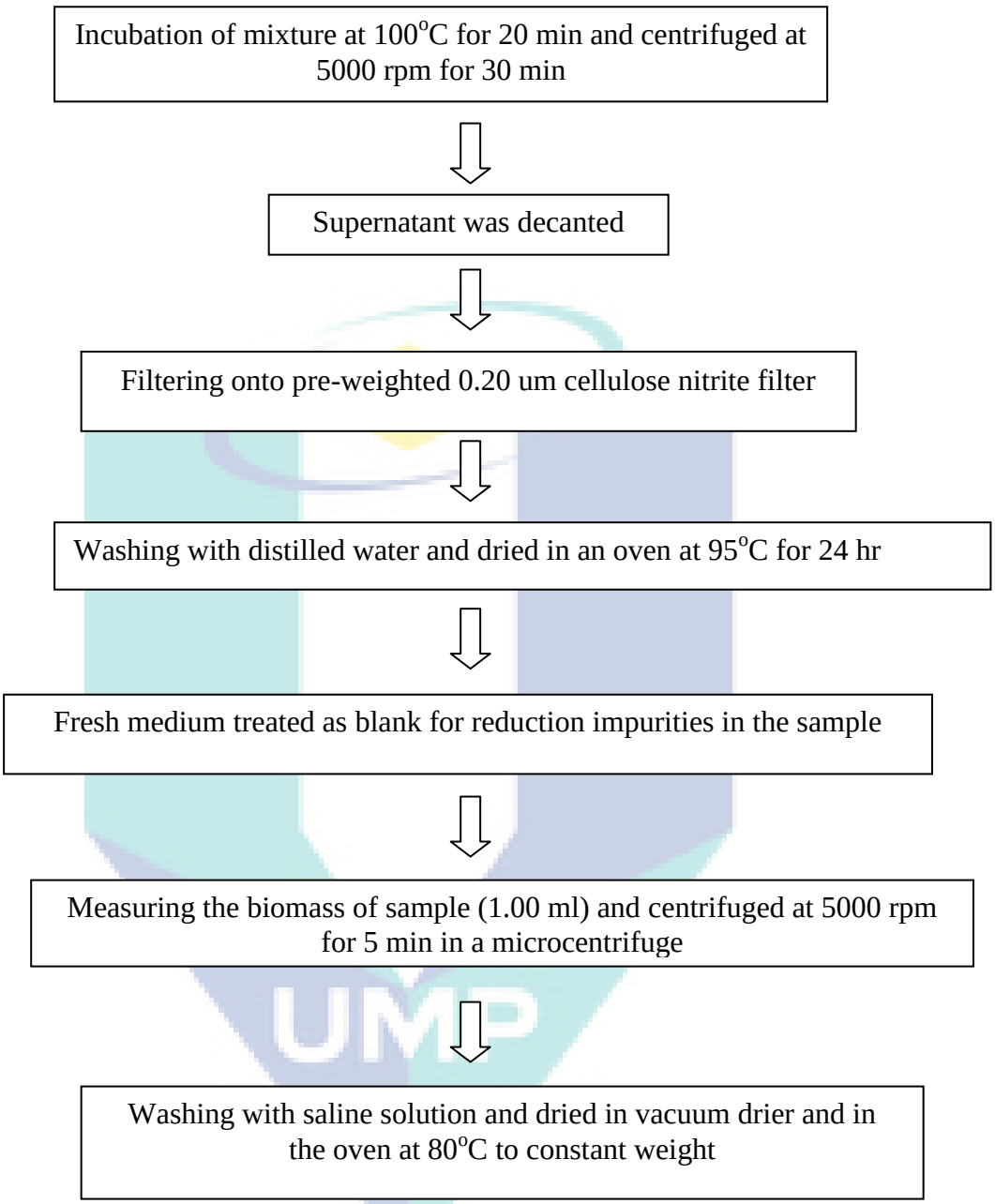


Figure 3.5: Flowchart of the determination of cell dry weight

3.6.7 Mercury Determination

A mercury analyzer system, RA-3000 (NIC, Japan), is an equipment that used to measure the mercury content in liquid samples taken from wastewater or in solid samples taken from soil by using the reducing vaporization with cold vapor atomic absorption spectrometry. Mercury compounds in the sample were first pretreated with strong acid and an oxidizing agent to change the compound into divalent mercury ions (Hg^{2+}). However, several safety precautions must be taken before the sample is measured in mercury analyzer. The maximum concentration of mercury that can be measured by the mercury analyzer is 15 ppb; hence before analyzing, the sample must first be diluted if the mercury content in the sample is high concentration.

A solution containing hydrogen sulphate (97%) and ultrapure water with 1:1 ratio is prepared. Then 40 ml of hydrogen sulphate (97%) is measured and mixed with 40 ml of ultrapure water using a measurement cylinder. The solution is then poured into a clean glass bottle. A mixture of stanum chloride and hydrogen sulphate is also prepared, where 2 g of stanum chloride is weighed in a beaker on digital balance. Then, 19 ml ultrapure water and 1ml of hydrogen sulphate (97%) are poured into a beaker. The mixture is then stirred with a glass rod until the solid stanum chloride completely dissolved.10 ml of the sample is poured into a test tube and using micropipette, 250 microlitre of stanum chloride mixture and hydrogen sulphate (1:1) is measured. Both the measured solution are added into the sample.

The test tube is then plugged into the socket of Mercury Analyzer test tube. The Mercury Analyzer software within the computer is run for 3 minutes before results are produced. Appendix 3.10 shows the mercury analyzer used in the experiment that is carried out in this section.

3.7 *P. putida* IN MEMBRANE BIOREACTOR

Before using Membrane bioreactor (MBR), the bioreactor must be pre-heated to the required temperature while allowing air to flow into the bioreactor. The pH of medium is adjusted to the desired value using sulfuric acid (H_2SO_4) and natrium hydroxide (NaOH). At the same time, the stirrer is kept running at constant speed. There are a series of steps need to be carried out before experiments can be performed in bioreactor. Sterilization of the bioreactor is the most important element must be done before *P. putida* can be grown in the bioreactor.

3.7.1 Sterilization of the Bioreactor

Contamination of the culture medium by other unwanted microbes from the raw medium or the environment would have an adverse effect on the fermentation process. Such contamination is usually due to insufficient sterilization of the vessel or could partly be caused by faulty seals, and incorrect handling of parts of the vessel.

Bioreactor is sterilized *in-situ* as follows:

i. Preparation of sterilization accessories.

If the pH-electrode had not been previously calibrated, it should be done before filling the culture medium into the vessel. Bioreactor is filled with 1.80 L of the growth media and all the probe cables are disconnected before sterilization. The pH probe is calibrated before sterilization using standard solutions at pH of 4, 7 and 10.

Two turbine impellers mounted on an agitator shaft is used for agitation. The bioreactor was equipped with temperature and pH control system. A sterilisable pH electrode is used to measure pH of the culture. The temperature of the culture is maintained using the built-in temperature control system by controlling the inflow and outflow of

cooling water through the water jacket available on the outer layer of the vessel. The oxygen probe must be calibrated after the bioreactor is sterilized.

All the reactor tubes were sealed with glass wool and the whole reactor including the glass vessel, head plate and its components was autoclaved at a temperature of 121^{o}C at pressure 15 psi for 25 minutes.

ii. Vessel filling and *in-situ* sterilization of media

Since the culture medium can be sterilized *in situ*, the 2 L medium was prepared as required and filled into the culture vessel through one of the top-plate ports. Note that some of the liquid would evaporate during the sterilization and exhaust via the inlet air and exhaust line. The exact loss of liquid due to evaporation must be evaluated empirically. The volume of the seed culture would compensate for some of the losses. Surplus water could be added before sterilization or some separately sterilized culture medium or water added afterwards. The culture vessel should not be sterilized when empty (Sheikh, 2006 and Ishenny, 2006). Appendix 3.11 shows the 2.00 L bioreactor used to grow the pure culture of *P. putida*.

3.7.2 Membrane Bioreactor Process

Membrane bioreactor is the combination of the suspended growth reactor and membrane filtration device into single unit process. The function of biomass separation membrane bioreactor is for biodegradation of organics combined in wastewater, and separation and retention of solids (Stepheson et al., 2000). The entire biomass is confined within the systems, providing both perfect control of residence time for the microorganisms in the reactors (sludge age) and the disinfection of the effluent (Judd, 2006). Meanwhile, membranes are often used as a replacement for sedimentation (clarifier), which is biomass separation within the bioreactor when coupled to biological process (Gao et al., 2009).

3.7.2.1 QuixStand Benchtop Membrane System

The membrane is at the heart of every membrane process and can be considered as a permselective barrier between two phases (Stepheson et al., 2000). In this study, Xampler hollow fiber microfiltration cartridge supplied by Amersham Bioscience was used in cross flow membrane system, Quixstand Benchtop. This system was used for the bioreactor to retain cells for the removal of mercury, and to remove turbidity and suspended solid. The specifications of the Xampler hollow fiber membrane (CFP-2-E-3MA) are as follows:

- Membrane configuration : Hollow Fiber (asymmetric porous)
- Membrane process : Microfiltration (Cross flow)
- Material : Polysulfone membrane
- Membrane area : 110cm^2 (0.01m^2)
- Separation principle : Sieving

The hollow fiber membrane with, 0.20 micron pore size,1.00 mm lumen diameter, is in the form of a self-supporting tube with a highly dense “skin” layer on the inside of the tube and sponge-like (Shamel, 1998). It is a highly open substructure with large voids on the shell side. The hollow fiber cartridge contains fibers running the length of the cartridge with a pair of shell-side ports and lumen or tube-side ports. Warm water is used for membrane rinsing at room temperature as the rinsing is less effective when cold water is used. When using new membrane cartridge, an addition of 100 000 ppb Sodium Hypochlorite (NaOCl) is added to the water for flushing purpose. Both retentate and permeate are discharge as schedule waste.

Membrane rinsing is continued for 90 minutes to ensure NaOCl has been thoroughly rinsed before introducing the process solution. Membrane cartridge has to be rinsed for at least 5 minutes at 5 psi inlet pressure before use. Appendix 3.12 shows QuixStand Benchtop Hollow Fiber Membrane System.

3.7.2.2 Flux Study with De-ionized (DI) Water

De-ionized water stored in the microfiltration reservoir was pumped into cartridge hollow fiber membrane with the valve fully open at the feed inlet while varying the TMPs. The temperature during the experiment is kept at ambient temperature (27^{o}C +/- 2^{o}C) the flow rate of water is measured using a stopwatch and graduated cylinder from permeate port at particular transmembrane pressures (TMP) generated from the pump.

$$\mathrm{TMP} = \frac{(P_{in} + P_{out})}{2} - P_0 \tag{3.1}$$

The performance or efficiency of a given membrane is determined by two parameters: its selectivity and the flow through the membrane which often denoted as the flux or permeation rate. Flux is defined as the volume of liquid flowing through the membrane per unit area and time (Mulder, 1991). SI unit used in this system is l/m^2/hr or LMH.

$$\text{Flux (LMH)} = \frac{\text{Flowrate (l/hr) x 0.06}}{\text{Membrane area (m2)}} \tag{3.2}$$

$$= \frac{\text{Flowrate x 0.06}}{0.01} \tag{3.3}$$

3.7.2.3 Membrane Bioreactor Operation

Membrane bioreactor (MBR) is combination of biological and physical processes to treat wastewater. In this case, the membrane unit is configured as external system independent of the bioreactor. There is recirculation loop as the separation occurs within the bioreactor itself while the microorganisms have the same residence time in the system.

MBR is the system which is reliable, easier, adaptable and flexible for wastewater application (Stephenson et al. and 2000, Bitton, 2005).

Figure 3.6 and Appendix 3.13 show the experimental setup of the membrane bioreactor system. The system consists of a bioreactor, reservoir, pump and hollow fiber cartridge (microfiltration), and the various parts are connected using tubes. All tubing and glassware used in this study are washed by a detergent solution, then immersed in 10% nitric acid for at least 24 hours, and finally are washed three times with distilled water. The bioreactor is equipped with temperature and pH control system. The growth of *P. putida* is measured using a sterilisable pH electrode. The built-in temperature control system used will maintain the temperature of the culture by controlling the inflow and outflow of cooling water through the water jacket available on the outer layer of the reservoir.

The 2L bioreactor was used for growing *P. putida* and removing of mercury process from wastewater is maintained at 37^{o}C. The reservoir is used to store the samples before introducing to the membrane system, the hollow fiber cartridge. *P. putida* was grown in an incubator at 30^{o}C before transferring into the reservoir of the bioreactor. The quantity of cell culture normally used is in the range of 3% – 10% (v/v) of medium volume (Standbury et al., 1984 and Ee, 2004). In this case, 0.2L of cell culture is added to the total system.

For membrane operation, firstly the cell sampling or drain valve is closed and ensured the cartridges of hollow fiber in the upper and lower manifolds are ensured to be secured. Then the tubes of the pump should be correctly positioned and tensioned within the pump head. Flexible tubing is connected from the retentate outlet on the upper manifold to one of the tubing barbs on the reservoir caps and another flexible tubing is directed from the upper permeate line to a collection reservoir to receive the product.

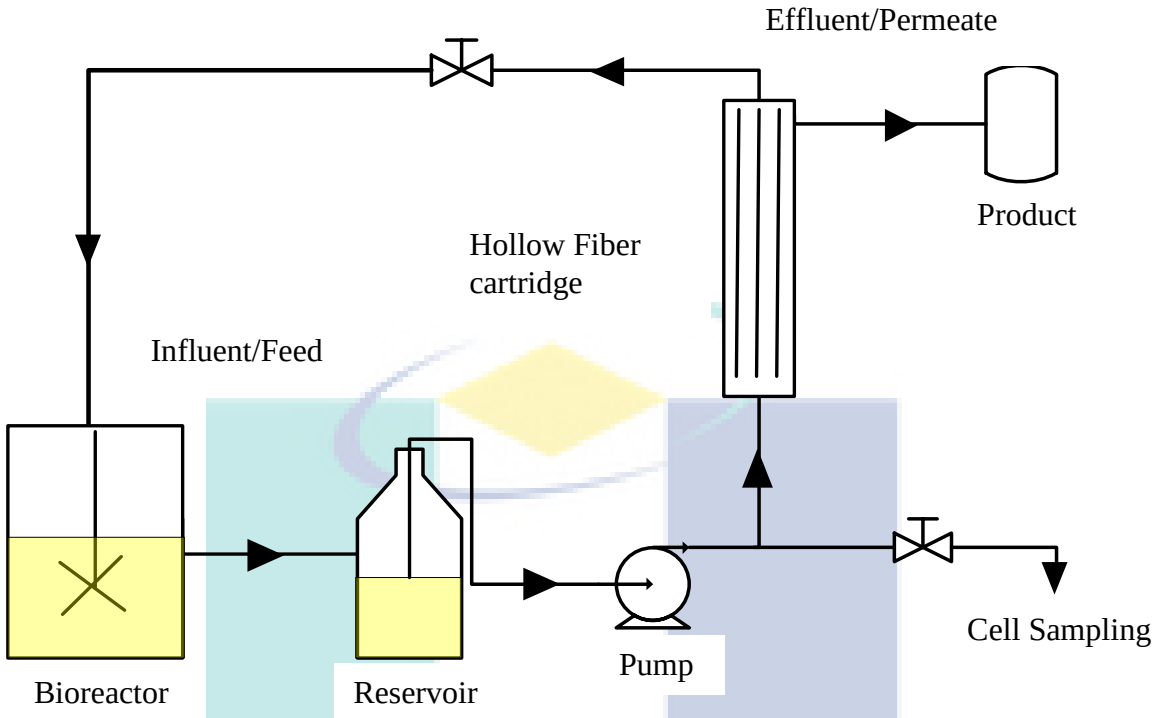


Figure 3.6: Diagram of Membrane Bioreactor (MBR)

The reservoir cap on the silicone gasket is repositioned and sanitary clamps were put in place. The backpressure tubing valve is opened for several times and the pump is operated at slow speed; 0.5 minutes is needed for the pressure to build up as the mechanically dampened pressure gauges responded slowly. As a result the speed of the pump increased slowly, the inlet pressure might build up and the data can be collected (Chung et al., 2005).

Meanwhile, the bioreactor was set up in "back-flush" configuration, where the feed stream flows from the outside of the hollow fibers and permeate is collected from the inside of the hollow fibers (Chen et, al. 1998 and Wagner-Dobler, 2003). This bioreactor is operated in a batch fluid recycle mode, where the retentate is circulated to the bioreactor for continuous operation and permeate as product is collected in another container. The circulation was achieved with the use of a pump and the flow rate of permeate is controlled by varying the pressure of the feed stream.

To reuse the hollow fiber cartridge, its membrane is cleaned with 20 mg/L NaOCl, thoroughly rinsed with de-ionized water, and stored accordingly (Chen et al. and 1998, Reardonet al., 2000).

3.8 PETROLUEM BASED INDUSTRIES WASTEWATER ANALYSIS

3.8.1 Temperature

The temperature of the sample was measured using dwi-function pH meter model Mettler Toledo Delta 320 with accuracy +/- 1^{o}C. The measurement was carried immediately after sample is collected. The electrode of the meter is immersed 10.00 cm below the surface of the sample for 2 - 5 minutes or until a stable reading is obtained.

3.8.2 Turbidity

Turbidity of the sample was determined based on the intensity of light scattered by particulate matters in the sample using turbidity meter model WTW Turb 350 IR. The unit of turbidity is Nephelometric Turbidity Unit (NTU) and turbidity ranges from 0 to 1111 NTU with the accuracy of +/- 0.01 NTU. 10 ml of sample is filled in the sample tubes, clear colorless glass that has been thoroughly cleaned of both the inside and outside before being used for testing. The sample tube is then placed in the equipment and the read button was pressed. The reading is recorded when a static value appeared on the screen.

A set of standard solution for calibration was also available ranging from 0.01, 10, 100 and 1000 NTU is also available and the calibration of the equipment is carried out once a week. The sample's turbidity value is measured in triplicate to obtain an average value.

3.8.3 pH

The pH meter model Mettler Toledo Delta 320 manufactured in the United Kingdom with a resolution of 0.01 is used to measure the pH of the sample. The measurement is carried out immediately after the sample is collection. The sample is then stirred to ensure homogeneity while the electrode of the pH meter is immersed 5 cm below the surface of the sample for 2 – 5 minutes or until a stable reading is obtained. Calibration of the pH meter is carried out once a week using buffer solution at pH 4, 7 and 10.

3.8.4 Total Suspended Solid (TSS)

Figure 3.7 shows the flowchart of the method of total suspended solid (TSS) analysis method. First, the filter disk is dried in an oven between 103°C to 105°C for 1 hour, cooled in a desiccator and weighed. The filtering apparatus and filter are then assembled and suction is performed. The filters are sealed by wetting with a small volume of distilled water to seal it. 10 ml of water sample is pipetted (mixed well to ensure homogeneity) onto the centre of filter disk in a flask, and the sample gently (under vacuum). Then the filters are washed three times successively with 10 ml of distilled water, allowing complete drainage between washings. Suction is continued for approximately 3 minutes after filtration the completion of the filtration process.

After that, the filters are carefully removed from the filtration apparatus and transferred to an aluminum weighing dish as a support. At least 1 hour drying time is needed between 103°C to 105°C in the oven. Next, the filter was cooled in a desiccator to balance the temperature and the sample is weighed once more. The drying, cooling, desiccating, and weighing are repeated until a constant weight is obtained. The tests for each sample are repeated at least once to check reliability of the data.

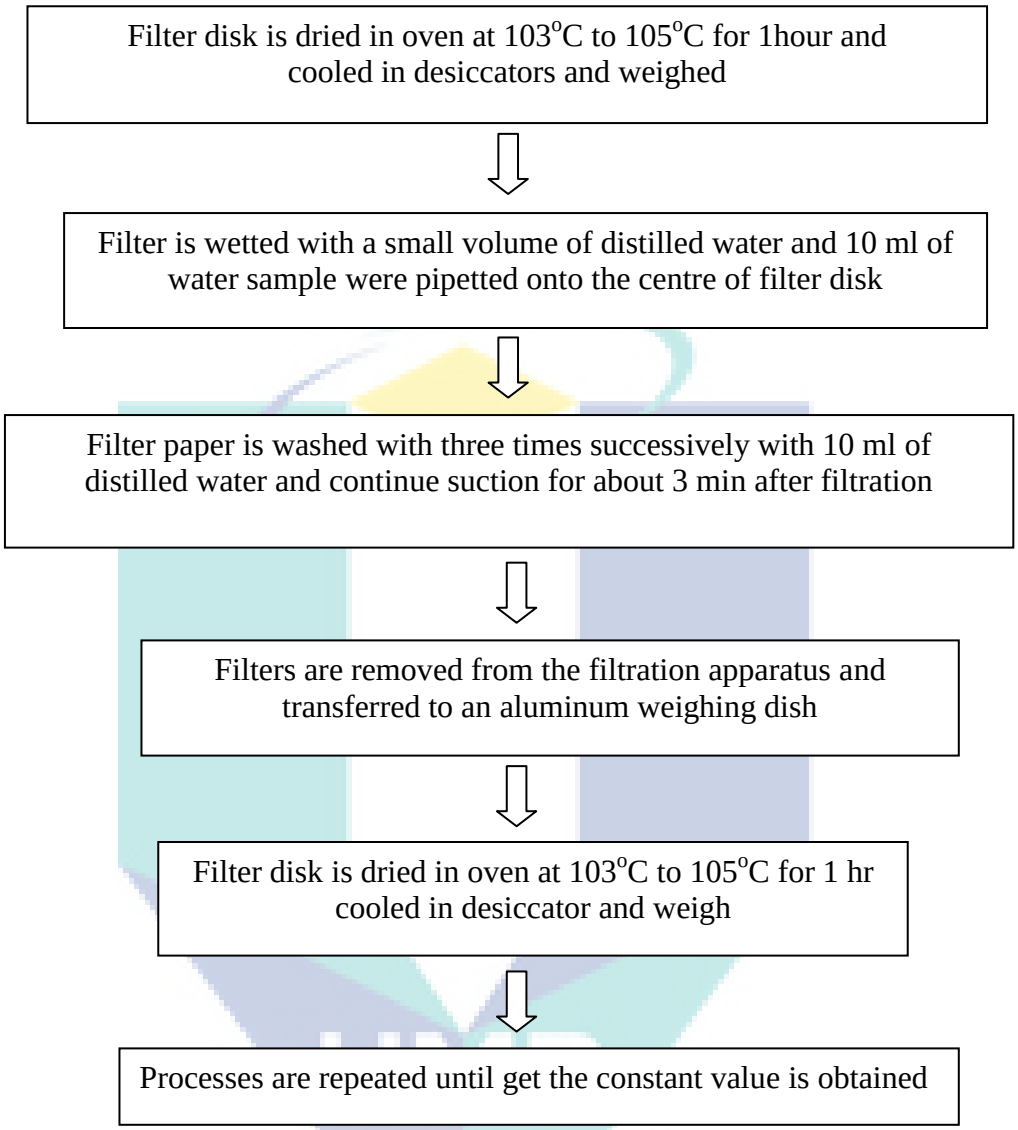


Figure 3.7: Flowchart of total suspended solid (TSS) analysis method.

3.8.5 Total Dissolved Solid (TDS)

Total dissolved solid is a measure of the combined content of all inorganic substances in a liquid in molecular, ionized or micro-granular (colloidal sol) suspended form. Generally the operational definition is that the solids must be small enough to survive filtration through a sieve the size of 2 micrometer. In this experiment, total dissolved solids are measured by conductivity method by using a TDS meter.

Electrical conductivity of water is directly related to the concentration of dissolved ionized solids in the water. Ions from the dissolved solids in water create the ability for that water to conduct an electrical current, which can be measured using a conventional conductivity meter or TDS meter.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 CASE STUDY OF MERCURY CONTAMINATION IN PETROLEUM BASED INDUSTRIAL WASTEWATER PLANTS

This section discusses on the finding of the case studies determined on mercury contamination in wastewater from two types of petroleum based industrial plants in Peninsular Malaysia. The main wastewater streams that derive from petroleum production and processing are produced water from both oil and gas production, and refinery wastewater. However, very small amounts of water are derived from gas processing and these are mainly water from separators at gas plants and condensed water from dehydration (U.S. EPA Report, 2001). To gauge the typical level of Hg contamination, wastewater samples are collected from two petroleum based industrial plants, Plant P1 and Plant P2 as elaborated in Chapter 3 on the case studies.

Appendix 41 shows the Third Schedule of Environmental Quality Act 1974, as a guide for data analysis on mercury concentration limitations, in which the Act are states that mercury concentration from Standard A is limited to 5 ppb while that of Standard B is 50 ppb. Standard A is applies to the industrial and development projects which are located within catchment areas or sampling point. However, Standard B is applied as the reference for data analysis in this study (EQA, 2011).

The case studies were conducted to identify and evaluate the baseline of mercury, Hg contamination in relationship with two different petroleum based industrial plants in the

East Coast and south-west of Peninsular Malaysia. These industries produce comprehensive range of petroleum products, most of which are consumed within Malaysia. The major activities of the companies are related to refining and manufacturing of petroleum products from crude oil that involves two major processes, which are separation process and conversion process. There are many processes available to the refiner and the final process chosen is determined by the required products (both quality and quantity) and the crude oil that is available (Kannan and Krishnamoorthy, 2005 and Pirrone and Mahaffey, 2006).

Petroleum products are made from crude oil and there are many types of crude oil which come from many different sources around the world. Selection of a suitable crude oil is a key part of the process that depends on many factors including quality, availability, volume and price (Al-Muzaini, 1997 and Shokrollahzadeh et al., 2008). The main processes are crude distillation unit, naphtha hydro-treating unit, catalytic, reforming unit with continuous catalytic regenerator, saturated gas concentration unit, sour water stripping unit, kerosene and heavy naphtha treating unit and mercury removal unit. The main products that are produced include liquefied petroleum gas (LPG), gasoline or petrol, jet fuel and gasoil or diesel (Pirrone and Mahaffey, 2006).

The mercury species that are present in crude oil and gas condensates are seldom accounted for in routine analytical methodology. Although only limited amounts of data are currently available, it appears that the distribution of mercury compounds in petroleum samples varies widely. The relative distribution of mercury compounds in wastewater is dependent on the sample source and history, and includes classes of compounds that have specific negative effects on people, equipment and catalysts (Campanella et al. 1986 and Alberti et al., 2006).

Mercury is universally found in petroleum processing system such as gas treatment system, heat exchangers, separators and wastewater. In addition, sour water that streams from the plants tend to attract mercury because of reactive mechanisms with the sulfides. Wastewater in plants that process feeds or crude oil containing mercury accumulate ionic mercury and other dissolved forms, and the concentration of the contaminants are

proportional to the its concentration in the feed (Wilhelm and Bloom, 2000). Even when the mercury concentration is relatively low, water streams that are by-products of equipment cleaning and water condensesation can contain high concentration of mercury.

In both petroleum based industrial wastewater treatment plants under study, analysis of mercury contamination is normally conducted during maintenance and repairs by contractor assigned by the company. Samples were collected from wastewater treatment plants for further analysis further and the summary of the findings are then used as a reference and guidance for subsequent studies which were carried in the laboratory as presented in appendix 4.2.

Table 4.1 shows the mercury concentration and pH value at plant P1 at location P1L1, which is located near the exit point of the wastewater treatment plant, in a period of 14 days. On the other hand, Table 4.2 shows the mercury concentration and pH value at location P1L2, which is located near the entry point of the wastewater treatment plant, in a period of 12 days. In general, the concentration fluctuated throughout the period of monitoring at both locations. For P1L1, the lowest reading recorded is 26.00 ppb, while its highest value recorded is 156.00 ppb and the average value for the 14 days monitoring period was 72.20 ppb.

Meanwhile, for P1L2, the lowest value of Hg concentration recorded is 16.00 ppb, while its highest value recorded is 386.00 ppb. Its average value for the 12 days monitoring period was 97.80 ppb. It is also observed that the pH values for both locations, P1L1 and P1L2, also fluctuated throughout the monitoring period, with values ranges from 4.14 to 6.56. From Table 4.1 and Table 4.2, it can be concluded that there is no specific relationship between the pH values and the concentrations of mercury in the wastewater observed during the monitoring period. Hence, it indicates that pH of the wastewater has no influence on the mercury concentration of the wastewater.

From the Table 4.1 and Table 4.2, as whole it can be seen that the Hg concentration at P1L2 is higher than the concentration at P1L1, which is at the exit point of the

wastewater treatment plant. Hence, it suggests the presence of natural microbes which partially consume the mercury in the wastewater and reducing it to a value approximately 26% from its original value. Nevertheless, the average concentration at the exit point at 72.20 ppb is still above the permitted level of Standard B imposed by the Department of Environmental (DOE), Malaysia. It is also important to note that the fluctuation of the Hg concentration in the inlet stream is due to the variety of wastewater sources coming into the treatment plant.

Table 4.1: Sampling at Plant 1 Location 1 (P1L1)

Day	pH	Hg Concentration ppb
1	4.50±0.27	26.00 ± 2.00
2	4.70±0.33	79.00±4.00
3	4.81±0.63	102.00±1.00
4	4.71±0.23	142.00±1.00
5	4.73±0.61	156.00±1.00
6	4.54±0.49	45.00±3.00
7	4.41±0.24	61.00±1.00
8	4.41±0.48	40.00±2.00
9	4.40±0.45	27.00±3.00
10	4.94±0.24	33.00±2.00
11	6.31±0.33	33.00±1.00
12	6.42±0.41	97.00±2.00
13	6.27±0.31	57.00±3.00
14	5.87±0.32	113.00±1.00

Table 4.2: Sampling at Plant 1 Location 2 (P1L2)

Day	pH	Hg Concentration Ppb
1	4.52±0.29	31.00±1.00
2	5.54±0.39	46.00±3.00
3	4.80±0.50	386.00±1.00
4	4.76±0.24	225.00±1.00
5	4.85±0.07	31.00±2.00
6	4.63±0.50	64.00±2.00
7	4.14±0.32	22.00±3.00
8	5.50±0.48	134.00±2.00
9	4.84±0.36	49.00±3.00
10	6.37±0.70	16.00±2.00
11	6.56±0.39	96.00±1.00
12	6.18±0.10	74.00±2.00

On the other hand, Figure 4.1 and Appendix 4.3 present the readings from the monitoring of wastewater from Plant P2 at six points within zone 1 of location P2L1, while Figure 4.2 and appendix 4.4 present the readings of mercury concentration at 34 points within zone 2 of location P2L2. Readings are collected at all six points of P2L1 in one day, while monitoring of mercury concentration at location P2L2 is conducted for a period of 5 days. Similar to the findings discussed above for plant P1, readings of Hg concentration at Plant 2 fluctuated throughout the monitoring period, with readings dropping to as low as 5.63 ppb (P2L2) and at another instant, the reading jumped to as high as 2318.00 ppb (P2L2). The average concentration at P2L1 was 269.00 ppb, while average concentration at P2L2 is recorded at 1056.00 ppb. Obviously, these average readings are far above the 50.00 ppb limit of Hg concentration allowed by the DOE. Close observation of the concentration data presented in the tables led to a conclusion that there are some sources of the wastewater generated in the premise of the plant contained very high concentration of mercury.

Based on the two case studies conducted at the two different plants, it can be concluded that the problem of mercury contamination in petroleum based industry is real and does exist in Malaysia. The level of contamination is very much dependent on the nature of its processing and the type of raw materials the plant used in its process. Plant P1, i.e. a gas processing plant that uses raw materials originated from sweet crude, showed relatively lower level of Hg contamination. On the other hand, plant P2, a petroleum refinery plant that uses raw materials originated from sour crude, showed relatively higher level of Hg contamination.

As a result, a decision was made to conduct the experimental study of *P. putida* treatment using two different ranges of Hg concentration, low range (1.00 to 19.00 ppb) and high range (1300.00 ppb to 4000.00 ppb). The two extreme conditions were chosen to examine the performance of *P. putida* in treating Hg contaminated wastewater under those conditions.

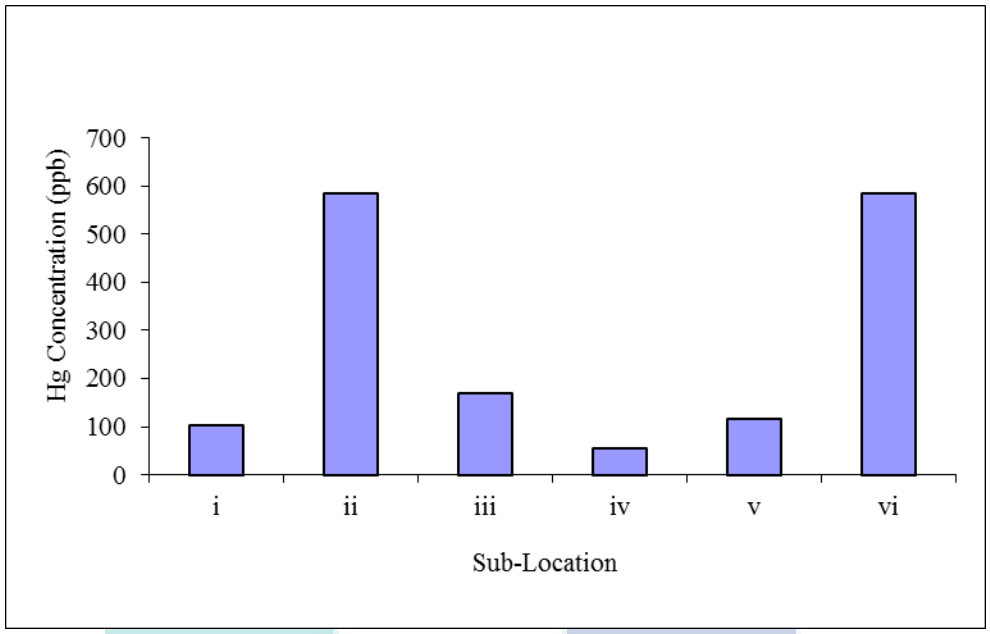


Figure 4.1: Survey of mercury contamination in wastewater from petroleum based industrial Plant 2 Location 1 (P2L1)

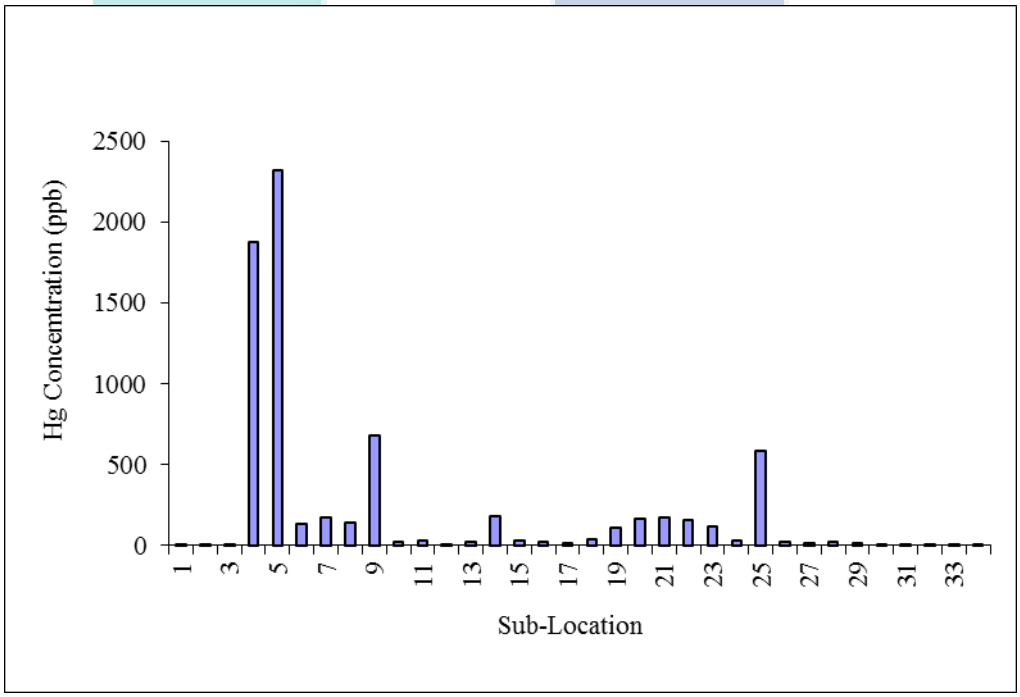


Figure 4.2: Case study on mercury contamination in wastewater from petroleum based industrial plant 2 (P2L2)

4.2 EFFECT OF INOCULUM ON GROWTH OF *P. putida* ATCC 49128

This part of the research is carried out first by observing the growth curve of *P. putida* ATCC 49128 in the Biotechnology Laboratory, Universiti Malaysia Pahang. In this study, the term of *P. putida* refers to *P. putida* ATCC 49128. As mentioned in Chapter 1, the growth curve displays the measurement of microbial cells growth, which is highly influenced by the seeding method employed.

4.2.1 Seeding of *P. putida*

The purpose of this experiment is to investigate the ability and the growth of *P. putida* profile in the laboratory. The experiment was carried out in an incubator shaker at a temperature of 37^{o}C with shaker speed of 180 rpm by adding *P. putida* in 20.00 ml nutrient broth (NB). *P. putida* has been incubated at 30^{o}C for 24 hours in an incubator oven as recommended by supplier, Merck (Malaysia) Sdn. Bhd before introducing to 180 ml of nutrient broth (NB) in the shake flask (Ee, 2004).

The data regarding the growth of *P. putida* are analyzed and the results are shown in Appendix 4.5. Figure 4.3 shows the profile of *P. putida* growth for approximately 11 hours obtained by measuring the optical density (OD) at 600 nm which represents the cell concentration (Shuler and Kargi, 2002).

When *P. putida* population is inoculated into fresh medium, growth usually begins only after a period of time based on the conditions that was applied. This interval may be brief or extended, depending on the history of the culture. However, if the culture is taken from an old culture and transferred in the original media, growth is not immediate (lag phase) usually occurs even if all the cells in the culture are able to reproduce. This is because the cells are depleted of the various essential constituents and time required for their synthesis (Brock et al., 2006).

The experiment was totally performed in liquid media by introducing *P. putida* in NB (liquid) tended to achieve the optimum growth rate. The same results have been reported in by other researchers (Lebeau et al., 2002).

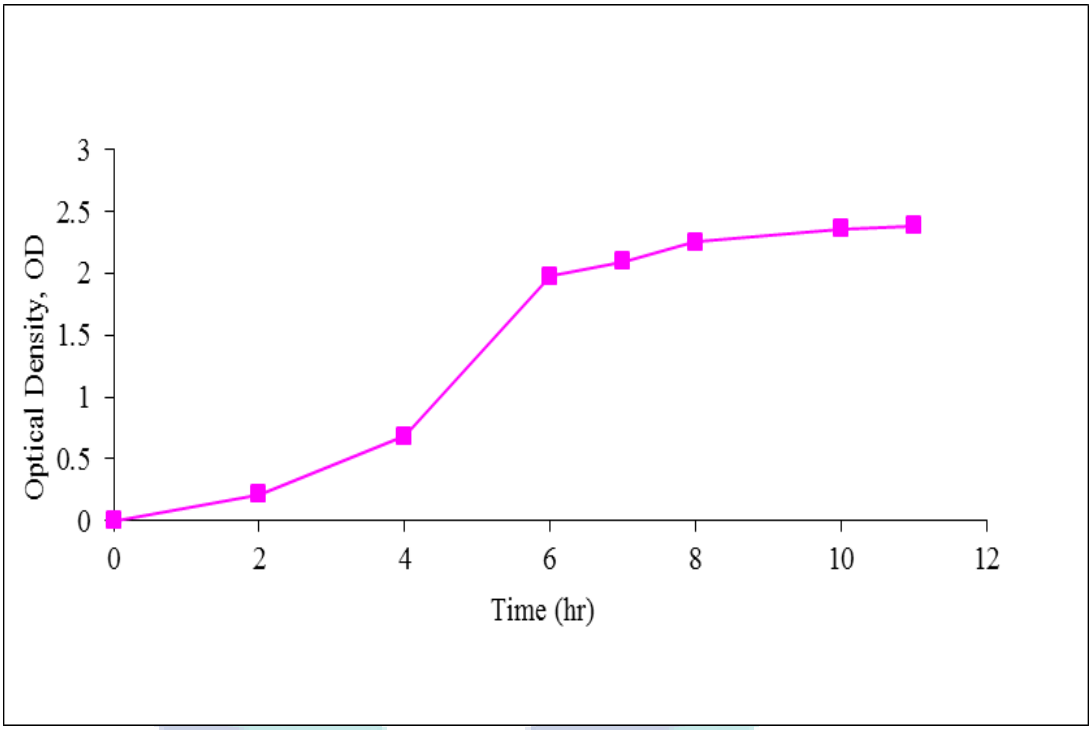


Figure 4.3: Seeding technique for *P. putida* growth curve for *P. putida* population

4.2.2 *P. putida* Growth Curve

Growth curve for *P. putida* grown for seeding process is shown in Figure 4.3, while the other parameters obtained presented in Appendix 4.6. The analysis was conducted by measuring the cell density or optical density (OD) to monitor the growth of *P. putida*. This method is based on the absorption of light by suspended cells and the optical density (OD) measurement of the culture medium provides a fast, inexpensive, and simple method of estimating cell density. The extent of light transmission in a sample chamber is a function of cell density and modulated by broth absorption and scattering. Hence, proper procedure entails using a wavelength that minimizes absorption by medium components and 600 nm

wavelength are often used. (Shuler and Kargi, 2002 and Brock et al., 2006). In the case of the control experiment, where de-ionized water instead of nutrient is used, no growth for *P. putida* was observed as shown for OD Control.

Generally, when an inoculum is introduced to fresh culture medium, the culture may not grow immediately at maximum rate, thus giving rise to a lag phase. From the results obtained, *P. putida* growth began to grow only after 1 hour of lag phase. This is essentially a period of adaptation for *P. putida* to adjust to the new environment and gave some indication of the state of the *P. putida* relative to nutrient. However, lag phase is considered a non-productive period of fermentation process and thus it is often desirable to minimize or control the duration of the lag phase (Lee, 2003).

A lag also ensues when the culture consists of cells that have been damaged but not killed by treatment and time is required for the cells to repair the damage. After that, *P. putida* stayed for 6 hours at exponential growth phase where *P. putida* grow at a balanced rate determined by specific growth rate, and cells are usually in their healthiest state during this period (Brock et al., 2006).

Finally, after 8 hours, the growth of *P. putida* enters the stationary phase as the availability of nutrient decrease. In this phase, some waste product of the organism could builds up in the medium to an excessive level. Also, even though many cell functions may continue, including energy metabolism and some biosynthetic process, the total number of cells remain reasonably constant (Shuler. and Kargi, 2002, Bitton, 2005).

From the experimental results, it was found that during the growth of *P. putida* the pH values fluctuated in the range of 6.20 to 7.60. From previous study, it was reported that most organisms grew actively at pH range of 2 to 3 and natural pH of the environment (between 5 and 9) (Brock et al., 2006). However, most of biological treatment of wastewater occurs generally at neutral pH and meet the demand of industries (Bitton, 2005). Hence, further study was carried out to determine the optimum pH value when using *P. putida* for mercury removal.

4.2.3 Growth Parameters for *P. putida*

Experiments on the *P. putida* growth were repeated for another 30 hours at pH of 7 and a temperature 37^{o}C in order to determine other parameters related to the growth. The results of the experiments are presented in Table 4.3 and the summary of the growth parameters are shown in Table 4.4. The growth parameters of *P. putida* are specific growth rate, μ (hr^{-1}), number of generation, $n = 3.3 \ln OD/ODo$, generation time, $g = t/n$ (hour) and growth rate constant, $k = \ln 2/g = 0.693/g$, (hr^{-1}). The specific growth rate value describes the increase of cell mass per unit time or the faster the cells growing, while the number of generation reflects the number cell reproduced. The generation time, on the other hand, describes the time required for formation of two cells from one or doubling time, and the growth rate constant is defined as the number of generation occurred per unit time in an exponential growth curve. When *n* and *t* are known, *g* and *k* can be calculated.

The results showed that the growth of *P. putida* population increased dramatically for the first 6 hours with OD increased from 0.21 to 1.98. The growth is a result of replication and change in the cell size (Bitton, 2005). This can be seen as per number of generation increased to 3.19 and the maximum of growth rate constant value is 0.37 during that time. Based on growth parameters results, *P. putida* grew and adapted well under this condition in suitable nutrient medium. As a result, microbial mass or population of *P. putida* increased with suitable time. Shuler and Kargi (2002) highlighted that the growth parameters will show the different microorganisms growing under the different culture conditions. This is often useful for optimizing culture conditions for a particular organism and also testing the positive or negative effect of some treatment on the bacteria culture.

Table 4.3: Data for parameters related to *P. putida* growth

Time (hr)	OD	Exponential cell growth (ln OD/OD_0)	Number of generation (*n*)	Generation time (*g*)	Growth Rate Constant (*k*)
2.00	0.21	5.36	0.00	-	-
4.00	0.68	6.53	1.67	2.40	0.29
6.00	1.98	7.59	3.19	1.88	0.37
7.00	2.10	7.65	3.28	2.14	0.32
8.00	2.25	7.72	3.38	2.37	0.29
10.00	2.36	7.77	3.44	2.90	0.24
11.00	2.39	7.78	3.46	3.18	0.22
26.00	2.51	7.83	3.53	7.36	0.09
28.00	2.59	7.86	3.58	7.83	0.09
30.00	2.57	7.85	3.57	8.41	0.08

Table 4.4: Summary of parameters related to *P. putida* growth

Growth Parameter	Min	Max
Specific Growth Rate, μ (hr^{-1})	0.96	-
OD	0.21	2.59
Exponential cell growth, (ln OD/OD_0)	5.36	7.86
Number Of Generation, *n*	1.67	3.58
Generation Time, *g* (hr)	1.88	8.41
Growth Rate Constant, *k* (hr^{-1})	0.08	0.37

In order to understand the *P. putida* growth, the growth cycle of a microbial population was conducted in an enclosed system such as shake flask, and this condition is called a batch culture. A typical growth curve for a population of cells was obtained, as illustrated in Figure 4.4.

In this case, there is no occurrence of lag phase. Since the physiological stage (age and size) of inoculum is an important factor in determining the length of growth lag, the duration of lag period could be eliminated by the addition of culture in the late exponential phase, as the intermediate products (Lee, 2003). The lag period increases when the

inoculum is at the stationary growth phase. This could be attributed to the re-organization necessary in the cells to reverse the change caused by cessation of growth.

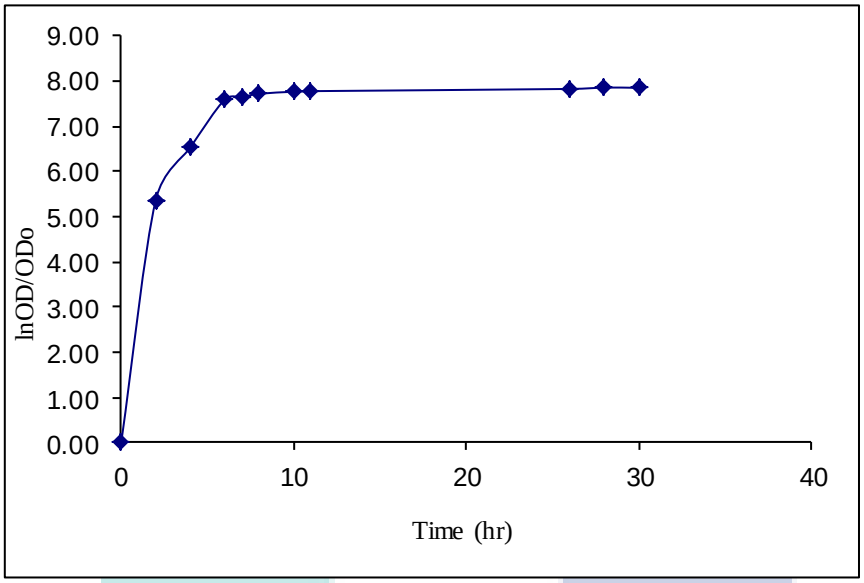


Figure 4.4: Typical of *P. putida* growth curve in the shake flask

During the first 6 hours of the 30 hour experiment carried out, the cell concentration is measured in OD and the exponential cell growth (ln OD/OD_0) shows maximum growth is 7.86. There are three stages in the growth phase, namely, the accelerating growth phase, the exponential (or logarithmic) growth phase and the decelerating growth phase. Normally, at the late lag period, the cells have adjusted to the new environment and begin to grow and multiply (accelerating growth phase), and eventually enter the exponential growth phase, where the cells grow and divide rapidly, at a relatively constant rate, as the exponential function of time.

After 6 hours, it is noted that the pattern of cell concentration is consistent and decreased slightly with time. During this period, the balanced growth occurred in which all components of the cell grow at the same rate. This occurred at the pH 7 where the increased

in the pH value is the result of the fast metabolism of *P. putida* (Wang and Loh, 1999a and Kresnowati et al., 2008).

The specific growth rate, μ is determined by plotting the exponential growth of *P. putida* against time, as shown in Figure 4.4, where cell concentrations were measured with time and it can be calculated from the slope of the plot. Thus, the specific growth rate, μ is found to be 0.96 hr^{-1}. The rate of exponential growth is influenced by environmental conditions such as temperature, composition of the culture medium and genetic characteristics of the organism itself (Brock et al, 2006).The result obtained is meeting to previous study where researchers have found that the specific growth rate, μ of *P. putida* for toxic organic waste such as benzene, toluene and phenol were in the range of 0.34 – 0.73, 0.42 – 0.86, 0.05 – 0.57 hr^{-1}, respectively (Abuhamed et al., 2003a).

Other results related to the growth of *P. putida* are shown in Appendix 4.7 - 4.9, Number of generation, *n* for *P. putida* is 1.67 to 3.58 while the generation time, *g*, which is the time interval between two cell divisions, is from 1.88 to 8.41 hours. The growth rate constant, *k* is in the range of 0.08 to 0.37 hr^{-1}, which a measure of how fast the cells are dividing in a culture for the first 2.00 to 6 hours from the 30 hours experiments. The finding is confirming with the results obtained by Abuhamed et al. (2003b) for the study of the growth of *Pseudomonas putida* F1 during benzene biodegradation. In the case of benzene, *k* has been found to be in the range of 0.12 to 3.36 hr^{-1}.

4.2.4 Determination of Standard Calibration Curve Using Cell Dried Weight

After obtaining the growth curve of *P. putida*, it was suggested that experiment on the standard growth curve by using dried cell weight is conducted in order to confirm the growth pattern by directly measuring cell density (OD).

So as to better understand the growth pattern, a calibration curve relating optical density (OD) to dry-weight measurement was conducted to observe the relationship for

both measurements. Such calibration curve can become non-linear at high OD value at 0.30 (Shuler and Kargi, 2002).

Determination of dry weight is most commonly used as a direct method for determination of cell mass concentration. Typically, samples of culture broth are centrifuged and filtered and washed. Then, the washed wet cell mass is dried and its weight measured (Ee, 2004).

From the observation of results presented in Appendix 4.10 and Figure 4.5, *P. putida* standard curve using cell dry weight method to be used as reference for dried weight. In this case the cell dried weight (g/L) is 0.39 times optical density. It shows that an increase in concentration of biomass will increase the optical density. Basically, optical density is based on the absorption of light by suspended cells in sample culture media and the intensity of transmitted light is measured using a spectrophotometer (Shuler and Kargi, 2002 and Ee, 2004).

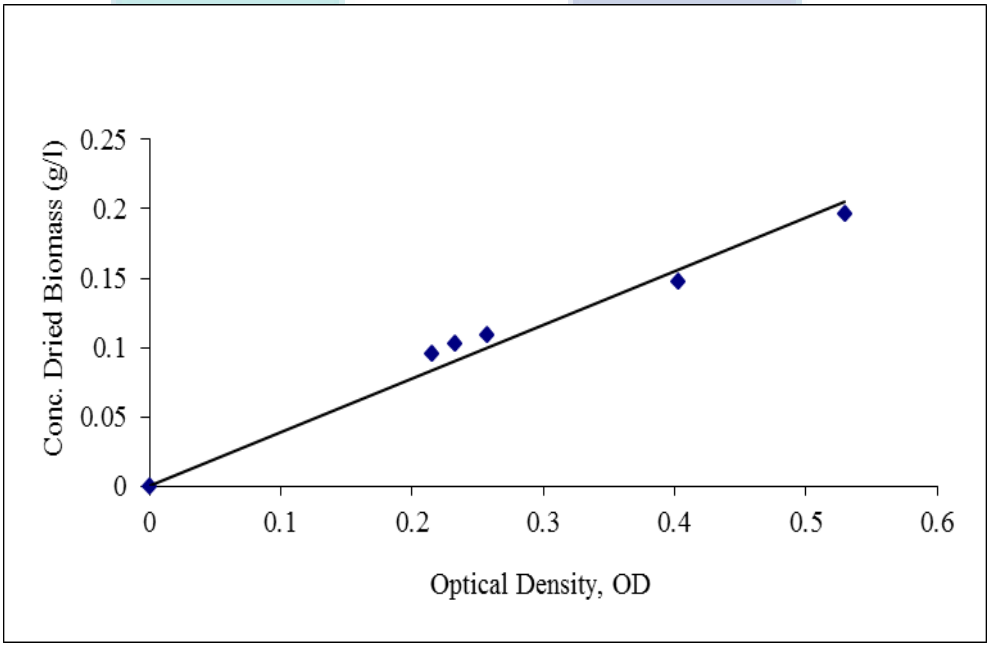


Figure 4.5: *P. putida* standard curve using cell dry weight method

4.2.5 *P. putida* Acclimatization

Acclimatization or acclimation is the process of an individual organism adjusting to gradual change in its environment which allows it to maintain performance across a range of environmental conditions. This phenomenon occurs in a short period of time (days to weeks), and within the organism's lifetime.

In this experiment, in order to achieve optimum growth rate, the effect of acclimatization time for *P. putida* was investigated in a microbiological incubator. The *P. putida* was cultured in a microbiological incubator for 24 hours (1 day), 48 hours (2 day) and 72 hours (3 day) at 30^{o}C as recommended by Merck (Malaysia) Sdn. Bhd. The summaries of results obtained from the acclimatization experiments are presented in Table 4.5, for better understanding, the growth curve for the *P. putida* population is shown in Figure 4.6.

From Figure 4.6, it is observed that the cultured *P. putida* that has been acclimatized for 24 hours adapted well to a new environment in the shake flask after the inoculum were incubated. Meanwhile, in the lag phase, *P. putida* needed 1 hour to grow and adapt after conducting the inoculation process. During this period, cells concentration increased slightly as it organizes their molecular constituents when transferred to a new medium.

In the first 2 to 5 hours, the growth rate of most of the bacteria increases, and as a result the cell density also increased. Consequently, the OD and the exponential *P. putida* growth are at the maximum values of 2.56 and 2.69, respectively. This phase is called exponential growth phase where the *P. putida* has adjusted to their environment and rapidly multiply. However, after this period, it is found that cell growth is constant and slightly decreased over time. This situation caused the pH value of the medium to slightly increased, as shown in Table 4.5, resulting in higher metabolism of *P. putida* in the system.

Table 4.5: Summary of parameters related to *P. putida* growth for 24 hours, 48 hours and 72 hours acclimatization time

Growth Parameter / Time (hr)	Min			Max		
	24	**48**	**72**	**24**	**48**	**72**
Specific Growth Rate ,μ (hr^{-1})	0.58	0.56	0.55	-	-	-
OD	0.17	0.17	0.17	2.57	2.09	2.14
Exponential cell growth (ln OD/OD_0)	0.05	1.79	2.00	2.70	2.49	2.52
Number of Generation, *n*	0.07	2.57	2.86	3.87	3.57	3.61
Generation Time, g(hr)	1.09	1.17	1.05	13.76	1.68	1.66
Growth Rate Constant, *k* (hr^{-1})	0.05	0.42	0.42	0.63	0.60	0.11
pH	6.24	6.59	6.60	7.46	7.42	7.47

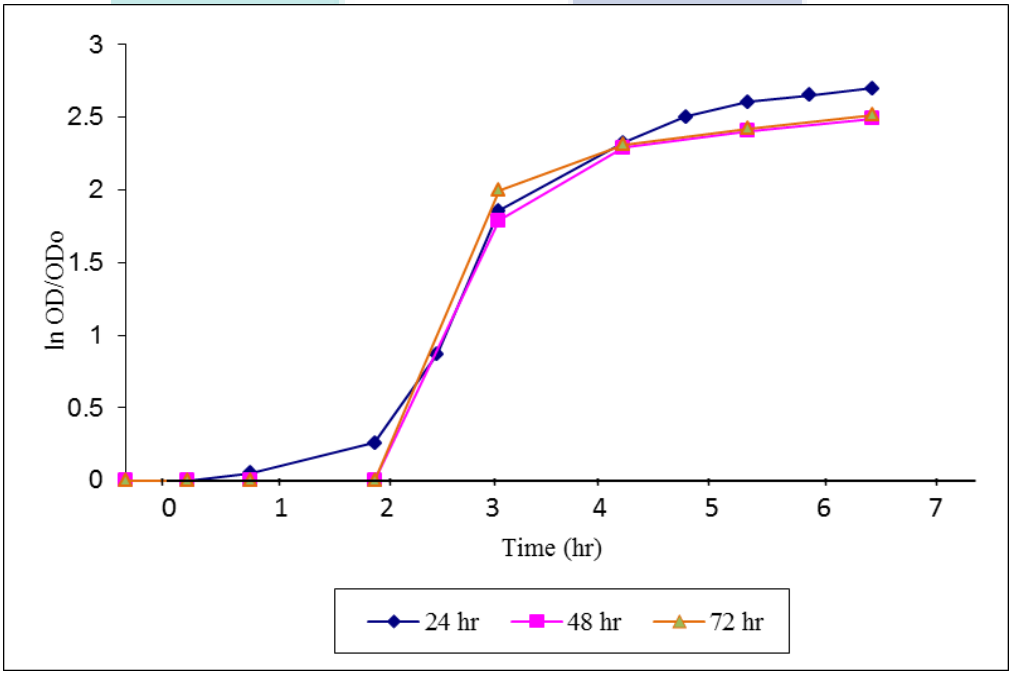


Figure 4.6: Effect of acclimatization time on *P. putida* growth

In this experiment, the specific growth rate, μ for the different acclimatization times were determined. The specific growth rate, μ represents the average growth rate of all cells present in a culture, but not necessary the maximum specific growth rate of an individual cells, as most microbial cultures are divided asynchronously (Lee, 2003). In this case the specific growth rate, μ of *P. putida* obtained from the experiments are 0.58 hr^{-1} for 24 hours acclimatization, 0.56 hr^{-1} for 48 hours acclimatization and 0.56 hr^{-1} for 72 hours. For the different acclimatization times of 24 hours, 48 hours and 72 hours, as shown in Figure 4.6, Appendix 4.11 and Appendix 4.12, the cell density (OD), the generation number, *n* and growth rate constant, *k* decreased to zero in the lag phase. In this condition, *P. putida* is considered in to be non-productive period and more time needed in order to adapt to the new medium before adjusting to new environments.

Other parameters related to the growth of *P. putida* are shown in Appendix 4.11 for number of generation, *n* for *P. putida*, Appendix 4.12 for growth rate constant, *k* and Appendix 4.13 for generation time, *g*. Based on the calculation, the maximum number of generation, *n* are 3.87 for 24 hours acclimatization, 3.57 for 48 hours acclimatization and 3.61 for 72 hours acclimatization. Based on the number of generation *n*, the approximated calculated maximum of generation time, *g* are 14.00 hours for 24 hours acclimatization, and 2.00 hours for both 48 hours acclimatization and 72 hours acclimatization. As a result, the growth rate constant, *k* is 0.01 hr^{-1} for 24 hours acclimatization, 0.60 hr^{-1} for 48 hours acclimatization and 0.66 hr^{-1} for 72 hours acclimatization.

Based on analysis of the effect of acclimatization times on the growth of *P. putida* and the behavior during the different growth phase as well as the growth related parameters, it can be concluded that *P. putida* requires 24 hours only to acclimatize to a new environment in order to achieve optimum growth and will minimize the time required to grow.

4.3 MERCURY REMOVAL IN SHAKE FLASK CULTURE

It is essential to conduct screening experiments that should focus on identifying vital parameters affecting the process, and to identify suitable scale-up factor for future actual bioreactor operation. In this research, screening of condition for maximum *P. putida* growth was conducted in a shake flask.

4.3.1 Effect of Orbital Shaker Speed

In investigating the effect of shaker speed on the growth rate of *P. putida*, the shake flasks were shaken horizontally in an orbital shaker. This experiment was carried out at 37°C (Alagappan and Cowan and 2003, Ee, 2004) and shaker was operated at 140, 180 and 200 rpm. The results of related growth parameters for *P. putida* are presented in Table 4.6 for the different shaker speeds of 140 rpm, 180 rpm and 200 rpm. It is noted that too high agitation speed could be harmful to the cells or the contents of the shake flasks might be spill (Feng et al., 2003).

Figure 4.7 shows a typical profile of *P. putida* growth at different shaker speeds with maximum optical densities of 1.90 (140 rpm), 1.88 (180 rpm) and 2.06 (200 rpm). As a result the maximum exponential growth calculated based on initial optical density are 1.83 (140 rpm), 1.94 (180 rpm) and 1.94 (200 rpm).

The effect of shaker speed is increasing the interfacial area between the media and the microbe in the aqueous phase (Ishenney, 2006). Shaker speed is actually related to the mass transfer area for the bacteria; higher shaker speed has resulted higher area of mass transfer, and as a consequence, higher rate of microorganism growth is obtained. Mass transfer area actually provides a medium for the bacteria to react, grow and metabolize (de-Bashan et al., 2012). The higher the mass transfer area of the media, the larger is the space available in the medium for the bacteria to metabolize which will result in an increase bacteria growth. Besides that, the increase in the shaker speed will reduce concentration

polarization, resulting in good mixing and better dispersion of the nutrient and microbes. Hence, this encourages better growth as well as increase area of mass transfer (Clarke and Corveia, 2008).

Furthermore, the variation of the shaker speed has resulted in the change of oxygen transfer rate (OTR) as well as dissolved oxygen concentration, which in turn affect the growth rate and activity of *P. putida* (Puthli et al., 2006 and Silva, et al., 2012)

Table 4.6: Summary of parameters related to *P. putida* growth in orbital shaker at 140, 180 and 200 rpm speed

Growth Parameter	**Min**			**Max**		
Speed (rpm)	**140**	**180**	**200**	**140**	**180**	**200**
Specific Growth Rate ,μ (hr^{-1})	1.04	1.17	1.07	-	-	-
OD	0.30	0.27	0.28	1.90	1.88	2.06
Exponential cell growth (ln OD/OD_0)	0.08	0.08	0.24	1.83	1.94	1.94
Number of Generation, *n*	0.12	0.11	0.34	2.63	2.78	2.78
Generation Time, g (hr)	1.01	0.75	0.64	4.25	4.66	1.48
Growth Rate Constant, *k* (hr^{-1})	0.01	0.01	0.01	0.68	0.88	1.09

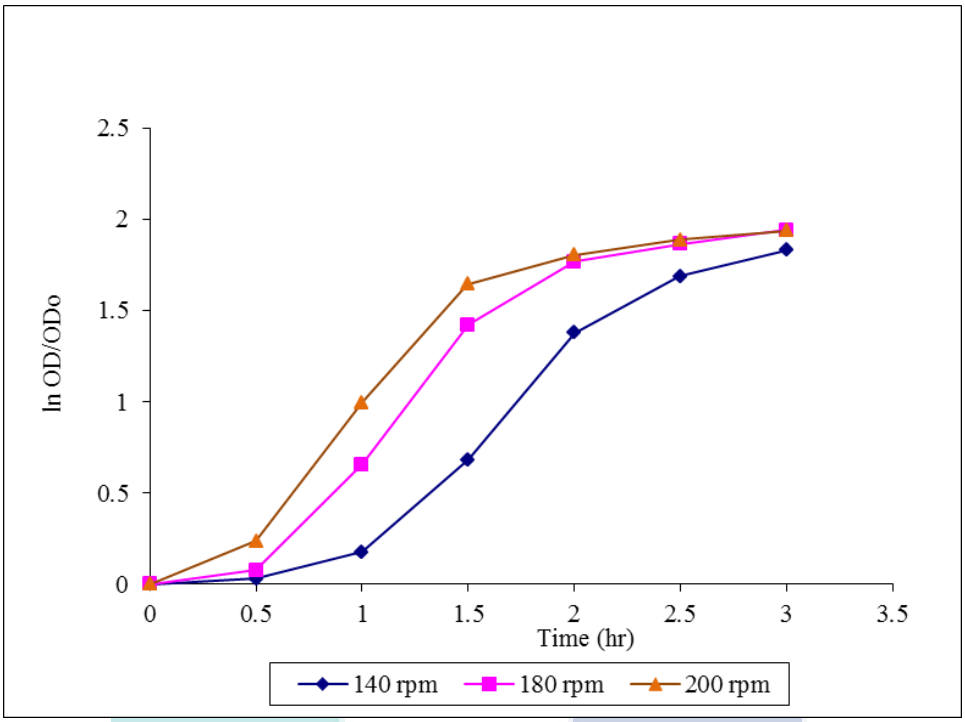


Figure 4.7 : Effect of orbital shaker speed on *P. putida* growth

For shaker speeds of 180 and 200 rpm, the growth of *P. putida* increased more rapidly compared to that at 140 rpm. At high shaker speeds, the growth of bacteria increased slightly higher in exponential phase. The specific growth rate, μ of *P. putida* for 140 rpm shaker speed is 1.04hr^{-1}, 1.17 hr^{-1} for 180 rpm and 1.07 hr^{-1} for 200 rpm. From the specific growth rate and parameters related to growth of *P. putida* results, it can be concluded that the shaker speed at 180 rpm is the optimum speed for *P. putida* growth.

The results obtained for other parameters that are related to the growth of *P. putida* are in the appendices as follows: number of generation, *n* - Appendix 4.14, generation time *g* - Appendix 4.15 and growth rate constant, *k* - Appendix 4.16. From the data obtained, it is found that the maximum number of generation, *n* obtained are 2.63 for 140 rpm, 2.78 for 180 rpm and 2.78 for 200 rpm. Also, the maximum generation time, *g* are 4.25 hours for 140 rpm, 4.66 hours for 180 rpm and 1.48 hours for 200 rpm, whereas the maximum

growth rate constant, *k* are 0.68 hr^{-1} for 140 rpm , 0.88 hr^{-1} for 180 rpm and 1.09 hr^{-1} for 200rpm.

Mortazavi, et al, (2005) reported that the bacteria grow more effectively when shaken due to the creation of dashing and, subsequently, more oxygen is present during the incubation period. Nevertheless, the growth rate will be decreased when the agitation speed is increased to 1000 rpm as at that speed shear rate would increase and could break the cell wall. However, hydrolysis rate will increase with increasing agitation speed (Calderbank, 1958). In addition, the high speed is not economical for the operation cost.

It is noted that, the results of *P. putida* growth are slightly better at 200 rpm especially for generation time, *g* and growth rate constant, *k*. However, shaker speed at 180 rpm was chosen for the subsequent experiments as it is more economical when applied in the industry and the speed produces the highest specific growth rate, μ. Therefore, the optimum shaker speed is 180 rpm and these results are supported by the previous study conducted by Mortazavi, et al., (2005).

4.3.2 Effect of Temperature

Temperature can affect living organism in two opposing ways. As temperature rises, chemical and enzymatic reaction in the cell proceeds at a more rapid rate, and growth becomes faster. However, above a certain temperature, particular protein may be irreversibly denatured. Thus, as the temperature is increased within a given range, growth and metabolic function increase up to a point where denaturation reactions set in. Above this point, cell functions fall sharply to zero (Brock et al., 2006).

Based on the results obtained from the study, further experiments are carried out to determine the effect of other parameters where the shaker is run at 180 rpm with 24.00 hours acclimatization time. In this experiment, the effect of temperature of the orbital shaker is investigated. The shake flasks are heated to temperature of 25°C, 30°C, 33°C,

37°C and 42°C. The results of the related growth parameters for *P. putida* during the growth are presented in Table 4.7 while Figure 4.8 and Appendix 4**.**17 show the effect of temperature on cell density and the exponential growth of *P. putida*. Since the maximum optical densities obtained were 0.66 (25^{o}C), 0.58 (30^{o}C), 0.76 (33^{o}C), 2.23 (37^{o}C) and 2.19 (40^{o}C), the maximum exponential growth for *P. putida* are identified as follows: 0.45 (25^{o}C), 0.62 (30^{o}C), 0.58 (33^{o}C), 1.84 (37^{o}C) and 1.84 (40^{o}C). These results occurred with the medium of pH 6 to 7 for 3.50 hours.

To better understand the growth rate in this experiment, the specific growth rate, μ for the different temperatures were determined. The highest specific growth rate, μ of 0.25 hr^{-1} is observed when the temperature is 37^{o}C. In addition, it is found that the durations required for *P. putida* to adapt to the environment which is at different temperature are as follows: where at, 2.50 hours (25^{o}C), 3.00 hours (30^{o}C), 2.50 hours (33^{o}C), 1 hour (37^{o}C and 42^{o}C). Hence, temperature at 37^{o}C and 42^{o}C showed the shortest duration required for *P. putida* to adapt to the new environment, which directly influenced the specific growth rate, μ. The decrease of specific growth rate, μ at high temperatures is due to the thermal denaturation of proteins, as well as changes in membrane structure, leading to alteration in cell permeability (Bitton, 2005). However, the factors determining an organism's minimum growth temperature are not clear. Perhaps the minimum temperature needed for the organism to grow is the temperature slightly the "freezing" point of the cytoplasmic membrane when the wall cannot function properly to transport nutrient (Brock et al., 2006).

Table 4.7: Summary of parameters related to *P. putida* growth at temperature 25°C, 30°C, 33°C, 37°C and 42°C

Growth Parameter Temp. (°C)	Min					Max				
	25	**30**	**33**	**37**	**42**	**25**	**30**	**33**	**37**	**42**
Specific Growth Rate ,μ (hr^{-1})	0.13	0.18	0.17	0.25	0.24	-	-	-	-	-
OD	0.024	0.31	0.42	0.35	0.36	0.66	0.58	0.76	2.23	2.19
Exponential cell growth (ln OD/OD_0)	0.197	0.00	0.00	0.00	0.00	0.45	0.62	0.58	1.84	1.84
Number of Generation, *n*	0.65	0.89	0.69	0.59	0.64	1.48	2.90	2.50	2.80	2.72
Generation Time, g(hr)	2.36	1.44	1.65	0.82	49.01	4.63	3.92	4.34	1.79	1.84

The summary of results of the other parameters related to the growth of *P. putida* obtained from using the various temperatures are as follows: number of generation, *n* is in Appendix 4.18, generation time, *g* in Appendi*x* 4.19, and growth rate constant, *k* in Appendix 4.20. It is observed that the highest growth rate for *P. putida* is achieved at 37°C although other related parameters did not achieve the highest value. In the lag phase, optical density are good at 37°C and 42°C and, at the same time, the growth pattern of *P. putida* increase at these temperatures. Every organism requires a minimum temperature to grow, below which growth can no longer occurs. An optimum temperature is the temperature when growth is most rapid but growth is no longer possible when the temperature is above a maximum (Brock et al., 2006). Also, no OD reading is observed at 25°C, 30°C and 33°C. This shows that the growth of *P. putida* is not immediate and longer time is needed to adapt to the new environment. Hence, it can be concluded that 37°C is the optimum temperature that can be used for *P. putida* to grow.

Furthermore, 37°C is a suitable temperature to be applied for petroleum based industries wastewater treatment as this temperature is more economical to use and is the same as the temperature of the surrounding environment in countries with tropical climate

like Malaysia. In addition, *P. putida* has the potential to be commercialized at petroleum based industries wastewater treatment plant without needing additional cost for heating or cooling system, as has been confirmed by Mortazavi (2005) and Pandey and Dwivedi (2006).

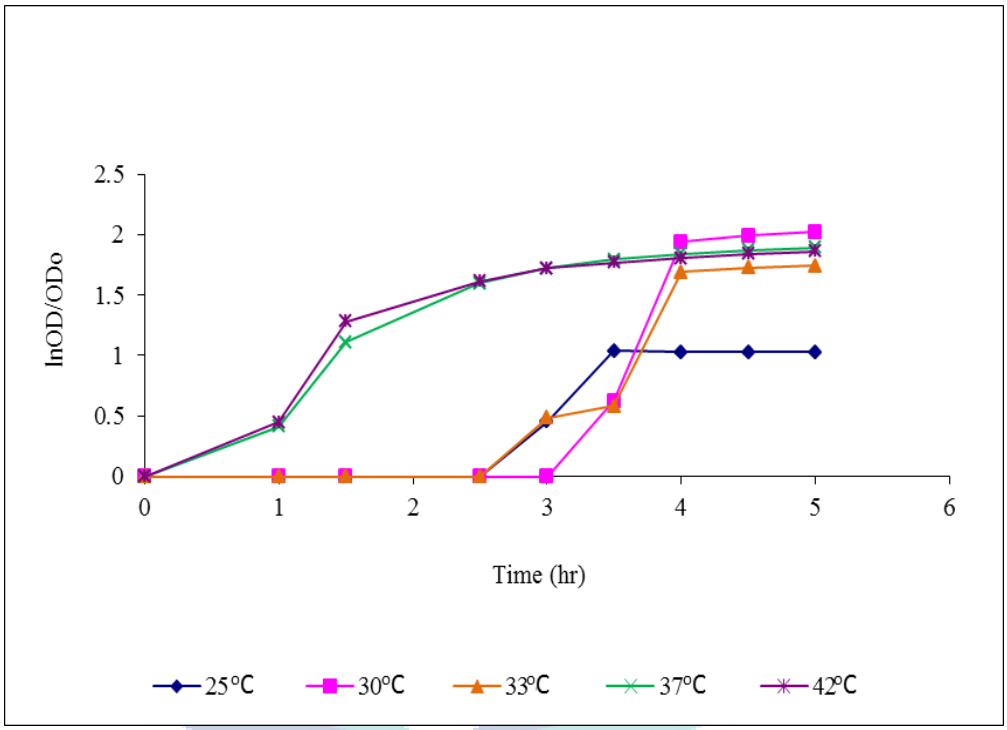


Figure 4.8: Effect of temperature on *P. putida* growth

4.3.3 Effect of pH

Each organism has a pH range within which growth is possible and usually has a well defined of the optimum pH value. Generally pH affects the activity of microbial enzymes. It affects the ionization of chemicals and thus plays a role in the transport of nutrients and toxic chemicals into the cells (Bitton, 2005). Most natural environments have pH values between 5 and 9 (Srivastava and Majumde, 2007).

The effect of pH on the growth of *P. putida* was investigated at three different pH values which are 4, 7 and 10 as well as a control sample with no pH adjustment which is

labeled as pH Control. Adjustment of pH is carried out using buffer, acidic, sulfuric acid (H_2SO_4) 0.10 M is used to adjust the acidity of the medium and sodium hydroxide (NaOH) 0.10 M is used the its alkalinity. The experiments were carried out for 3.00 hours at optimum parameters, which was identified from earlier studies (temperature at 37^oC, 180 rpm for shaker speed and 24.00 hours acclimatization time). The related growth parameters for *P. putida* results obtained from the experiments are shown in Table 4.8. Figure 4.9 shows the effect of pH on the growth of *P. putida*. The maximum OD obtained are 0.34 (pH 4), 2.00 (pH 7), 1.50 (pH 10) and 2.00 (pH Control). Also, the maximum exponential growths for *P. putida* were determined as follow, 0.73 (pH 4), 2.78 (pH 7), 2.58 (pH 10) and 2.72 (pH Control). As a result, the specific growth rate, μ for the different pH value are are 0.26 hr^{-1} for pH 4, 1.56 hr^{-1} for pH 7, 0.93 hr^{-1} for pH 10 and 1.41hr^{-1} as pH Control.

Table 4.8: Summary of parameters related to *P. putida* growth at pH 4, pH 7, pH 10, pH Control

Growth Parameter pH	Min				Max			
	4	**7**	**10**	**Control**	**4**	**7**	**10**	**Control**
Specific Growth Rate ,μ (hr^{-1})	0 .26	1.56	0.93	1.41	-	-	-	-
OD	0.16	0.12	0.11	0.13	0.34	2.00	1.50	2.00
Exponential cell growth (ln OD/OD_0)	0.23	2.22	0.55	0.060	0.73	2.78	2.58	2.72
Number of Generation, *n*	0.33	3.18	0.80	0.086	1.04	3.98	3.70	3.90
Generation Time, (hr)	2.88	0.16	0.75	0.49	4.08	0.75	1.34	6.82

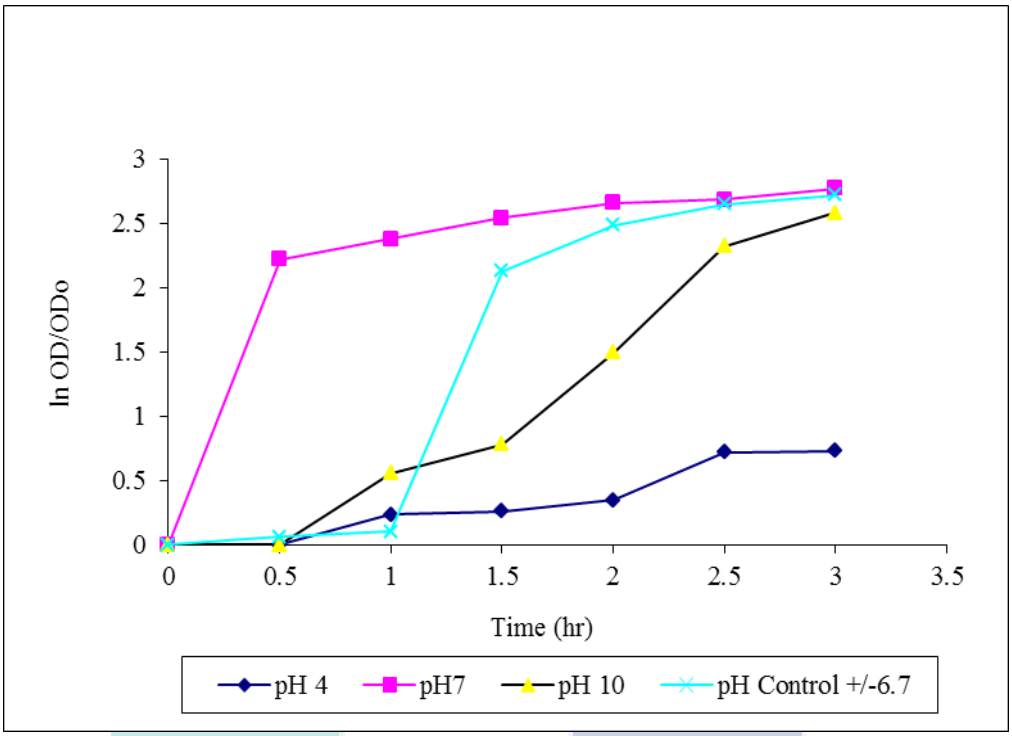


Figure 4.9: Effect of pH on *P. putida* growth

The results of other parameters related to the growth of *P. putida* are shown in Appendix 4.21 to Appendix 4.23. From the results it was found that the maximum value number of generation, *n* obtained is 1.04 for pH 4, 4.00 for pH 7, 3.70 for pH 10 and 3.90 for pH Control while the generation time, *g*, is 4.08 hours for pH 4, 0.75 hours for pH 7, 1.34 hours for pH 10 and 6.82 hours for pH Control and the growth rate constant, *k* was 0.24 hr^{-1} for pH 4, 4.41 hr^{-1} for pH 7, 0.78 hr^{-1} for pH 10 and 1.41 hr^{-1} for pH Control.

In order to confirm that pH 7 is the most suitable pH for the growth of *P. putida*, a zoom in experiment was conducted to observe the growth behavior at a narrower range of pH, i.e. at pH 6, 6.5,7, 7.5 and 8. As shown in Figure 4.10, the growth patterns at pH 6.5, 7.0 and 7.5 were found to be of optimum cell activity and growth rate. pH 6 and 8, on the other hand, showed relatively lower cell activity and growth rate in comparison. Hence, it conforms with the suggestion made previously in the section above that pH 7 is the optimum pH for the growth of *P. putida*. Nevertheless, pH 7 is chosen for further study

because it is practical for measurement purpose and more applicable for industry application.

This finding is due to the fact that when pH is raised to neutrality, the cytoplasmic membranes of strongly acidophilic bacteria (organisms that grow best at low pH) were destroyed and the cells lysed. This indicates that high concentrations of hydrogen ions are required for membrane stability (Brock et al., 2006).

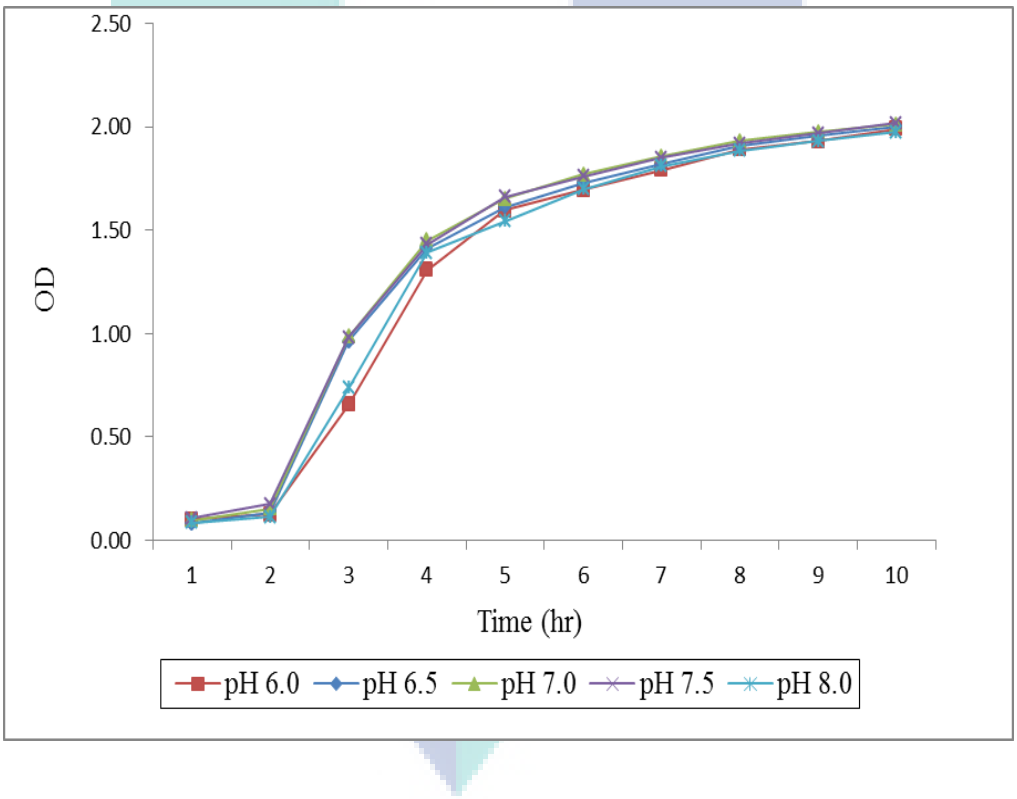


Figure 4.10: Effect of pH on *P. putida* growth at 6.0, 6.5, 7.0, 7.5, 8.0

The reduction in any metal removal with increasing pH beyond its optimum value has been attributed to reduced solubility and precipitation. Nevertheless, in the control experiment where pH of the medium is adjusted in the range of pH 6.5 to pH 7.5, *P. putida* showed an increase in growth and the other related parameters. The finding is consistent

with an earlier study (Mortazavi et al., 2005), where the results revealed that, when applied to mercury removal, the optimum pH for the growth of *P. puitida* could go up to 8. The activity of binding sites is also depending on pH due to the negatively charged carboxyl groups which are responsible for the binding metal via ion exchange mechanism (Vijayaraghavan and Yun, 2008).

Furthermore, biological treatment of wastewater occurs generally at neutral pH and commonly, the optimum pH for bacterial growth is around 7 (Bitton, 2005). It was also reported by previous researchers that *P. putida* was optimal for biodegradation of phenol at pH 6.80 – 7.00 (Chung et al., T. 2003; Tsai and Juang, 2006 and Srivaastava and Majumde, 2007).

4.3.4 Effect of Nutrient Concentration

For successful culture of a given microorganism, it is necessary to understand its nutritional requirements and the supply of the nutrients in the proper form and proportions in the culture medium. Different organisms need different sets of nutrient and not all nutrients in the same amount (Brock et al., 2006).

In the study on the effect of nutrient concentration, experiments were conducted for 6 hour with all other parameters set at optimum conditions (i.e. temperature of 37^{o}C, pH 7, shaker speed of 180 rpm and 24 hours of acclimatization in incubator). The nutrient concentration was varied from 4 g/L, 8 g/L, 12 g/L and 16 g/L in 0.50 L de-ionized water. The results obtained from the experiments of the parameters related to the growth of *P. putida* are summarized in Table 4.9. In addition, the comparison of the growth curves for *P. putida* population over nutrient concentrations is illustrated in Figure 4.10.

Table 4.9: Summary of parameters related to *P. putida* growth at nutrient concentration 4 g/L, 8 g/L, 12 g/L and 16 g/L

Growth Parameter / Nutrient Concentration (g/L)	Min				Max			
	4	**8**	**12**	**16**	**4**	**8**	**12**	**16**
Specific Growth Rate, μ (hr^{-1})	1.24	1.51	1.13	1.13	-	-	-	-
OD	0.04	0.03	0.08	0.09	2.03	2.37	2.17	2.47
Exponential cell growth (ln OD/OD_0)	1.22	0.92	1.43	1.24	3.95	4.34	3.36	3.31
Number of Generation, *n*	1.75	1.32	2.05	1.77	5.67	6.21	4.82	4.75
Generation Time, g (hr)	0.53	0.47	0.49	0.53	1.09	0.97	1.24	1.26

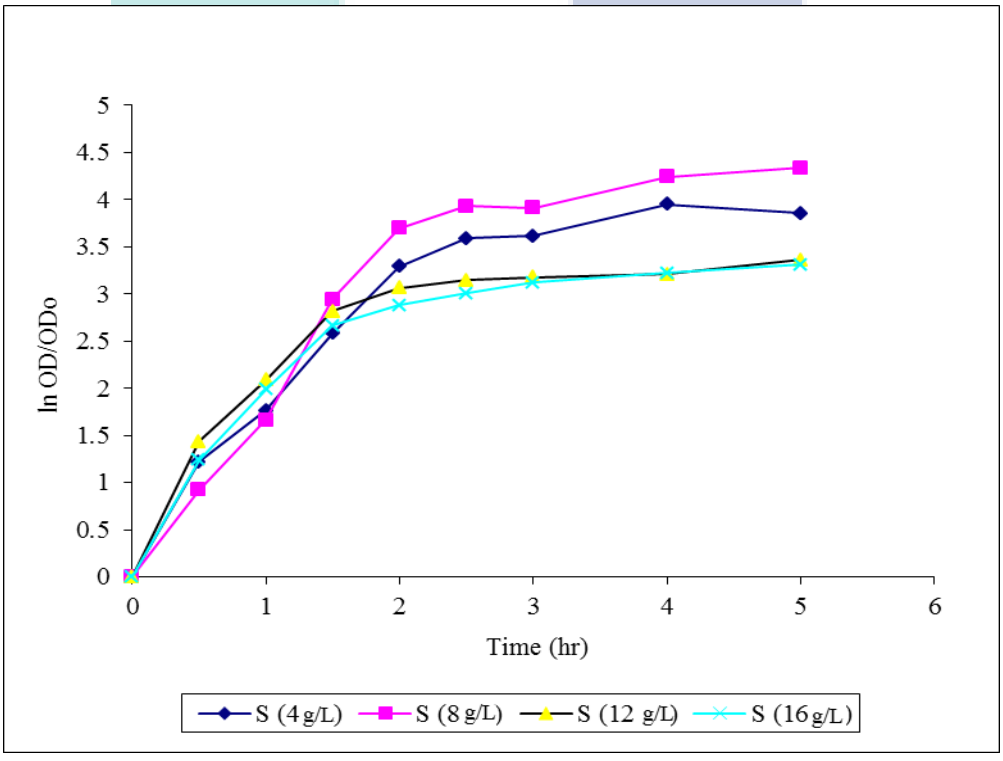


Figure 4.11: Effect of nutrient concentration on *P. putida* growth

From Figure 4.10, it is observed that, for all concentrations, *P. putida* immediately adapted to a new environment in the lag phase. For the first 1.67 hours, most of the cells showed an increasing growth rates and as a result, the cell density also increases. Consequently, OD and the exponential cell growth show maximum results of 2.03 for nutrient concentration 4 g/L, 2.37 for 8 g/L, 2.17 for 12 g/L and 2.47 g/L for 16 g/L.

However, compared to the exponential growth, initial growth is 3.95 for 4 g/L, 4.34 for 8 g/L, 3.36 for 12 g/L and 3.31 g/L for 16 g/L. The results showed significantly higher values than the results obtained from experiments conducted earlier. These phenomena occurred due to application of the optimum operation parameters identified were applied in the experiment. Based on the analysis of the effect of nutrient concentration for *P. putida* growth behavior in shake flask, the specific growth rate μ is determined, and as shown in Figure 4.11, are as follows: 1.24 hr^{-1} for 4 g/L, 1.51 hr^{-1} for 8 g/L, 1.13 hr^{-1} for 12 g/L and 16 g/L as.

The other parameters related to the growth of *P. putida* are shown in Appendix 4.24 for number of generation, *n* for *P. putida,* Appendi*x* 4.25 for generation time, *g* and Appendix 4.26 for growth rate constant, *k*. It can be seen from the graph that the maximum number of generation, *n* obtained are 5.67 for 4 g/L, 6.21 for 8 g/L, 4.82 for 12 g/L and 4.75 for 16g/L. Based on the number of generation *n*, the calculated the generation time, *g* is 1.09 hours for 4 g/L, 0.97 hours for 8g/L, 1.24 hours for 12 g/L and 1.26 hours for 16 g/L. As a result, the growth rate constant, *k* is 1.47 hr^{-1} for 4 g/L, 1.47 hr^{-1} for 8 g/L, 1.40 hr^{-1} for 12 g/L and 1.32 hr^{-1} for 16 g/L.

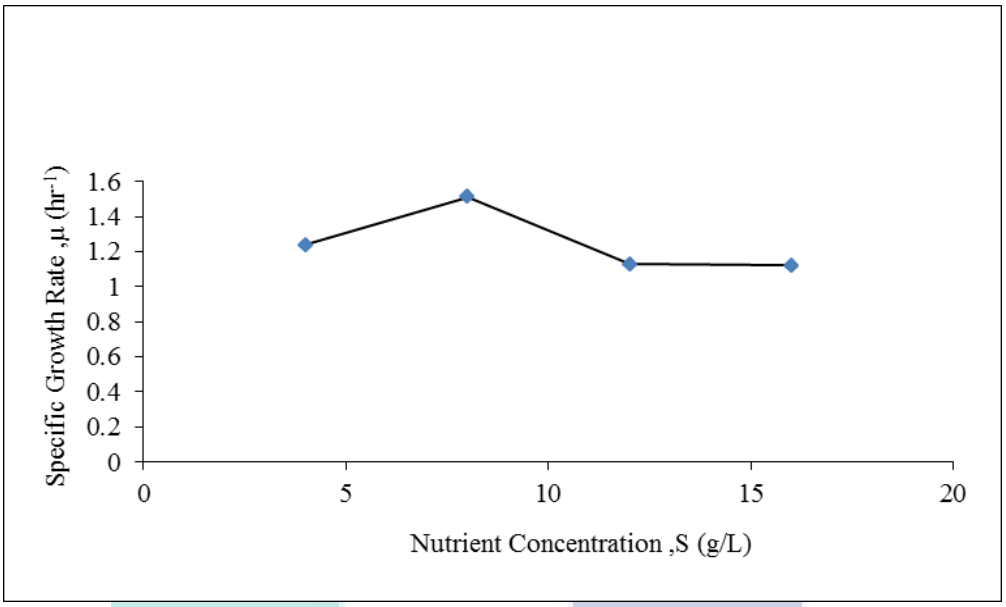


Figure 4.12: Effect of nutrient concentration on specific growth rate of *P. putida*

Most of the organism activities are results of their responses to nutrients as well as other growth factors. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and enzymes formation (Shuler and Kargi, 2002). In comparing the cell growth profile and the specific growth rate, μ and other parameters related to *P. putida* at different nutrient concentrations were conducted. Figure 4.11 clearly shows clearly that nutrient concentration at 8 g/L yielded the optimum specific growth rate (i.e optimum growth of cells).

However, it has been previously reported that a very high concentration of nutrient will inhibit the growth of *P. putida* cell because high substrate concentrations may cause inhibition in some enzymatic reactions known as substrate inhibition (Keweloh and Heipiepe, 1996, Shuler and Kargi, 2002).This study is also supported by another research (Schmidt and Alexander, 1985) that shows the decreased of pure culture due to the dependence on nutrient concentration.

Experiments on the effect of nutrient concentration on cell density of *P. putida* were carried out for at concentration of 2 g/L, 4 g/L, 6 g/L, 8 g/L as shown in Figure 4.12. In order to identify the maximum specific growth rate, μ_m and the saturation or Monod constant, *Ks* as per Monod Model, the specific growth rate, μ needs to be identified as the growth rate is dependent on the substrate concentration and from the Monod equation for biomass growth shown in Equation 4.1, μ_m and *Ks* can be identified:

$$\mu = \frac{\mu_m S}{K_s + s} \tag{4.1}$$

where,

- μ_m is the maximum specific growth rate, hr^{-1}
- K_S is the saturation or Monod constant, g/L
- *S* is the concentration of the growth limiting substrate, g/L

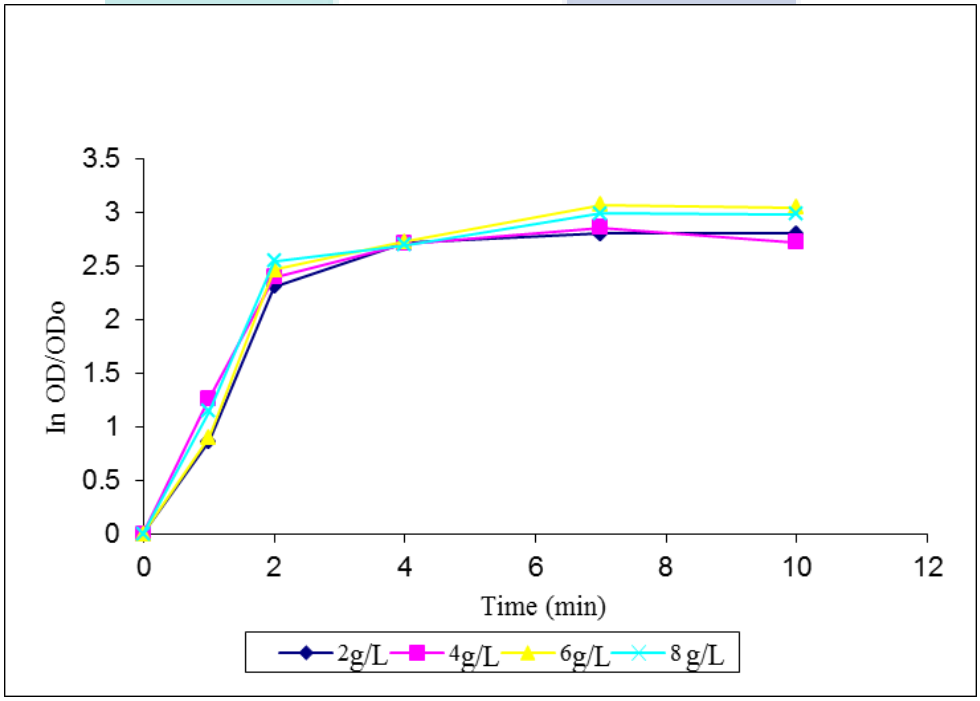


Figure 4.13: Effect of nutrient concentration on the growth of *P. putida*

The specific growth rate**,** μ of *P. putida* obtained from experiments using different concentrations of batch nutrient are presented in Table 4.10. The graph of the specific growth rate**,** μ and substrate or nutrient concentration is plotted in Figure 4.13. By linearizing Equation 4.1, K_s and μ_{max} could be calculated. From the graph, it is found that K_s is 1.65 g/l and μ_{max} is 0.26 hr^{-1}. The maximum specific growth rate, μ_{max} is the maximum grow rate achievable when the concentration of the nutrient is not limiting the growth of *P. putida*; meanwhile, Monod constant, K_S refer to the concentration of the nutrient at which the specific growth rate is half the maximum value and an affinity the organism has for the nutrient (Bitton, 2005).

The value of μ_{max} and K_S is dependt on the organism, the growth limiting nutrient, fermentation medium and environmental factors such as pH and temperature. The value of μ_{max} ranges between 0.01 to 3.00 hr^{-1} (Brock et al., 2006). The K_s value in this part of research is consistent with the finding by Lee, et al. (2003), where K_s was found to be 0.08 g/L for *P. putida* during experiments performed on the effect of additional source on naphthalene biodegradation.

Table 4.10: Specific growth rate, μ of *P. putida* at different nutrient concentration

Nutrient, *S* (g/L)	**Specific Growth Rate, μ (hr^{-1})**
2	0.69
4	1.19
6	1.23
8	1.27

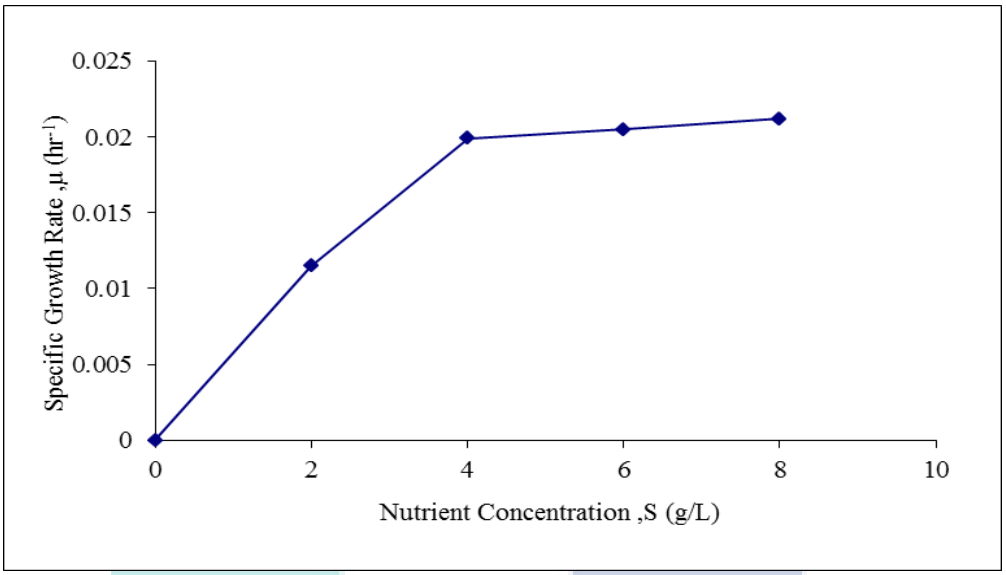


Figure 4.14: Effect of nutrient concentration on *P. putida* specific growth rate, μ at 2g/L, 4g/L, 6g/L and 8g/L

Lineweaver-Burke Plot

For estimation of the kinetic parameters, Monod's equation can be linearized using the Lineweaver-Burke (Double-reciprocal) equation:

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}[S]} + \frac{1}{\mu_{max}} \qquad (4.2)$$

From the study carried out, the graph of 1/μ and 1/substrate or nutrient concentration is plotted yielding linear line as shown in Figure 4.14 for Lineweaver-Burke Plot. From the graph obtained from the experiments, it is found that K_s value for *P. putida* in the experiment conducted is 4.51 g/L and μ_{max} is 0.04 hr^{-1}. This value is still within the range of value μ_{max} as reported by Brock et al., (2006) in which different strains of bacteria were used thst yielded values in the range of 0.01 to 3.00 hr^{-1} while the value reported by Okpokwasili and Nweke (2005) was 0.45 hr^{-1} for toluene biodegradation and 0.20 – 0.90 hr^{-1} for phenol biodegradation (Li, et al. 2010). K_s represents the affinity of microorganism

for the substrate. However, data points at low substrate concentrations would influence the slope and intercept more than those at high substrate concentrations (Shuler and Kargi, 2002 and Ishenny, 2006).

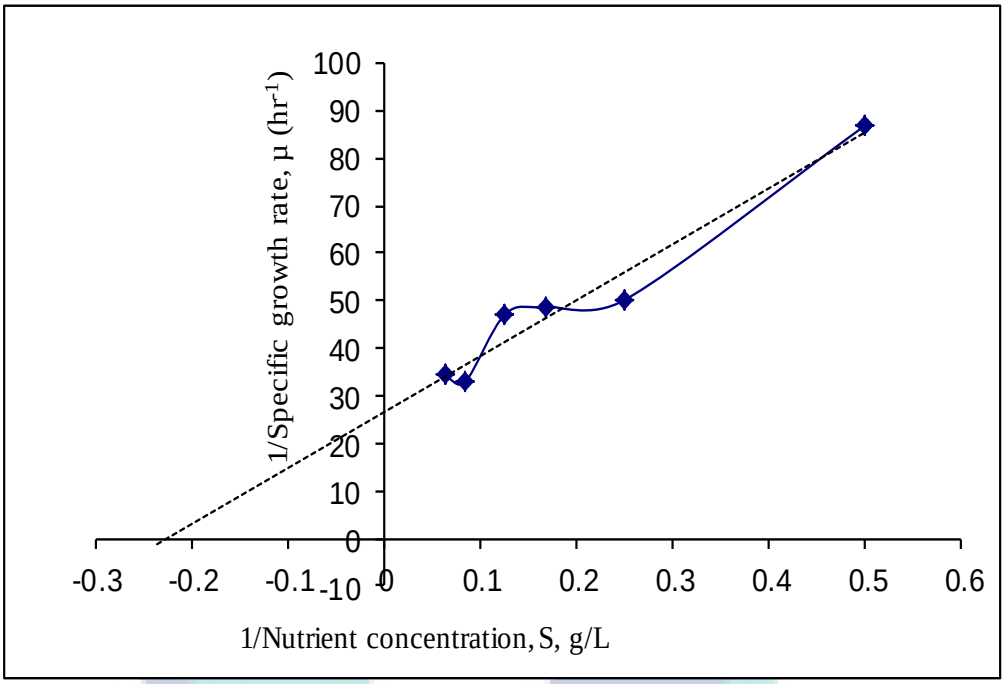


Figure 4.15: Effect of nutrient concentration on *P. putida* specific growth rate, μ at 2 g/L, 4 g/L, 6 g/L and 8 g/L using Lineweaver-Burke Plot

4.3.5 Optimum Condition of *P. putida* in Shake Flask

The purpose of the experiment carried out earlier is to determine the optimum operating parameters of *P. putida* that can be used for mercury removal in wastewater from petroleum based industries. A **s**ummary of the optimum operating conditions for *P. putida* growth behavior in shake flask is presented in Table 4.11 and these values are used for the next experimental work.

Table 4.11: Optimum Operating Condition of *P. putida* in Shake Flask

No.	Optimum Operating Condition	Value
1.	Acclimatization Time	24 hrs
2.	Orbital Shaker Speed	180 rpm
3.	Temperature	37^{o}C
4.	pH	7
5.	Nutrient Concentration	8 g/L

4.3.6 Effect of Mercury Concentration on *P. putida* Growth

A series of experiments using different concentration of Hg were conducted to study the effect of Hg on growth of *P. putida*. Samples used in the experiments are as follows: Sample A - *P. putida* in nutrient broth (NB) were mixed with fresh NB (8 g/L); Sample B - *P. putida* in NB were grown in NB with 6.00 ppb Hg solution; Sample C - *P. putida* in NB were grown in NB with 1.00 ppb Hg solution while sample D, *P. putida* in NB were grown in NB with 19.00 ppb Hg solution. The study was performed at optimum operating condition in shake flask with 24 hours acclimatization time, orbital shaker speed of 180 rpm, temperature at 37^{o}C, pH 7 and nutrient concentration at 8 g/L.

Table 4.12: Effect of Low Mercury Concentration (ppb) on *P. putida* growth behavior for 24 hours

Initial Hg Concentration, (µg/L)	OD_0 Initial	OD_{max} (4 hr)	OD (24 hr)	Biomass Concentration, (g/L)	Final Hg Concentration, (µg/L)	Hg % Removal	µg Hg /gcell
0.00	0.00	0.53	0.39	0.02	0.00	0.00	0.00
1.00	0.00	0.50	0.08	0.03	0.01	99.00	0.33
6.00	0.00	0.37	0.03	0.01	0.01	99.83	1.00
19.00	0.00	0.30	0.12	0.04	0.27	98.58	6.75

In this case, percentage of mercury removal can be determined directly by using Equation 4.3:

$$\text{Percentage of Hg Removal} = \frac{A-B}{A} \times 100\% \quad (4.3)$$

where,

- A is initial Hg Concentration (ppb)
- B is final Hg Concentration (ppb)

From Table 4.12, it can be seen that the OD decreased from 0.53 after 4 hours to 0.05 after 24 hours for the control sample with no mercury added. This shows the normal behavior of *P. putida* growth in batch system when the nutrient is introduced only at an early stage. Cell density may increase for the first 4 hours and after which it starts to reduce due to the decreasing of nutrient concentration and from the study conducted earlier that showed the growth rate of *P. putida* is dependent of nutrient concentration.

The reason for the termination of growth may either be exhaustion of an essential nutrients or accumulation of toxic products. If an inhibitory product is produced and accumulated in the medium, the growth rate will slow depending on inhibitor production at a certain level of inhibitor concentration (Bull and Whitten, 1974 and Bitton, 2005).

For culture with mercury concentration of 1.00 ppb and after 4 hours of experiment, the maximum optical density, OD_{max} is 0.50. Also, the cell density is further decreased to 0.08 after 24 hours. As a result, the mercury concentration has decreased from 1.00 ppb to 0.01 ppb and the percentage mercury removal is 99% and the ratio of mercury mass over cell mass is 1.00 µg Hg /gcell.

For culture with mercury concentration of 6.00 ppb, the maximum optical density, OD_{max} obtained was 0.37 and the cell density decreased to 0.03 after 24 hours of experiment. It can be seen that, mercury concentration decreased from 6.00 ppb to 0.01 ppb

with percentage mercury removal is 99.58 %. In this case, the ratio of mercury mass over cell mass is 0.33 µg Hg /gcell.

Finally, the culturing with 19.00 ppb mercury concentration, the results showed that the maximum optical density, OD_{max} is 0.30. After 24 hours, the cell density is reduced to 0.12. In this case, the percentage of mercury removal was 98.50% which is just slightly lower than the above two experiments carried out earlier. As a result the ratio of mercury mass over cell mass is increased dramatically i.e. 6.75 µgHg /gcell.

Since full mercury retention at low concentration was obtained from a 24.00 hours inoculation, it may be concluded that the microbial community present and the activity of detoxification was occur. The detoxification mechanism for mercury is based on the unique peculiarities of this metal: the electrochemical potential of Hg^{2+}/ Hg^{0} at pH 7 is +430mV, which means living cells are able to reduce Hg^{2+} to elemental form Hg^{0}, which is non-toxic to human and microorganism (Singh et al., 2008). Although micro-organism cannot destroy metals but they can alter their chemical properties via a surprising array of mechanism that can be used to treat toxic metal contamination involving highly specific biochemical pathways that have evolved for their protection (Lloyd and Lovly, 2001).

At very low concentrations of metal ions, the ratio of sorptive surface area to the total metal ions available is high (Mortazavi, et al. 2005). Thus, there is a greater chance for metal removal. When mercury concentration is increased, binding sites become more quickly saturated as the amount of biomass concentration remained constant.

Devars et al. (2000) and Mortazavi et al. (2005) reported that the bacteria resistance to mercury is related to enzymatic reduction of ionic mercury, Hg^{2+} to water insoluble metallic volatile mercury, Hg^{0}. Mercury detoxification process originated from mer operon located on either plasmids or transposable elements in the mercury resistant microorganisms. Specific transport of bulk mercury across the cell membrane is achieved by two mer operon genes merP and merT, which express cysteine-rich proteins to deliver ambient mercuric toward intracellular mercuric reductase for subsequent reduction of

mercuric ions to volatile Hg^0. Since the melting point of mercury is extraordinary low (melting point -39^oC, boiling point 357^oC), therefore elemental mercury does not remain inside the cell but leave it by passive diffusion and is then either volatilized into the air or precipitates due to its low solubility in water.

The discharge limit for mercury for industrial wastewater is 50.00 ppb for Standard B as DOE as required by (EQA, 2011), however, some local water authorities at some other countries demanded the limit to be 10.00 ppb. This is of crucial importance for a potential industrial application of the microbial mercury remediation technology.

The results obtained from the experiments are in agreement with study conducted by Wagner-Dobler et al. (2000). In this case, mercury removal at 36.00 ppb in industrial wastewater was conducted over a period of 10 hours by using different species of *Pseudomonas* i.e. *P. stuitzeri* and *P. fulva* and it was reported that the efficiency of and the percentage of removal was between 95% - 99%.

4.3.7 Mercury Removal by *P. putida* in Orbital Shaker at Optimum Operating Condition

Mercury removal was conducted at optimum operating conditions and when the growth of *P. putida* is high. The experiments were conducted for 28 hour, employing the yield of optimum operating condition in a shake flask with 24 hours acclimatization, orbital shaker speed 180 rpm, temperature 37^oC, pH 7 and nutrient concentration 8 g/L. The results of the growth of *P. putida* and the corresponding mercury removal for 1000 ppb mercury concentration are shown in Table 4.13.

Figure 4.15 presents the plot of *P. putida* growth behavior and mercury removal against time. It can be observed from the graph that *P. putida* grew immediately after inoculation. In this case, an exponentially growing culture was transferred into the medium under the same condition of growth. A lag phase does not occur and exponential growth begins immediately. Usually the interval may be brief or extended, depending on the

history of culture and growth condition. A constant mercury concentration of 79 ppb is obtained after 1 hour and the ratio of mercury mass over cell mass is increased dramatically (1215.00 ugHg /gcell) within this period of exponential growth phase. With decreasing mercury concentration, *P. putida* showed an increasing growth rate and the cell density increase in the first three hours, i.e. 0.33 g/L, and the maximum OD and exponential cell growth obtained are 0.86 and 1.89. However, cell growth is constant and slightly decreased over time after this period.

The parameters related to the growth of *P. putida* and mercury removal are summarized in Table 4.14. Based on the results, the specific growth rate, μ is 0.70 hr^{-1} as determined from the growth curve shown in Figure 4.15. This result is lower than the specific growth rate obtained from the earlier experiment at optimum condition without mercury in the sample.

The other parameters related to the growth of *P. putida* are as follows: number of generation, *n* is shown in Appendix 4.27, generation time, *g* is shown Appendi*x* 4.28, and growth rate constant, *k* is shown in Appendix 4.29. It can be seen from the graph that the number of generation, *n* obtained is 2.00. Based on the number of generation *n*, the calculated generation time, *g* is 14.64 hours. Consequentlyt, the growth rate constant, *k* is 0.78 hr^{-1}.

Table 4.13: The Growth Kinetics of *P. putida* in mercury removal at 1000 ppb concentration

Time (hr)	Optical Density (OD)	Biomass concentration (g/L)	Hg concentration (ug/L)	µgHg/ gcell	% Hg removal
0.00	0.00	0.00	1000	0.00	0.00
1.00	0.17	0.06	79	1215.00	92.10
2.00	0.63	0.24	56	230.00	94.40
3.00	0.86	0.33	43	130.00	95.70
20.00	0.58	0.22	34	153.00	96.60
23.00	0.51	0.20	24	121.00	97.60
28.00	0.50	0.19	20	105.00	98.00

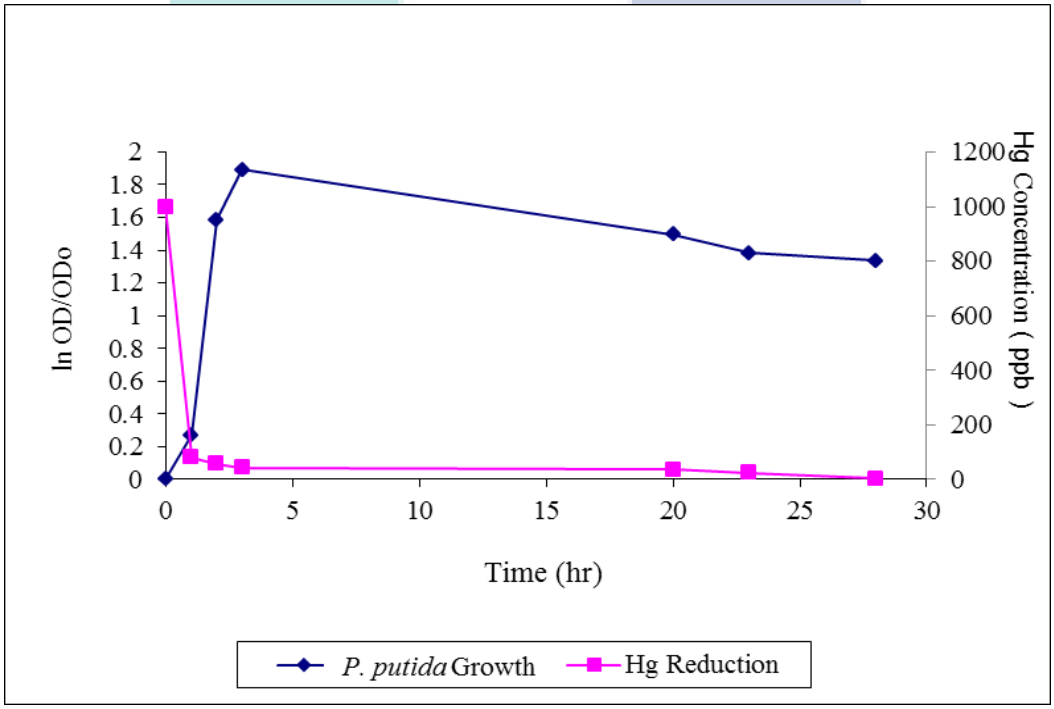


Figure 4.16: Mercury (1000 ppb) removal by *P. putida* in shake flask at optimum condition

Table 4.14: Mercury (1000 ppb) removal by *P. putida* in orbital shaker at optimum operating condition

Growth Parameter	Min	Max
Specific Growth Rate, μ (hr^{-1})	0.70	-
OD	0.13	0.86
Exponential cell growth (ln OD/OD_0)	0.27	1.89
Number Of Generation, *n*	0.39	1.98
Generation Time, *g* (hr)	0.88	14.64
Growth Rate Constant, *k* (hr^{-1})	0.06	0.78
Hg Removal (%)	-	98.00

Green-Ruiz (2006) has showed that the maximum achievable percentage of mercury removal at 1000 ppb concentration by *Basillus sp* is 88%. However, the percentage of mercury removal achieved in this experiment is better at 92.1 % for the first 1 hour and 98% after 28 hours. Also, the cell density decreased to 0.49 compared to the maximum cell density, but cell density of 0.86 were detected after 3 hours of experiment in the study conducted by Mortazavi et al. (2005). This is because with the increase in cell concentration, the percentage removal increases as the number of possible binding sites is increased.

At low concentration of metals ions, the ratio of sorptive surface area to total available metal ions available is high and thus, there is a greater chance for highly toxic water-soluble ionic mercury been taken up by *P. putida* and reduced to insoluble metallic mercury through intracellular enzyme mercuric reductase, encoded by merA gene (Wagner-Dobler et al., 2000). Metallic mercury subsequently diffuses out of cells. The reduction process can be continuously performed within a submersed microbial and resulting in accumulation of metallic mercury within bioreactor. The percentage of mercury removal for mercury concentration of 10000 ppb was 80%, as reported by Mortazavi et al. (2005).

4.4 MERCURY REMOVAL in 2L BIOREACTOR

A 2L batch mode bioreactor with closed-system is used in this study where the sterile nutrient solution is inoculated with *P. putida* under optimum operating conditions. The culture is incubated as before, prior to the transfer into the bioreactor. In this bioreactor operation, effects of agitation speed and aeration rate are examined to understand the effects on the microbial growth behavior and the removal of mercury from the suspension.

4.4.1 Growth of *P. putida* in 2L Bioreactor

The growth curve for *P. putida*, growing in 2L bioreactor is shown in Figure 4.16. In the same manner, the other parameters related to *P. putida* growth obtained are presented in Table 4.15. Experiments were conducted for 51 hours at optimum operating parameters obtained from earlier shake flask experiments carried out in the shake flask with 24 hours acclimatization, agitator speed at 180 rpm, temperature at 37^{o}C, pH 7 and nutrient concentration at 8 g/L. However, some of the operating conditions and other growth related parameters are to be studied further in bioreactor 2L.

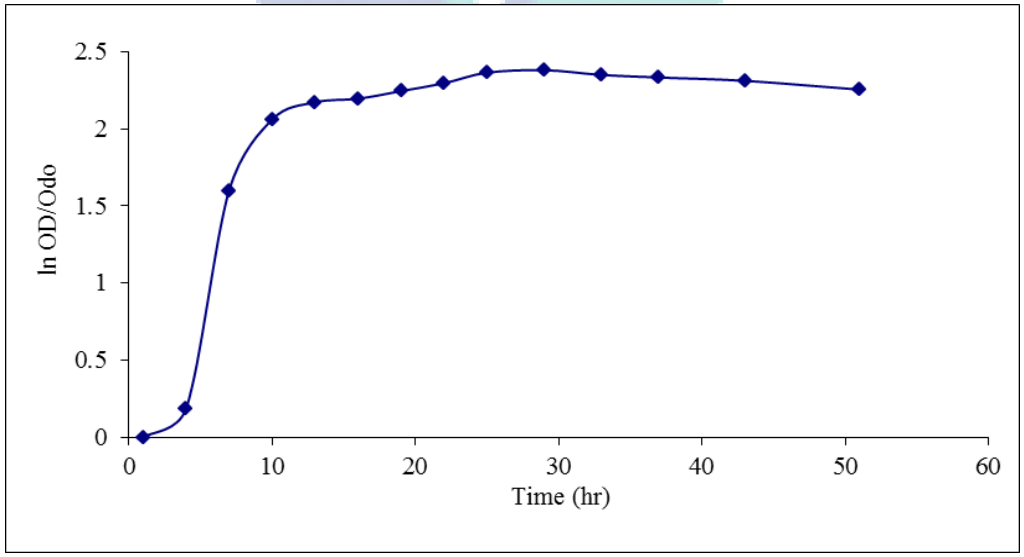


Figure 4.17: *P. putida* growth curve in 2L bioreactor

Table 4.15: Summary of parameters related to *P. putida* growth in 2L bioreactor

Growth Parameter	Min	Max
Specific Growth Rate, μ (hr^{-1})	0.22	
OD	0.22	2.94
Exponential cell growth (ln OD/OD_0)	0.19	2.38
Number Of Generation, *n*	0.27	3.41
Generation Time, *g* (hr)	0.05	0.26
Growth Rate Constant, *k* (hr^{-1})	2.63	13.61

According to these results, the maximum cell density is 2.94 after 10 hours of experiment but the exponential growth is slightly lower at 2.38. The specific growth rate, μ is determined at the exponential growth of *P. putida*, where cell concentrations measured against time, can be calculated from the slope obtained from the graph. The specific growth rate, μ is 0.22 hr^{-1} , is lower than that achieved in the shake flask at 0.96 hr^{-1}, conducted during the initial study. This shows that the cells are reproduced faster in the shake flask compared to growth in bioreactor. Furthermore, growth in the bioreactor only began after the lag phase has lapsed and also because the inoculum was taken from the culture in the stationary phase before transferring to the medium. Some of the cells are depleted and needed time to re-synthesize. The results discussed above were average from three sets of experiments, which showed minimal variation. However, the growth rate obtained is almost similar to the value of 0.20 hr^{-1} suggested by Naik et al. (2006) in their studies employing a stirred tank bioreactor.

The other parameters related to the growth of *P. putida* are shown in Appendix 4.30 to 4.32. The maximum number of generation, *n* obtained is 3.41 while the maximum generation time, *g* was 0.26 hours and the maximum growth rate constant, *k* is 13.61 hr^{-1}. As stated by De and Ramaiah (2006), the generation time for *P. putida* was 48 hours in the presence of 1000 ppb Hg concentration. Meanwhile, the growth of pseudomonad isolated from Chennai harbor was much faster at, two generations within the first 6 hours at 50000 ppb Hg concentration.

4.4.2 Effect of Agitator Speed

Agitation brings about proper mixing of the fermentation broth and has a tremendous effect on productivity of the system. These effects may be beneficial or deleterious like rupture of the cell wall, change in cell morphology, variation in the efficiency of the product formation, substrate utilization and rate of growth. Additionally excess foam is generated at higher mixing speed. Investigations on the growth of *P. putida* for mercury removal from petroleum based industries wastewater are carried out at different agitator speeds (100, 180, 200 rpm) for about 19 hours. The previous optimum operating parameters were adopted, which are 24 hours acclimatization time, temperature 37^{o}C, pH 7 and nutrient concentration 8 g/L. The summary of the results are shown in Table 4.16 and Figure 4.17.

Table 4.16: Effect of agitation on *P. putida* growth (100 rpm, 180 rpm, 200 rpm)

Growth Parameter	**Min**			**Max**		
Agitation speed (rpm)	100	180	200	100	180	200
Specific Growth Rate ,μ (hr^{-1})	0.15	0.25	0.11	-	-	-
OD	0.23	0.22	0.22	1.16	2.07	1.24
Exponential cell growth (ln OD/OD_0)	1.26	0.19	0.01	1.65	2.24	1.73
Number of Generation, *n*	1.80	0.27	0.01	2.36	3.22	2.58
Generation Time, g(hr)	1.70	3.05	7.11	10.56	14.94	307.00
Growth Rate Constant, *k* (hr^{-1})	0.06	0.05	0.01	0.41	0.23	0.10

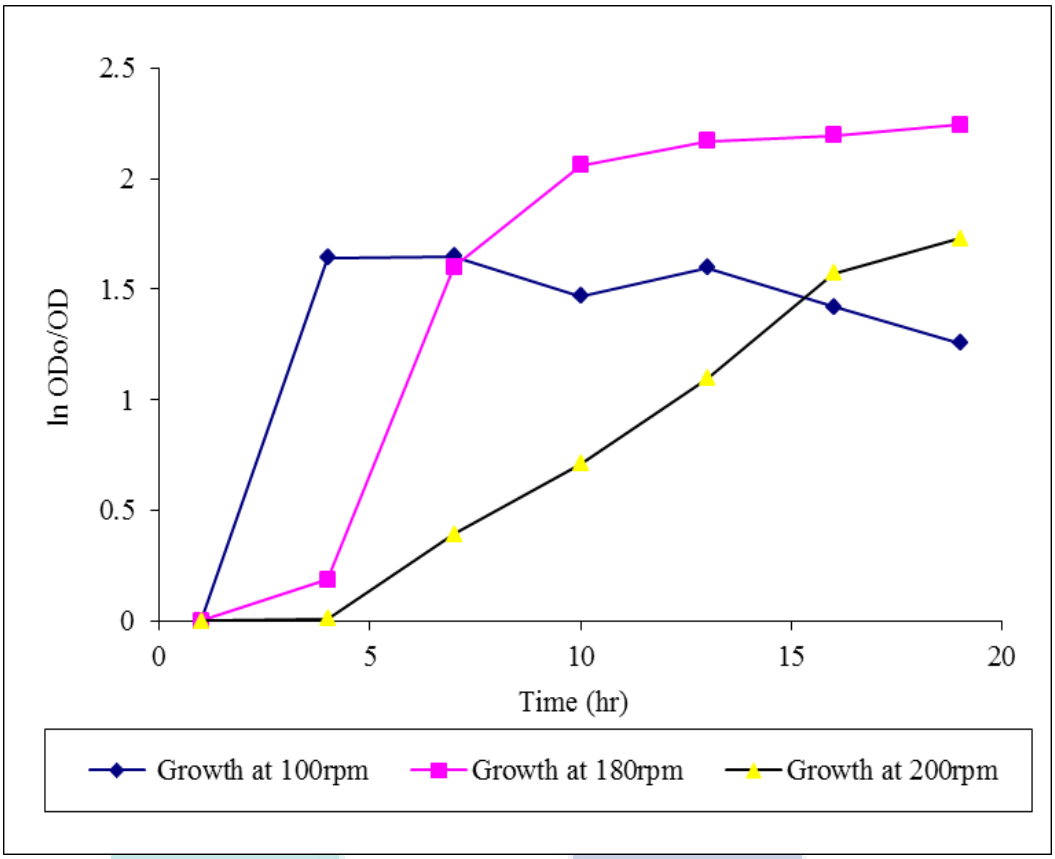


Figure 4.18: Effect of agitator speed to *P. putida* growth in 2L bioreactor

At 100 rpm, cell growth and the activity were found to be comparatively low (OD maximum is 1.16 and the exponential growth was 1.65 and the specific growth rate, μ is 0.15 hr^{-1}). This could be attributed to the lack of oxygen available for the *P. putida* due to the insufficient mixing (low agitation rate). The dissolved oxygen in the culture broth decrease rapidly at the beginning of inoculation (Wagner-Dobler et al., 2000).

The cell when grown at 200 rpm supposedly should show faster growth with the early induction of stationary phase, however it does not happen. In this situation, the cell could have sheared more easily which adversely affected the activity of *P. putida*. From the results obtained, the optical density at initial stage is 0.22 and the maximum growth activity is 1.24, and the exponential growth is 1.72 which is slightly higher than that obtained at 100 rpm. However, the specific growth rate, μ at 0.11 hr^{-1} is lower than obtained at 100 rpm.

Calderbank (1958) reported that the increased of agitation speed would increase hydrolysis rate. Thus, the growth rate would be decreased and this may due to the change of substrate utilization, rupture of the cell wall or change in the cell morphology.

Similar activities are found when cultivation was carried out at 180 rpm. The cell activity at 180 rpm is marginally higher and obtained earlier with OD maximum, exponential growth and specific growth rate, μ of 2.07, 2.24 and 0.25 hr^{-1} respectively.

The other parameters related to the growth of *P. putida* are shown in Appendices 4.33 to 4.35. From the results it is found that the number of generation, *n* is 3.22 while the generation time, *g* is 14.94 hours and the growth rate constant, *k* is 0.23 hr^{-1}. Hence an agitation speed of 180 rpm is chosen as the optimal agitation speed.

4.4.3 Effect of Aeration Rate on the Growth of *P. putida* in Bioreactor

Aeration is essential for metabolic activities of aerobic organisms. Aeration also brings about mixing of the bioreactor contents thereby increasing the efficiency of the bioreactor. The dissolved oxygen concentration in a suspension of respiring microorganisms depends on the rate of oxygen diffusing to the microorganism and its consumption by the microorganism. During fermentation, the transfer of oxygen occurs from the air bubbles into the medium, then is transferred to the cell and ultimately to the site of reaction within the cell. Thus oxygen transfer from air bubbles, through liquid medium, to microbial cell has great importance. The more the oxygen transfer the more is oxygen available to the organism. Oxygen transfer can be increased by increasing the aeration rate.

Table 4.17: Effect of aeration rate on *P. putida* growth in bioreactor (0.25 vvm, 0.5 vvm, 0.75 vvm)

Growth Parameter	**Min**			**Max**		
Aeration rate (vvm)	0.25	0.50	0.75	0.25	0.50	0.75
Specific Growth Rate, μ hr^{-1}	0.24	0.21	0.12	-	-	-
OD	0.10	0.22	0.53	2.28	2.37	2.42
Exponential cell growth (ln OD/OD_0)	0.34	0.19	0.63	3.09	2.38	1.52
Number of Generation, *n*	0.50	0.27	1.24	4.43	3.41	2.18
Generation Time, g(hr)	2.26	4.18	2.42	6.10	14.94	17.78

In this study, the effect of increasing aeration rate was checked by sparging air to the reactor at different rates of 0.50 L/min (0.25vvm), 1.00 L/min (0.50 vvm) and 1.50 L/min (0.75 vvm). The experiments are also carried out at the optimum operating parameters determined earlier (24 hours acclimatization time, temperature at 37^{o}C, pH 7, nutrient concentration at 8g/L and agitation speed at 180 rpm) for more than 30 hours. Summary of the results are shown in Table 4.17.

It is observed that increasing the aeration rate caused the decline in the specific growth rate, μ and the exponential growth. On the other hand, for 0.75 vvm aeration rate, the initial OD at 0.53 and OD maximum at 2.42 showed higher results as shown in Table 4.17. A lower aeration rate of 0.25 vvm is found to be optimum for higher cell activities with initial OD at 0.10 and OD maximum at 2.28, and better performance of the exponential growth at 3.09 as well as the specific growth rate at 0.24 hr^{-1} as observed in Figure 4.18. However, the cells that are provided with aeration at 0.50 vvm showed longer life span than that provided with other aeration rates, by measuring optical density with specific growth rate, μ 0.21 hr^{-1} which is comparable to 0.25 vvm aeration rate as shown in Figure 4.19.

The other parameters related to *P. putida* growth are shown in Appendices 4.36 to 4.38. The number of generation, *n* obtained is 4 for 0.25 vvm, 3.4 for 0.50 vvm and 2.2 for 0.75 vvm. As a result, the maximum generation time, *g* is 6.10 hours for 0.25 vvm, 14.94 hours for 0.50 vvm and 17.78 hours for 0.75 vvm while the maximum growth rate constant, *k* is 0.31 hr^{-1} for 0.25 vvm, 0.23 hr^{-1} for 0.50 vvm and 0.29 hr^{-1} for 0.75 vvm. Therefore, the aeration rate of 0.50 is the optimum result obtained compared to the other two aeration rate, 0.25 vvm and 0.75 vvm.

Zlokarnik (1978) and Silva et al., (2012) reported that volumetric oxygen transfer will be affected by factors such as operating condition (speed of stirrer, gas flow), geometry of the reactor (stirring system, capacity, method of aeration) and material (diffusion constant, surface tension, specific gravity, viscosity, presence of dissolved substance such as salt).

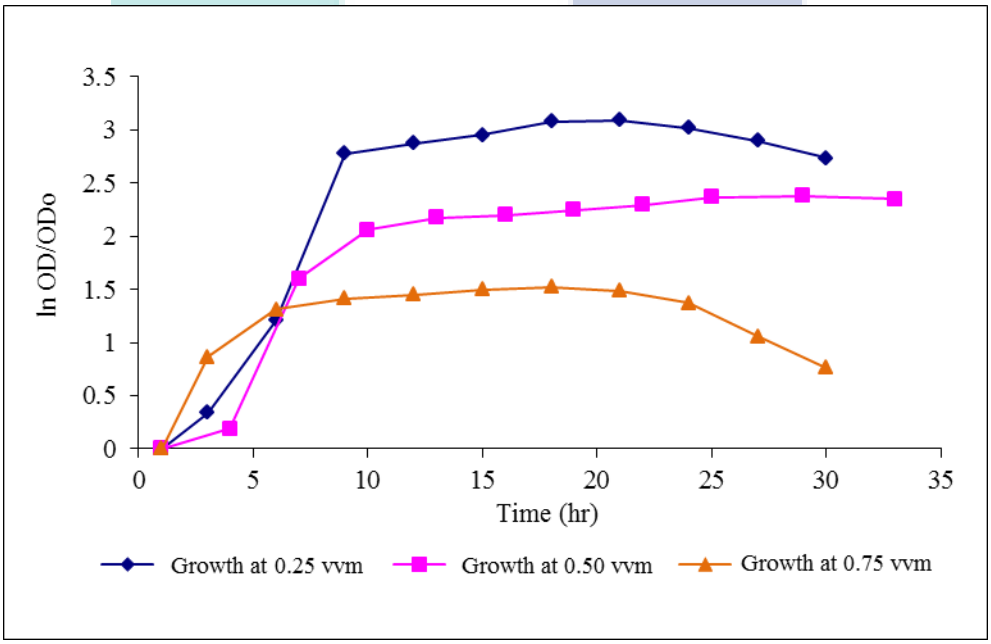


Figure 4.19: Effect of of aeration rates on the growth of *P. putida*

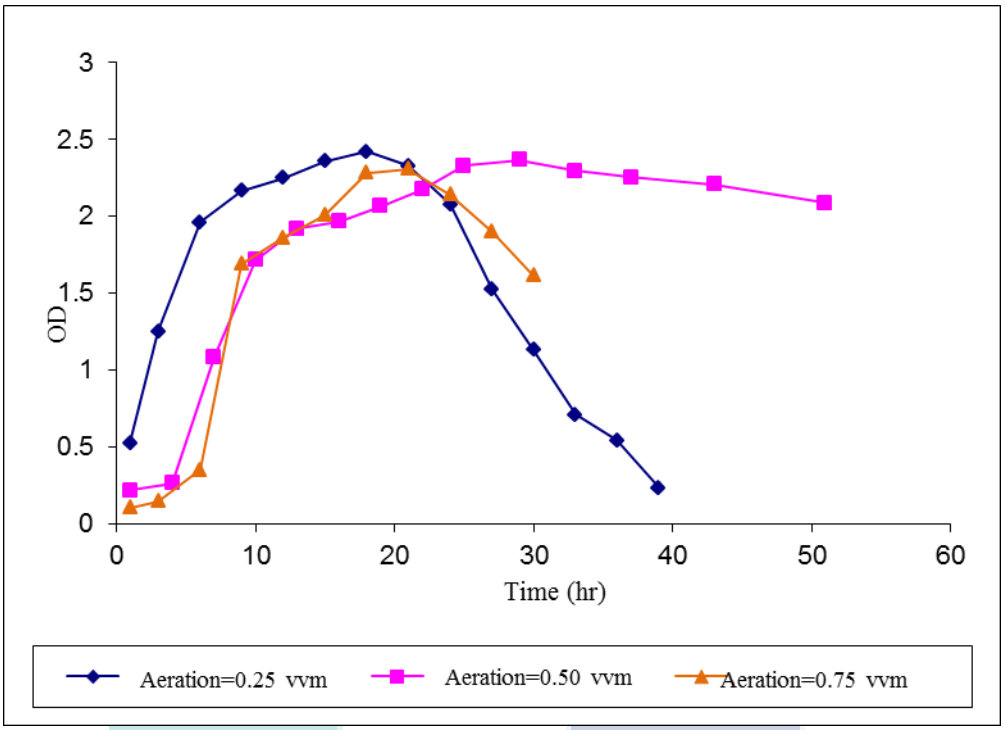


Figure 4.20: Effect of aeration rates on the growth of *P. putida* (OD vs Time)

4.5 MERCURY REMOVAL IN MEMBRANE BIOREACTOR USING MODEL WASTEWATER

Membrane bioreactor (MBR) is the combination of biological degradation process and membrane separation by using hollow fiber microfiltration into a single process where suspended solid and microorganisms responsible for the biodegradation are separated from the treated water by a membrane filtration unit. The entire biomass is confined within the systems, providing both perfect control of residence time for the microorganisms in the reactors (sludge age) and the disinfection of the effluent (Gao et al., 2009 and Judd, 2006).

4.5.1 Mercury Removal Using Hollow Fiber Microfiltration Membrane

Crossflow filtration is a process in which the formation of filter cake is limited by flow of suspension parallel to filtration surface and, because this system is pressurized,

water is forced through the filter. In this system particles deposited on the filter medium are swept away by the crossflow velocity actions. This parallel flow produces shear and lift forces on the particles as they become attached to the filter medium.

At the beginning of the investigation of mercury removal in membrane bioreactor, the experiments are conducted on de-ionized water in order to understand and observe the membrane performance in terms of flux and pressure applied. Before running the experiment, warm water is used to rinse the membrane with the addition of 100 000 ppb NaOCl to flush the water. Microfiltration membrane (pore size, 0.05 - 10.00 µm) from polysulfone polymer in hollow fiber cartridge, Xampler Cartridge (CFP-2-3MA) with pore size 0.20 µm and membrane area 110.00 cm^2 (0.01 m^2) in the Quixstand system is used in this study.

The plot of permeate flux as a function of inlet pressure (P_{inlet}) for de-ionized water is shown in Figure 4.20. From the data, it is observed that the permeate flux was seen to start at 512.70 L/m^2h with P_{inlet} of 3.00 psi and then increased rapidly increased to 2372.70 L/m^2h with 19 psi.

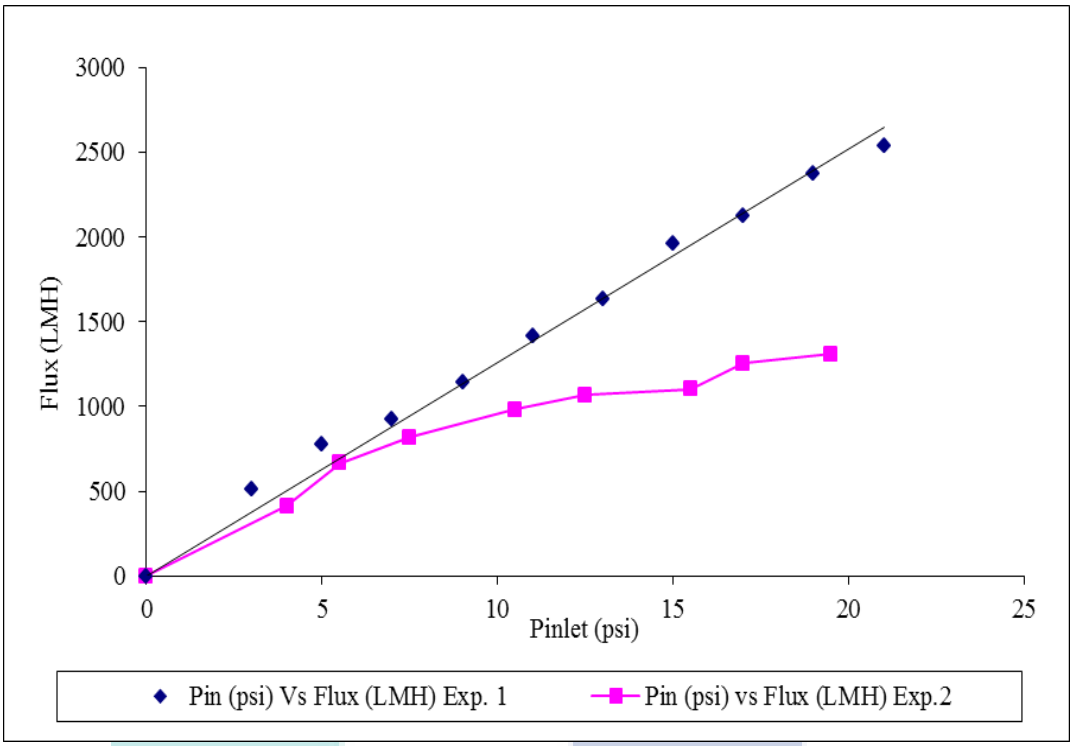


Figure 4.21: Permeate flux for de-ionized water (Exp. 1) and Hg solution at 14000 ppb (Exp. 2) with respect to inlet pressure, P_{inlet}.

Figure 4.20 also shows the effect of adding mercury solution of concentration 14 000 ppb to P_{inlet} and permeate flux. It is found that the initial permeate flux is 414.50 L/m^{2}h with P_{inlet} of 4.00 psi and the flux kept increasing up to 1309.10 L/m^{2}h with 19.5 psi running pressure. The results indicate that by adding high mercury concentration, the permeate flux dropped almost half (45%) as compared to the permeate flux of de-ionized water at the same P_{in}. It is noted that, during the filtration process, mercury ion is retained at the membrane. As a result, the accumulation is visualized as concentration polarization which increased the total filtration resistance, reduced the flux and altered the membrane separation properties due to pore plugging, pore narrowing or the growth of a fouling layer (Stephenson et al., 2000 and Dizge et al, 2011).

The drop in the flux also may be attributed to either a decrease in the useful membrane area (due to clogging of membrane pores) as mentioned earlier or an increase in

hydraulic resistance to filtration. This increase in hydraulic resistance can be caused either by narrowing of the pores (in depth clogging) or by concentration polarization on the membrane surface. This total hydraulic resistance comprises resistance caused by internal membrane fouling, and resistance caused by deposition of particles and/or colloids on the membrane surface (Al-Malack and Anderson, 1996). In this case, the membrane cleaning process was applied by using back-flushing method as well as chemical cleaning with 100 000 ppb NaOCl to minimize fouling effect and the drop of the flux value.

Nevertheless, the cross-flow filtration is a process design where a tangential flow is forced along the filter surface (Al-Malack and Anderson, 1996a). Not only diffusive but also convective back-transport in the optimal situations can keep the boundary layer above the surface of the filter free from accumulated foulants (Melin et al., 2005). Generally, however, the cross-flow is not capable of keeping the filter free from accumulated foulants and some material gathers in the vicinity of the membrane or adsorbs onto the membrane. Some adsorption is even inevitable.

The efficiency of crossflow microfiltration is primarily a function of operating parameters. This efficiency is measured by the filtrate flow rate (flux) and its quality. Besides that, Crossflow velocity, transmembrane pressure, temperature, pore size of the membrane, and feed concentration were reported to affect the performance of crossflow microfiltration (Milisic, 1986, Mulder and 1991, Ripperger, 1989).

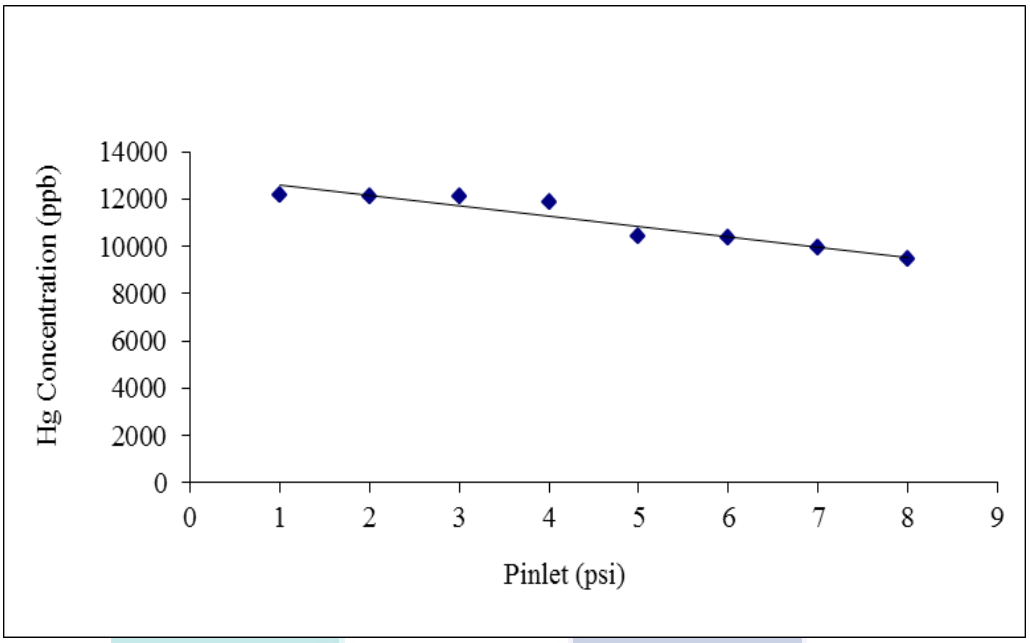


Figure 4.22: Mercury (14000 ppb) removal using membrane microfiltration system

The removal efficiency of mercury solution (14000 ppb) started at about 13% with 4.00 - 7.50 psi inlet pressure. By the end of the run, it had increased to approximately 33.3% with 9 – 15 psi inlet pressure as presented in Figure 4.21. The mercury concentration in the permeate is still more than 9000 ppb at the end of the experiment. It is clear that the membrane microfiltration alone is not capable of removing mercury at high concentration. Micheals (1989) reviewed the factors influencing the selection of crossflow microfiltration and stated that the properties to be considered in the filter media selection and testing should include: chemical compatibility, cleanability, service life, flux and cost.

The improvement in the process performance could largely be attributed to the introduction of a pretreatment process such as a biological treatment, like the membrane bioreactor to be implemented. The used of membrane filtration in this case is for the separation of cells, cell debris and particles from a solution. Besides that, the function of the membrane is to separate the treated wastewater from the active biomass in an aerobic bioreactor (Brady et al., 1994 and Chung et al., 2005), to reduce turbidity by removing

residual suspended solids, and to reduce bacteria to condition the water for effective disinfection (Stephenson et al., 2000).

Microfiltration (MF) membranes are used for the separation of cells, cell debris and particles from a solution (Porter et al., 2005). Polymeric MF membranes are much porous than UF membranes, commonly are of symmetric structure with rougher surface and tortuous paths rather than defined pores. MF membranes are in general are characterized by their nominal) pore size, ranging from 0.10 to 5.00 microns (Broom et al., 1994 and Al-Malack and Anderson, 1996a). In this study, MF membranes have been used in the MBR. The membrane functions to separate the treated wastewater from the active biomass in an aerobic bioreactor (Brady et al., 1994 and Chung et al., 2005).

In advanced treatment applications, MF has been most commonly used as a replacement for depth filtration to reduce turbidity, remove residual suspended solids, and reduce bacteria to condition the water for effective disinfection (Stephenson et al., 2000). The characteristics and typical operating information for MF membrane technologies used for wastewater including operating pressures and flux rates are stated in Appendix 2.13

Porous MF membranes are capable of removing only suspended materials, generally down to around 0.05 µm in size. Due to the operational cost, it is the porous membranes that are used in MBRs to retain the suspended solid materials, mainly biomass, within the reactor treatment without requiring further purification. (Meng et al., 2005).

4.5.2 Effect of Mercury Concentration on Growth of *P. putida*

Most of mercury remediation through common physico-chemical technologies is ineffective, especially when metal ion concentration in aqueous solution is in the range of 10 000 ppb to 100 000 ppb (Volesky, 2001). Bioremediation is often considered a cost effective and environmental friendly method and is gradually making inroads for environmental clean-up application (Wang and Chen, 2006).

From the studies carried out on mercury contaminated wastewater at the two petroleum based industrial wastewater plants, the maximum mercury concentration measured are 1877 ppb and 2318 ppb. In this study, these concentrations are utilized as a reference in the preparation synthetic or model wastewater. Mercury concentration in model wastewater was prepared at the range of 1000 ppb and 3000 ppb but the actual readings recorded by the mercury analyzer are 1300 ppb and 3000 ppb. Hence, the concentration of mercury the model wastewater is still within the range of mercury contamination at the two petroleum based industrial plants.

The effect of *P. putida* on mercury (1300 ppb) was investigated for more than 48 hours at optimum operating condition with 24 hours at 30^{o}C in incubator oven, agitator speed of 180 rpm, temperature of 37^{o}C, pH 7, nutrient concentration of 8 g/L and aeration of 0.50 vvm for 14 hours. Results of parameters related to *P. putida* growth behavior are presented in Table 4.18 and a plot of *P. putida* growth and mercury removal is presented in Figure 4.22. In the lag phase, it can be seen that *P. putida* immediately grew after inoculation. It is also noted that, mercury is reduced dramatically (88.46%) in this experiment. Summary of the results are shown at Table 4.18.

Table 4.18: Mercury (1300 ppb) removal by *P. putida* in bioreactor

Growth Parameter	Min	Max
Specific Growth Rate, μ, hr^{-1}	0.09	-
OD	0.40	2.57
Exponential cell growth, (ln OD/OD$_0$)	0.01	0.27
Number Of Generation, *n*	0.011	0.90
Generation Time, *g* (hr)	18.76	63.00
Growth Rate Constant, *k* (hr^{-1})	0.01	0.04
Hg Removal (%)	88.46	-

As the mercury concentration is further decreased over time, *P. putida* showed increasing growth behavior and activity with higher cell density observed for the first 10 hours. Consequently, the OD and maximum exponential cell growth with 2.57 are obtained

with initial growth of 0.40. With the increasing cell density during the exponential phase, the specific growth rate, μ is 0.09 hr^{-1}.

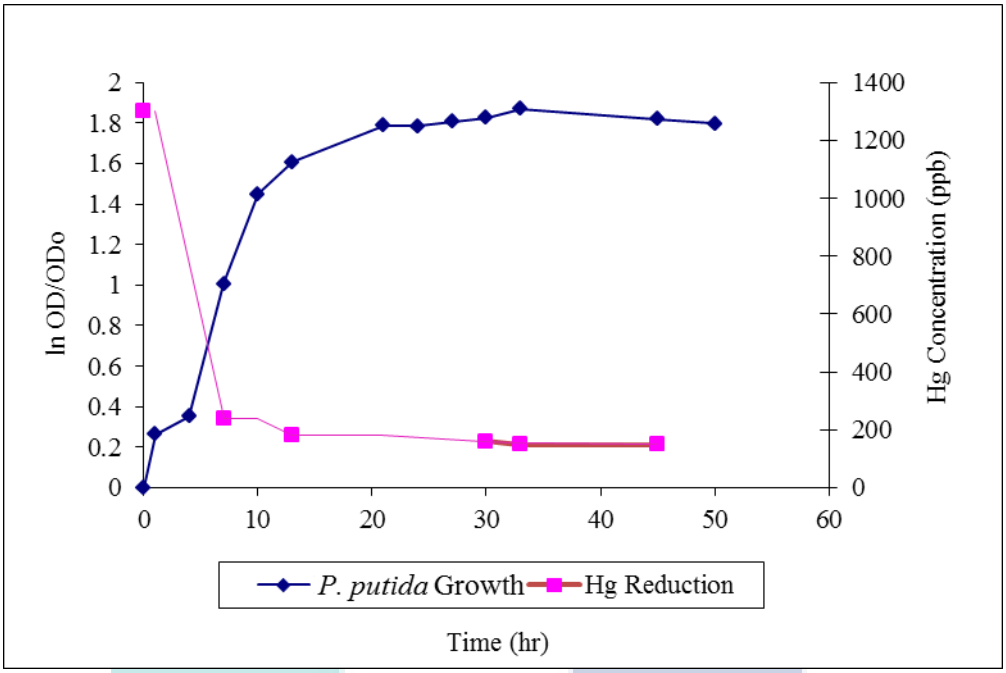


Figure 4.23: Effect of mercury (1300 ppb) to the growth of *P. putida*

Based on the results obtained on the growth behavior *P. putida* in the 2L bioreactor, it can be seen from Appendix 4.39 that number of generation, *n* obtained is 0.90. Then, based on the number of generation *n*, the generation time, *g* is calculated as 63.00 hours (Appendix 4.40). As a result, the growth rate constant, *k* is 0.04 hr^{-1} (Appendix 4.41).

Table 4.19: Mercury (3 000 ppb) removal by *P. putida* in bioreactor

Growth Parameter	Min	Max
Specific Growth Rate, μ (hr^{-1})	0.20	-
OD	0.24	3.00
Exponential cell growth, (ln OD/OD_0)	0.06	1.88
Number Of Generation, *n*	0.64	0.90
Generation Time, *g* (hr)	4.65	11.51
Growth Rate Constant, *k* (hr^{-1})	0.06	0.15
Hg Removal (%)	94.00	-

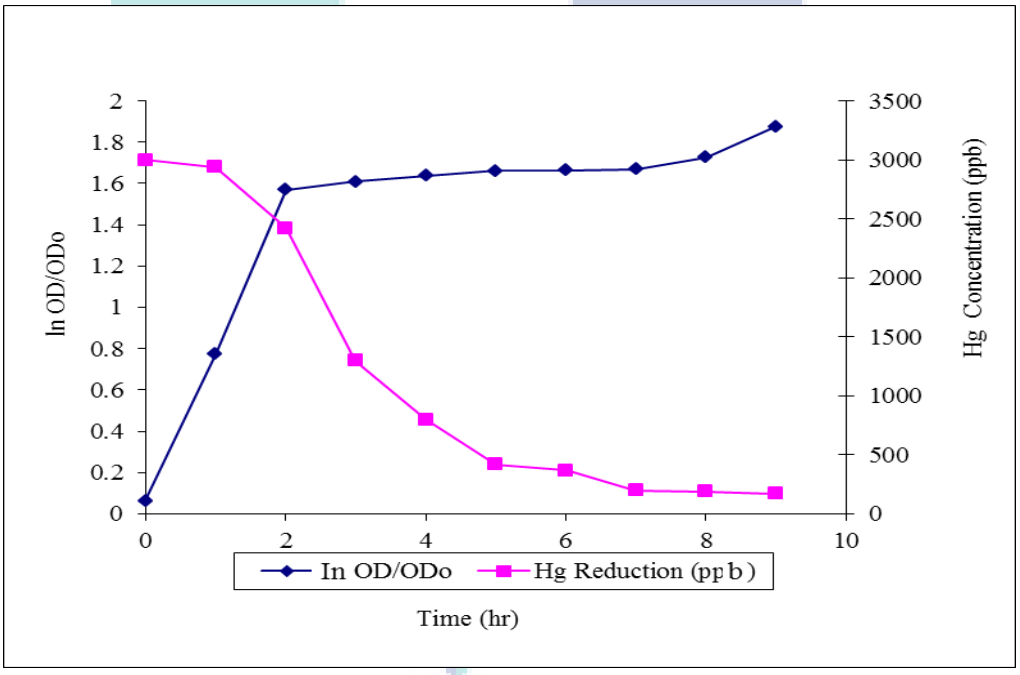


Figure 4.24: Effect of mercury (3 000 ppb) to the growth of *P. putida*

The effect of mercury removal at 3000 ppb concentration to *P. putida* growth over time is shown in Table 4.19. Experiments are conducted at optimum operating condition for less than 15 hours. Figure 4.23 shows the *P. putida* growth behavior and the reduction of

mercury concentration. Similarly as observed from previous study, there is no occurrence of lag phases occurred, *P. putida* immediately grow exponentially after inoculation. However mercury levels decreased to almost 94% after less than 8 hours. With the decrease in mercury concentration, *P. putida* shows an increasing growth and the maximum cell density is obtained after 2 hours of experiment. As a result, the maximum exponential cell growth is 1.88. Furthermore, it is found that after this period, cell growth is constant and slightly increased over time.

The specific growth rate, μ is 0.20 hr^{-1}, which is higher than the previous experiment. Consequently, it is found from the graph that the maximum number of generation, *n* obtained is consistently at 0.90 (Appendix 4.42) the generation time, *g* is 11.51 hours (Appendix 4.43) and the maximum growth rate constant, *k* is 0.15 hr^{-1} (Appendix 4.44). These parameters indicate slightly better performance at 88.46% compared to that obtained from the study carried out for 1300 ppb mercury a concentration i.e. but it was still considered good performance.

From both studies, the investigation obviously demonstrates that using higher mercury concentration at certain level will result in an improvement in the percentage of mercury removal by *P. putida*. Meanwhile, the percentage of mercury removal for mercury concentration of 10 000 ppb is 80% as reported by Mortazavi et al., (2005). Comparing with the results reported in the literature review, the performance at 88.64% mercury removal for 1300 ppb and 94% removal for 3000 ppb are still acceptable. Meanwhile, Green-Ruiz (2006) reported that for mercury concentration between 1000 ppb to 2500 ppb, the mercury removal performance of bioremediation using *Bacillus sp*. was in the range of 78% to 88%.

4.6 MERCURY REMOVAL FROM PETROLEUM BASED INDUSTRIES WASTEWATER BY *P. putida*

Today, stringent legislation requires intensive treatment of petroleum based industries wastewater in order to fulfill the discharge limit requirement. Therefore, studies

on the treatment of petroleum based industries wastewater at two different plants were carried out by using mercury resistant microorganism i.e. *P. putida* in an effort to develop an environmental friendly, cost effective, integrated, end-of-pipe remediation technology.

4.6.1 Plant 1

Plant 1 is located in the East Coast approximately 110.00 km south of Kuala Terengganu and in an industrialized area dedicated to oil and gas facilities and was the first production division with the commencement of gas production. This industry has been designed to accommodate gas and condensate and it is built with a facility to receive slugs and separation of two phases of fluid received from the Platform Collector.

Here, the analysis whether or not actual petroleum based industrial plant 1 effluents could be treated with *P. putida* was carried out by determining the mercury concentration in the treated wastewater. A very low mercury concentration of 0.01 ppb was found in the wastewater at location 1 (P1L1), when the sampling was carried out during normal production. Therefore another 1000 ppb mercury solution was added into the sample to wastewater and a study on mercury removal at 1000 ppb was performed at pH 7 with temperature at 37^{o}C and experiment was conducted in orbital shaker at 180 rpm for 4 hours.

In view of characteristics of petroleum based industries wastewater, the study was carried out in a shake flask at the optimum operating condition as previously described. The solution containing mercury was adjusted at the desired pH and three types of samples were prepared as follows:

- Sample A: 20 ml (Nutrient broth, NB + *P. putida*) added in to 180 ml NB
- Sample B: 20 ml (Nutrient broth, NB + *P. putida*) added in to 180 ml wastewater
- Sample C: 20 ml (Nutrient broth, NB + *P. putida*) added in to 180 ml wastewater with 1000 ppb mercury

Table 4.20: Growth behavior of *P. putida* in different media condition for sample A, sample B and sample C.

Growth Parameter	**Min**			**Max**		
Sample	A	B	C	A	B	C
Specific Growth Rate ,μ hr^{-1}	0.83	0.47	0.68			
OD	0.07	0.08	0.06	1.52	0.46	0.86
Exponential cell growth, (ln OD/OD_0)	0.25	0.06	0.08	3.07	1.73	2.66
Number of Generation, *n*	0.36	0.08	0.11	4.40	2.48	3.81
Generation Time, g(hr)	0.68	1.54	0.64	1.41	5.90	1.14
Growth Rate Constant, *k* (hr^{-1})	0.49	0.11	0.16	1.01	0.45	1.09
Hg Removal(%)			84 (4 hr)			90.5 (96 hr)

From the results obtained for sample A, sample B and sample C as shown in Table 4.20, a growth behavior of *P. putida* in different media condition can be produced as presented in Figure 4.24. In the lag phase, *P. putida* took quite sometimes to grow after inoculation especially in the petroleum based industries wastewater (sample B and sample C). It is therefore the maximum optical density, OD for sample A shows that the highest value i.e. 1.52 followed by sample C i.e. 0.86 and finally sample B i.e. 0.462. As a result, the exponential growth obtained was 3.01 for sample A, 2.66 for sample C and 1.73 for sample B. It is evident that the presence of mercury in the sample wastewater affects the kinetics of *P. putida* with higher value of specific growth rate, μ of 0.67 hr^{-1} for sample C and 0.47 hr^{-1} for sample B. In this case, sample A, as the control experimental, has the highest specific growth rate, μ, of 0.83 hr^{-1}.

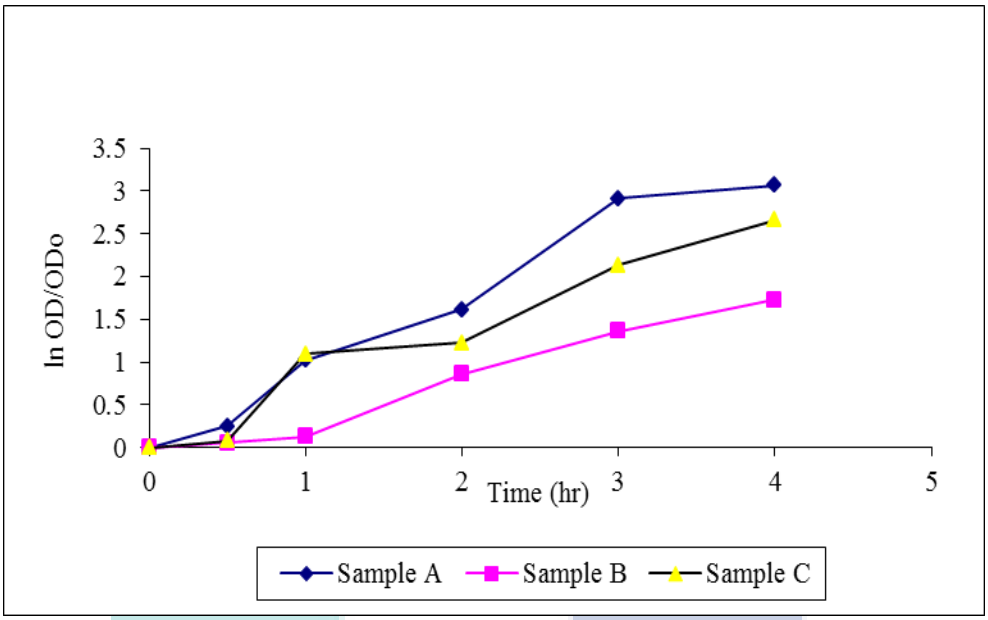


Figure 4.25: The growth of *P. putida* in petroleum based industries wastewater

On further observation of the *P. putida* growth behavior in petroleum based industries wastewater, it can be seen from Appendix 4.45 that the maximum number of generation, *n* obtained is 4.40 for sample A, 2.48 for sample B and 3.81 for sample C. The maximum generation time, *g* is 1.41 hours for sample A as presented at Appendix 4.46, 5.90 hours for sample B and 1.14 hours for sample C. As a result, the growth rate constant, *k* is 1.01 hr^{-1} for sample A, 0.45 hr^{-1} for sample B and 1.09 hr^{-1} for sample C, as shown in Appendix 4.47.

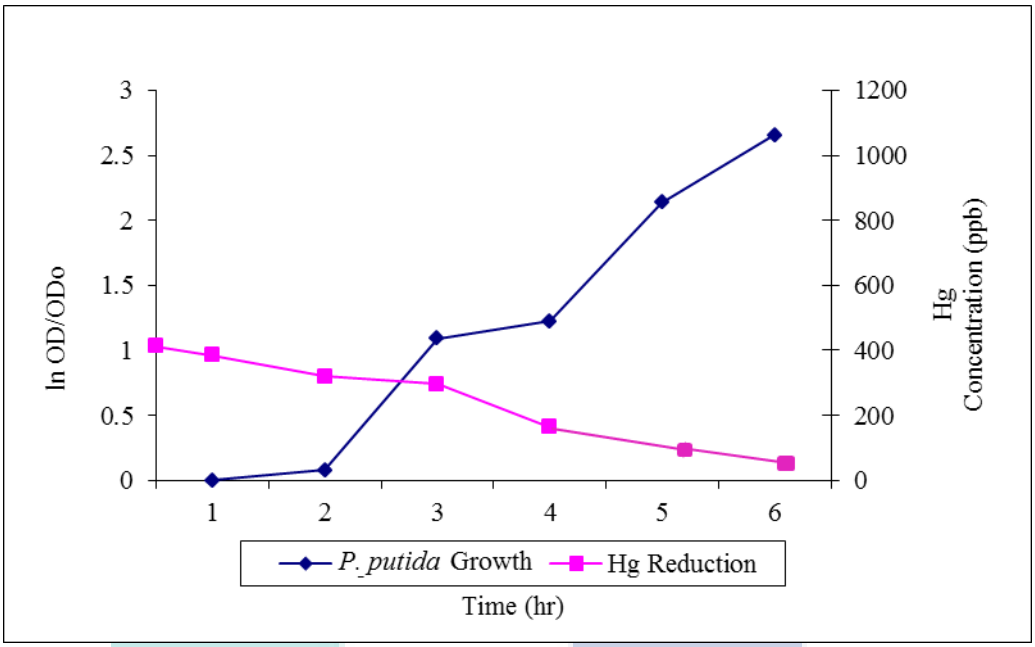


Figure 4.26: Mercury (1000 ppb) removal from petroleum based industries wastewater by *P. putida* at optimum operating condition in the shake flask

Figure 4.25 illustrated the mercury removal from petroleum based industries wastewater by *P. putida*. The efficiency of mercury removal increased from 84 % after 4.00 hours of experiment to 90.5% after 96 hours (Appendix 4.48). These results agree with the results reported by Green-Ruiz (2006), where he disclosed that the removal of mercury from wastewater appeared to be more efficient at a lower metal concentration (92% for 250 ppb) than at the highest (68.5% for 10 000 ppb). In addition, Volesky (1990) reported that alternative conventional techniques may be ineffective for low mercury concentration in the wastewater that is less than 100 000 ppb.

4.6.2 Plant 2

The petroleum based industrial Plant 2 is a refinery plant which was located 90 km from Kuala Lumpur at Southern of Peninsular Malaysia and the main processes carried out at the plant are crude distillation unit, naphtha hydro-treating unit, catalytic, reforming unit with continuous catalytic regenerator, saturated gas concentration unit, sour water stripping

unit, kerosene and heavy naphtha treating unit and mercury removal unit. The three main components of wastewater are large volume of cooling water, proves wastes from refineries and sanitary waste. In this case study, the samples were collected at the same location as in the previous case studies; the collections were conducted during the normal production and the actual wastewater samples were used for this study.

Contaminated water containing mercury often varies in chemicals and physical parameters, such as pH and the presence of organic and inorganic components. All this factors can affect mercury removal. An ideal mercury cleanup process, therefore, requires removal of mercury at low concentrations and flexibility in dealing with variations in ambient conditions.

In this experiment, the wastewater parameters such as pH, temperature, total dissolved solid (TDS), suspended solid (SS) and turbidity are first determined for petroleum based industrial Plant 2. Then, the actual sample of petroleum based industries wastewater are treated to determine the mercury retention efficiency and wastewater parameters of the microbial detoxification system.

Based on petroleum based industries wastewater analysis, the wastewater is found to contain 22.00 ppb mercury at pH 6.09, temperature at 25^{o}C, with total dissolved solid (TDS) was 260.00 mg/L, total suspended solid (TSS) at 27.33 mg/L and turbidity of 68.30 NTU. Although the concentration of mercury is lower than the requirement of DOE under Standard B at 50 ppb, this concentration is higher than Standard A at 5 ppb. However, to obtain actual wastewater from petroleum based industries with high mercury concentration during the normal production as required for purposed of this experiment, is considered impossible due to the well maintained system operation with the mercury removal unit (MRU) is place.

In order to study the mercury (22 ppb) removal from petroleum based industries wastewater by *P. putida*, the experiments were conducted in a shake flask at optimum operating condition as previously described for 52 hours at optimum condition for the

growth of *P. putida*. Composition of wastewater samples from the petroleum based industrial Plant B prepared are as follow:

(i) *P. putida* + Wastewater
(ii) *P. putida* + Nutrient Broth (NB)
(iii) Wastewater only
(iv) Wastewater + Nutrient Broth (NB)
(v) Wastewater + Nutrient Broth (NB) + *P. putida*

Table 4.21: Mercury removal by *P. putida* from petroleum based industrial Plant P2 wastewater

Growth Parameter	**Min**					**Max**				
Sample	i	ii	iii	iv	v	i	ii	iii	iv	v
Specific Growth Rate, μ(hr^{-1})	0.02	0.05	0.02	0.11	0.11	-	-	-	-	-
OD	0.14	0.18	0.01	0.01	0.01	0.55	2.34	0.03	2.28	2.68
Exponential cell growth, (ln OD/OD_0)	0.45	0.11	0.00	0.26	0.88	1.40	2.55	0.90	5.43	5.59
Number of Generation, *n*	0.64	0.16	0.00	0.38	1.25	2.00	3.65	1.29	7.78	8.01
Generation Time, *g* (hr)	0.08	0.05	0.00	0.10	0.04	26.63	18.44	4.46	7.98	6.60
Growth Rate Constant, *k* (hr^{-1})	1.56	2.26	0.00	0.52	6.30	8.86	1.35	0.15	6.88	17.88
Hg Removal (%)	94.09	-	12.73	56.82	97.27	-	-	-	-	-

The results of the experiments for sample (i), sample (ii), sample (iii), sample (iv) and sample (v) are presented in Table 4.21. The growth behavior of *P. putida* in these media condition is shown in Figure 4.26. These results are obtained when the medium is at pH of about 7 and temperature at 37^{o}C. As a result, the specific growth rate, μ obtained are 0.02 hr^{-1} for sample (i), 0.05 hr^{-1} for sample (ii), 0.02 hr^{-1} for sample (iii), 0.12 hr^{-1} for sample (iv) and 0.11 hr^{-1} for sample (v).

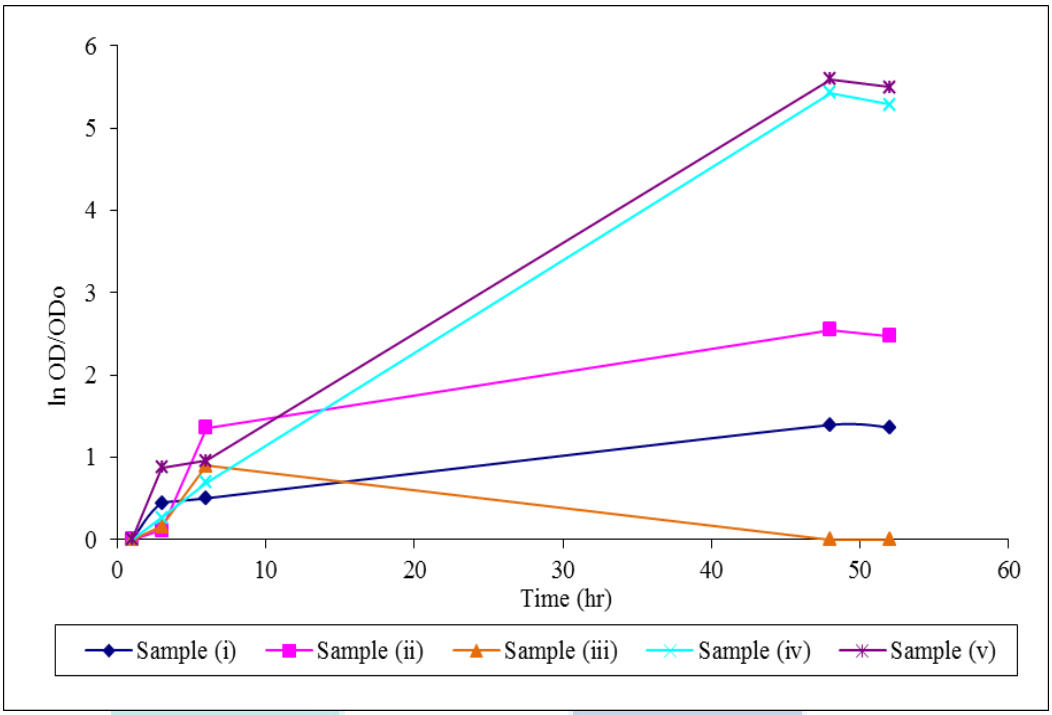


Figure 4.27: *P. putida* and other natural microbe growth in petroleum based industries wastewater

The summary of the other parameters related to the growth of *P. putida* are illustrated in Appendix 4.49 for number of generation, *n*, Appendix 4.50 for generation time, *g* and Appendix 4.51 for growth rate constant, *k*.

The results indicate that in the presence of nutrient broth with 8 g/L concentration samples (ii), (iv) and (v) showed high cell activity with increasing optical density and the exponential growth especially for samples (iv) and (v) where the nutrient was added in wastewater sample. Moreover, it is noted that the pattern of results obtained from sample (iv) and (v) are quite similar and are consistent with the cell growth behavior as has been indicated. However based on data analysis, mercury removal for sample (iv) is 56.82% compared 97.27% for sample (v).

In this situation, the efficiency of mercury removal in sample (iv) at 56.80 % is lower than sample (v) at 97.27%, as observed in Figure 4.27. High efficiency of mercury removal is noted in sample (i) and (v) at 94.09% and 97.27% whereby both samples were added with *P. putida*. It is also observed that higher removal of mercury and higher *P. putida* growth are seen in sample (v). These findings show that substrate interaction is involved in the experiment, and also proves the function of *P. putida* in removing mercury ion by bioremediation process. Furthermore, the increasing *P. putida* growth is due to nutrient being consumed during the mercury detoxification phase. These observations are consistent with previous studies using *Pseudomonas aeruginosa* (Chang and Hong, 1994).

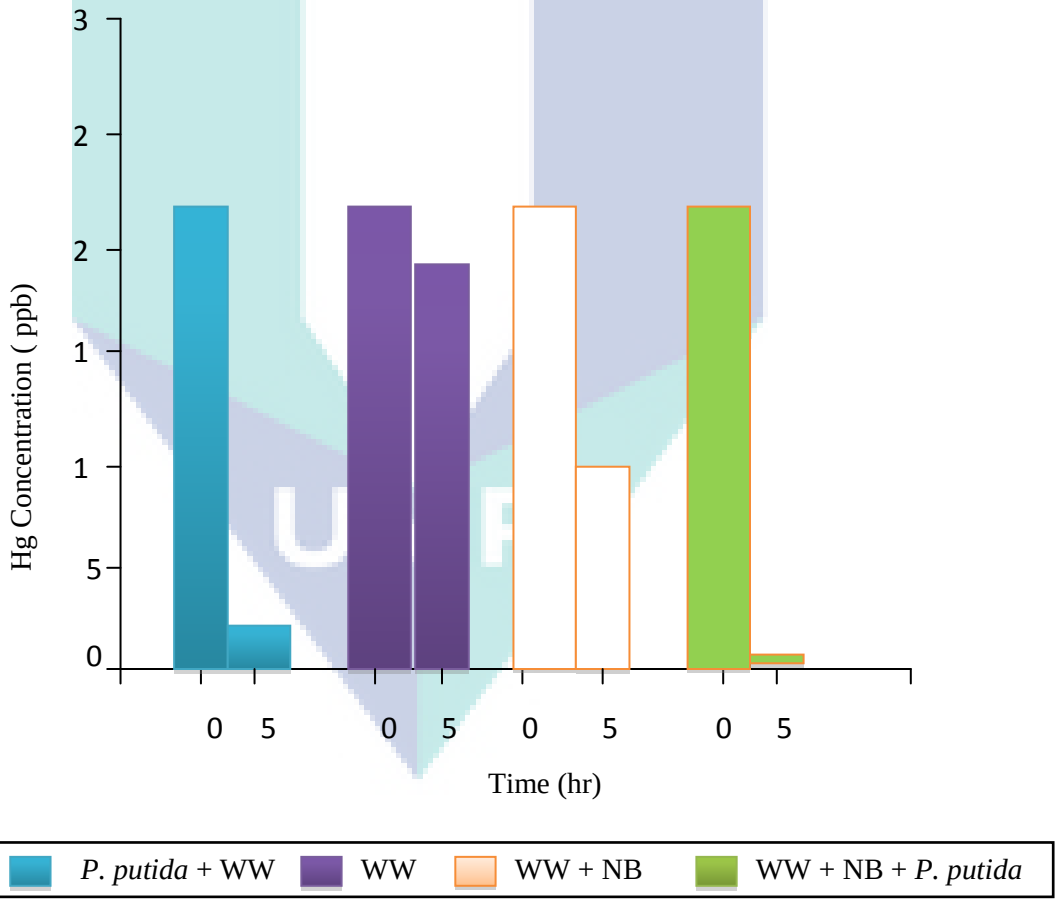


Figure 4.28: Mercury removal by *P. putida* in petroleum based industries wastewater

Using *P. putida*, mercury removal is highly selective, efficient and can minimize the volume of mercury contaminated by-products without requiring other method of disposal that cause extra operational cost. Mercury removal by *P. putida* involves the phenomenon of bioaccumulation and biosorption based on the state of biomass. Bioaccumulation is defined as the phenomenon of living cells, whereas biosorption mechanism is based on the use of dead biomass. The uptake of mercury as toxicants can be considered as bioaccumulation. The mercury can be transported into the cell, accumulated intracellularly, across the cell membrane and through the cell metabolic cycle (Malik, 2004).The reduction of Hg^{2+} to eliminated mercury, Hg^{0} is carried out by mercury reductase (Mer A) enzymes released by *P. putida* (Barkay et al., 2003). Hg^{0} is subsequently removed either through mercury vapor sorption onto various materials or filtration process.

Furthermore, research has revealed that inactive microbial biomass can passively bind metal ions involved in the biosorption process (Veglio and Beolchini, 1997). The bacteria cell wall is the first component that comes into contact with mercury ion, where the solutes can be deposited on the surface or within the cell wall structure (Beveridge and Murray, 1976, Doyle et al., 1980). Since the mode of solute uptake by inactive cells is extracellular, the chemical functional groups of the cell wall play vital roles in biosorption. However, most the mechanism for biosorption has not been discussed in the reports of previous studies.

4.6.3 Petroleum Based Industries Wastewater Quality Analysis

Appendix 4.52 illustrates the degree of water quality parameters presented by Metcalf and Eddy (2003) and it shows the degree of concentration for total dissolved solid and suspended solid. The table is used as a reference of indicator of water quality for this study as well as the requirement by Environmental Quality Act (EQA) 2011, Environmental Quality Sewage and Industrial Effluents for Standard B.

i. Total Dissolved Solid

The effect of *P. putida* growth on the total dissolved solid in the wastewater of petroleum based industries and the degree of concentration is presented in Table 4.22. As previously described, total dissolved solid is a measure of the combined content of all inorganic substances contained in the liquid in molecular, ionized or micro-granular (colloidal sol) suspended form.

Table 4.22: Petroleum based industries wastewater (WW) total dissolved solid with respect to the treatment

Sample Petroleum Based Industries Wastewater (WW)	Before Treatment (mg/L)	After Treatment (mg/L)	Degree of Concentration (Metcalf and Eddy, 2003)
(i) *P. putida* + WW	260.00	430.00	Low
(ii) *P. putida* + NB	260.00	1800.00	High
(iii) WW	260.00	250.00	Very low
(iv) WW + NB	260.00	1860.00	High
(v) WW + NB + *P. putida*	260.00	2150.00	Very high

These results indicated that with the supplementation of nutrient broth (NB) to the system, the total dissolved solid showed an increasing trend as a function of time throughout the study, showing a significant change in the sample media cell as illustrated in Figure 4.28. However, it is noted that for sample (iii), total dissolved solid in the media almost reach a stable value of 250.00 mg/L.

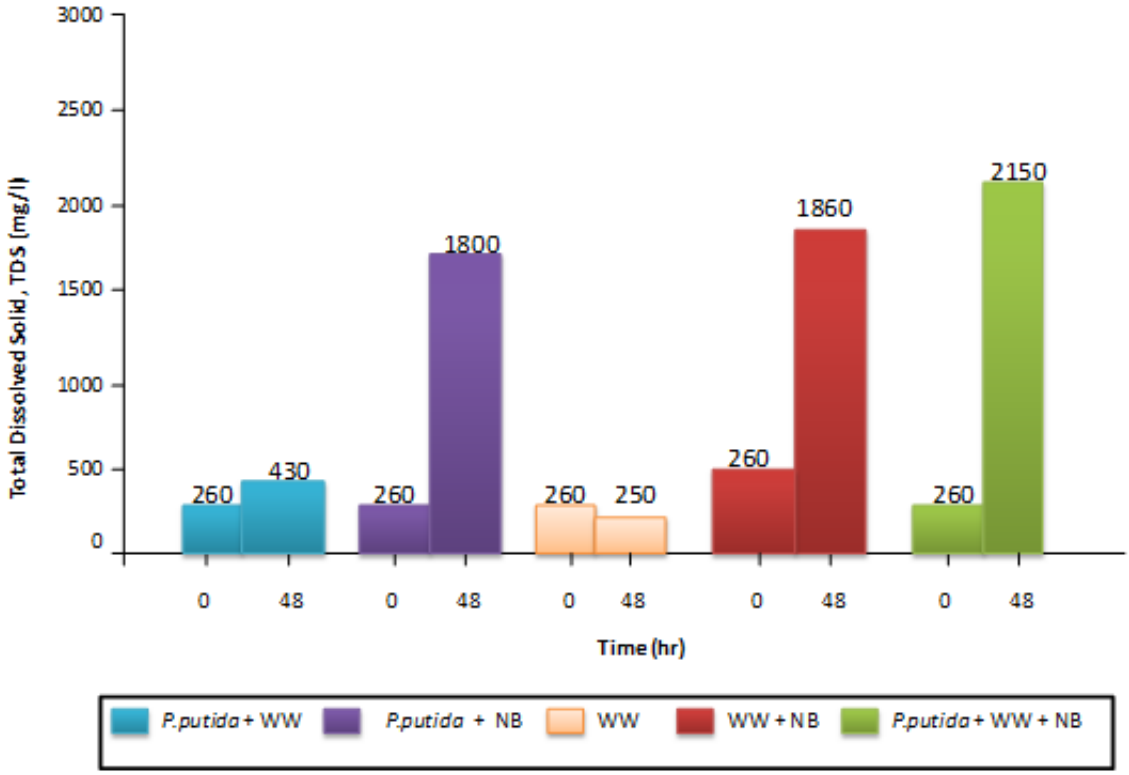


Figure 4.29: Effect of *P. putida* growth to total dissolved solid of petroleum based industries wastewater

Meanwhile sample (i) showed a slight increased of total dissolved solid to 430.00 mg/L. High total dissolved solid levels of 2150.00 mg/L is observed in sample (v), followed by sample (iv) and (ii). Referring to the degree of concentration proposed by Metcalf and Eddy (2003), sample (v) shows a very high concentration, followed by sample (iv) and (ii), while sample (i) is low and finally sample (ii) was very low. The results indicate that the phenomenon of bioaccumulation and biosorption obviously occurred in sample (v). Therefore, the increase of living cells and inactive biological materials can be seen from the increase of total dissolved solid in the media (Vijayaraghavan and Yun, 2008).

The nutrients are formed of natural organic matter (NOM) and the addition of NB to media contributed to the increasing total dissolved solid. Thus, the less organic matter in

the water, the better is the water quality. This finding shows the need a filtration system such as membrane filtration in order to improve the quality of the water.

ii. Suspended Solid (SS)

Suspended solid is defined as the solid discharged to water and settled downstream, the rate depending on the size of the particles and the turbulence. Settlement especially occurs where the turbulence is the lowest (Gray, 2005). Solids suspended in water may consist of inorganic or organic particles or immiscible liquids. However, if the concentrations are enhanced through, this can lead to alterations to physical, chemical and biological properties of the water body (Bilotta and Brazier, 2008).

Physical alteration caused by suspended solids include reduced penetration of light, temperature changes, and infilling of channels and reservoirs when solids are deposited. These physical alteration are associated with undesirable aesthetic effects (Lloyd and Lovly, 2001), higher costs of water treatment, reduced navigability of channels and decreased the longevity of dams and reservoirs. Furthermore, where the suspended solids have high organic content, the *in-situ* decomposition can deplete levels of dissolved oxygen in the water, producing a critical oxygen shortage which can lead to fish kills during low-flow conditions.

Table 4.23: Petroleum based industries wastewater (WW) suspended solid with respect to the treatment

Sample of Petroleum Based Industries Wastewater (WW)	Before Treatment (mg/L)	After Treatment (mg/L)	Degree of Concentration (Metcalf & Eddy, 2003)
(i) *P. putida* + WW	27.33	0.54	Very low
(iv) WW + NB	27.33	1.62	Very low
(v) WW + NB + *P. putida*	27.33	1.12	Very low

Table 4.29 shows the suspended solids in wastewater over running time of 48.00 hours. It indicates that the suspended solid decreased from 27.33 mg/L to almost below 2.00 mg/L for sample (i), (iv) and (v) after treatment. The suspended solids in the rest of the samples were unchanged as illustrated in Figure 4.29. The value of suspended solids obtained are categorized as very low (Metcalf and Eddy, 2003) since it is lower than the requirement by Environmental Quality Act (EQA) 2011, Environmental Quality Sewage and Industrial Effluents for Standard B which is 100.00 mg/L.

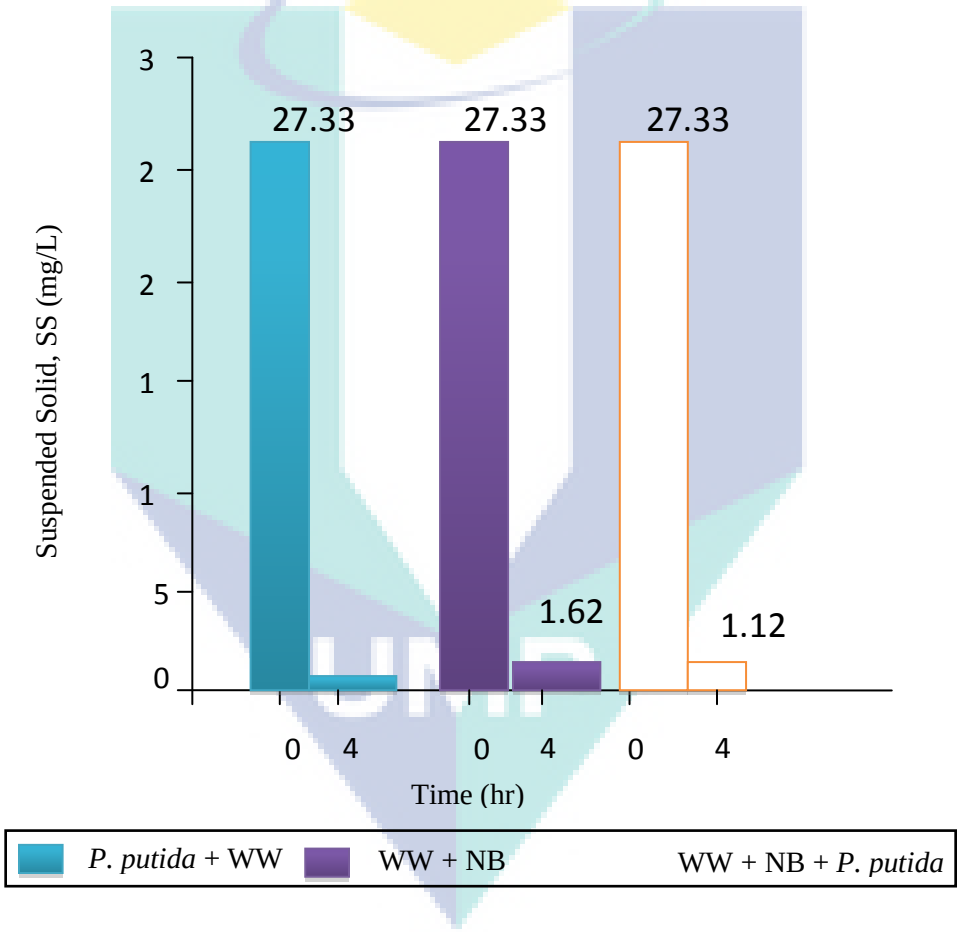


Figure 4.30: Effect of *P. putida* growth to suspended solid of petroleum based industries wastewater

P. putida and NB have been shown here to have a significant impact on wastewater by improving removal of suspended solids in the range of 96% – 98%. More degradation of suspended organic solids in samples (iv) and (v) could be achieved due to the increasing

of *P. putida* growth and living cells in the wastewater sample. Lloyd and Lovly (2001), reported that total suspended solid more than 8 mg/L will affect 3% – 13% reduction of primary productivity on organism. However, this study shows that suspended solid after the treatment by adding NB and *P. putida* is 1.12 mg/L or 95.9% removal and by adding NB is 1.62 mg/L or 94.07% removal. However, the result showed the suspended solid reduced at 98.02% for sample (i) due to the decreased nutrient concentration in the sample that resulted in low living cells activities. As a conclusion, these findings are still lower than was reported by Lyold and Lovly (2001) and under very low indication as stated by Metcalf and Eddy (2003).

High levels of suspended solids in transport by fast flow rates can act to scour these organisms away from streambed substrates as well as being abrasive and damaging to photosynthetic structures of organisms (Alabaster and Lyold, 1982). Suspended solids can indirectly affect the abundance of phytoplankton, periphyton and macrophytes through acting as a vector of nutrient such as phosphorus (Heathwaite, 1994), and toxic compounds such as mercury in pesticides from land surface to the water body (Kronvang et al., 2003).

iii. Turbidity

Turbidity is the murkiness in the water caused by colloidal (1 to 100 nm particles) and other suspended particles, such as clay, sand, silt, organic matter of plant and animal origin, planktons, and other microscopic organisms. Turbidity particles can be waterborne pathogens or particles harboring them (Sarai, 2006).

Turbidity is the measure of light scattering properties of water. It is typically measured using *in-situ* equipment which record the attenuation (i.e. attenuance turbidimeters - measure the loss in intensity of narrow parallel beam or dual beams) or scattering (i.e. nephelometric turbidimeters – measure light scattered at an angle to the beam), of a radiation beam (Lewis et al., 1996). Nephelometric turbidimeter has been most widely used, recording turbidity data in nephelometric turbidity units (NTU).

The effect of *P. putida* growth and the activity regarding to turbidity of petroleum based industries wastewater is illustrated in Figure 4.30 where the experiments were carried out for 48 hour in the shake flask at optimum operating condition.

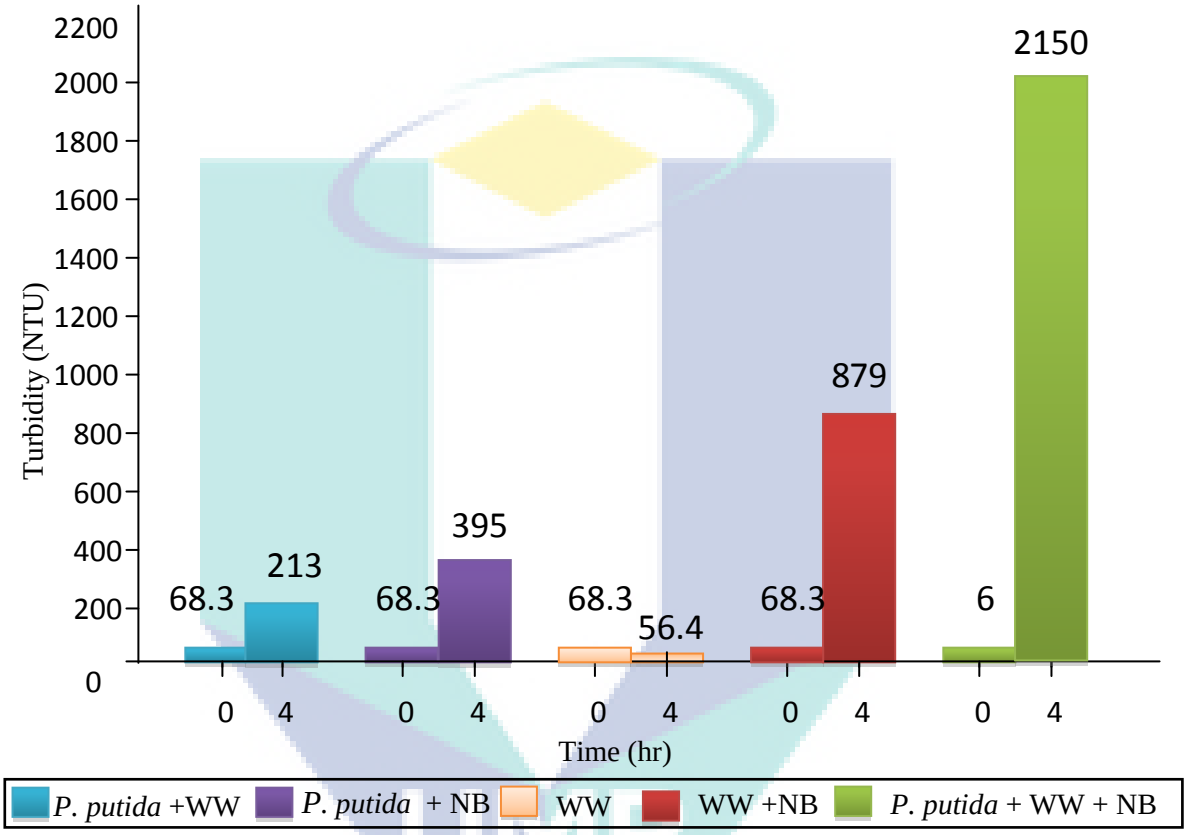


Figure 4.31: Effect of *P. putida* growth to turbidity of petroleum based industries wastewater

Turbidity below 0.30 NTU indicates very clear water and possibly the absence of any waterborne life cell or pathogens. The less the turbidity, the less the number of particles and less is the possibility of the presence of life cell or pathogen (Singh et al., 2008). The results show all the samples wastewater contained life cells which contribute to high turbidity of 68.30 NTU. In the presence of *P. putida* and NB, sample (v) shows the highest increment of turbidity value by more than 30 times. This is followed by sample (iv) that increased by approximately 13 times increased where only nutrient broth is introduced to

the sample. Turbidity value sample (ii) is 6 times increased and sample (i) were slightly increased by 3 times. Meanwhile sample (iii) remained unchanged as observed in Table 4.24 and this also can be clearly seen in Figure 4.30 as well. From Table 4.24, as presented by Metcalf and Eddy (2003) shows that the degree of turbidity is considered in the very low range.

Table 4.24: Petroleum based industries wastewater (WW) turbidity with respect to the treatment

Sample Petroleum Based Industries Wastewater (WW)	Before Treatment (NTU)	After Treatment (NTU)
(i) *P. putida* + WW	68.30	213.00
(ii) *P. putida* + NB	68.30	395.00
(iii) WW	68.30	56.40
(iv) WW + NB	68.30	879.00
(v) WW + NB + *P. putida*	68.30	2150.00

Turbidity readings are influenced by particle size and shape of suspended solids, the presence of *P. putida*, the presence of dissolved humic substance and the presence of minerals substance. Consequently, a high turbidity reading can be recorded without necessarily involving a high suspended solids concentration. Therefore, the exact cause of turbidity could not be known just by relying on turbidimeter data (Bilotta and Brazier, 2008). But in this case, the turbidity caused by NB and *P. putida* which corresponds well with the observation of a high number of suspended solids in the wastewater. This demonstrated that turbidity of the sample is largely caused by total dissolved solids and total suspended solids (Lapinski and Tunnacliffe, 2002). However, the amount of solids in suspensions will depend on the degree of agitation of the wastewater sample.

The result indicates that the phenomenon of bioaccumulation and biosorption also obviously occurred in the sample (v). Therefore, the increase in the number of living cells and inactive biological materials can be seen from the increase in the total dissolved solid in the media (Vijayaraghavan and Yun, 2008).

4.7 MERCURY REMOVAL FROM ACTUAL PETROLEUM BASED INDUSTRIES WASTEWATER BY *P. putida* IN MEMBRANE BIOREACTOR

From previous findings, mercury can be successfully removed by *P. putida*; however, high growth of *P. putida* could affect the amount of suspended solid in the wastewater. Hence, a separation method to remove the suspended solid is proposed and in this study, a membrane bioreactor is employed.

4.7.1 Mercury Removal and *P. putida* Growth Behavior

Petroleum based industries wastewater sample from location Plant P1L1 is added with 4000.00 ppb mercury and is treated for 120 hours by *P. putida* in a membrane bioreactor operated at optimum condition as described earlier. In addition to the previous operating parameters, flux of 120 LMH is observed with inlet pressure (P_{inlet}) of 11 psi, (P_{outlet}) is 1 psi and TMP is 5 psi. The feed flowrate is 0.22 L/min for the hollow fiber membrane polysulfone with membrane area of $0.011m^2$ and 0.20 um of pore size. Livingston (1994) reported that the function of the membrane is to separate wastewater from cell medium (*P. putida*), where biodegradation takes place under controlled conditions and to clarify by dead-end filtration. Thus, an attempt is made to enhance *P. putida* growth activities process using microporous hollow-fiber membrane. Cells immobilized on microporous hollow fiber have been employed for biotechnology application such as cell culture and biodegradation (Chung et al., 2005).

Owing to the high surface area per unit module volume, hollow fibers serve as better supports than other beads. With varied porous structures, microporous hollow fibers act as the barriers to prevent mass movement but allow restricted or regulated passage of one or more species through the pores (Juang & Wu, 2006). However, the accumulation of biomass on the membrane surface can strongly affects the membrane performance (Chang and Kim, 2005) and depending on biomass concentration, the permeate flux increases with the increase transmembrane pressure (Alberti et al., 2006).

Observation from this study showed that when the bioreactor operated in the proximity or in the stationary phase of the curve the amount of excess sludge is considerably reduced. In this condition the substrate removal efficiency is very high and thus confirming that one of the advantages of using membrane bioreactor (MBR) rather than conventional activated sludge lies in the possibility of operating with high biomass concentrations with lower reactor volumes, high removal efficiency and low waste sludge amount.

Table 4.25: Parameter related *P. putida* growth behavior and activity during mercury removal in membrane bioreactor

Growth Parameter	Min	Max
Specific Growth Rate, μ (hr^{-1})	0.0277	-
OD	0.713	2.155
Exponential cell growth, (ln OD/OD_0)	0.912	1.106
Number Of Generation, *n*	1.307	1.585
Generation Time, *g* (hr)	25.000	80.328
Growth Rate Constant, *k* (hr^{-1})	0.009	0.170
Hg Removal (%)	99.6 for 6 hr	99.8 for 120 hr and 99.9 after membrane

Shown in Table 4.25 are results obtained for *P. putida* growth related parameter in the membrane bioreactor system. It is found that the cell exhibits a typical batch growth curve of microbial culture, which is divided into the lag, exponential, stationary and death phases. In the lag phase, cells immediately show an exponential growth phase with a sharp increase in cell density in the first 6 hours with a fast decline in mercury concentration as a result of mercury removal by *P. putida* activities (Figure 4.31). After that, the cells consistently grow in the range of optical density 1.99 - 2.02, at minimum mercury concentration of 16.40 – 6.00 ppb. The highest cell density obtained is 2.155 with exponential growth of 1.11 and specific growth rate, μ of 0.03 hr^{-1}.

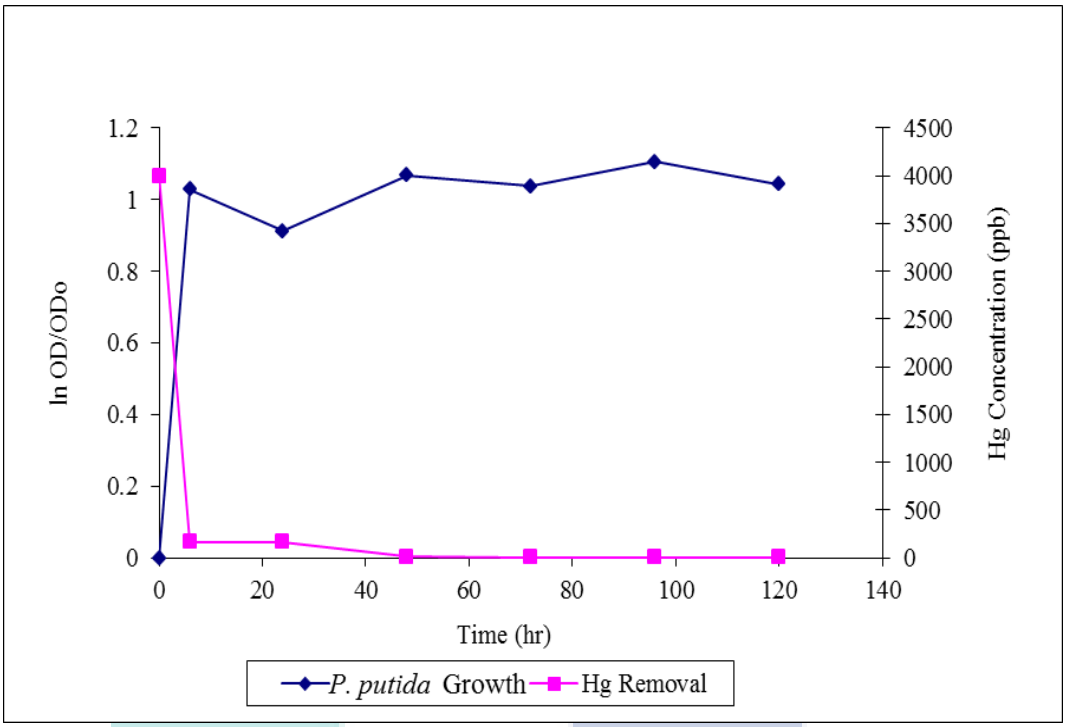


Figure 4.32: Mercury (4000 ppb) removal by *P. putida* in membrane bioreactor

The dramatic increase in mercury biosorption has been observed at pH 7.40. (Bogdanova et al., 1988). Meanwhile, Fourest and Roux (1992) observed precipitation of heavy metals, including zinc, nickel and lead, when pH values are above 7.50, therefore, the precipitation of mercury at high values is examined. In all cases, the optimal mercury uptake by biomass was at pH 7.40. It is expected that the adsorption of metals decrease at low pH value because of competition for binding sites between cations and protons (Sahoo and Das, 1992). When the pH value is higher than 7, hydroxo species of the metals can be formed and do not bind to the adsorption sites on the surface of the adsorbent (Kacar et al., 2002).

Based on the analysis of the data obtained from the experiments, it is found from the graph that the maximum number of generation, *n*, obtained is 1.59 as shown in Appendix 4.53, the generation time *g* of *P. putida* cell, is 80.33 hours as shown in

Appendix 4.54 while the growth rate constant *k* is 0.17 hr^{-1} as shown Appendix 4.55 which is a way of measuring how fast the cells are dividing in a culture and defined on the basis of generation time or doubling time.

The microfiltration membranes applied in MBRs have proven to achieve consistently high removal rates for microbiological parameters such as total coliforms, faecal coliforms even bacteriophases (Stephenson, et al. 2000). Biomass immobilized on microporous hollow fibers could degrade phenol at level 1 g/L when the solution does not have extreme values of pH and/or high salinity. For example, 1.50 g/L of phenol can be fully removed by *P. putida* within 58.00 hours in the cellulose acetate hollow fiber (Zhu, et al. 2000). Even when the initial phenol level increased to 3.50 g/L, only 250 hours is required for complete removal by *P. putida* in polysulfone hollow fibers (Loh and Yu, 2000). In this regard, microporous membrane bioreactor is promising for practical if the substrates were not so toxic even at high levels and the solution conditions were relatively mild such as in a situation with moderate pH and low salinity (Juang and Tsai, 2006).

Table 4.26: Results of parameter related *P. putida* growth behavior and activity during mercury removal in membrane bioreactor

Time (hr)	Optical Density (OD)	Biomass concentration (g/L)	Hg concentration (µg/L)	ugHg/ gcell	% Hg removal
0.00	0.00	0.00	4000.00	-	-
6.00	1.995	0.77	16.00	20.78	99.6
24.00	1.775	0.68	16.00	23.30	99.6
48.00	2.076	0.80	12.00	15.00	99.7
72.00	2.013	0.78	8.00	10.25	99.8
96.00	2.155	0.83	6.00	7.23	99.8
120.00	2.022	0.78	6.00	7.64	99.8
After Membrane	0.00	0.00	3.60	-	99.9

From Table 4.26**, t**he overall percentage of mercury removal in membrane bioreactor is very high, which is more than 99% and the highest removal efficiency is

99.9% for petroleum based industries wastewater with 4000 ppb mercury concentration that was treated for 120 hours. In this case, results indicated that mercury level at 3.60 ppb in treated petroleum based industries wastewater is below than the discharge limit required by DOE for Standard B (50 ppb) and even better than the requirement for Standard A of 5 ppb (DOE, 2011). The study also has shown that the percentage of mercury removal efficiency in bioreactor is 99.6 % for first 6 hours and 99.8% for 120 hours before the sample is introduced to a membrane system. In this case, 6 hours was the optimum time for the mercury removal with the ratio of mercury mass over cell mass is 20.78 µgHg/ gcell for *P. putida*.

From the investigation, it is confirmed that the function of microfiltration membrane system is introduced to retain *P. putida* in bioreactor. As a result, the cross-flow membrane filtration system would allow maximum microbe-metal interaction and reduced the diffusional barrier for the metal and produced high cell densities can be produced. In addition, cell harvesting and metal clean up could be processed in one membrane system. Chen et al. (1998) and Chung et al. (2003) reported that the evidence from the SEM image that *P. putida* cells are indeed entrapped and grow in microvoids of the hollow fibers.

In this case, the reduction of ionic mercury to water insoluble metallic mercury, which is catalyzed by mercury resistant bacteria, is the mechanism by which mercury is removed from a solution. While volatilization of the reduced Hg^0 from batch microbial cultures were captured as metallic mercury within the bioreactor (Wagner-Dobleret al., 2000b).

Elemental mercury produced by microbial reduction diffuses out of the cells and can easily be volatilized into atmosphere. Remediation technologies based on mercury volatilization have been explored (Fry et al., 1992; Saouter et al., 1994 and Gadd, 2000), but have never proceeded beyond laboratory scale, because collecting the volatilized mercury is tedious and expensive on a technical scale.

Studies conducted at National Research Institute for Biotechnology, United State (Brunke et al. 1993) showed that the elemental mercury formed could be retained in a packed bed bioreactor. The packed bed consisted of inert porous carrier material that was covered by a biofilm of mercury-resistant bacteria. Characklis (1981) has described four stages of the bioflm development: surface contact by mass transfer, cell adherence, biofilm growth and biofilm stripping.

The solubility of elemental mercury in water is 60.00 µg/L (Barkay et al., 2003), which is the minimum concentration of mercury that can be obtained in the effluent of this type of mercury retention bioreactor. In order to reach the wastewater discharge limit reliably, a polishing step, such as carbon filtration is necessary (Wagner-Dobler, 2003).

4.7.2 Petroleum Based Industries Wastewater Quality Analysis

Typical quality of feed (petroleum based industries wastewater) and membrane bioreactor permeate (water quality) as a function of time throughout the study is shown in Table 4.27. Figure 4.32 and Figure 4.33 indicate petroleum based industries wastewater turbidity and suspended solid at the initial stage with *P. putida*. Figure 4.34 shows the results of comparison between turbidity and suspended solids after mercury removal by *P. putida* in the membrane bioreactor. Suspended solid in petroleum based industries wastewater is consistently less than 0.30 mg/L although cell density showed further enhancement of the growth with an increase in optical density and turbidity fluctuated from 82.20 to 91.90 mg/L. In this situation, it is noted that mercury concentration decreased at a very fast rate in the first 6 hours of the operation when the membrane bioreactor is operated at optimum operating condition.

Table 4.27: Mercury removal from petroleum based industries wastewater by *P. putida* in membrane bioreactor

Time (hr)	Turbidity (NTU)	Suspended Solid (mg/L)	OD	Biomass concentration (g/L)	Hg Concentration (µg/L)
0.00	88.20	0.13	0.00	0.00	4000.00
6.00	94.50	0.20	2.00	0.77	16.00
24.00	90.40	0.25	1.78	0.68	16.00
48.00	84.60	0.23	2.08	0.80	12.00
72.00	82.10	0.23	2.01	0.78	8.00
96.00	78.50	0.27	2.16	0.83	6.00
120.00	91.90	0.22	2.02	0.78	6.00
After Membrane	5.32	0.09	0.00	0.00	4.00

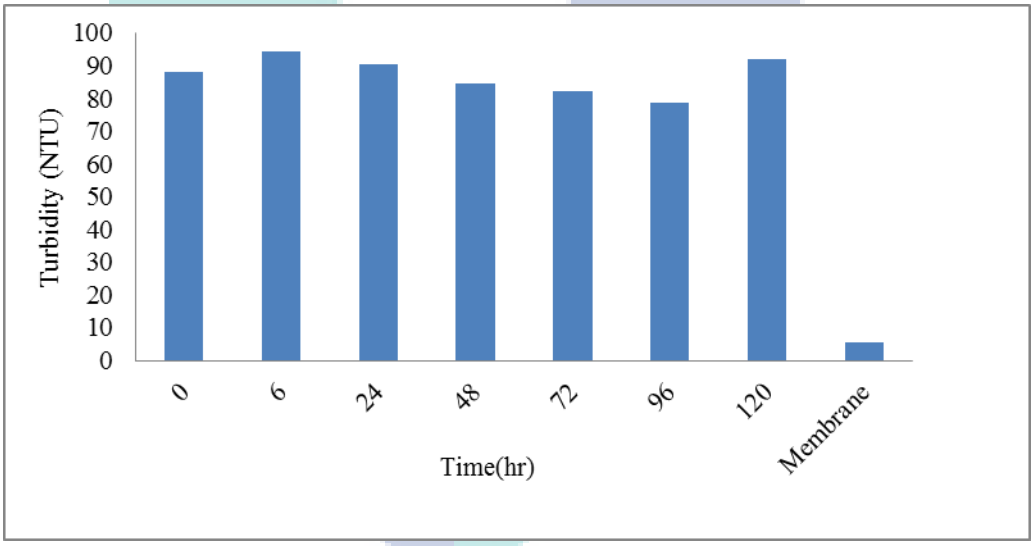


Figure 4.33: Turbidity in membrane bioreactor after mercury removal by *P. putida*

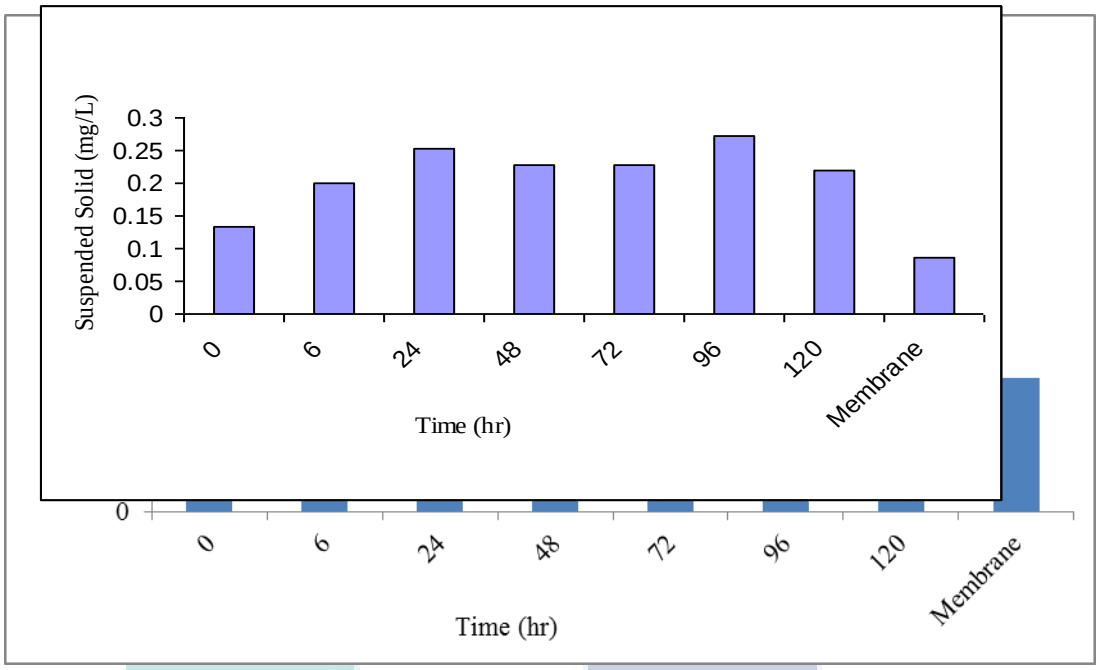


Figure 4.34: Suspended solids in membrane bioreactor after mercury removal by *P. putida*

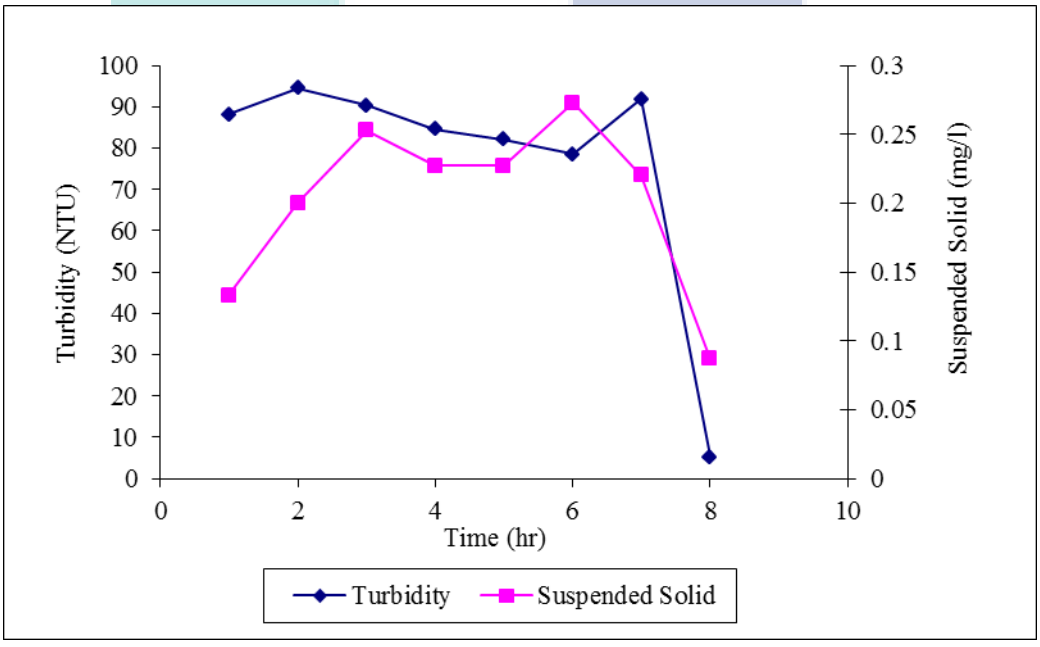


Figure 4.35: Comparison of turbidity and suspended solids in membrane bioreactor after mercury removal by *P. putida*

Based on the results analysis on effect of mercury removal to turbidity and suspended solids of petroleum based industries wastewater in membrane bioreactor, it is found that the percentage of turbidity removed in the membrane microfiltration phase is 94.2 %, while the percentage of suspended solids removed is 60.4%. Consequently, it can also be seen that there is no cell content in the permeate as the measured optical density is 0.00.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSIONS

The results of case studies for the Hg concentrations found at Location 1 (P1L1) and Location 2 (P1L2) of the Plant 1 are 156.00 ppb, and 386.00 ppb, respectively. Meanwhile, at Location ((Plant 2) , the maximum reading is 586.00 ppb and 2318.00 ppb at 34 different Sub-Location and these readings are higher than Department Of Environmental (DOE), Malaysia requirement for Standard B which is 50.00 ppb. Based on the data obtained from this study, the highest reading of Hg concentration from this survey (2318.00 ppb) has been applied as a model wastewater which is to be treated by *P. putida.*

The optimum operating condition for the growth behavior of *P. putida* in a shake flask such as acclimatization time (24.00 hours), orbital shaker speed (180 rpm), temperature (37^{o}C), pH (7) and nutrient concentration (8 g/L) have been used for mercury removal for model wastewater which containing mercury in the range of 1000 – 4000 ppb, and actual petroleum based industries wastewater. As a result, the parameters related to cell growth are found to be as follows: OD_{max} = 2.17; exponential growth = 4.34; specific growth rate, μ = 1.51 hr^{-1}; number of generation, n = 6.20; generation time, g = 58.00 hours and growth rate constant, k = 0.024 hr^{-1}. In addition, the maximum specific growth rate, μ_{max} (0.26 hr^{-1}) and Monod constant, K_s (1.65) are identified as per Monod Model.

In the case of removal at low concentration mercury from the model wastewater, by applying the optimum operating condition in the shake flask, it is found that the efficiency

of mercury removal are 99 % (1.00 ppb) of mercury concentration, 99.83% for 6.00 ppb and 98.58% for 19.00 ppb. The effect of mercury removal at 1000.00 ppb is observed for 28.00 hours and the following parameters are found: the OD_{max} = 0.89; exponential growth = 1.90; specific growth rate, μ = 0.700 hr^{-1}; number of generation, n = 2.00; generation time, g = 53.00 hours; growth rate constant, k = 0.01 hr^{-1}. The percentage of mercury removal is 92% for 1 hour and 98% for 28 hours.

The removal of mercury (1300 ppb and 300 ppb) in the model wastewater has been succesfully achieved. High-efficiency removal of mercury by mercury-resistant bacteria, *P. putida* was shown with the overall levels of mercury removal with 86% for 1300 ppb and 94% for 3000 ppb mercury concentration. The efficiency of mercury removal from actual petroleum based industries wastewater with the presence of 1000 ppb Hg increased from 84% after 4 hours to 90.5% after 96 hours. However, the overall percentage of mercury removal from the sample with 4000 ppb mercury in the bioreactor is 99.6 % for the first 6 hours and 99.8% removal after passing through the microfiltration membrane system. These findings indicate that the microbial detoxification system for mercury is highly effective.

The investigation clearly demonstrates that the *P. putida* growth and activity in bioreactor can be enhanced by optimizing the operating parameters and the use of microfiltration membrane. By retaining *P. putida* in the bioreactor, this would result in maximum microbe-mercury interaction and could reduce diffusional barrier for mercury hence producing high cell densities. At the same time, the elemental mercury formed could be retained in the system. Using membrane bioreactor will result in an improvement in the overall performance of the microfiltration process and the wastewater quality. In addition, the turbidity is improved by 94.2% (5.32 NTU) and suspended solid removed by 60.4% (0.09). With this minimum concentration of mercury in petroleum based industries wastewater after improving the wastewater quality, the effluent can be discharged from wastewater treatment plant, hence meeting the limit of DOE requirement.

The mercury remediation technology described here offers a highly efficient way to extract mercury from polluted wastewater especially from petrochemical industries. It is environmentally friendly, since it works at ambient temperature, requires little electrical energy and no extra chemicals. Operating costs are also very low; for every 100 m^3 of wastewater cleaned, approximately RM 70.00 is spent on nutrients to feed the bacteria. It is proven to effective on a technical scale and to be robust against a variety of stresses occurring at factory testing site. The technology is applicable to wastewater from petroleum based industry plants and to other types of mercury polluted water, such as groundwater, soil wash water, and mining wastes.

It is also important to note that occasionally the wastewater from these industries show mercury readings above the permitted level. Hence, the proposed treatment can be used as a remedy. The proposed method can be used in existing wastewater treatment plants with minor modification which is by adding NB and *P. putida* into the pond and a filtration system before discharging the final effluent.

5.2 Recommendations and Further Studies

The following areas are recommended for further studies:

i) The use of membrane bioreactor pilot plant scale to treat large volumes of petroleum based industries wastewater and continuously operating for long periods using on-line mercury analyzer in order to obtain more efficient and accurate results. Furthermore, a study on the optimum parameters for the membrane bioreactor process including type of membranes, operating conditions and analysis with economical assessment are necessary.

ii) Study of the effects of salt concentration and upper limit of microbial reduction activities and biofilms, which were observed during the several months of reactor operation in this study.

iii) Study of the application of the submerged membrane bioreactor technology for the treatment of petroleum based industries wastewater.

iv) Study on the effect of concentration of oil and grease, the efficiency of the biological process, the membrane fouling and the need for pre-treatment before introducing to the membrane bioreactor system.

APPENDICES

APPENDIX A: FIGURES AND TABLES IN CHAPTER 2

Appendix 2.1: General properties of mercury

General properties of mercury	
Name	Mercury
Symbol	Hg
Number	80
Chemical series	Transition Metals
Group	12
Period	6
Block	D
Appearance	silvery
Standard atomic weight	200.59, $g \cdot mol^{-1}$
Electron configuration	[Xe] $4f^{14}$ $5d^{10}$ $6s^2$
Electron per shell	2, 8, 18, 32, 18, 2

Appendix 2.2: Physical properties of mercury

Physical properties of mercury						
Phase				liquid		
Density (near r.t.)				(liquid) 13.534 $g \cdot cm^{-3}$		
Melting point				234.32K		
				-38.83°C		
				-37.89°F		
Boiling point				629.88 K		
				356.73°C		
				674.11°F		
Critical point				1750 K, 172.00 MPa		
Heat of fusion				2.29 $kJ \cdot mol^{-1}$		
Heat of vaporization				59.11 $kJ \cdot mol^{-1}$		
Heat capacity				(25°C) 27.983 $J \cdot mol^{-1} \cdot K^{-1}$		
Vapor pressure						
P(Pa)	1	10	100	1 k	10 k	100 k
at *T*(K)	315	350	393	449	523	629

Appendix 2.3: Atomic properties of mercury

Atomic properties of mercury	
Crystal structure	Rhombohedral
Oxidation states	2, 1 (mildly basic oxide)
Electronegativity	2.00 (Pauling scale)
Ionization energies	1st: 1007.1 kJ/mol 2nd: 1810 kJ/mol 3rd: 3300 kJ/mol
Atomic radius	150 pm
Atomic radius (calc.)	171 pm
Covalent radius	149 pm
Van der Waals radius	155 pm

Appendix 2.4: Miscellaneous of mercury

Miscellaneous of mercury	
Magnetic ordering	diamagnetic
Electrical resistivity	(25 °C) 961 nΩ·m
Thermal conductivity	(300K) 8.30 $W \cdot m^{-1} \cdot K^{-1}$
Thermal expansion	(25°C) 60.4 $\mu m \cdot m^{-1} \cdot K^{-1}$
Speed of sound	(liquid, 20 °C) 1451.4 m/s

Appendix 2.5: Selected isotopes of mercury

Isotopes of mercury					
iso	NA	half-life	DM	DE(MeV)	DP
^{194}Hg	syn	444 y	ε	0.040	^{194}Au
^{195}Hg	syn	9.9 h	ε	1.510	^{195}Au
^{196}Hg	0.15%	Hg is stable with 116 neutrons			
^{197}Hg	syn	64.14 h	ε	0.600	^{197}Au
^{198}Hg	9.97%	Hg is stable with 118 neutrons			
^{199}Hg	16.87%	Hg is stable with 119 neutrons			
^{200}Hg	23.1%	Hg is stable with 120 neutrons			
^{201}Hg	13.18%	Hg is stable with 121 neutrons			
^{202}Hg	29.86%	Hg is stable with 122 neutrons			
^{203}Hg	syn	46.612 d	β^-	0.492	^{203}Tl
^{204}Hg	6.87%	Hg is stable with 124 neutrons			

Appendix 2.6: Parameter Limits of Effluent of Standards A and B Under Environmental Quality Act 1974, (Amendment 2011)

Parameter	**Unit**	**Standard**	
		A	B
temperature	C	40.00	40.00
pH value		6.00 - 9.00	5.50 - 9.00
BOD_5 at 20^oC	mg/L	20.00	50.00
Suspended solids	mg/L	50.00	100.00
Mercury	mg/L	0.005	0.005
Cadmium	mg/L	0.01	0.02
Chromium, hexavalent	mg/L	0.05	0.05
Chromium, trivalent	mg/L	0.05	0.10
Arsenic	mg/L	0.05	0.10
Cyanide	mg/L	0.10	0.50
Lead	mg/L	0.20	1.00
Copper	mg/L	0.20	1.00
Manganese	mg/L	0.20	1.00
Nickel	mg/L	0.20	1.00
Tin	mg/L	0.20	1.00
Zinc	mg/L	1.00	1.00
Boron	mg/L	1.00	4.00
Iron (Fe)	mg/L	1.00	5.00
Silver	mg/L	0.10	1.00
Aluminium	mg/L	10.00	15.00
Selenium	mg/L	0.02	0.50
Barium	mg/L	1.00	2.00
Fluoride	mg/L	2.00	5.00
Formaldehyde	mg/L	1.00	2.00
Phenol	mg/L	0.001	1.00
Free chlorine	mg/L	1.00	2.00
Sulphide	mg/L	0.50	0.50
Oil and grease	mg/L	1.00	10.00
Amminiacal nitrogen	mg/L	10.00	20.00
colour	mg/L	100.00	200.00

Appendix 2.7: Environmental and Occupational Health Standards for inhalation exposure to mercury vapor

Environmental and Occupational Health Standards for Inhalation Exposure to Mercury Vapor	
AGENCY	MERCURY CONCENTRATION (µg/m)
OSHA Ceiling Limit[2]	100.00
NIOSH REL[3]	50.00
ACGIH TLV[4]	25.00
ATSDR MRL [5]	0.20
ATSDR Action Level for clean up	1.00
EPA Rfc[6]	0.30

[1]micrograms per cubic meter (µg/m)

[2]Ceiling Limit = the concentration of mercury vapor cannot exceed this limit at any time

[3]REL = Recommended Exposure Limit, a time-weighted average for an 8-hour day

[4]TLV = Threshold Limit Value, a time-weighted average for an 8-hour day

[5]MRL = Minimal Risk Level

[6]Rfc = Reference Concentration

Appendix 2.8: Mercury in Produced Water (U.S. EPA, 2001)

Location Discharge	**Ppb (Total Hg)**
Gulf of Mexico Ocean	0.64 <0.01
Gulf of Mexico Ocean	0.40 <0.01
Gulf of Mexico Coastal	0.007 – 27.00
North Sea Brent	<3.00
North Sea Northern	<3.00
North Sea Central	<3.00
North Sea UK	<1.00
North Sea Dutch	4.00

Gulf of Mexico Coastal	<0.01 – 0.20

Appendix 2.9: Pollutant concentrations for a typical refinery wastewater (Wilhelm and Bloom, 2000)

	Value (mg/L)
Trace Metals	
Arsenic	0.0050
Chromium	0.0680
Copper	0.0180
Mercury	0.0009
Nickel	0.0100
Selenium	0.0172
Zinc	0.0610
Trace Organics	
Benzene	0.0005
Toluene	0.0008
Acenaphthene	0.0011
Benz[*a*]anthracene	0.0004
Benzo[*a*]pyrene	0.0007
Chrysene	0.0003
Phenanthrene	0.0002
Pyrene	0.0005
2,4-Dimethylphenol	0.0022

Appendix 2.10: Typical materials used for the manufacture of membrane with the characteristic and typical application.

Type of Membrane Material	Characteristics	Membrane Process Application
Cellulose acetate	Inexpensive Hydrophilic Good resistance to fouling Tendency to hydrolyzed Easily destroyed by the microbes	Reverse osmosis and Ultrafiltration
Polyamide thin film composites	Can withstand pH range of 2 to 12 Operating temperature up to 70^{o}C Very low tolerance to free chlorine Can foul easily	Reverse osmosis and Ultrafiltration
Polysulphone	Has a good chemical and thermal stability up to 80^{o}C Tolerates pH from 1.5 to 12	Ultrafiltration and microfiltration

Appendix 2.11: Membrane Configurations

Configuration	Area/volume Ratio (m^2/m^3)	Cost	Turbulent Promotion	Advantages	Disadvantages	Applications (most important first)
Plated cartridge	800 - 1000	Low	Very poor	Robust construction compact design	Easily fouled Cannot be cleaned	Dead end MF
Plate-and-frame	400 - 600	High	Fair	Can be dismantled for cleaning	Complicated design Cannot be back flushed	UF, RO
Spiral-wound	800 - 1000	Low	Poor	Low energy cost robust and compact	Not easily cleaned Cannot back flush	RO, UF
Tubular	20 – 30	Very high	Very good	Easily mechanically cleaned tolerates high TSS water	High capital and membrane replacement cost	Cross-flow filtration, high TSS waters
Capillary tube	600 - 1200	Low	Good	Characteristics between tubular and hollow fiber		UF
Hollow fibre	5000 - 40000	Very low	Very poor	Can be back flushed Compact design Tolerates high colloid levels	Sensitive to pressure shocks	MF, RO

Appendix 2.12: Summary of different membrane processes, characteristics and typical applications

Process	Principle method Of particle retention	Particle size removed (um)	Transmembrane pressure, TMP (bar)	Typical application
Microfiltration (MF)	Sieving action	0.1 – 10	< 2	Removal of particles from liquid and gas streams in chemical, biological, pharmaceutical and food industries. Clarification and sterile filtration of heat sensitive solutions and beverages. Waste water treatment
Ultrafiltration (UF)	Sieving action	0.001 – 0.02	1 - 7	Pulp and paper industries Clarification of juices and wines in the food and dairy plants industry Manufacturing of antibiotics Treatment of blood and plasma in the biological and pharmaceutical industry Desalination of brackish water and sea water
Reverse osmosis (RO)	Related to their size, shape, ionic charge and interactions	0.0001 – 0.001	30 - 100	Production of pure water for variety of industries Concentration of solutions of food products, pharmaceutical solutions and chemical streams Waste water treatment

Appendix 2.13: Characteristics and typical operating information for MF membrane

Characteristics	Description
Membrane driving force	Hydrostatic pressure difference
Typical separation mechanism	Sieve (streaming)
Pore size (nominal)	0.10 – 5.00 microns
Permeate description	Water + dissolved solutes
Typical constituents removed	TSS, turbidity, some bacteria and viruses
Operating pressure, kPa	07 – 100
Rate of flux, m^3/m^2h	0.0169 – 0.0667
Configuration	Plate and frame, spiral wound, hollow fiber

Note: Modified from Metcalf and Eddy, (2003)

Appendix 2.14: Advantages and disadvantages of MF membrane

Advantages	Disadvantages
New membrane design allows use of lower pressures; system cost may be competitive with conventional wastewater treatment processes	May need pre-treatment to prevent fouling; pre-treatment facilities increase space needs and overall costs
Smaller space requirements (footprint); membrane equipment requires 50 to 80% less space than conventional plants	May require residuals handling and disposal of concentrate
Reduce labour requirements; can be automated easily	Flux (the rate of feedwater flow through the membrane) gradually declines over time. Recovery rates may be considerably

	less than 100%
Remove protozoan cysts, oocysts, and helminthova; may also remove limited amounts of bacteria and viruses	Scale formation can be a serious problem. Scale-forming potential difficult to predict without field testing
Reduce the amount of treatment chemicals	Require replacement of membranes about every 3 to 5 years Lack of a reliable loe-cost method of monitoring performance

Note: Modified from Metcalf and Eddy (2003)

Appendix 2.15: Characteristic of *Pseudomonas putida* (*P. putida*)

Scientific classification	
Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Pseudomonadales
Family:	Pseudomonadaceae
Genus:	*Pseudomonas*
Species:	*P. putida*
Binomial name	*Pseudomonas putida*

APPENDIX B: FIGURES AND TABLES IN CHAPTER 3

Appendix 3.1: Freeze-dried of *P. putida* ATCC: 49128

Appendix 3.2: Process of preparing *P. putida* nutrient using hotplate

Appendix 3.3: Laminar flow cabinet (ESCO), for culture preparation

Appendix 3.4: Sampling or handling requirement (Standard Methods APHA.AWWA,WPCF, 1985)

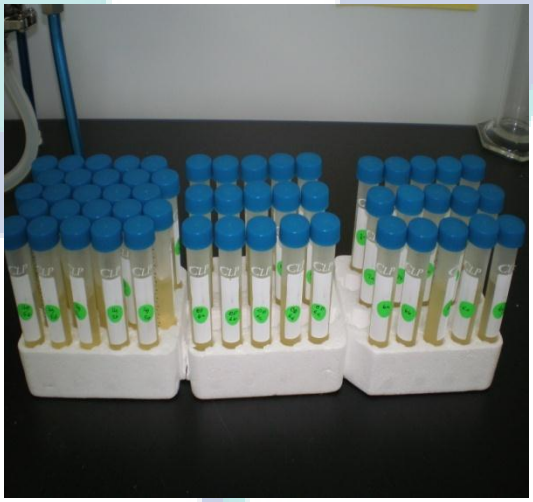
No	Determination	Container	Min. Sample Size (ml)	Preservation	Maximum storage Recommended/ Regulatory
1.	BOD	P,G	1000	Refrigerator	6hr/48hr
2.	COD	P,G	100	Analyze immediately or add H_2SO_4 to pH <2	7d/28d
3.	pH	P,G	-	Analyze immediately	2hr/2hr
4.	Temperature	P,G	-	Analyze immediately	-/-
5.	Turbidity	P,G	-	Analyze same day, store in dark up to 24h	24hr/48hr

P: Plastic (Polypropylene, polyethylene)

G: Glass

Appendix 3.5: Autoclave (H+PVarioklav Stream Sterilizer ESCO) for sterilization

Appendix 3.6: Samples of *P. putida* with nutrient and mercury are ready to be analyzed



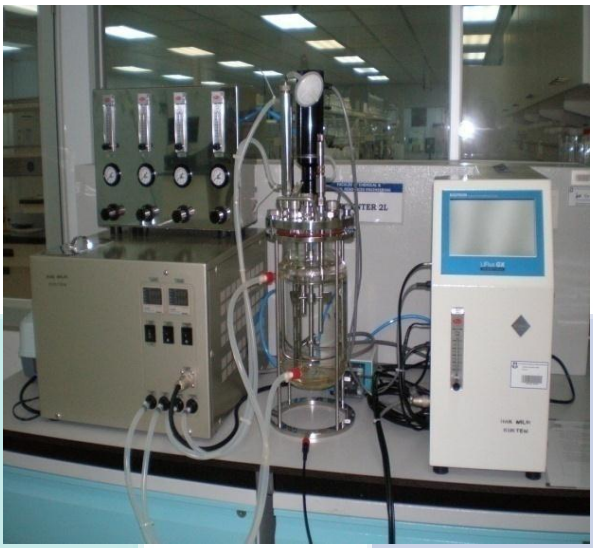
Appendix 3.7: UV Spectrophotometer for the growth of *P. putida* monitoring

Appendix 3.8: Refrigerated Centrifuge (5810 R) for cell dried weight test

Appendix 3.9: Samples in oven for cell dried weight test

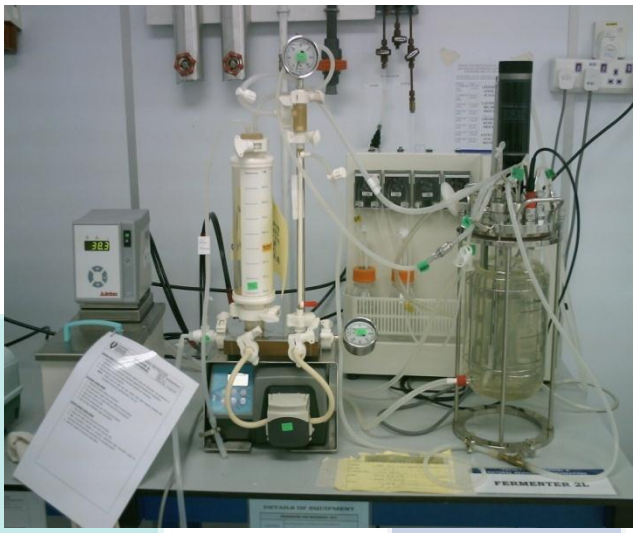
Appendix 3.10: Mercury Analyzer, RA-3000 (NIC, Japan) for mercury measurement

Appendix 3.11: Bioreactor 2.00 L for the growth of *P. putida*



Appendix 3.12: QuixStand Bench top hollow fiber membrane system

Appendix 3.13: Membrane Bioreactor (MBR)

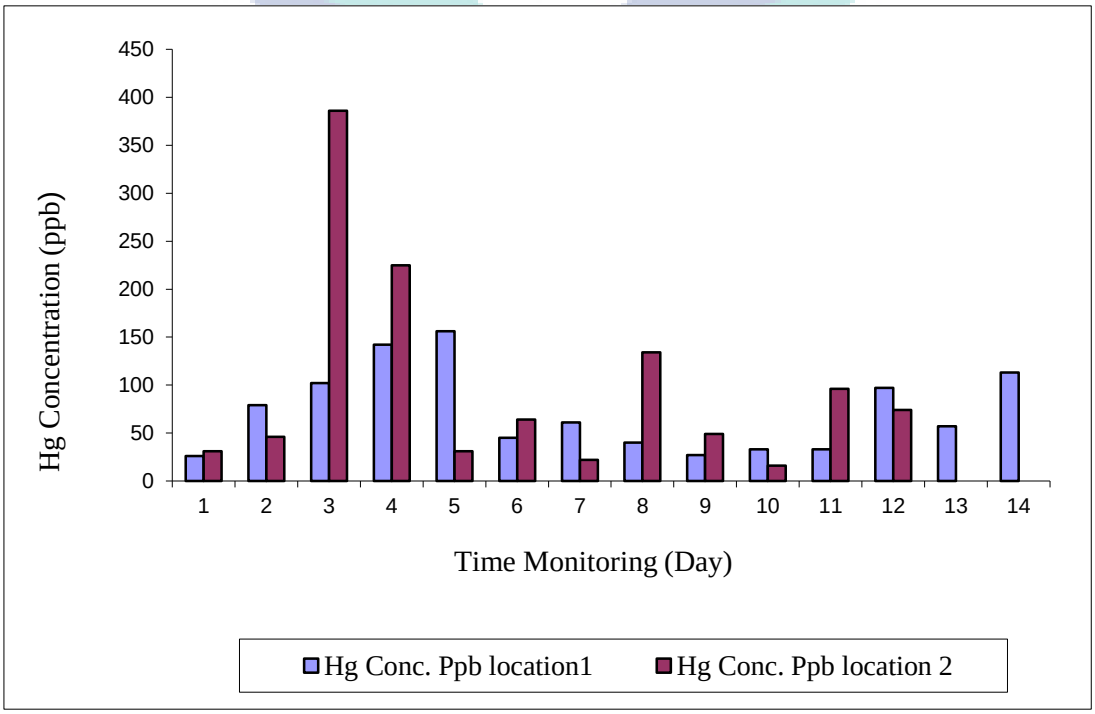


APPENDIX C: FIGURES AND TABLES IN CHAPTER 4

Appendix 4.1: Environmental Quality Act (EQA), under Environmental Quality (Industries Effluent), Regulations 2011

Characteristic	Limit
Temperature	40°C
pH value	5.5 – 9.0
BOD at 20°C	50 mg/L
COD	100 mg/L
Suspended solids	100 mg/L
Oil and grease	10 mg/L
Mercury	0.05 mg/L

Appendix 4.2: Survey of mercury contamination in wastewater from petroleum based industrial Plant 1 at Location 1 (P1L2) and Location 2 (P1L2)



Appendix 4.3: Sampling at Plant 2 Location 1 (P2L1)

Location	Hg Concentration (ppb)
i	102.00
ii	584.00
ii	170.00
iv	55.00
v	117.00
vi	586.00

Appendix 4.4: Sampling at Plant 2 Location 2 (P2L2) at different sub-locations during 5 days monitoring

	Sample	**Hg Concentration. (ppb)**
1st Day	1	9.88±3
	2	10.80±1
	3	8.48±2
	4	1877.00±3
	5	2318.00±2
	6	129.75±3
2nd Day	7	172.55±3
	8	144.85±3
	9	677.54±1
	10	23.13±3
	11	27.65±2
	12	10.89±3
	13	19.12±1
	14	177.89±1
	15	28.18±3
	16	23.61±1
	17	15.36±3
	18	42.42±3
3rd Day	19	112.66±2
	20	168.52±1
	21	170.05±1
	22	155.21±4
	23	117.48±3
	24	31.65±3
	25	586.00±2
	26	19.95±1
	27	11.47±3
	28	21.16±3
	29	13.99±2

	30	5.63±1
4th Day	31	9.83±3
	32	7.78±2
5th Day	33	6.53±2
	34	8.74±2

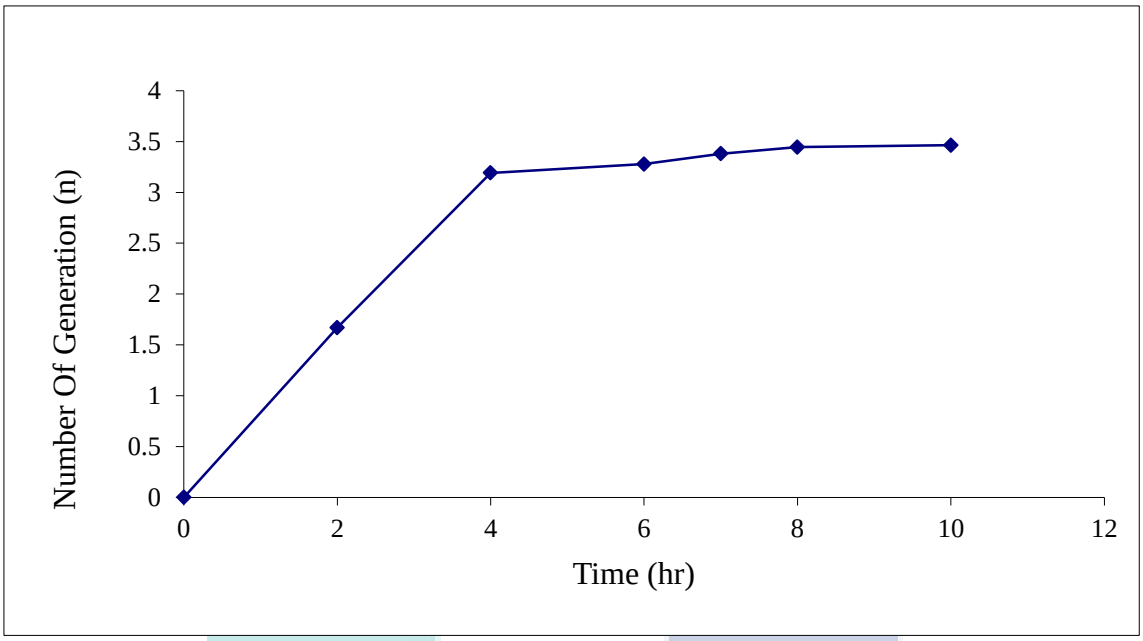
Appendix 4.5: Data of seeding technique for growth curve for *P. putida* growth population

Time (hour)	OD, Control	OD
2.00	0	0.21
4.00	0	0.68
6.00	0	1.98
7.00	0	2.10
8.00	0	2.25
10.00	0	2.36
11.00	0	2.39

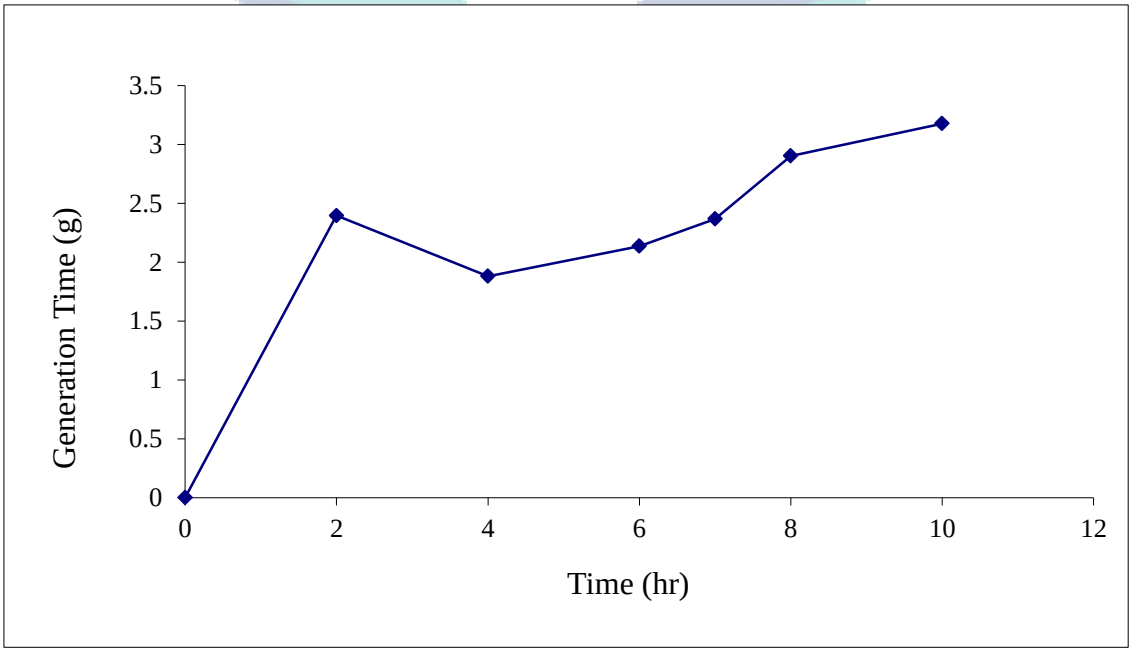
Appendix 4.6: Determination of *P. putida* growth curve

Time (hour)	OD (600 nm)	pH	ln OD/OD_0
0.00	0.17	6.98	0.00
0.50	0.17	6.85	0.01
1.00	0.18	6.88	0.04
2.00	0.22	6.75	0.25
2.50	0.41	6.53	0.87
3.00	1.11	6.30	1.85
4.00	1.77	6.24	2.32
4.50	2.11	6.62	2.50
5.00	2.35	7.09	2.60
6.00	2.46	7.23	2.65
6.50	2.57	7.46	2.70
6.80	2.66	7.61	2.73

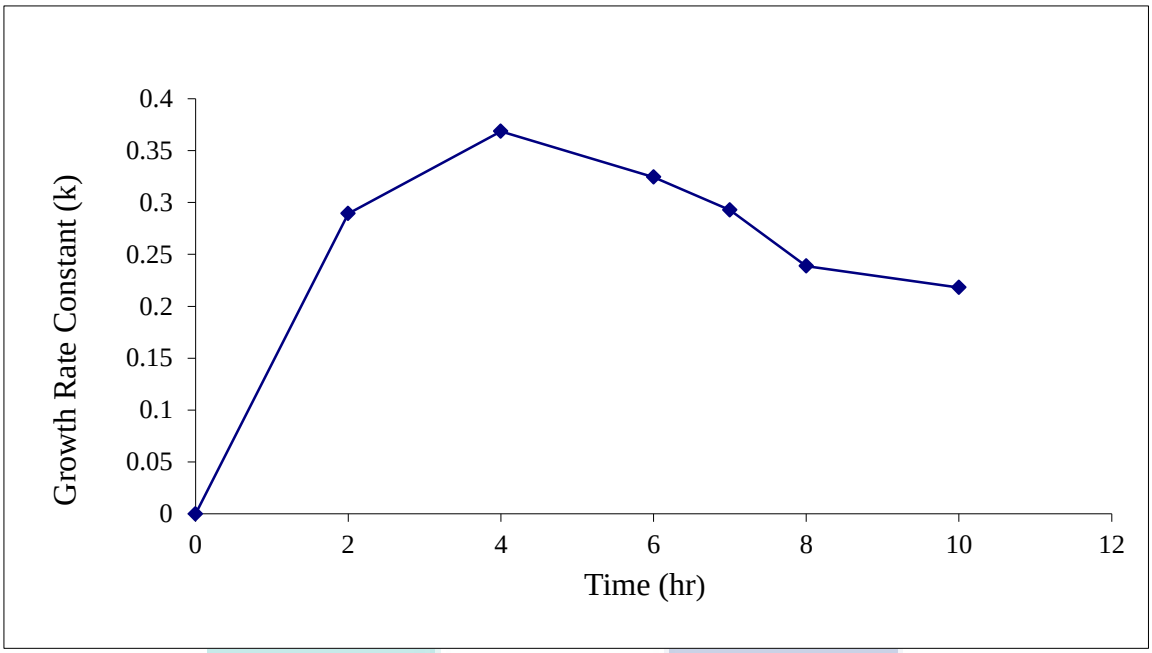
Appendix 4.7: Number of generation, *n* for *P. putida*



Appendix 4.8: Generation time, *g* for *P. putida*



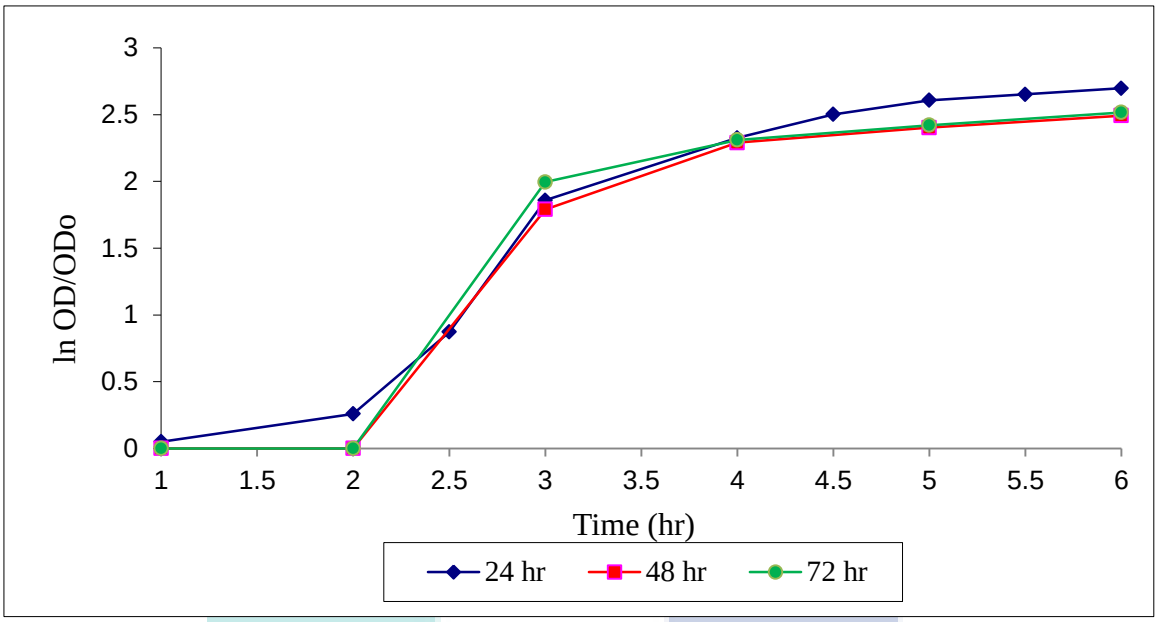
Appendix 4.9: Growth rate constant, *k* for *P. putida*



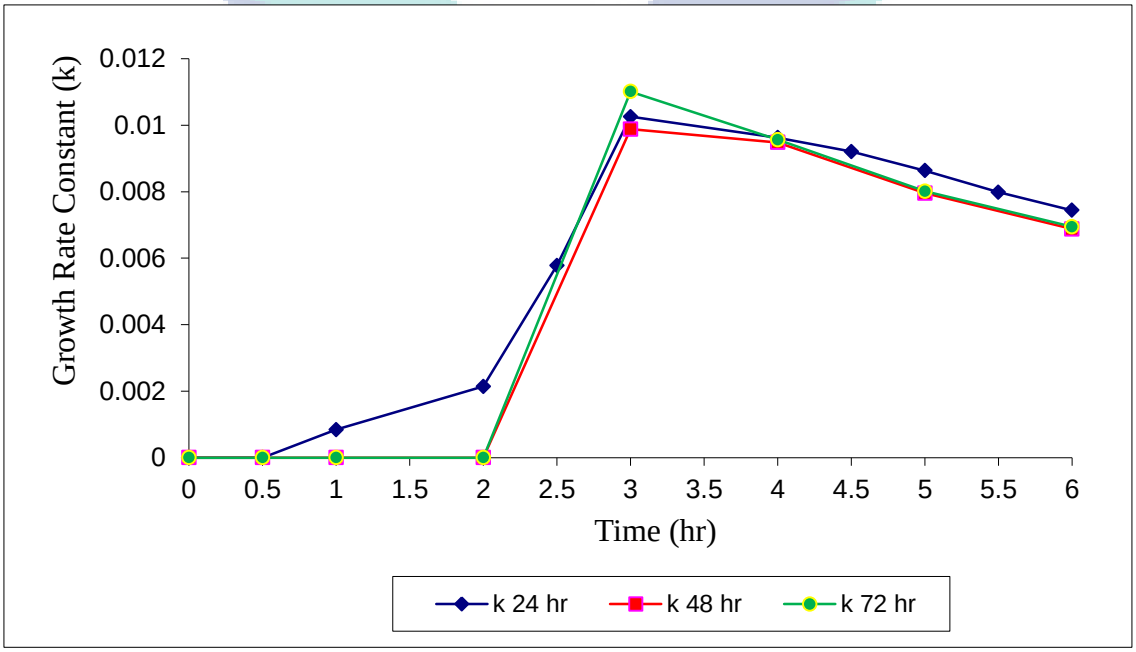
Appendix 4.10: *P. putida* standard growth curve using cell dried weight

Sample Number	Weight (g)	Weight Cell (g)	Cell Weight (g)	Total Volume (L)	Concentration Biomass (g/L)	Optical Density (OD)
1	0.95	0.95	0.001	0.013	0.10	0.21
2	0.94	0.94	0.001	0.013	0.10	0.23
3	0.94	0.94	0.001	0.010	0.11	0.26
4	0.95	0.95	0.002	0.010	0.15	0.40
5	0.95	0.96	0.002	0.010	0.20	0.53

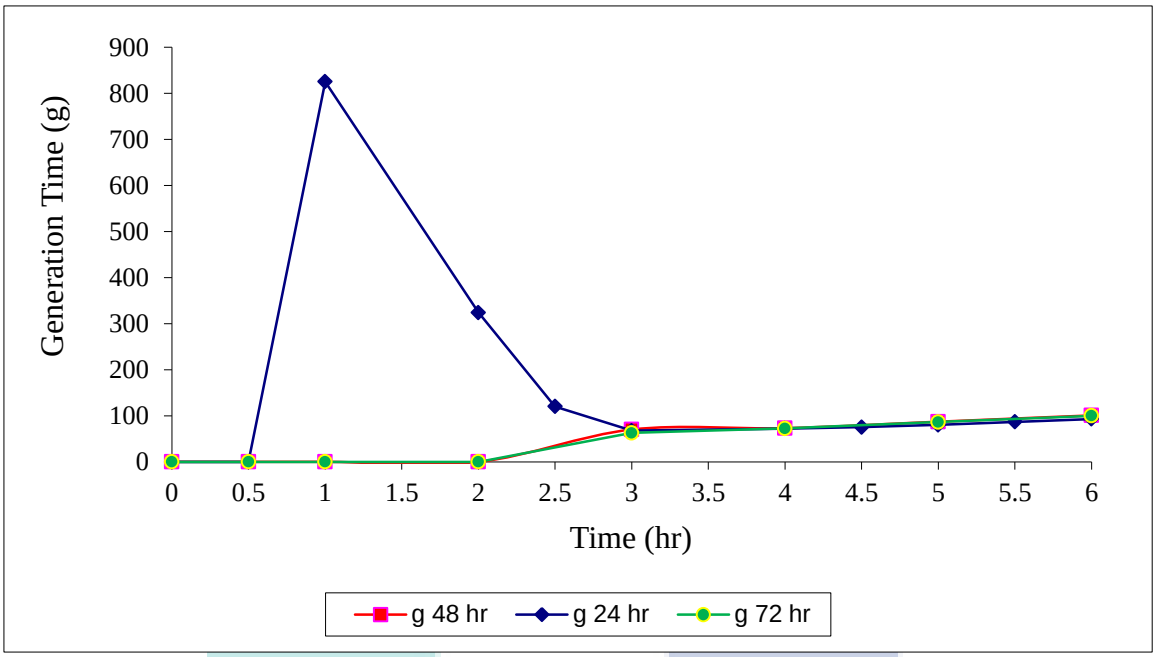
Appendix 4.11: Effect of acclimatization time to number of generation, *n P. putida*



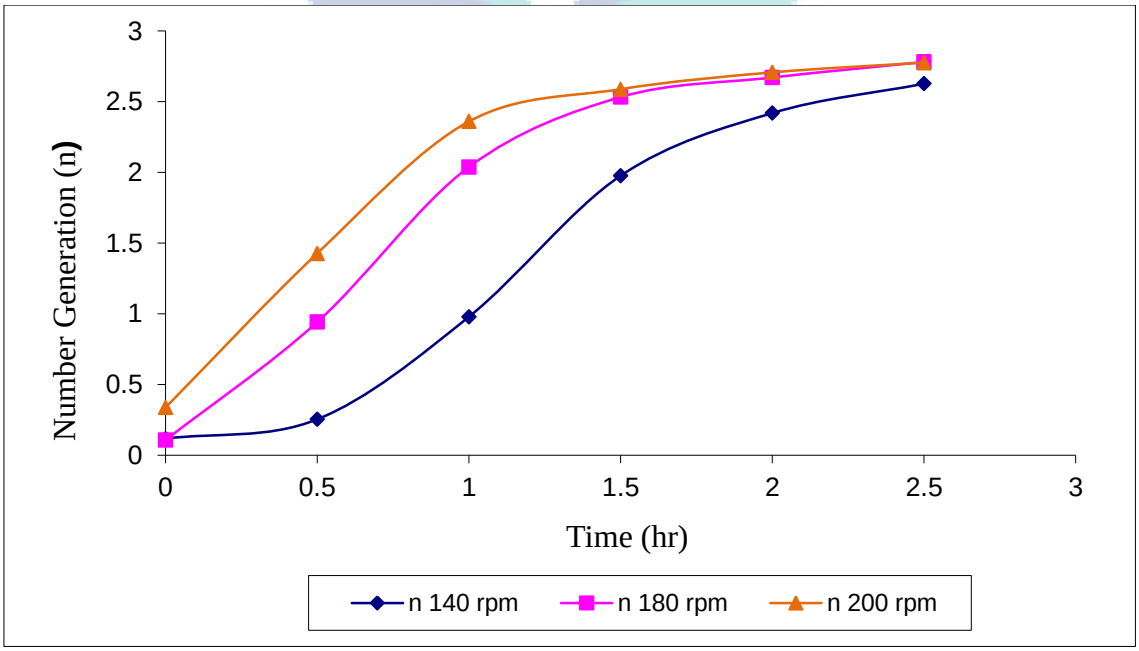
Appendix 4.12: Effect of acclimatization time to growth rate constant, *k P. putida*



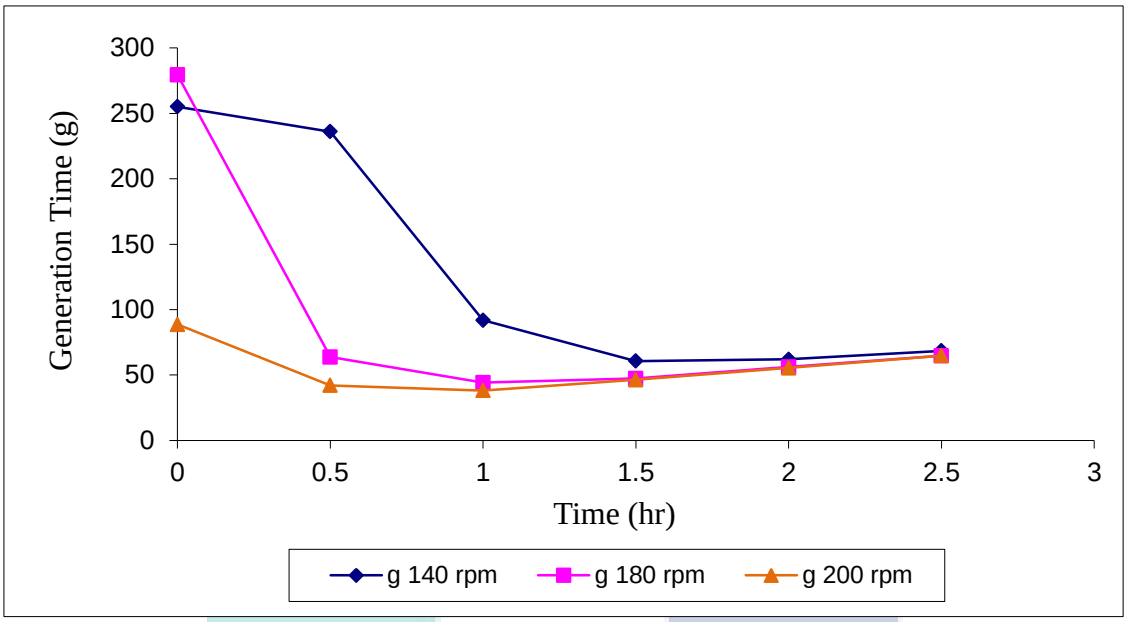
Appendix 4.13: Effect of acclimatization time to generation time, *g P. putida*



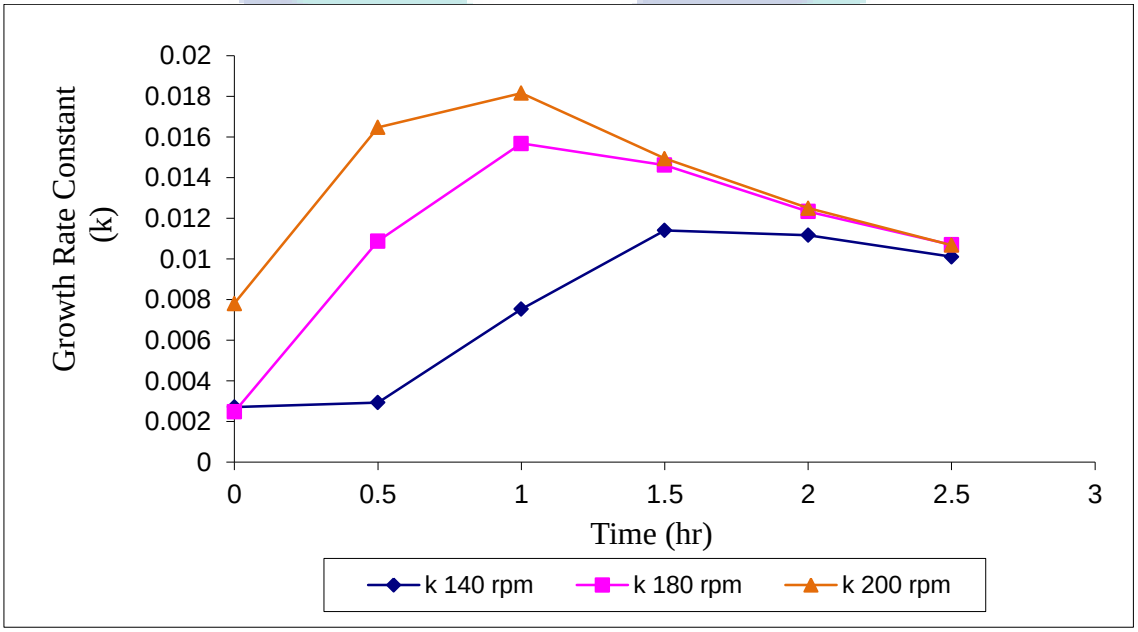
Appendix 4.14: Effect of orbital shaker speed to number of generation, *n P. putida*



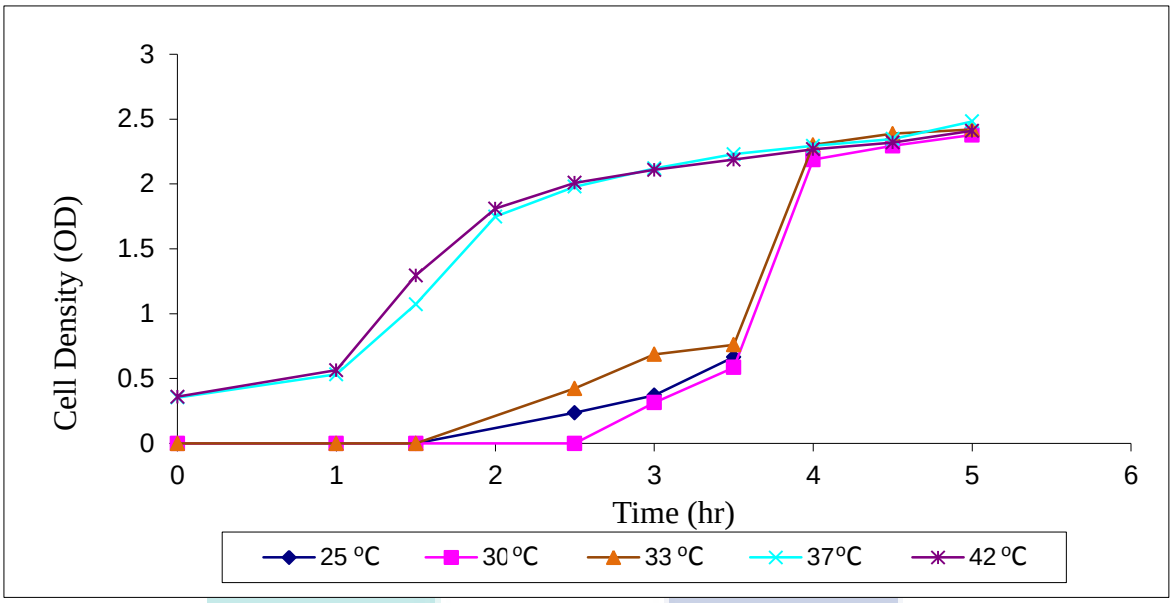
Appendix 4.15: Effect of orbital shaker speed to generation time, *g P. putida*



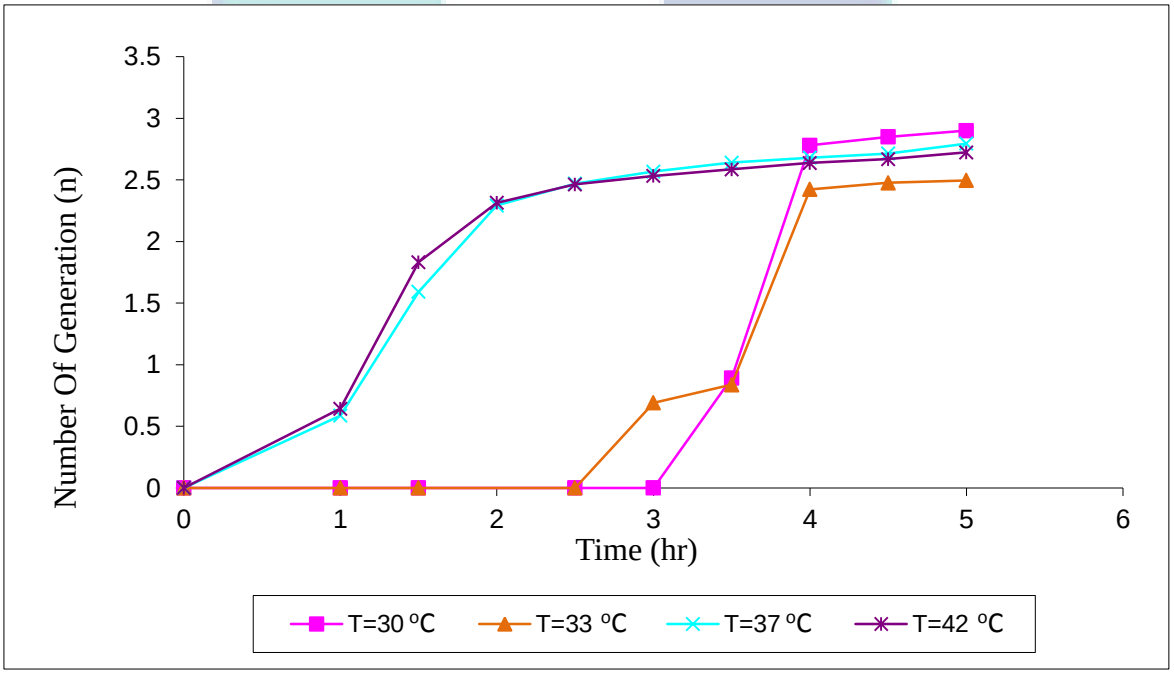
Appendix 4.16: ffect of orbital shaker speed to growth rate constant, *k P. putida*



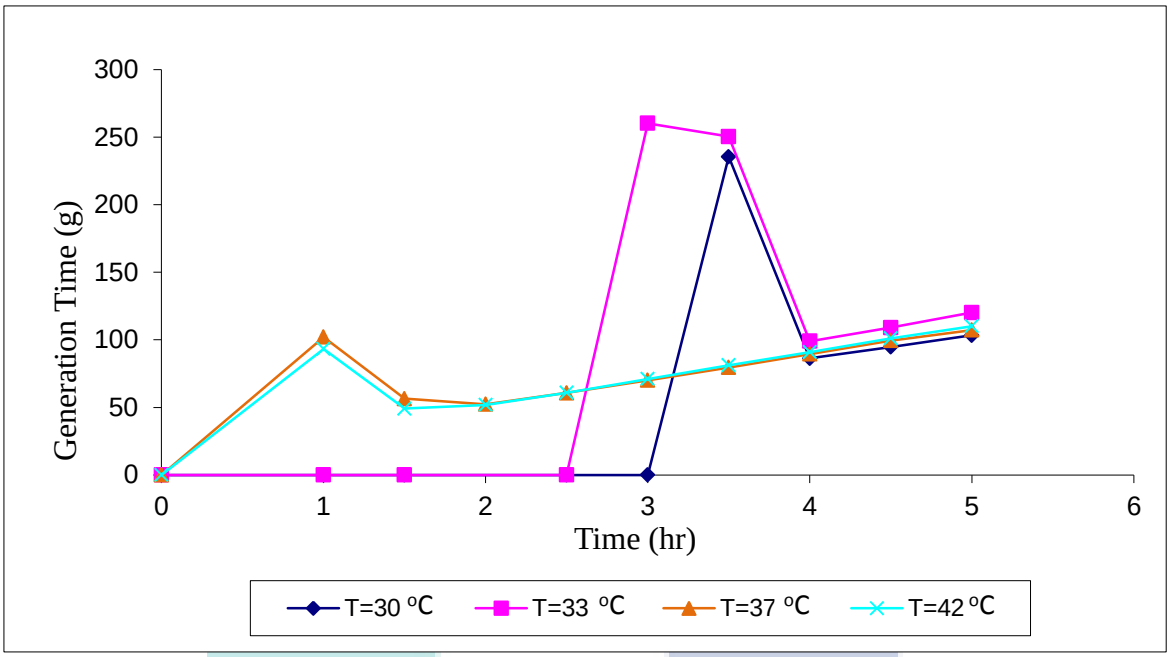
Appendix 4.17: The effect of temperature on cell density of *P. putida*



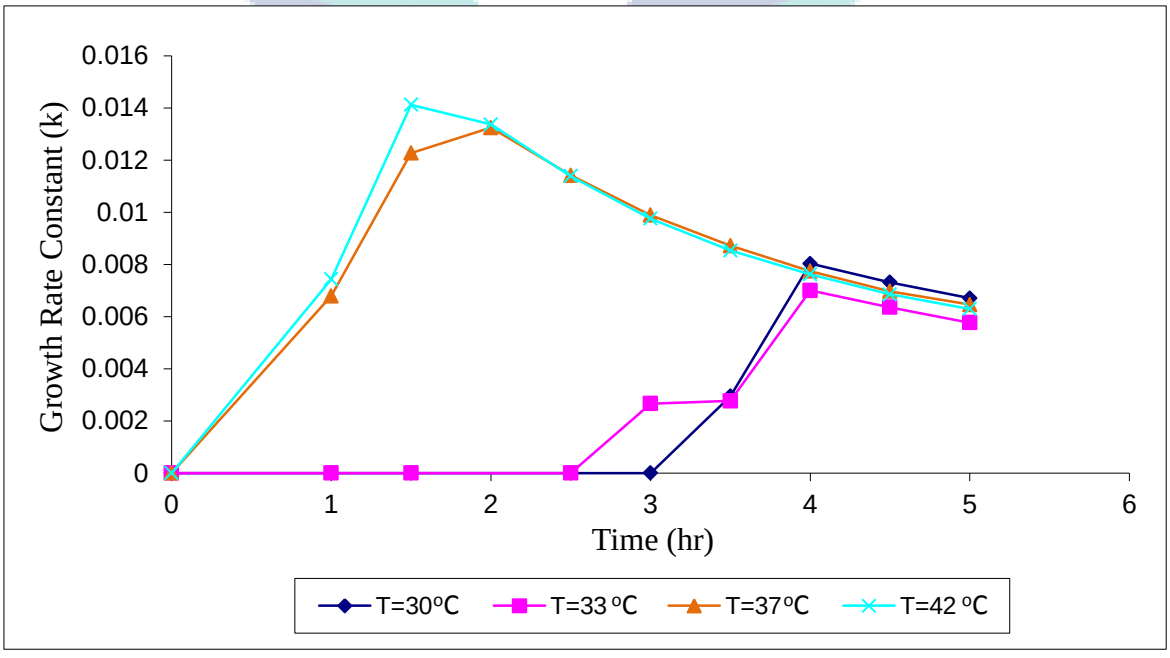
Appendix 4.18: Effect of temperature to number of generation, *n P. putida*



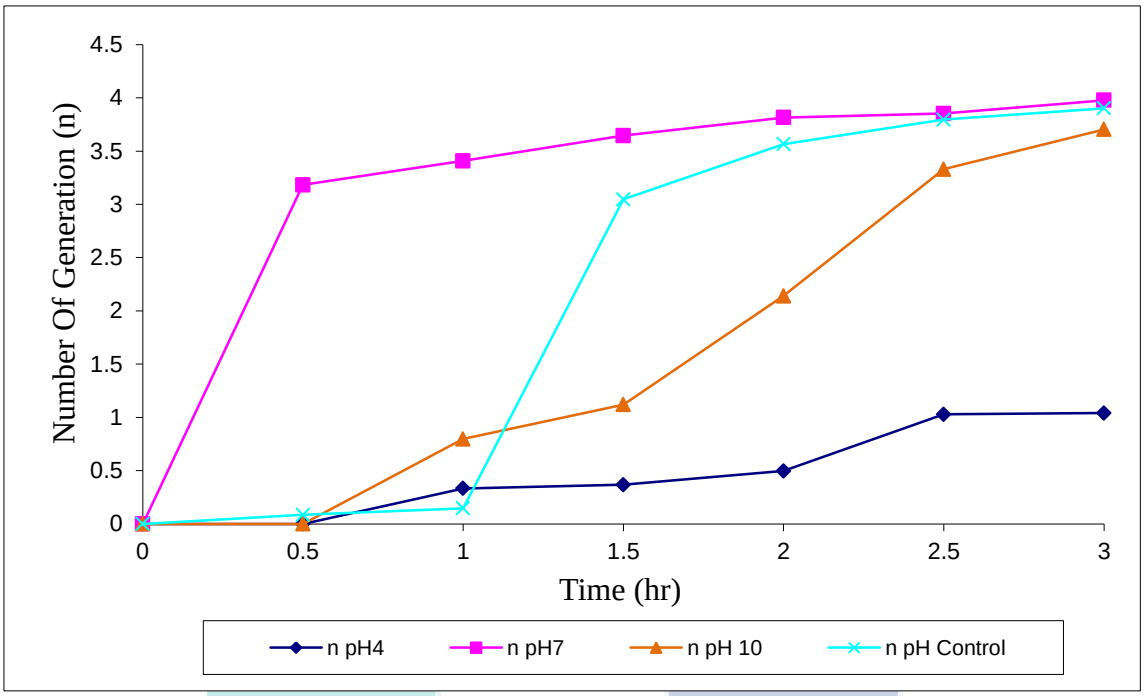
Appendix 4.19: Effect of temperature to generation time, *g P. putida*



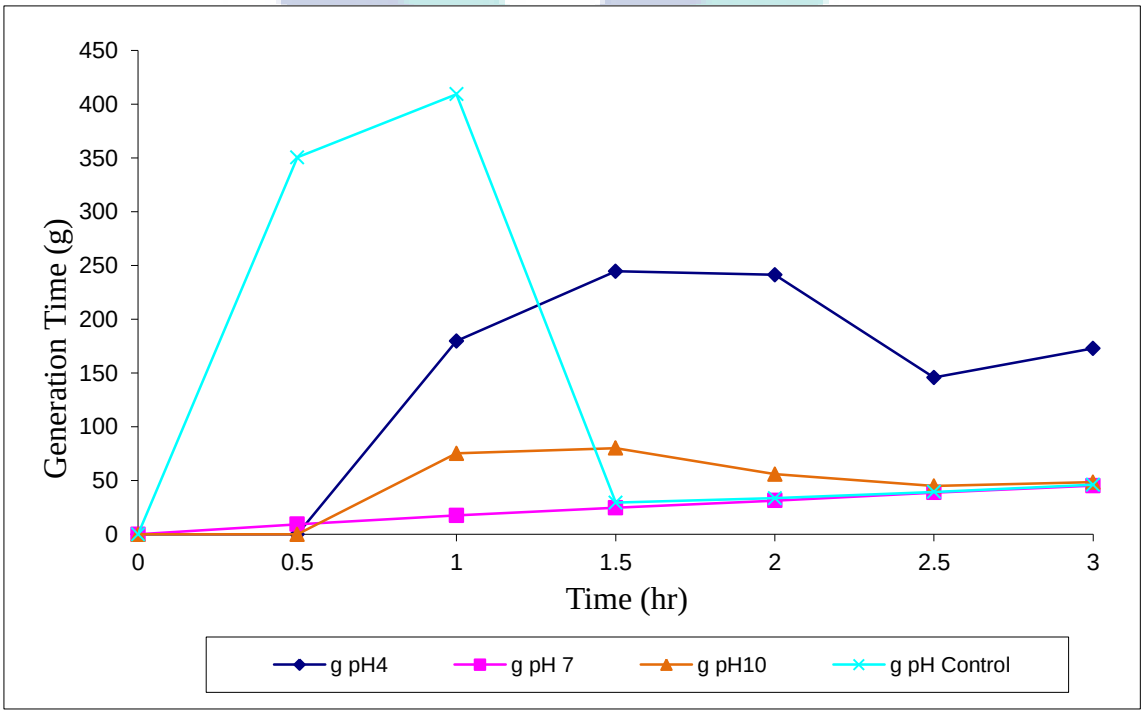
Appendix 4.20: Effect of temperature to growth rate constant, *k P. putida*



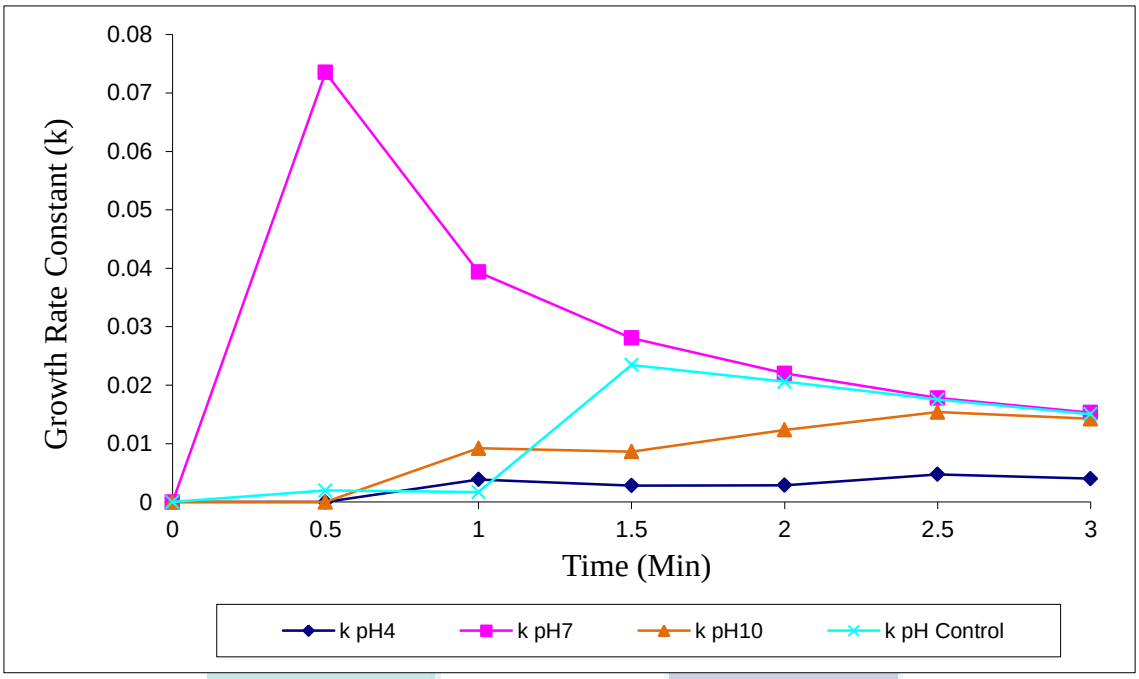
Appendix 4.21: Effect of pH to number of generation, *n P. putida*



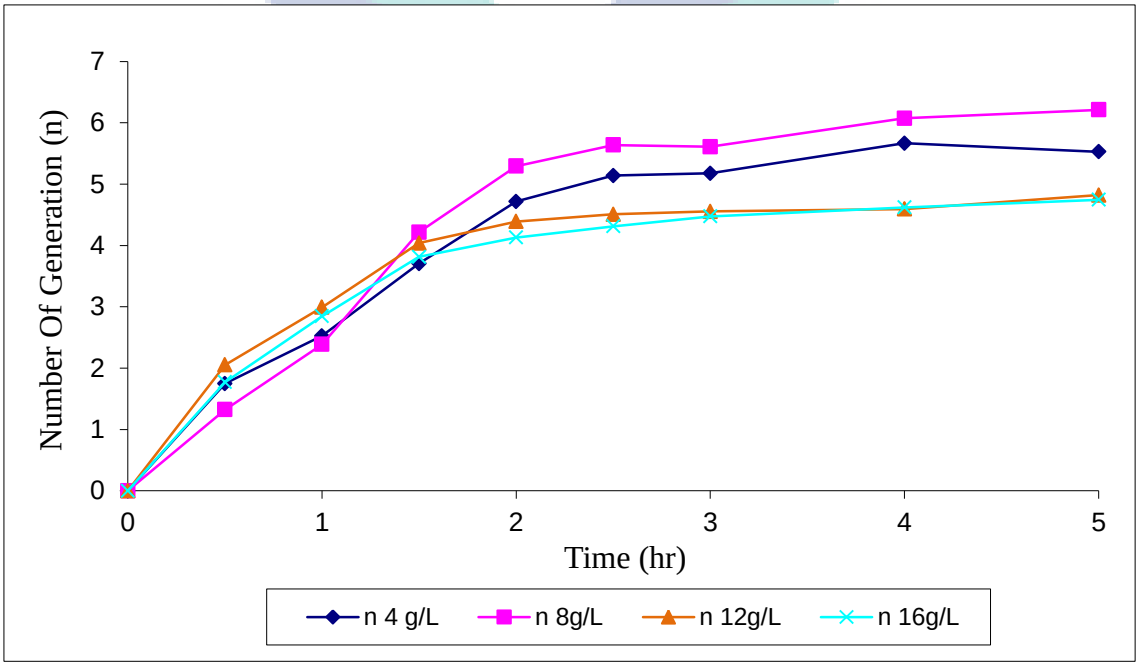
Appendix 4.22: Effect of pH to generation time, *g P. putida*



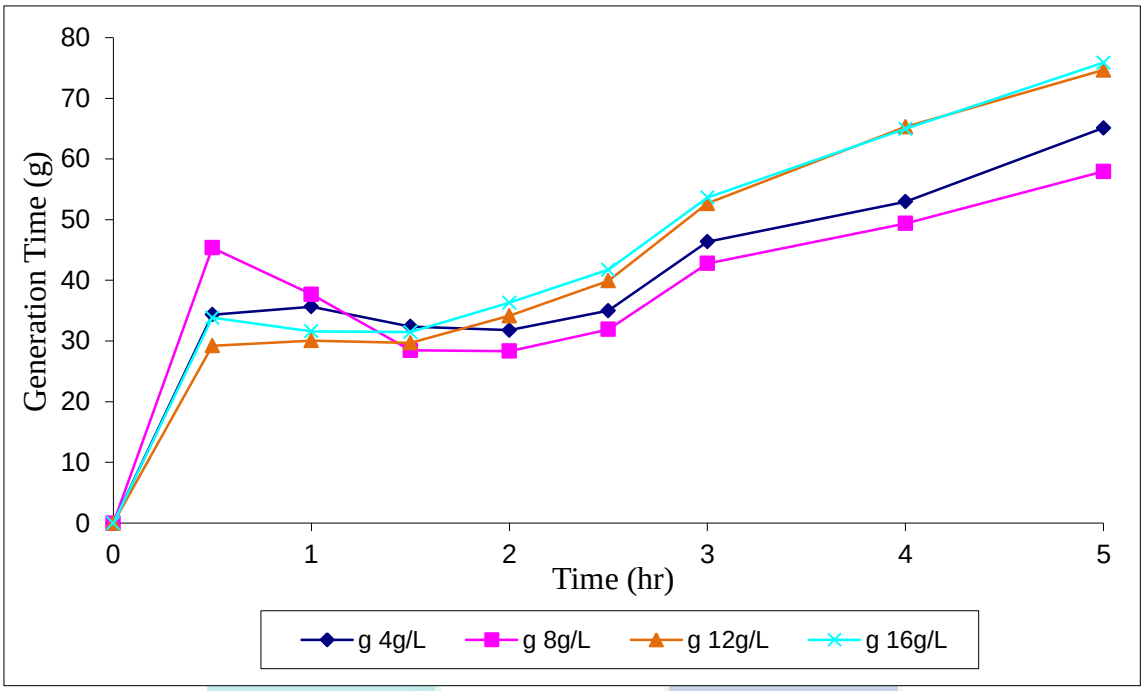
Appendix 4.23: Effect of pH to growth rate constant, *k P. putida*



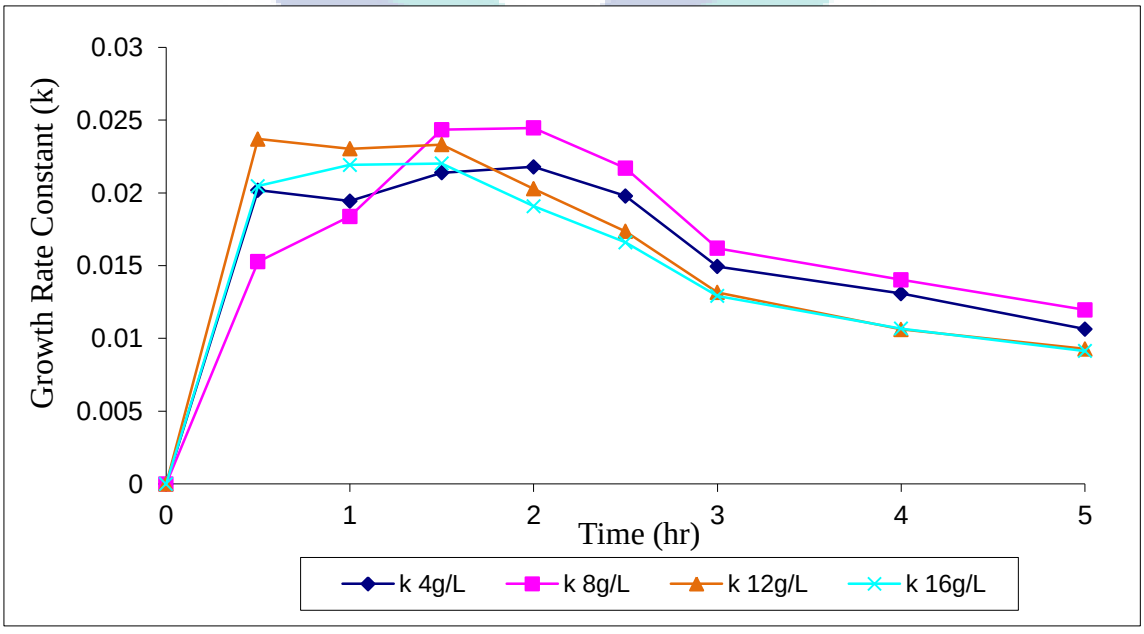
Appendix 4.24: Effect of nutrient concentration to number of generation, *n P. putida*



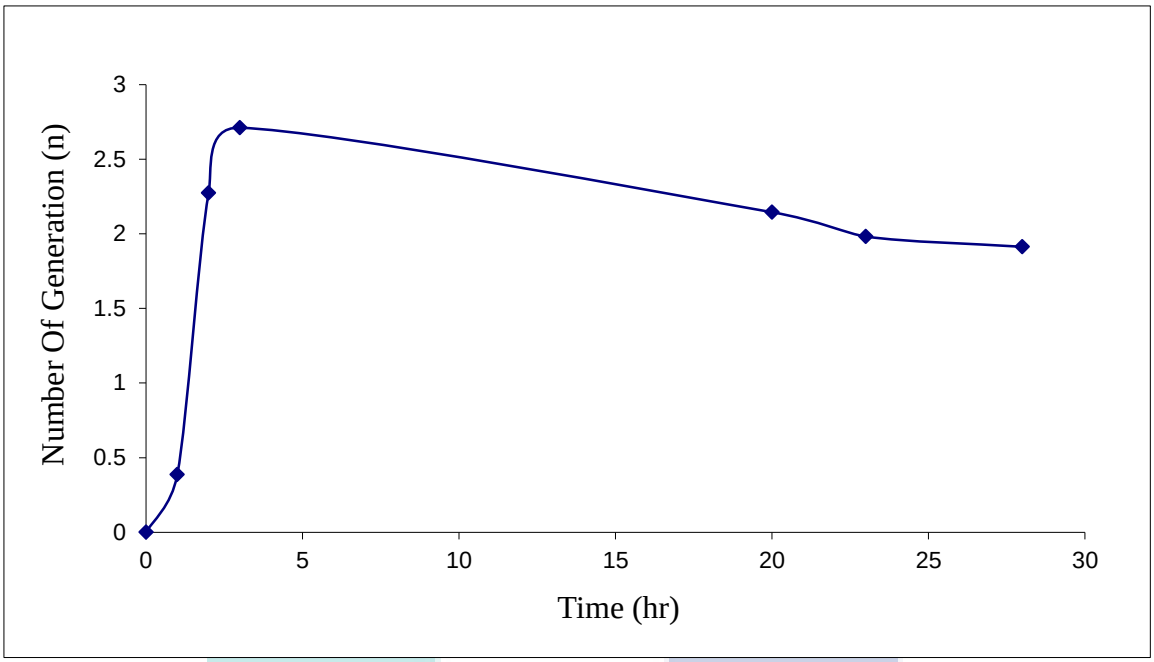
Appendix 4.25: Effect of nutrient concentration to generation time, *g P. putida*



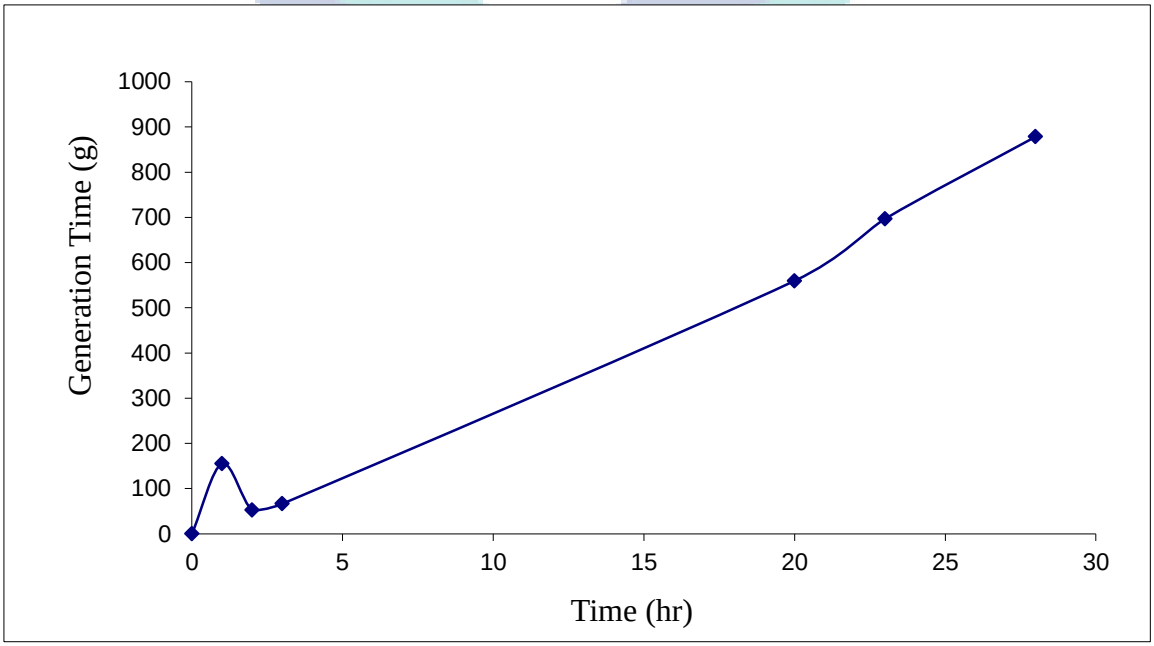
Appendix 4.26: Effect of nutrient concentration to growth rate constant, *k P. putida*



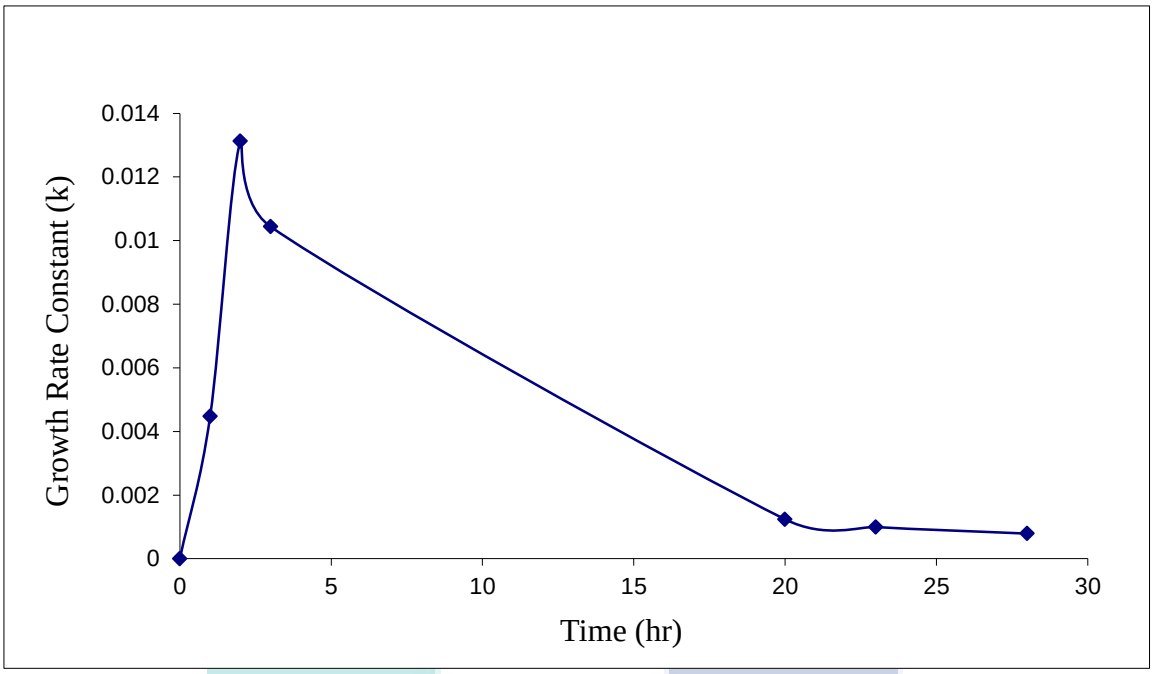
Appendix 4.27: Number of generation, *n* for *P. putida* during mercury (1000 ppb) removal



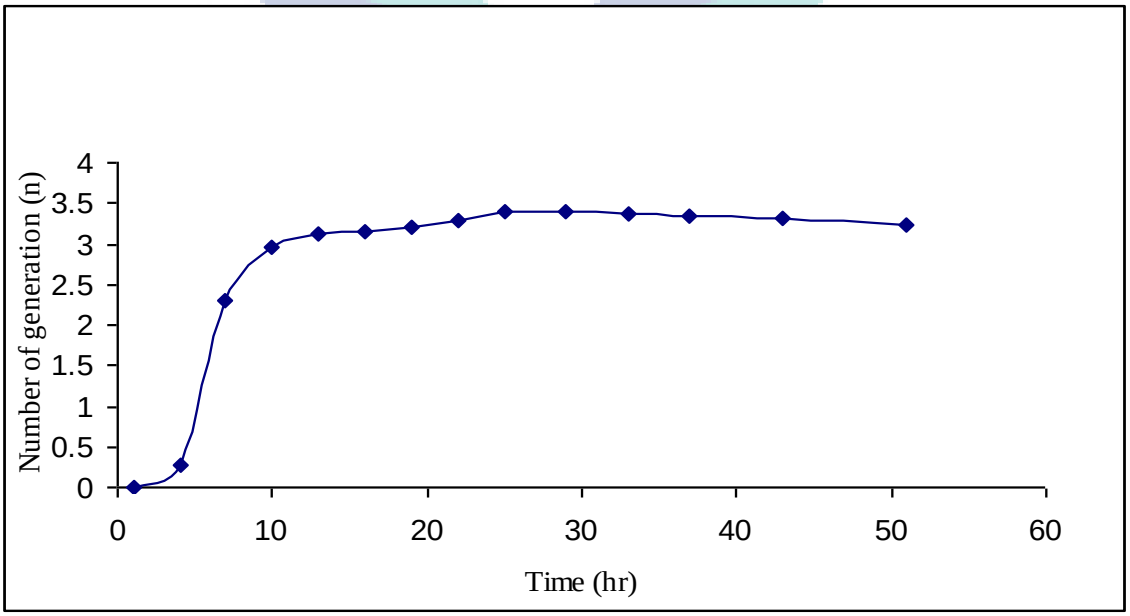
Appendix 4.28: Generation time, *g* for *P. putida* during mercury (1000 ppb) removal



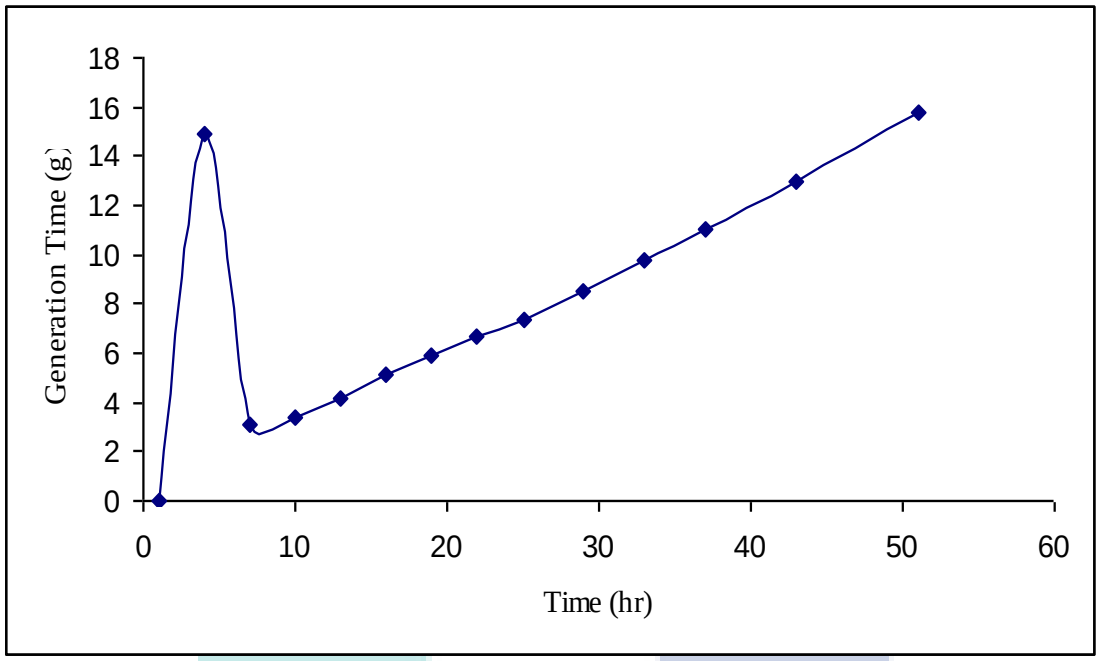
Appendix 4.29: Growth rate constant, *k* for *P. putida* during mercury (1000 ppb) removal



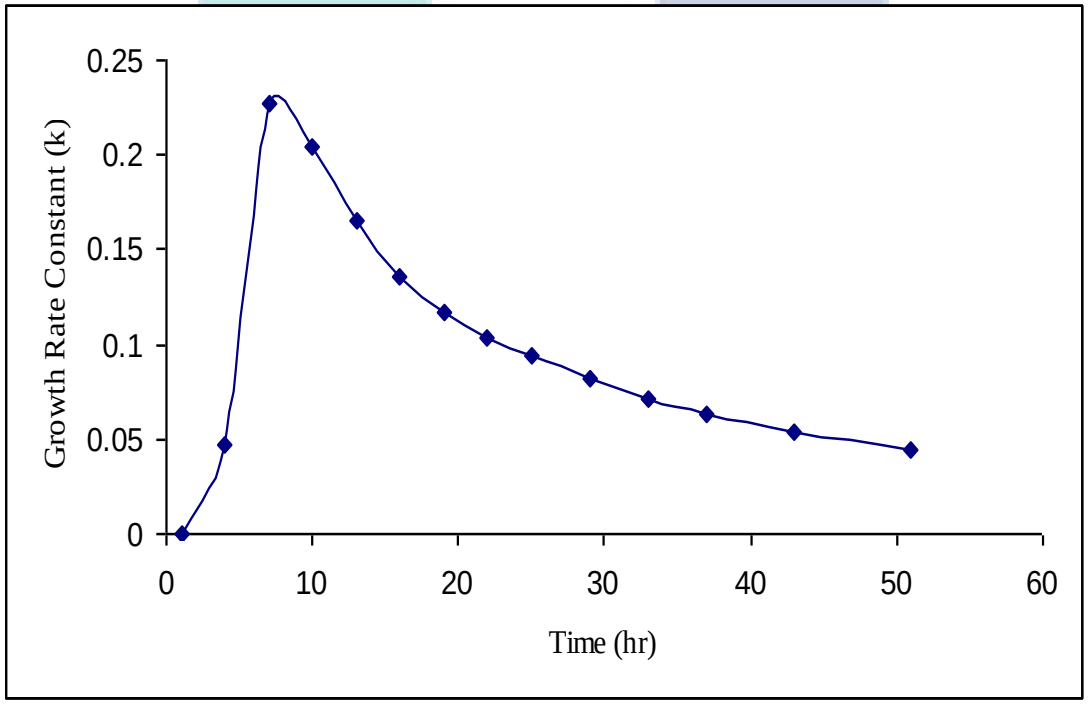
Appendix 4.30: Number of generation, *n* for *P. putida* in bioreactor



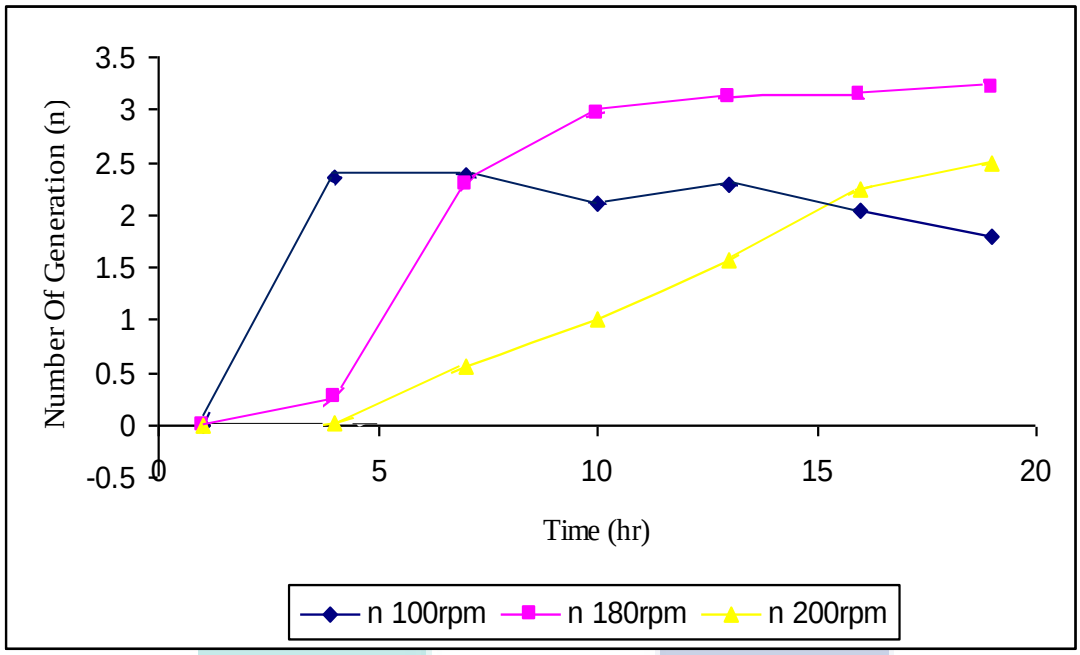
Appendix 4.31: Generation time, *g* for *P. putida* in bioreactor



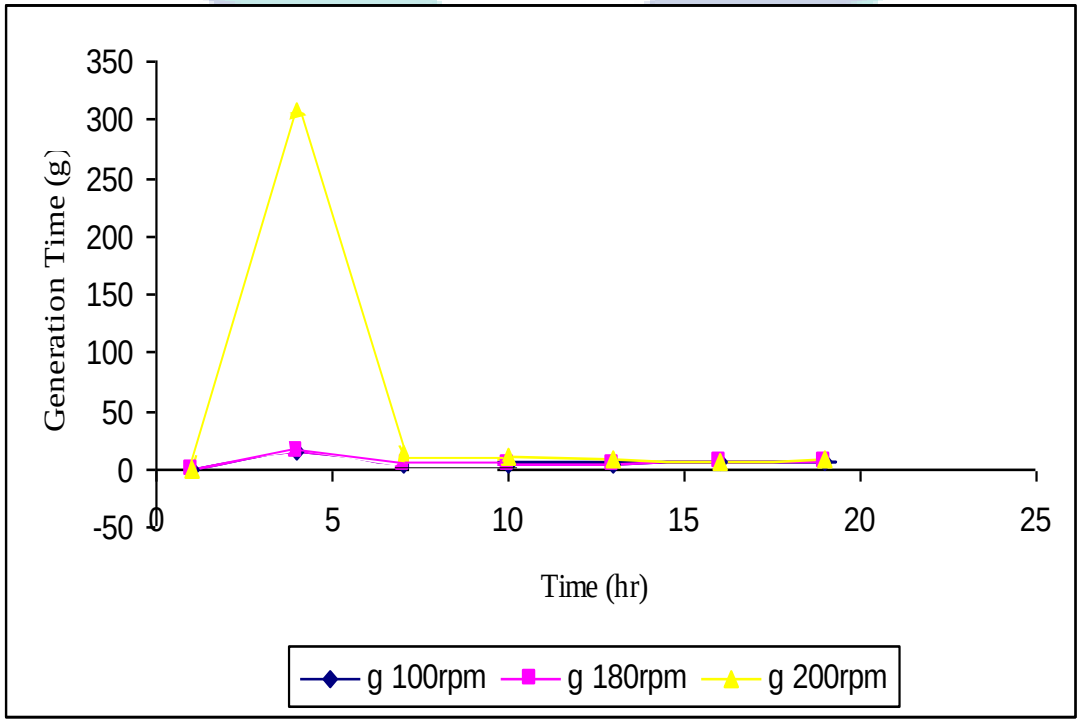
Appendix 4.32: Growth rate constant, *k* for *P. putida* in bioreactor



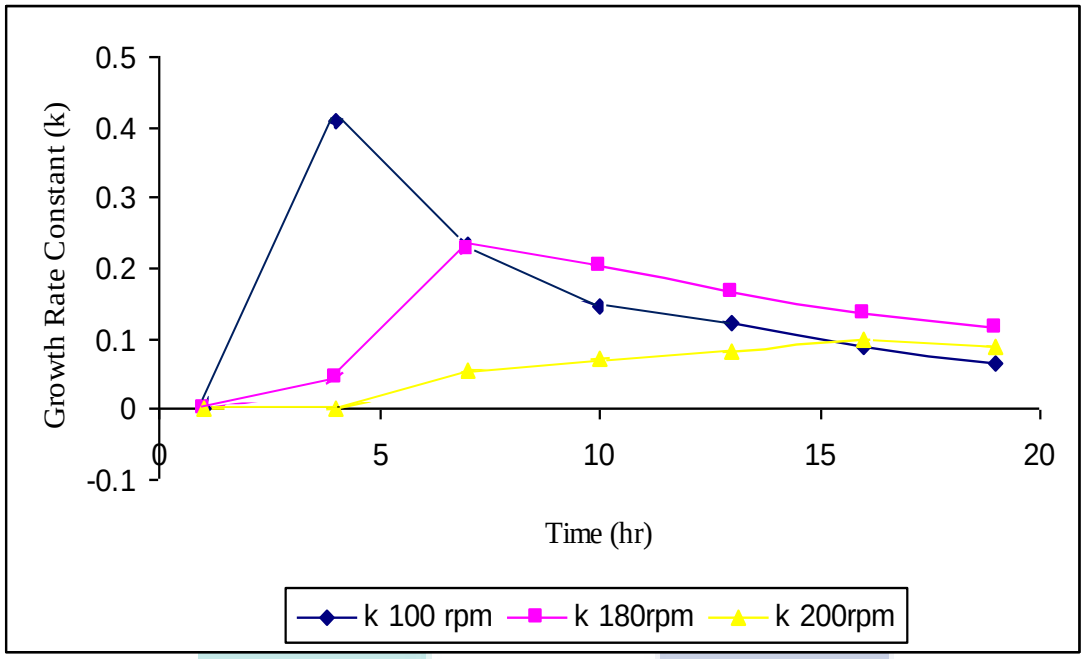
Appendix 4.33: Effect of agitation speed to number of generation, *n*



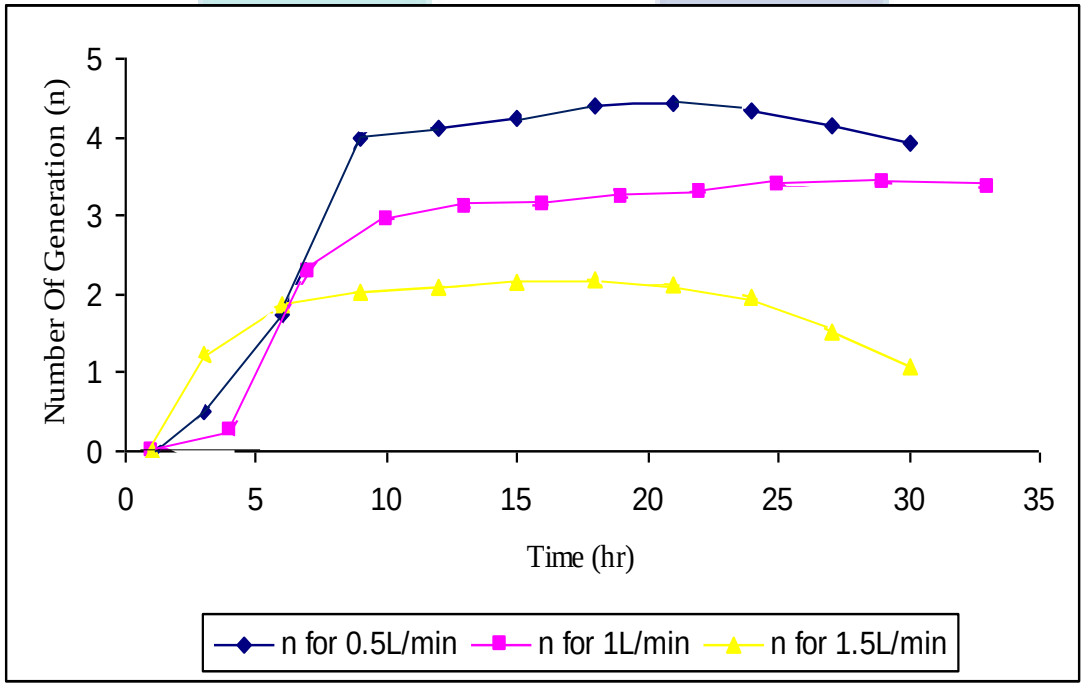
Appendix 4.34: Effect of agitation speed to generation time, *g*



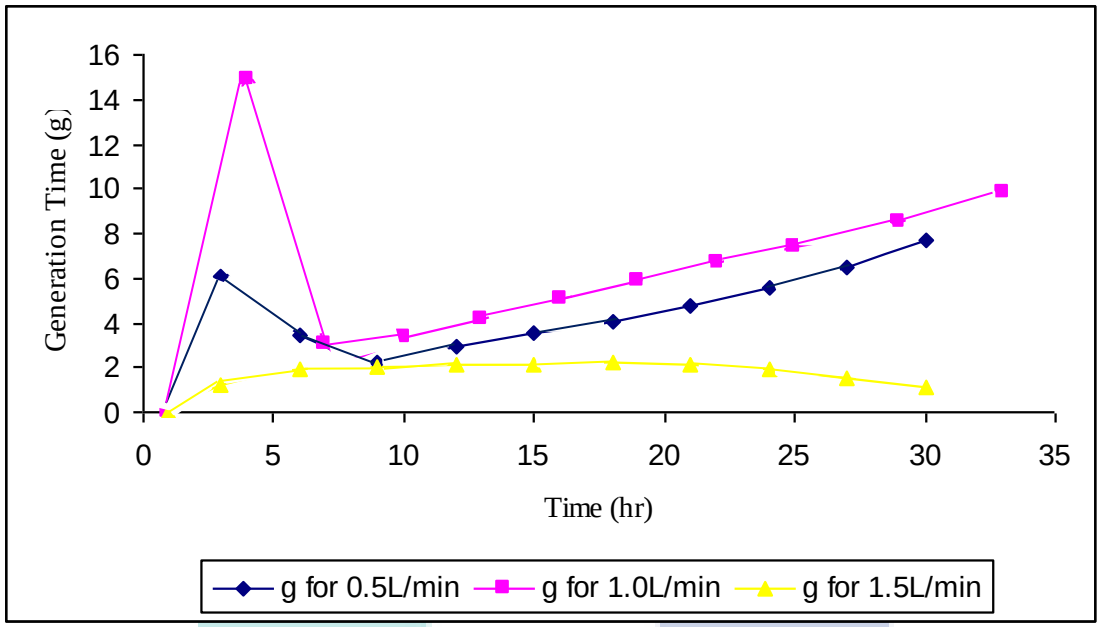
Appendix 4.35: Effect of agitation speed to growth rate constant, *k*



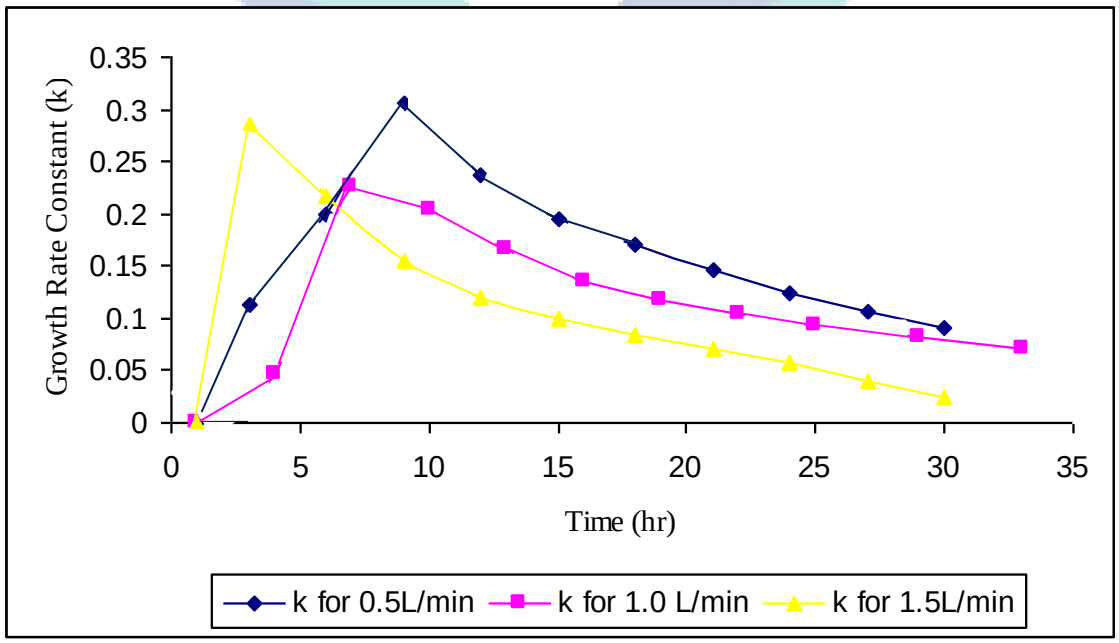
Appendix 4.36: Effect of aeration rates on number of generation, *n* for *P. putida*



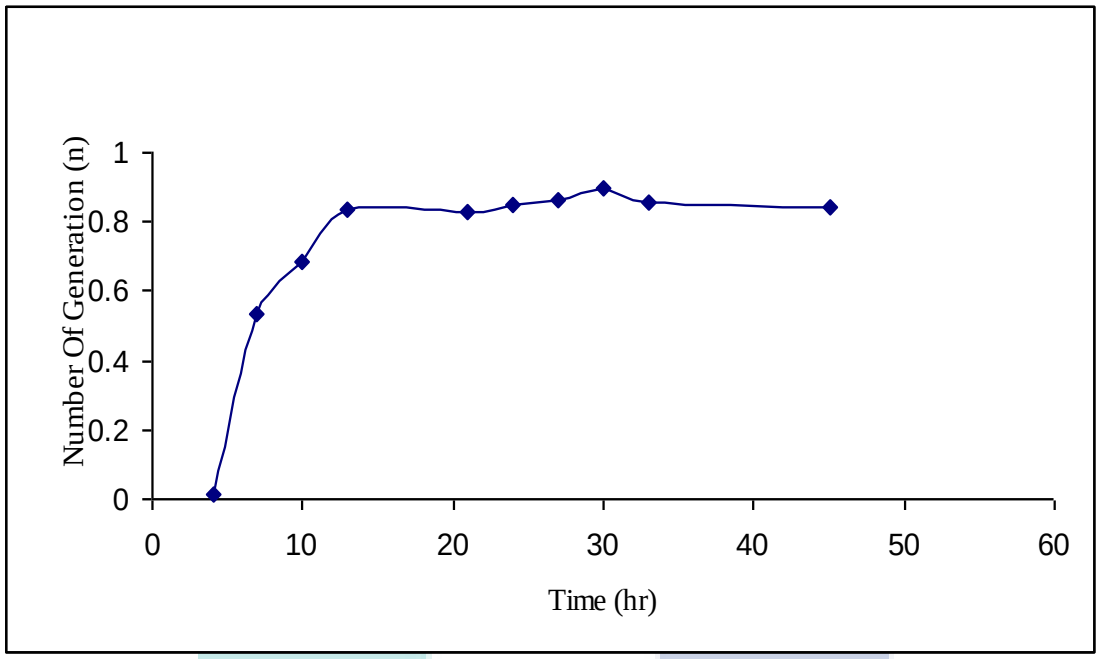
Appendix 4.37: Effect of aeration to generation time, *g* for *P. putida*



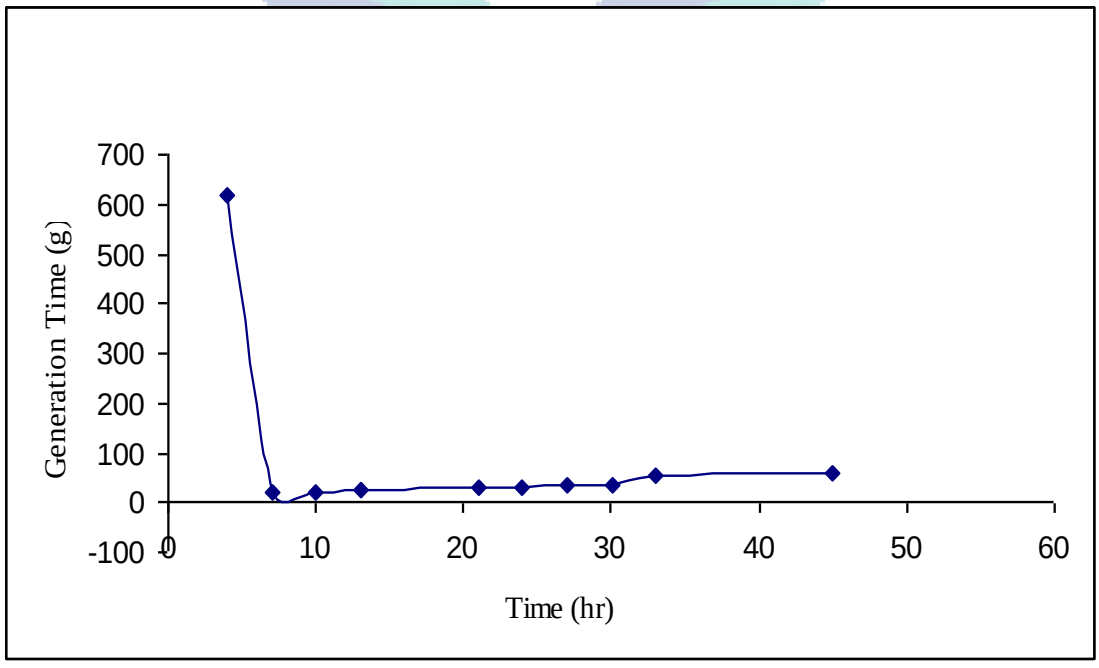
Appendix 4.38: Effect of aeration to growth rate constant, *k* for *P. putida*



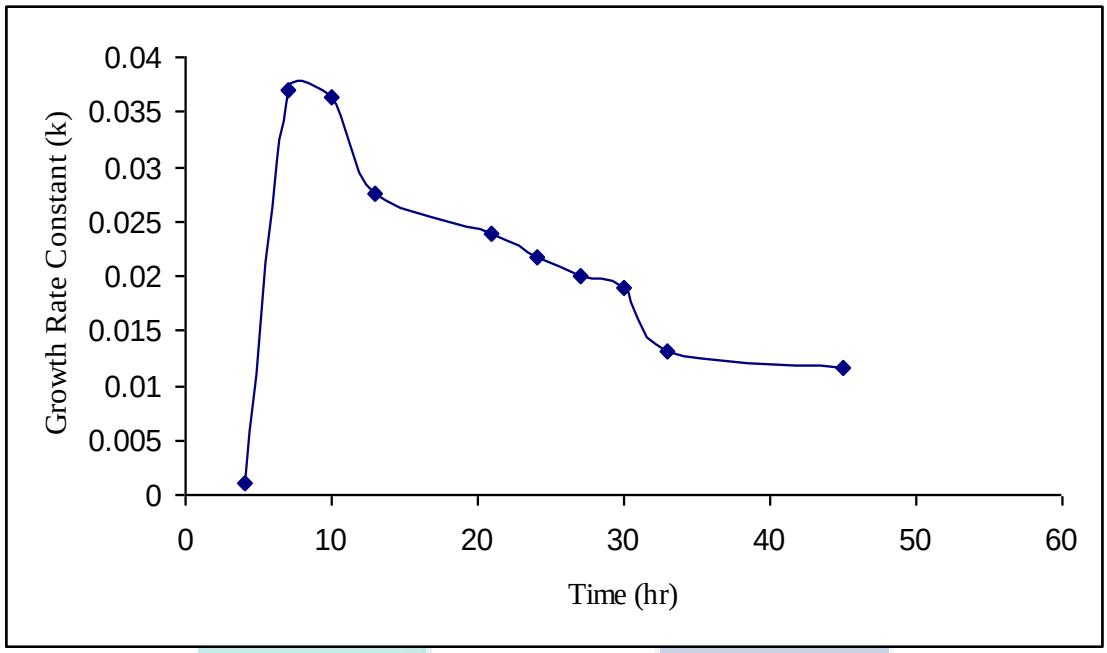
Appendix 4.39: Effect of Hg (1300 ppb) to number of generation, *n P. putida*



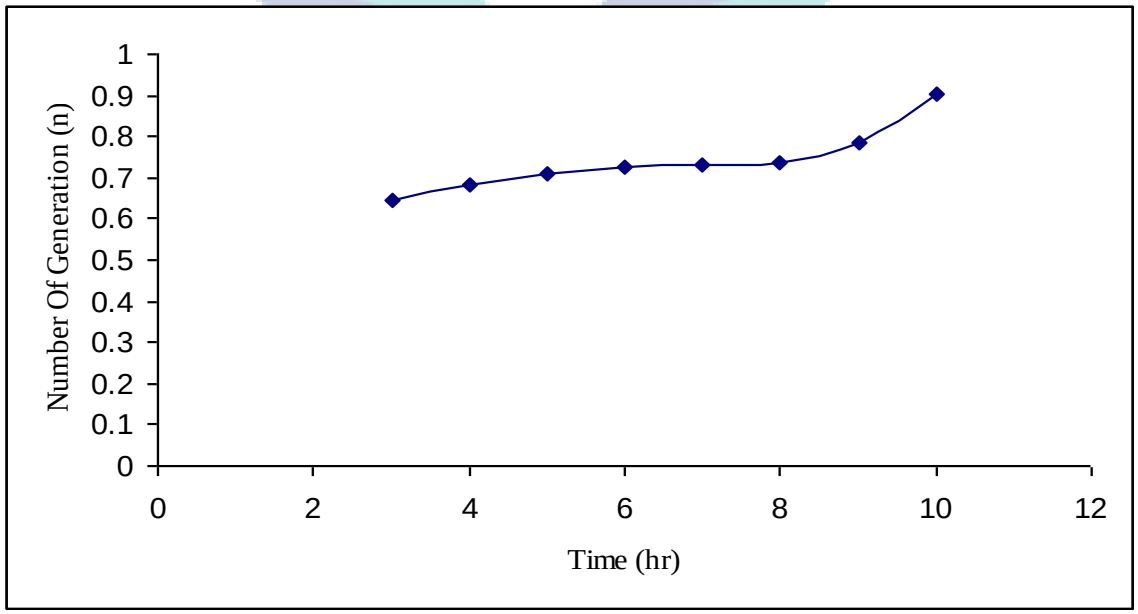
Appendix 4.40: Effect of Hg (1300 ppb) to generation time, *g P. putida*



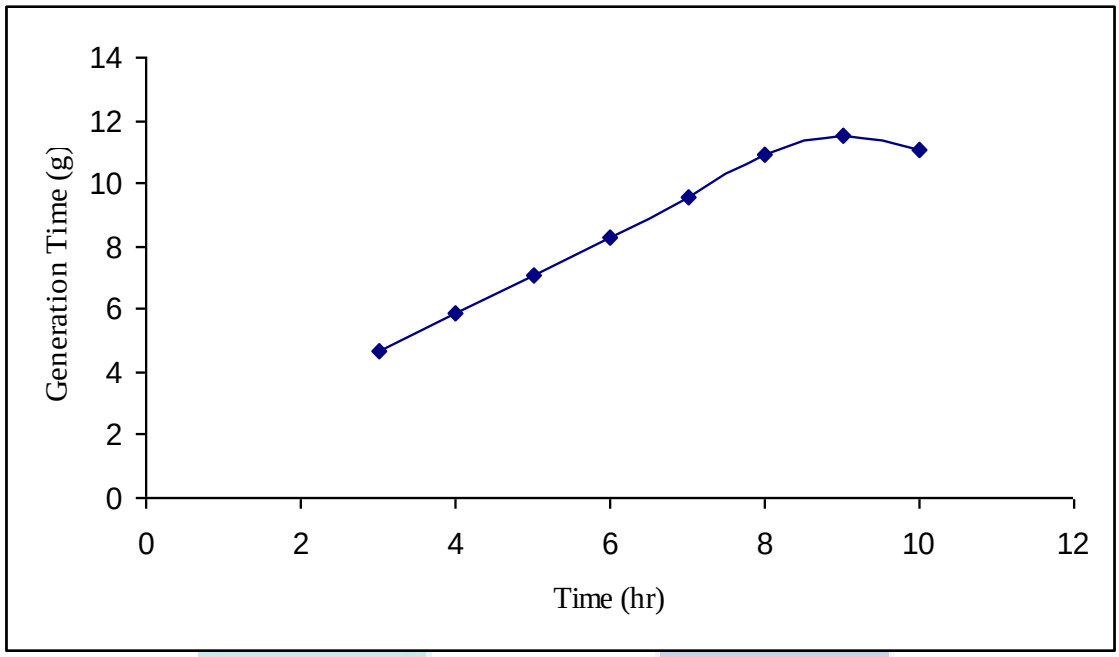
Appendix 4.41: Effect of Hg (1300 ppb) to growth rate constant, *k P. putida*



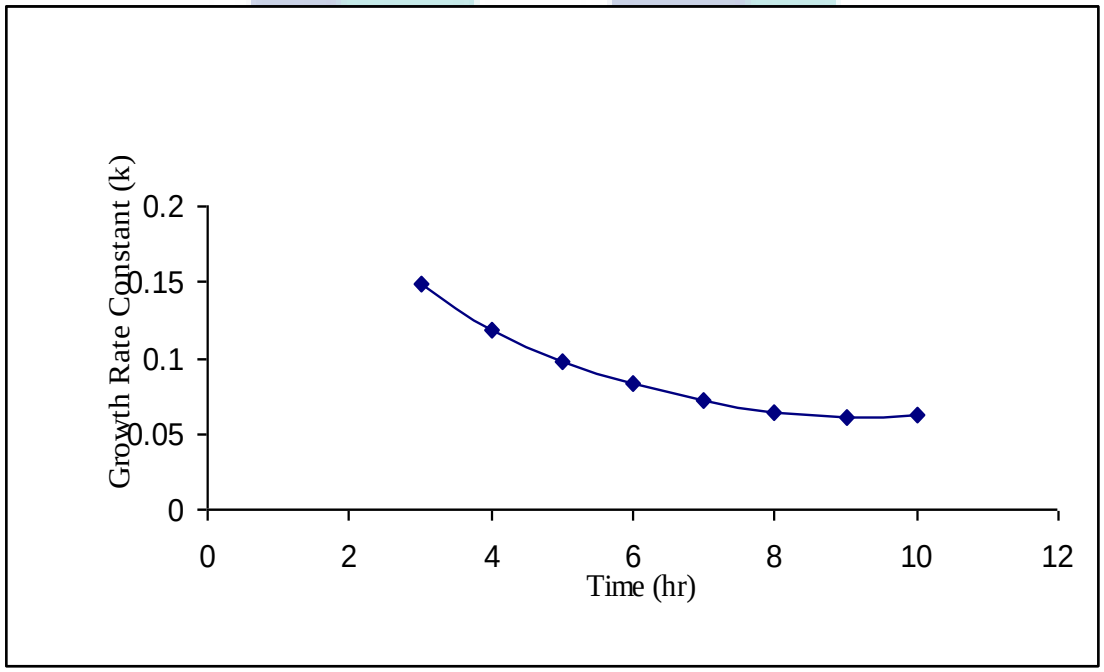
Appendix 4.42: Effect of Hg (3000 ppb) to number of generation, *n P. putida*



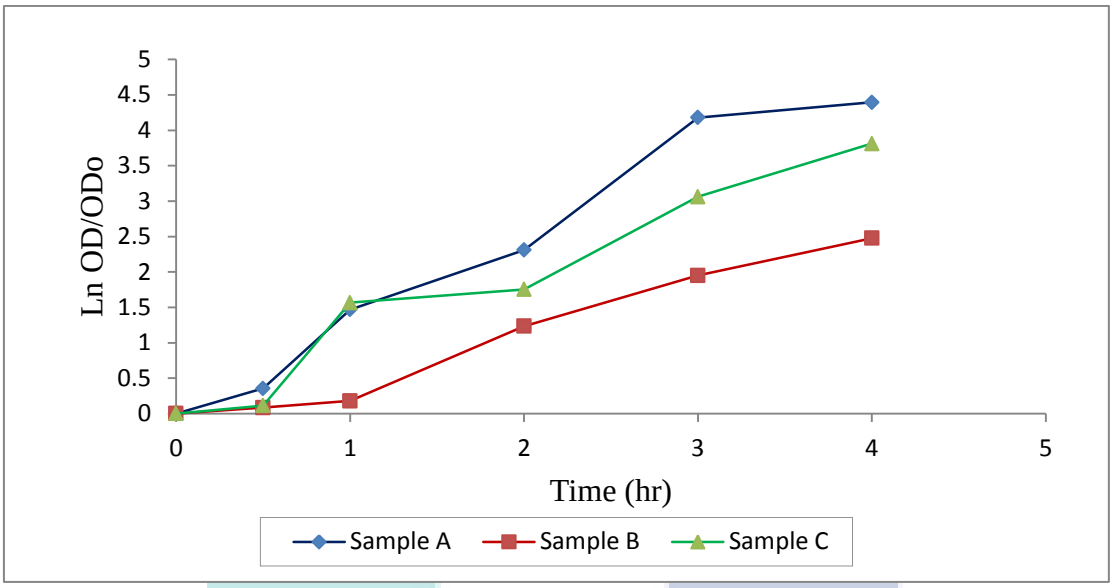
Appendix 4.43: Effect of Hg (3000 ppb) to generation time, *g P. putida*



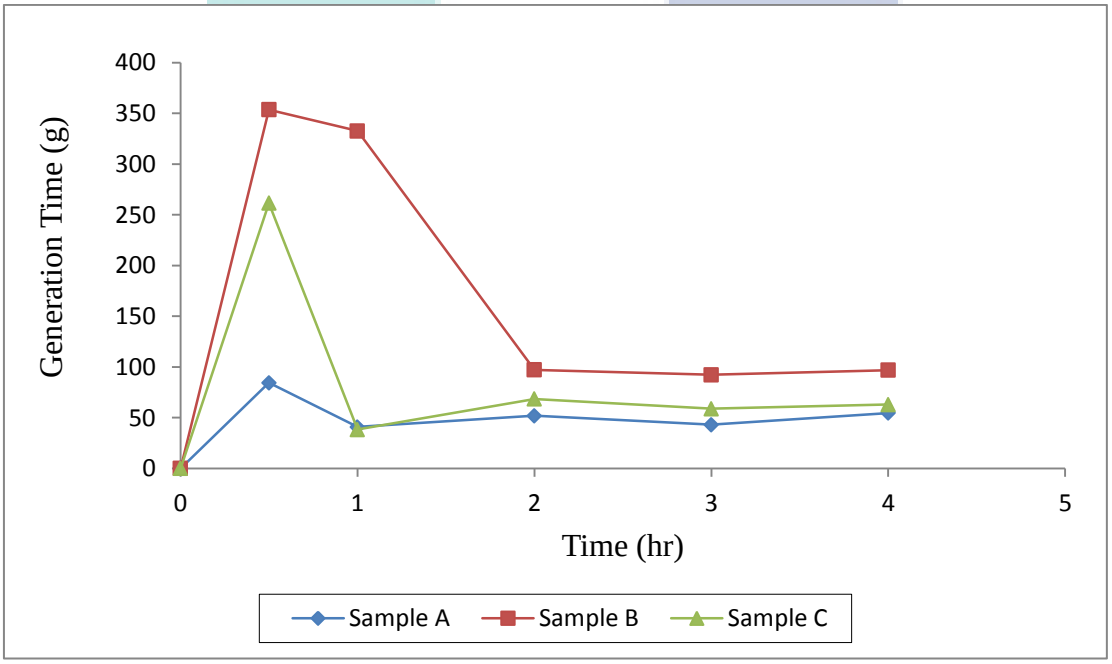
Appendix 4.44: Effect of Hg (3000 ppb) to growth rate constant, *k P. putida*



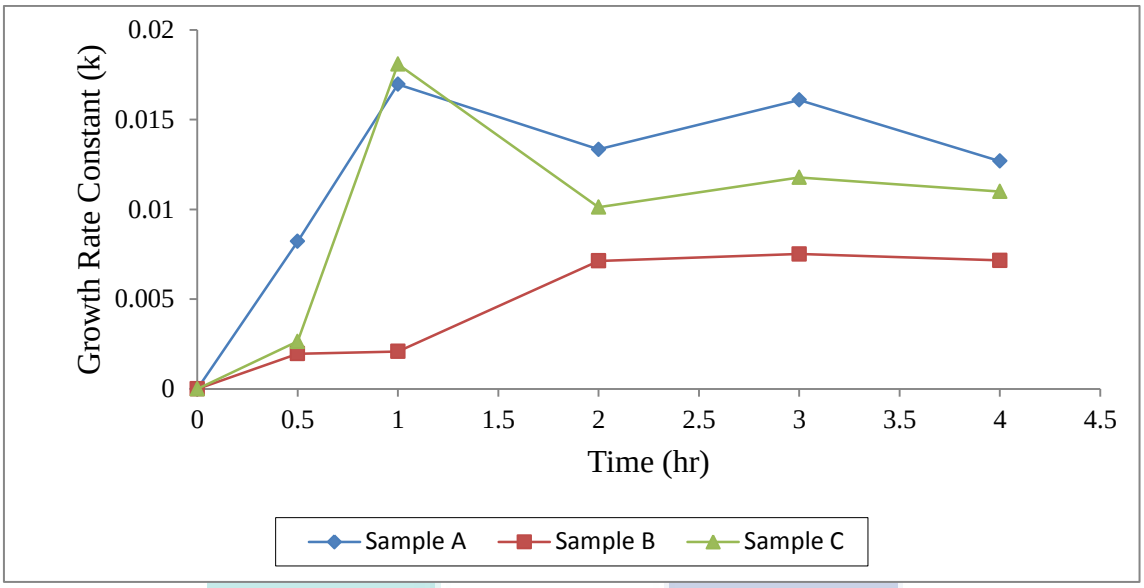
Appendix 4.45: Number of generation, *n* of *P. putida* in different sample media



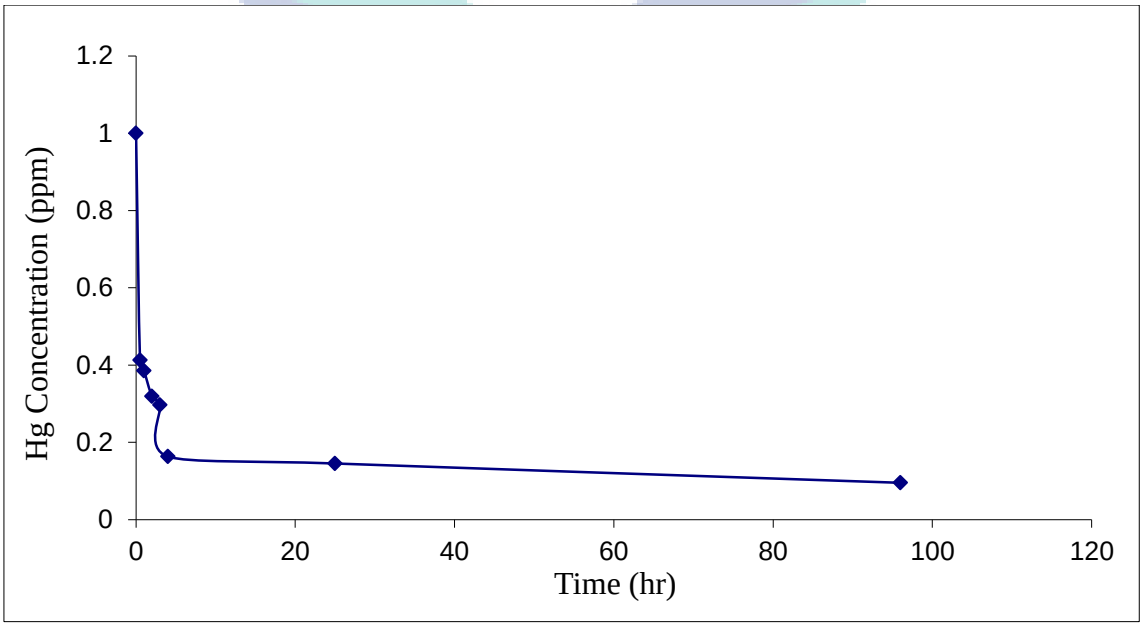
Appendix 4.46: Generation time, *g* of *P. putida* in different sample media



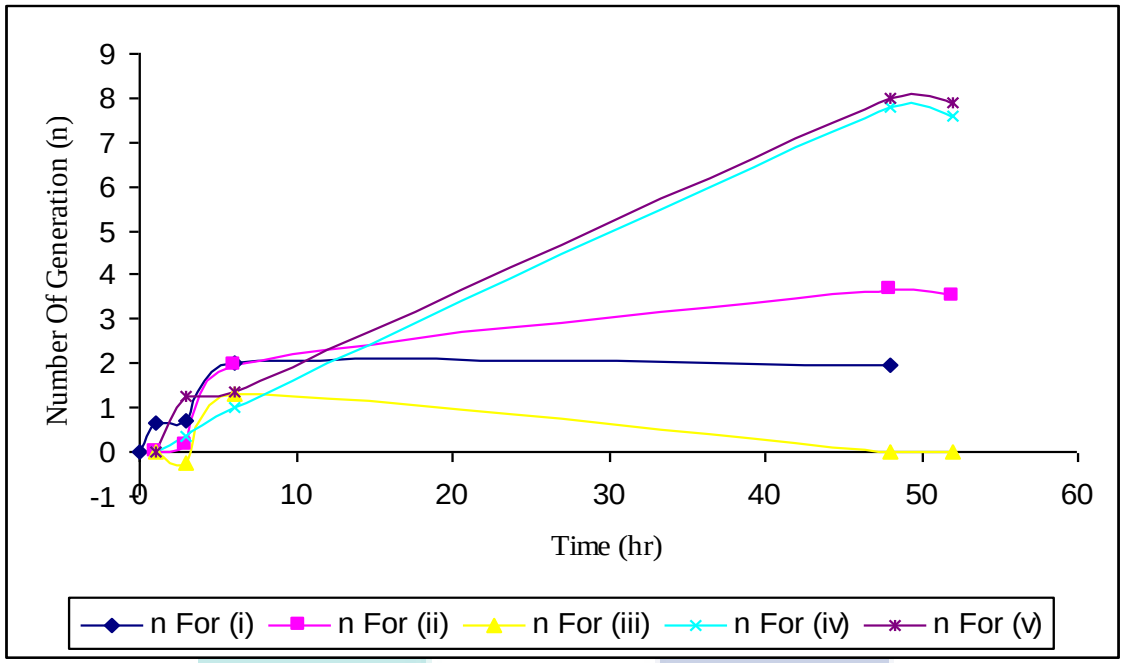
Appendix 4.47: Growth rate constant, *k* of *P. putida* in different sample media



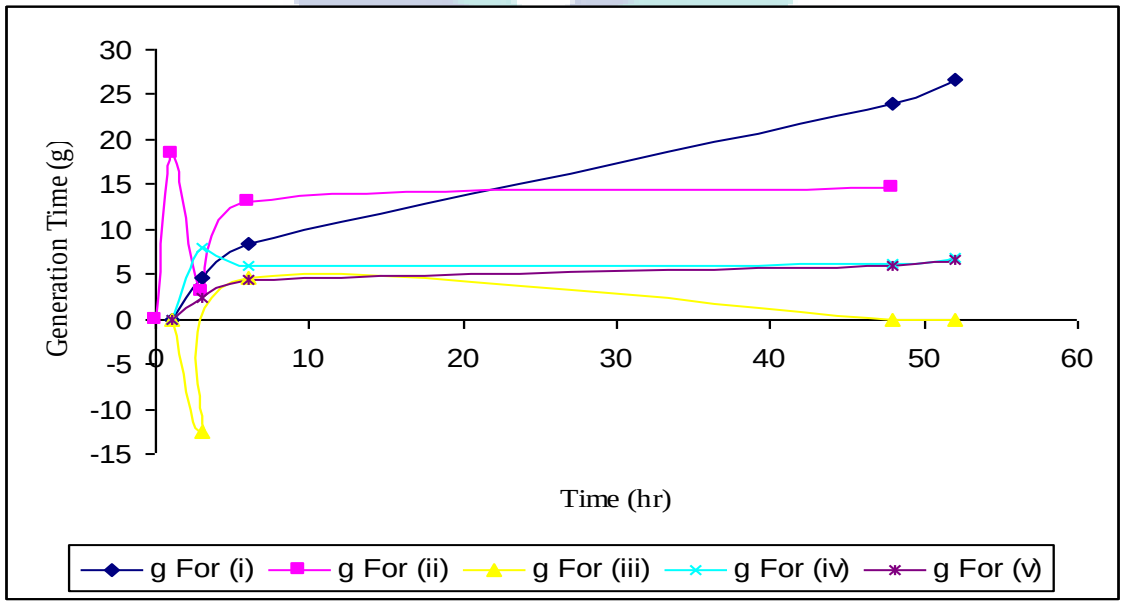
Appendix 4.48: Mercury concentration reduction in petroleum based industries wastewater for 96 hr in the shake flask



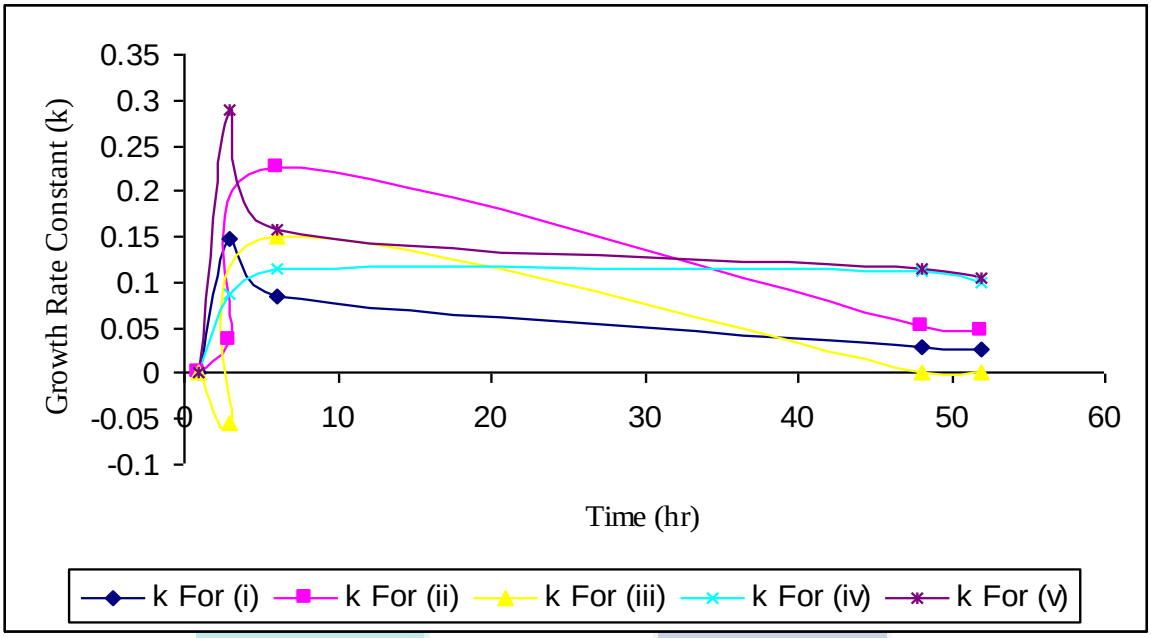
Appendix 4.49: Number of generation, *n* for *P. putida* and other microbes in petroleum based industries wastewater



Appendix 4.50: Generation time, *g* for *P. putida* and other microbes in petroleum based industries wastewater



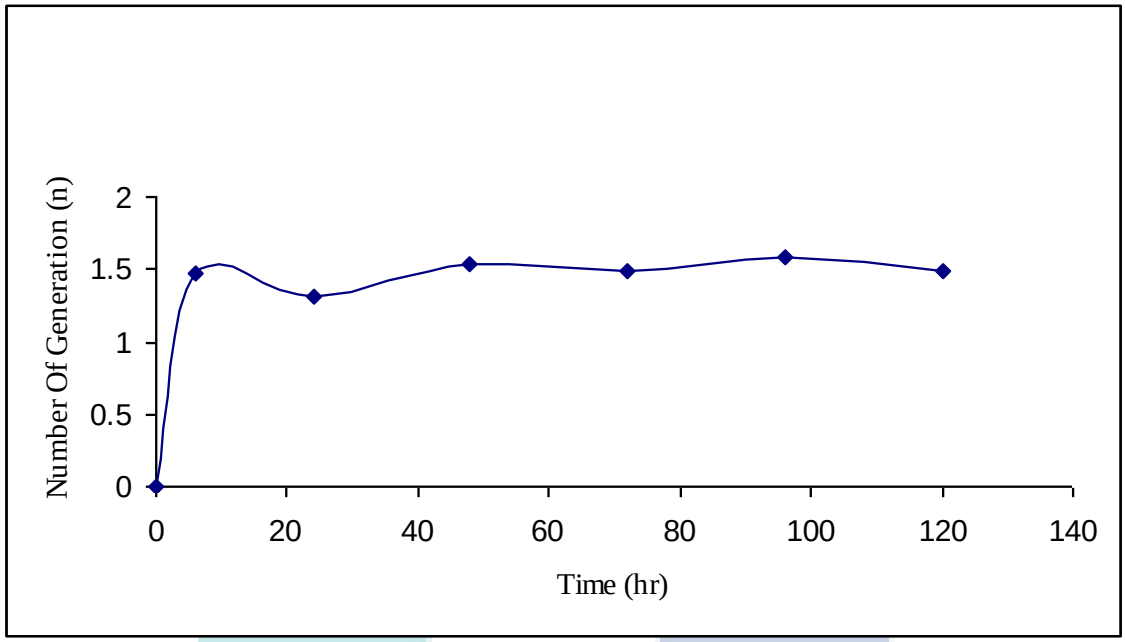
Appendix 4.51: Growth rate constant, *k* for *P. putida* and other microbes in petroleum based industries wastewater



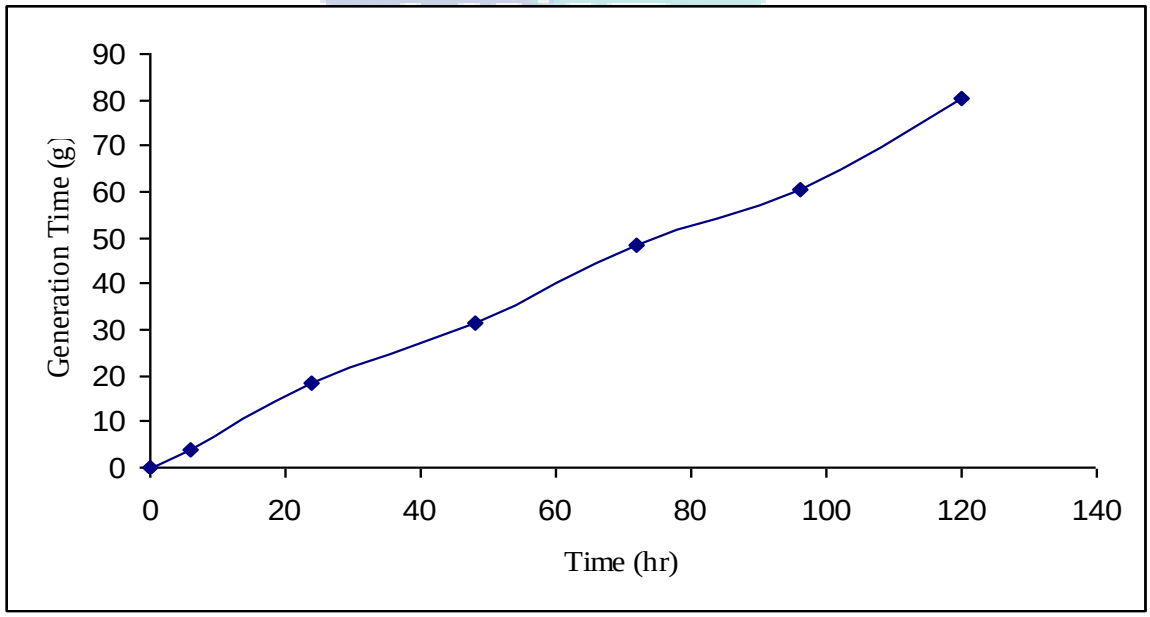
Appendix 4.52: The degree of water quality parameters presented by Metcalf and Eddy, 2003

Parameter	High Concentration (mg/L)	Medium Concentration (mg/L)	Low Concentration (mg/L)
Total solids	1200.00	720.00	350.00
Suspended solids	350.00	220.00	100.00

Appendix 4.53: Number of generation, *n* of *P. putida* in membrane bioreactor during mercury removal



Appendix 4.54: Generation time, *g* of *P. putida* in membrane bioreactor during mercury removal



Appendix 4.55: Growth rate constant, *k* of *P. putida* in membrane bioreactor during mercury removal