BIOREMEDIATION OF OIL FROM DOMESTIC WASTEWATER USING MIXED CULTURE: EFFECTS OF INOCULUM CONCENTRATION AND AGITATION SPEED

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I declare that this thesis entitled "*Bioremediation Of Oil From Domestic Wastewater Using mixed Culture: Effects Of Inoculum Concentration And Agitation Speed*" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Date : 14 MAY 2008

Special Dedication of This Grateful Feeling to My…

Beloved father and mother;
Mohd Zuhan Abd Rahman and Was Mastura Wan Mahmood

Loving brothers and sisters;
Azura, Zuriati, Azim, Amirul, Zuhairi and Nur Alim

For Their Love, Support and Best Wishes.

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ABSTRACT

The volume of oil in domestic wastewater is increasing each year due to the urbanization and industrial development all around the world. It is concerned that the increasing of oil in the wastewater could cause severe impact to the environment and to human health. Bioremediation of oil from domestic wastewater using mixed culture is being studied to overcome this problem. Microorganisms from local palm oil plant are utilised for this study. The ability of the microorganisms to degrade the oil is observed by investigating effect of concentration of the inoculum (g/ml) and the agitation speed (rpm) on oil removal. The optimum condition for these microorganisms to degrade oil is aimed for the highest volume of oil degraded. From the result obtained it is show that agitation with the speed of 150 rpm give the best condition for oil removal while the addition of 4g/110ml of inoculum concentration over wastewater and oil volume give the optimum oil removal. Higher concentration of inoculum cause high oil removal but at highly concentrated inoculum could cause reverse effect. Therefore high agitation also contributes to higher oil removal.

ABSTRAK

Isipadu minyak di dalam sisa air setempat telah meningkat setiap tahun berikutan perbandaran dan perkembangan industri yang pesat di seluruh dunia. Perkara trersebut telah mendatangkan kerisauan kerana peningkatan jumlah minyak di dalam sisa air boleh menyebabkan impak yang teruk kepada alam sekitar dan juga manusia. Walaupun boleh diuraikan secara semulajadi, minyak memerlukan masa yang lama untuk diuraikan. . Kebolehan microorganisma untuk menguraikan minyak diperhatikan melalui kajian kesan kepekatan inoculum (g/ml) dan kelajuan putaran (rpm) terhadap penguraian minyak. Keadaan optima untuk microorganisma menguraikan minyak adalah dicari untuk penguraian minyak terbanyak. Daripada keputusan yang diperolehi menunjukkan kelajuan 150 rpm memberikan keadaan terbaik bagi penguraian minyak manakala penambahan 4g/110ml (w/v) kepekatan inoculum terhadaapa air buangan dan minyak memberkana keadaan terbaik bagi penguraian minyak. Pada kepekatan yang tinggi menyebabkan penguraian yang banyak terhadap minyak namum pada kepekatan yang terlalu tinggi menyebabkan kesan yang sebaliknya. Namun begitu, kelajuan putaran menyumbangkan kepada penguraian minyak yang lebih tinggi.

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LIST OF ABBREVIATIONS

BOD – biological oxygen demand

COD – chemical oxygen demand

g - gram

l – liter

ml – milliliter

O&G- oil and grease

rpm – rotation perminute

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

In modern societies proper management of wastewater is a necessity, not an option. Wastewater is usually classified with characteristics compatible with municipal wastewater is often discharged to the municipal sewers. Many industrial wastewaters require pretreatment to remove non-compatible substances prior to discharge into the municipal system. Lipid (characterized as oils, greases, fats, and long-chain fatty acids) are one of the important organic composition of wastewater. Their amount in municipal wastewater is approximately 30-40% of the total chemical oxygen demand. (Chipasa & Medrzycka, 2006)

The concern over the behavior of lipid in biological treatment system has led to many studies, which have been evaluated their removal extensively. Oil wastes either of petroleum or vegetable origin are considered as serious types of hazardous pollutants in aquatic environments, due to their highly toxicity to the aquatic organisms (Mendiola *et al.* 1998). Wastewaters containing fat and oils were traditionally treated physically, which is currently considered insufficient if the fat is in its dispersed form. Biological treatment has been found to be the most efficient method for removing fat, oil and grease by degrading them into miscible molecules. Therefore, manipulation of microorganisms for treatment and bioremediation purposes affords a very efficient tool for purifying contaminated effluents and natural water (Glazer & Nikaido 1995). The use of lipase enzymes (triacylglycerol acylhydrolases) that are produced by all organisms may solve that problem, where they may catalyze the synthesis or hydrolysis of fats (Shabtai & Wang 1990). Under certain conditions, it is possible to isolate bacterial strains that are capable of degrading lipids by using a selective medium containing a source of lipid. Those lipid-degrading bacteria often produce extracellular lipase enzymes, where these enzymes are generally inducible in the presence of different inducers such as olive oil, palm oil, and oleic acid (Shabatai 1991; Shabatai & Daya-Mishre 1992; Sigurgisledottir *et al*. 1993). The synthesis and secretion of extracellular lipases by microorganisms appear to be controlled in a variety of ways (Jeager et al. 1994; Samkutty *et al.* 1996). However, microbial lipases constitute an important group of biotechnologically valuable enzymes (Wooley & Petersen 1994; Suzuk *et al*. 1998). Most of the well-studied microbial lipases are inducible extracellular enzymes, and they are synthesized within the cell and exported to its external surface or environment (Kosugi *et al*. 1988; Shabatai & Daya-Mishre 1992).

Vegetable oil is oil been removed from a plant or the seeds of a plant by pressing the seeds and extracting the oil with steam or water. Lipids are one of the most important components of vegetable oil and many synthetic compounds and emulsions and mostly found in pharmaceutical and cosmetic industrial effluents. Further, lipids constitute one of the major types of organic matter found in municipal wastewater (Quemeneur and, Marty Y; 1994).

The amount of lipid-rich wastewater increases every year due to urbanization and the development of factories. Suspended lipids can be readily removed from wastewater by physical methods. Nevertheless, chemically and/or physically stabilized lipid/water emulsions should be managed in an appropriate manner. This is necessary because lipids that pass through physicochemical treatment processes contribute to the levels of biological oxygen demand (BOD) and chemical oxygen demand (COD) in the effluents

(Chang *et al*; 2001). Thus, biological treatment processes will be employed in this study to remove the emulsified lipids from wastewater.

1.2 PROBLEM STATEMENT

During the last decade, the use of vegetable oils has increased worldwide. According to the US Department of Agriculture (USDA), in 2002/03, the world oilseed production rose to 328.9 million metric tons from 324.4 million in the previous year, (USDA, 2003). This intense activity applies to the transport, storage, handling, and processing of large amounts of these products and, consequently, there is a high possibility that accidental spills may occur. For this reason, in 1994, the US Environmental Protection Agency (USEPA) determined that, in accordance with the Oil Pollution Act of 1990 (OPA), animal fats and vegetable oils (AFVO) should not be exempted from regulations governing the cleanup of oil spills, (USEPA, 1994).

When a spill takes place, the physical properties and chemical composition of the discharged substance determine its fate in the environment. From the physical point of view, both types of oils (petroleum and non-petroleum) have analogous characteristics, i.e., they are insoluble in water and have lower specific gravity and higher viscosity than water. Consequently, when released into the water column, the mechanical behaviours of the oils are very similar. Because of the insolubility and lower density, oils form a layer on the surface of the water such that oxygen exchange between gas and liquid phases decreases. As a result, the water becomes depleted of dissolved oxygen, and aquatic life is potentially smothered. In the case of vegetable oils, suffocation is exacerbated as a consequence of their high BOD values when microbial degradation takes place, (Groenewold *et al*., 1982). Owing to their viscous nature, oils contaminate the feathers of birds and the fur of mammals, causing hypothermia, loss of buoyancy, and alterations in metabolism. Oils can also coat food and, therefore, cause starvation of the fauna or its poisoning because of the ingestion of polluted feed, (Crump-Wiesner and Jennings, 1975).

The fact is that the use of selected bacterial strains, with broad substrate range and high metabolic rates, frequently has failed in natural environments. The selected microorganisms that have beneficial traits for biodegradation must also be able to overcome biotic and abiotic stresses in the environment in which they are introduced (Alexander, 1999). Macnaughton *et al*. (1999) have demonstrated the early disappearance of the components of microbial consortia introduced in a natural environment polluted with hydrocarbons. Hence, the maintenance of sufficient activity of an inoculant population over a prolonged period after release often represents the main hurdle in the successful use of inoculants in bioremediation (Sanjeet *et al*., 2001)

Through this research we hope to acquire optimum condition which can be employed to degrade oil. Consideration will be taken upon the effect of agitation speed and inoculum concentration on the oil removal.

1.3 SCOPE OF RESEARCH

1. Bioremediation of domestic waste water which is cooking oil will be conducted using collected mixed culture.
2. This experiment will involve 2 different parameters which is the effect of 1) inoculum concentration 2) agitation speed (rpm).
3. The removal of waste water will be analyzed.

1.4 OBJECTIVE OF THE PROJECT

- The aim of this study is to determine the optimum conditions of bioremediation process for the removal of oil from domestic wastewater.
- Hence, the objective of the research are:

1. To study the effect of agitation speed towards the removal of oil-contaminating waste water.
2. To study the effect of different inoculum concentration on the removal of oil and grease in wastewater by mixed culture.

CHAPTER 2

LITERATURE REVIEW

2.1 BIOREMEDIATION - AN INTRODUCTION AND APPLICATION

Bioremediation, defined as the use of microorganisms to degrade environmental contaminants (Atlas and Cerniglia, 1995; Boopathy, 2000) has proved to be a useful tool in removing oil. Since crude oils contain such a wide range of molecular structures, it was postulated that mixed cultures capable of rapidly degrading crude oil might have broader applications in the general biotransformation of hazardous hydrophobic environmental contaminants. Their use eliminates the high cost implications of pure culture installations, provides greater metabolic diversity and a process able to degrade a variety of oily waste sludge and a process able to degrade a variety of oily waste sludge (Ward *et al*., 2003).

Bioremediation methods use microorganisms that occur naturally in the environment and degrade (mineralize) contaminants to less toxic or harmless products like carbon dioxide and water. Biological processes have been used successfully to remediate soils contaminated with petroleum hydrocarbons and their derivatives (Mulligan, Yong & Gibbs; 2001).

Bioremediation is an attractive approach of cleaning up petroleum hydrocarbons because it is simple to maintain, applicable over large areas, cost-effective and leads to the complete destruction of the contaminant (Frankenberger,1992). To warrant a practical application, any bioremediation process should demonstrate that removal of contaminants is the primary effect of biodegradation, and that the degradation rate is greater than the natural rate of decontamination. One of the difficulties of developing bioremediation strategies lies in achieving as good or better results in the field as in the laboratory (Juhasz *et al*., 2000).

Strategies for inexpensive and natural bioremediation include natural attenuation, biostimulation, bioventing, bioaugmentation, landfarming, composting, and phytoremediation (Skipper, 1999). Diesel oil bioremediation in soil can be promoted by stimulation of the indigenous microorganisms, by introducing nutrients and oxygen into the soil (biostimulation) (Seklemova *et al*., 2001) or through inoculation of an enriched microbial consortium into soil (bioaugmentation) (Richard and Vogel, 1999; Barathi and Vasudevan, 2001).

Various microorganisms have been used for the treatment of grease-containing restaurant wastewater and other grease-containing wastewaters. A method was proposed for the treatment of waste by the direct cultivation of lipophilic yeasts into the waste (Anon, 1994). Wakelin and Forster (1997) investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases. They cultivated pure and mixed microbial flora (known to produce lipases and other enzymes), and found that *Acinetobacter sp*. was the most effective of the pure cultures, typically degrading 60–65% of the fatty material, whose initial concentration had been 8 g/L. *P. aeruginosa* LP_{602} was tested for its ability to treat this type of wastewater (Dharmsthiti and Kuhasuntisuk, 1998). When these cells were added to the wastewater at approximately 107 CFU (colony forming units)/mL, they dominated the whole system, and no other microorganisms were found in detectable numbers. Indeed, the BOD was reduced by 94.1% after an 8-day incubation period. The lipid content in the wastewater was also rapidly reduced, and all the lipid content was removed within the first 5 days of treatment. Crude lipase from *P. aeruginosa* LP_{602} (3.5 U/ mL) was added to this same lipid-rich restaurant wastewater in a ratio of 1:1 and incubated with shaking at 37 ºC. The lipid content (approximately 200 mg/L) was reduced by 70%, to less than 10 mg/L, during the first 24 h and was not detected after 48 h. Tano-Debrah *et al*. (1999) developed an inoculum with high fat and oil degrading activities that consisted of a mixed culture of 15 bacterial isolates from various fatty wastewater samples taken from grease-traps of restaurants in Japan. All isolates were able to degrade various fats and oils to some degree. However, the extent of degradation varied for each fat/oil tested, being lower for shea fat (fat obtained from nuts of the tree *Butyrospermum parkii*). The variation in the composition of the fat/oil mixture caused variations in the development of the inoculum bacteria. The authors suggested that this variation highlights the need to identify more microorganisms with degrading activities in order to optimize and broaden the applications of lipid biodegradation technology.

Mongkolthanaruk and Dharmsthiti (2002) evaluated a mixed culture composed of *P. aeruginosa* LP_{602}, *Acinetobacter calcoaceticus* LP_{009} (both lipase-producing bacteria) and *Bacillus sp*. B_{304} (an amylase and protease producing bacterium) to lower the biochemical oxygen demand (BOD) value and lipid content of lipid-rich wastewater. It was demonstrated that this consortium of 3 bacterial cultures could be used successfully to treat lipid-rich wastewater. The BOD and the lipid content were reduced from 3600 mg/L and 21,000 mg/L, respectively, at day 0, to less than 20 mg/L within 12 days under aerobic conditions. Before these products can be widely applied however, it needs to be examined whether there are hazardous species present and whether they have any adverse environmental effects, especially when used in places with conditions that differ considerably from the conditions under which the bacteria were isolated.

Another alternative method to deal with high fat content in wastewater is the use of surfactants to facilitate the biodegradation by dissolving the fat and oils. Biosurfactants can easily be incorporated directly into the biological process, thus eliminating the need for additional processes and resulting in lesser operational costs. Nakhla *et al*. (2003) demonstrated the effectiveness of a biosurfactant derived from cactus (BOD-Balance) in improving the anaerobic digestion of high strength oil and grease laden rendering wastewater within a mesophilic temperature range. The biosurfactant applied at 500 mg/L to the raw wastewater resulted in substantial improvement in the overall anaerobic biodegradability with total and soluble COD reduction of 62% and 74%, respectively. After reduction of the O&G content by dissolved air flotation, the biosurfactant appears not to offer any further lipid degradation. However, the economic viability of using the biosurfactant with a cost of just 7–9 US dollars per liter (data supplied by the authors) should be considered.

Lipases have also been used for the degradation of wastewater contaminants from olive oil processing. De Felice *et al*. (2004) cultivated the yeast on wastewater from an olive oil mill under batch culture conditions. They found that the yeast was capable of reducing the COD value (100–200 g/L) by 80% in 24 h and producing a useful biomass of 22.45 g/L as single cell protein and enzyme lipases. Similar results were described by Scioli and Vollaro (1997), who reported lipolytic activity of 770 U/L in the fermentation medium after 24 h. Lanciotti *et al*. (2005) evaluated the ability of several *Y. lipolytica* strains to grow in olive mill wastewater and to metabolize its lipidic fraction. All strains studied were able to produce extra cellular lipases. Some strains, in addition to a high lipase activity, induced a COD reduction with respect to the uninoculated wastewater (control) ranging from 20% to 40% after 72 h at 25 ºC.

As noted by Lanciotti *et al*. (2005), the biological characteristics of a microorganism proposed for use in wastewater treatment should be considered carefully. Indeed, it is desirable to adopt species that are Generally Regarded As Safe (GRAS). Furthermore it is preferable that the species have limited nutritional requirements and have the ability to antagonize the growth of pathogenic species as well as the capacity to adapt to stringent environmental conditions (i.e. low temperatures and water activity, high concentrations of toxic substances).

The impact of the enzymatic hydrolysis of fat particles on the efficiency of a downstream anaerobic digestion process was evaluated by Masse´ *et al*. (2003).

Slaughterhouse wastewater containing fat particles was pretreated with 250 mg/L of pancreatic lipase PL-250 and delivered to an anaerobic sequencing batch reactor operated at 25 ºC. Approximately 35% of the neutral fat was hydrolyzed during pretreatment. However, the pretreatment presented only a small overall effect on the fat particle digestion, marked by a decrease of about 5% (3 h) in the digestion time to achieve 80% of reduction in the neutral fat and LCFA concentrations.

2.1.1 ENVIRONMENTAL FACTORS

2.1.1.1 Nutrients

Although the microorganisms are present in contaminated soil, they cannot necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be stimulated. Biostimulation usually involves the addition of nutrients and oxygen to help indigenous microorganisms. These nutrients are the basic building blocks of life and allow microbes to create the necessary enzymes to break down the contaminants. All of them will need nitrogen, phosphorous, and carbon. Carbon is the most basic element of living forms and is needed in greater quantities than other elements. In addition to hydrogen, oxygen, and nitrogen it constitutes about 95% of the weight of cells. Phosphorous and sulfur contribute with 70% of the remainders. The nutritional requirement of carbon to nitrogen ratio is 10:1, and carbon to phosphorous is 30:1. (Vidali, 2001).

2.1.1.2 Environmental Requirements

Microbial growth and activity are readily affected by pH, temperature, and moisture. Although microorganisms have been isolated in extreme conditions, most of them grow optimally over a narrow range, so it is important to achieve optimal conditions. Temperature affects biochemical reactions rates, and the rates of many of them double for each 10 °C rise in temperature. Above a certain temperature, however, the cells die.

Plastic covering can be used to enhance solar warming in late spring, summer, and autumn. Available water is essential for all the living organisms, and irrigation is needed to achieve the optimal moisture level. The amount of available oxygen will determine whether the system is aerobic or anaerobic. Hydrocarbons are readily degraded under aerobic conditions, whereas chlorurate compounds are degraded only in anaerobic ones. To increase the oxygen amount in the soil it is possible to till or sparge air. In some cases, hydrogen peroxide or magnesium peroxide can be introduced in the environment. Soil structure controls the effective delivery of air, water, and nutrients. To improve soil structure, materials such as gypsum or organic matter can be applied. Low soil permeability can impede movement of water, nutrients, and oxygen; hence, soils with low permeability may not be appropriate for *in situ* clean-up techniques. (Vidali, 2001).

2.1.2 BIOREMEDIATION STRATEGIES

Different techniques are employed depending on the degree of saturation and aeration of an area. In situ techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance. Ex situ techniques are those that are applied to soil and groundwater at the site which has been removed from the site via excavation (soil) or pumping (water). Bioaugmentation techniques involve the addition of microorganisms with the ability to degrade pollutants.

2.1.2.1 Bioaugmentation

Bioremediation frequently involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit: 1) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and 2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degrades if the land treatment unit is well managed.

Bioaugmentation purpose is to enhance natural biodegradation is a useful alternative (Vogel, 1996; Jansson *et al*., 2000; Cunningham *et al*., 2004). Bioaugmentation has met with varying degrees of success (Crawford and Mohn, 1985; Brodkorb and Legge, 1992; Leavitt and Brown, 1994; Vogel, 1996; Atlas and Bartha, 1992) and there has been a considerable debate over the efficacy of this methodology.

2.1.3 ADVANTAGES OF BIOREMEDIATION

• Bioremediation is a natural process and is therefore perceived by the public as an acceptable waste treatment process for contaminated material such as soil. Microbes able to degrade the contaminant increase in numbers when the contaminant is present; when the contaminant is degraded, the biodegradative population declines. The residues for the treatment are usually harmless products and include carbon dioxide, water, and cell biomass.

• Theoretically, bioremediation is useful for the complete destruction of a wide variety of contaminants. Many compounds that are legally considered to be hazardous can be transformed to harmless products. This eliminates the chance of future liability associated with treatment and disposal of contaminated material.

• Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible.

• Bioremediation can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation.

• Bioremediation can prove less expensive than other technologies that are used for clean up of hazardous waste. (Vidali, 2001)

2.2 OILY WASTEWATER

Oil wastes either of petroleum or vegetable origin are considered as serious types of hazardous pollutants when they find their way into aquatic environments, since they are highly toxic to the aquatic organisms and can completely damage the ecology of beach areas (Mendiola *et al*. 1998). Wastewaters containing fat and oils were traditionally treated physically, which is currently considered insufficient if the fat is in a dispersed form. Biological treatment has been found to be the most efficient method for removing fat, oil and grease by degrading them into miscible molecules. Therefore, manipulation of microorganisms for treatment and bioremediation purposes affords a very efficient tool for purifying contaminated effluents and natural water (Glazer & Nikaido 1995). The use of lipase enzymes (triacylglycerol acylhydrolases) that are produced by all organisms may solve that problem, where they catalyze the synthesis or hydrolysis of fats (Shabtai & Wang 1990). Under certain conditions, it is possible to isolate bacterial strains that are capable of degrading lipids by using a selective medium containing a source of lipid. Those lipid-degrading bacteria often produce extracellular lipase enzymes, where these enzymes are generally inducible in the presence of different inducers such as olive oil, palm oil, oleic acid and Tween 20 (Shabatai 1991; Shabatai & Daya-Mishre 1992; Sigurgisledottir *et al*. 1993). The synthesis and secretion of extracellular lipases by microorganisms appear to be controlled in a variety of ways (Jeager *et al.* 1994; Samkutty *et al.* 1996). However, microbial lipases constitute an important group of biotechnologically valuable enzymes (Wooley & Petersen 1994; Suzuk *et al*. 1998). Most of the well-studied microbial lipases are inducible extracellular enzymes, and they are synthesized within the cell and exported to its external surface or environment (Kosugi *et al*. 1988; Shabatai & Daya-Mishre 1992).

2.3 EFFECT OF AGITATION SPEED

Environmental condition could affect the production of extracellular proteolytic enzymes. Agitation rates have been shown to affect protease production in various strains of bacteria (Pourrat *et al*). In the present investigation, some bacteria grown in culture media with 4% inoculum size showed maximum protease activity at 170 rpm agitation speed after 48 of incubation. At this speed, aeration of the culture medium was increased which lead to sufficient supply of dissolved oxygen in the media (Kumar, C.G. and H. Takagi, 1999) Nutrient uptake by bacteria also will be increased resulting in increased protease production. At 200 rpm, protease activity was found to be reduced. This was perhaps due to denaturation of enzymes caused by high agitation speed (Lee *et al*). Agitation speed 0f 100 and 200 rpm affected the growth of the organism considerably. At 100 rpm, insufficient aeration and nutrient uptake perhaps caused the inability of bacteria to grow efficiently. At 200 rpm, however excessive aeration and agitation could occur which led to cell lysis and increased cell permeability due to abrasion by shear force. (Darah, I. and C.O. Ibrahim, 1996).