CHARACTERIZATION, IDENTIFICATION AND APPLICATION OF *Acinetobacter baumannii* SERDANG 1 FOR PHENOL BIODEGRADATION

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ZAILATUL HANI BINTI MOHAMAD YADZIR

A thesis submitted to the Postgraduate Studies programme as a requirement for the degree of Master of Science

DEPARTMENT OF CHEMICAL ENGINEERING
UNIVERSITI TEKNOLOGI PETRONAS
OCTOBER 2007

Specially dedicated to.....

My Father and Mother,

MOHAMAD YADZIR BIN HJ TAIB

SALINA BINTI HJ SOIB

My Sisters,

HAFIZATUL HANIDA

NAZIATUL AZUA

NUR IZZAH

NUR IZZATI

My Beloved Love,

HARMAN BIN RAMLI

with you all....

I have the strength to stand at all....

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CHARACTERIZATION, IDENTIFICATION AND APPLICATION OF *Acinetobacter baumannii* SERDANG 1 FOR PHENOL BIODEGRADATION

By

ZAILATUL HANI MOHAMAD YADZIR

August 2007

Chairman: Dr Mohd Azmuddin Abdullah, PhD

Department: Chemical Engineering

Phenolic compounds has long been recognized as one of the most recalcitrant and persistent substance in petroleum refinery effluents. The potential of microorganisms to catabolize and metabolize aromatic compounds has been recognized as effective means of disposing the hazardous wastes. A locally isolated bacterial strain Serdang 1 was investigated for phenol degradation. Based on Biolog ™ Identification System and a BLASTN search of GenBank, the complete sequences of bacteria strain Serdang 1 shared 98% similarity with *Acinetobacter baumannii*. The bacteria strain Serdang 1 was redesignated *Acinetobacter baumannii* Serdang 1 and was deposited at the GenBank under the accession number EF525671. Phenol biodegradation by free and Ca-alginate

immobilized cells was investigated in batch system and continuous packed-bed reactor. The optimal conditions for immobilization of cells were: 3% (w/v) of both sodium alginate and calcium chloride solution; 2 mm bead diameter and initial cell loading (ICL); 400 beads/flask. Optimum initial pH and temperature of phenol degradation were determined at pH 7.5 and 30°C, respectively for both free and immobilized cells. The best nitrogen source for both free and immobilized cells was ammonium sulphate at 1 g/L. Comparison of freely suspended and Ca-alginate immobilized cells on phenol degradation were carried out. The immobilized cell was able to degrade phenol at initial phenol concentration up to 2g/L, whilst free cells did not grow at all. Repeated use of immobilized cells showed that they could be used up to five cycles without loss of activity. Degradation of phenol in a packed-bed column (2.5 cm x 40 cm) with immobilized bacteria in alginate beads was carried out with phenol in minimal salts media, pre-aerated with air at 0.1ml/min. A flow rate of 1.5 ml/min and a bed height to diameter ratio of 15.2 was optimum to achieve 76.7% phenol removal of 200 mg/L phenol concentration.

Abstrak tesis yang dikemukakan kepada Senat Universiti Teknologi Petronas sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGECAMAN, PENGENALAN DAN APLIKASI *Acinetobacter baumannii* SERDANG 1 UNTUK BIODEGRADASI FENOL

Oleh

ZAILATUL HANI MOHAMAD YADZIR

August 2007

Pengerusi: Dr Mohd Azmuddin Abdullah, PhD

Jabatan: Kejuruteraan Kimia

Fenol telah lama dikenalpasti sebagai salah satu sebatian yang tegar dan kekal di dalam sisa penapisan petroleum. Keupayaan mikroorganisma untuk memetabolismakan sebatian aromatik adalah kaedah yang berkesan untuk menghapuskan sisa berbahaya ini. Bakteria tempatan strain Serdang 1 telah dipencilkan untuk kajian biodegradasi fenol. Berdasarkan Sistem Pengenalan Biolog dan pencarian pada GenBank, jujukan sepenuhnya bakteria strain Serdang 1 mempunyai 98% kesamaan dengan strain *Acinetobacter baumannii.* Strain bakteria Serdang 1 ini telah dinamakan sebagai *Acinetobacter baumannii* Serdang 1 dan didaftar di dalam GenBank di bawah nombor penambahan EF525671. Biodegradasi fenol oleh sel bebas dan sel tersekatgerak di dalam alginat telah dikaji di dalam sistem kultur sesekelompok dan di dalam reaktor suapan. Keadaan optimum bagi

sel tersekat gerak adalah: 3% (berat/isipadu) bagi kedua-dua larutan alginat dan kalsium klorida; 2 mm diameter gel dan muatan sel awal; 400 biji/kelalang. Keadaan optimum bagi biodegradasi fenol adalah pada pH 7.5 dan suhu 30°C bagi kedua-dua sel bebas dan sel tersekat gerak. Sumber nitrogen terbaik bagi kedua-dua sel bebas dan sel tersekat gerak adalah ammonium sulfat pada kepekatan 1 g/L. Perbandingan biodegradasi fenol antara sel bebas dan sel tersekat gerak telah dilakukan. Sel tersekat gerak mempunyai keupayaan untuk menguraikan fenol sehingga pada kepekatan 2 g/L, sedangkan sel bebas tidak dapat hidup. Pengulangan penggunaan sel tersekat gerak menunjukkan bahawa mereka dapat digunakan sebanyak lima kali tanpa kehilangan aktiviti. Biodegradasi fenol di dalam kolum dasar padatan (2.5 cm x 40 cm) berisi sel tersekatgerak telah dilakukan dengan menggunakan fenol di dalam media garam minima, yang telah diudarakan terlebih dahulu pada 0.1 ml/min. Kadar aliran 1.5 ml/min dan nisbah tinggi dasar kepada diameter pada 15.2 adalah optimum dengan pengurangan fenol sebanyak 76.7% daripada kepekatan fenol 200 mg/L.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

%	percentage
°C	Celsius temperature (centigrade temperature)
µg	Microgram
µl	Microliter
bp	Basepair
cfu	colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene-diamine-tetraacetic acid
g	gram
kb	Kilobase pair
L	liter
M	Molar
mg	milligram
$MgCl_2$	Magnesium chloride
ml	milliliter
mM	milimolar
pH	puissance hydrogen (Hydrogen-ion concentration)
rpm	round per minute
TBE	Tris-Base-EDTA-buffer
Tris-HCL	Tris (hydroxymethyl) aminomethane hydrochloride
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

Petroleum refineries and petrochemical plants are the hearts and souls of petroleum industries. Benefits are derived by conversion of crude oil into a wide range of products such as petroleum fuels, lubricants, bitumen, waxes, plastics and resins, synthetic rubber, materials for agriculture including agrochemicals and cleaning agents.

While undoubtedly petroleum-based industries are imminent contributors for national gross products and economic well beings, they are also associated with pollution of wastes generated world–wide (Ruiz-Ordaz *et al.,* 2001; Chang *et al.,* 1998). Prominent among these are phenol and its derivatives. Levels of 20-200 mg/l, far surpassing permitted phenol level in the environment have been reported from petroleum refineries (World Bank Group, 1998). In Malaysia, the Environmental Protection Act (1974) establishes the limit of a phenol concentration of 0.001 mg/L for Standard A, 0.1 mg/L for standard B, and 5 mg/L other than standard A and B, as the limit for wastewater discharges into inland waters. Based on the report by the Department of Environment (DOE) in 2005, phenol levels exceeding permissible level has also been recorded at solid landfill, urban/suburban, agricultural areas, industrial areas, ex-mining areas and rural areas.

Phenols are distributed throughout the environment as artificial or natural mono-aromatic compounds and could pose serious ecological problems

(Fava *et al.,* 1995). Being water-soluble, phenol is likely to eventually reach downstream drinking water sources and can cause severe odor and taste problems even at low concentrations (Collins and Daugulis, 1997b). The presence of phenol in drinking water and irrigation water represents serious health hazards to humans, animals, plants and microorganisms (Sharma *et al.*, 1997; Salonen *et al.*, 1989; Shailubhai, 1986). A concentration of 1 μg/L has been described permissible for drinking waters (WHO, 1994). Phenol concentrations greater than 50 ppb are toxic to some form of aquatic life and ingestion of 1 g of phenol can be fatal in human beings (Seetharam and Saville, 2003). As a result, the disposal of phenol has become a global concern (Percival and Senior, 1998). Effluents from phenol- generated industries require proper treatment before they can be discharged into natural sinks such as rivers, lakes or lagoons.

Different methods have been proposed to remediate sites contaminated with phenols. Among them is the use of activated carbons (Dabrowski *et al.,* 2005; Snoeyink *et al,* 1969). However, the higher the quality of activated carbon, the more expensive is the cost. This includes both chemical and thermal regeneration of the poisoned carbon, making it with additional effluent and considerable adsorbent loses (Dursun and Tepe, 2005). Other strategies for phenol remediation involve chemical oxidation, solvent extraction or irradiation, oxidation by ozone, ion exchange and biodegradation (Nuhoglu and Yalcin, 2005; Annadurai *et al.,* 2002; Garcia *et al.,* 2000; Alexander, 1999; Saha *et al.,* 1999). Of these, biodegradation has become an increasingly popular strategy as it is environmentally-friendly and more economical

(Alexander, 1999). Biodegradation offers the possibility of complete mineralization of phenol (Kobayashi and Rittmann, 1982).

Several studies have shown that phenol can be degraded by a wide variety of microorganisms, including pure bacterial cultures of *Acinetobacter calcoaceticus* (Paller *et al.*, 1995), *Alcaligenes eutrophus* (Léonard and Lindley, 1998; Hughes *et al.*, 1984), *Bacillus stearothermophilus* (Buswell, 1975), *Burkholderia cepacia* G4 (Schröder *et al.*, 1997; Solomon *et al.*, 1994; Folsom *et al.*, 1990), *Nocardia sp.* (Rizzuti *et al.*, 1979), *Nocardioides sp.* (Cho *et al.*, 2000), *Pseudomonas pickettii* (Fava *et al.*, 1995), *Pseudomonas putida* (Götz and Reuss, 1997; Hinteregger *et al.*, 1992; Bettmann and Rehm, 1984; Hill and Robinson, 1975; Yang and Humphrey, 1975; Feist and Hegeman, 1969) *Pseudomonas resinovorans* (Dikshitulu *et al.*, 1993), *Ralstonia eutropha* (Léonard *et al.*, 1999) and *Rhodococcus sp.* (Straube, 1987) and the fungal species of *Fusarium sp.* (Anselmo *et al.*, 1985) and *Aureobasidium* (Cheetham and Bucke, 1984). Several reports have included yeasts as also capable of breaking-down phenol such as *Candida spp.* (Krug and Straube, 1986; Hofmann and Krüger, 1985; Neujahr *et al.*, 1974) and *Trichosporon cutaneum* (Spånning and Neujahr, 1991; Gaal and Neujahr, 1979; Neujahr and Gaal, 1973). Others have reported degradation of phenol by mixed bacterial cultures (Farrell and Quilty, 1999; Mörsen and Rehm, 1990; Pawlowsky and Howell, 1973).

Phenol toxicity studies suggest that bacteria can adapt to low phenol concentrations, but the overall phenol biodegradation drops with increasing phenol

concentrations (Dean-Ross, 1989). At toxic concentrations, phenol inhibits microbial growth (Chung *et al.,* 1998; Ahmed, 1995; Fava *et al.,* 1995; Zaisev, 1995) and cause cell-lysis (Ruiz-Ordaz *et al.,* 1998). It is necessary to construct a barrier between microorganisms and the toxic phenol concentration to protect microorganisms from being damaged as well as to maintain a continuous cell growth and phenol degradation (Chung *et al.,* 1998). Immobilization of microorganisms behind a porous boundary is an established technique. Cell entrapment, in which the living cells are enclosed in a polymeric matrix which allows the diffusion of substrates into the cells and products away from the cells, is one of the most used techniques (Chung *et al.,* 1998; Wu and Wisecarver, 1992; Hobson and Millis, 1990; Yang and Wang, 1990; Kolot, 1988). Cell entrapment in calcium alginate for example is versatile, simple, cheap and non-toxic to biomolecules and microorganisms (Mörsen and Rehm, 1987). There is a possibility of repeated use of immobilized cells as cells are protected from immediate direct contact with high concentration of toxic pollutants (Chung *et al.,* 1998). Phenol degradation and purification of phenol containing waste waters using *Pseudomonas putida* (Aksu and Bulbul, 1999), *Acinetobacter* sp. strain W-17 (Beshay *et al.,* 2002) and *Ralstonia eutropha* (Dursun and Tepe, 2005) immobilized in Ca-alginate beads have been reported.

The objectives of this study are:

1. To isolate, characterize and identify the phenol-degrading bacteria from Malaysian soil.

2. To compare the activities of freely and Ca-alginate immobilized cells on phenol biodegradation in a batch system.

3. To establish important operating conditions for continuous phenol degradation in a packed-bed reactor.

CHAPTER II

LITERATURE REVIEW

2.1 Phenol as a "Friendly-Chemical"

Phenol, C_6H_5OH is an aromatic molecule containing a hydroxyl group attached to a benzene ring structure (Figure 2.1). Common names for phenol include benzene phenol, benzenol carbolic acid, hydrobenzene, monohydroxybenzene, monophenol, phenic acid, phenol alcohol, phenyl hydroxide and phenylic acid (Environment Canada, 1998a).

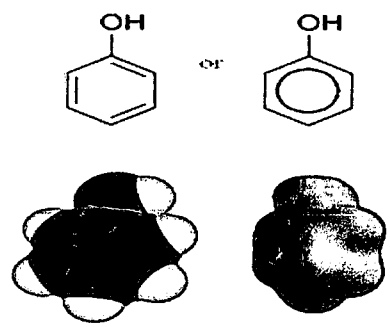


Figure 2.1: The structure of phenol.

Phenol has a molecular weight of 94.11 gm/mole (Lide, 1993). Its Chemical Abstracts Service (CAS) number is 108-95-2. It forms white to colourless crystals with a melting point of 43°C (WHO, 1994; Budavari *et al.*, 1989). The crystals turn pink or red on exposure to air and light and hastened in the presence of alkalinity or impurities (Windholz, 1983). In molten state, it is a clear, colorless liquid with low viscosity. It has

relatively high water solubility and is soluble in most organic solvents such as aromatic hydrocarbons, alcohols, ketones, ethers, acids, halogenated hydrocarbons (Kirk and Othmer, 1980; Lide, 1993). The solubility is however limited in aliphatic solvents (WHO, 1994). Phenol has a characteristic acrid smell and a sharp burning taste. The odor threshold of phenol in air is 0.04 ppm (v/v), with a strong sweet odor reported (ATSDR, 1989; Amoore and Hautala, 1983) and in water between 1 ppm and 7.9 ppm (w/v) (Baker *et al.*, 1978; Amoore and Hautala, 1983).

The annual productions of phenols are estimated around 1.25 x 10^9 kg (Béchard *et al.*, 1990). In 1995, the total annual capacity of phenol production approaches 2.03 billion kg (CMR, 1996). The most commonly used production method for phenol is from cumene (isopropylbenzene) (IARC, 1989) and from chlorobenzene and toluene. Phenol is used in the production of adhesives, explosives, coke, fertilizers, illuminating gas, paints and paint removers, rubber, asbestos goods, wood preservatives, textiles, drugs, pharmaceutical preparations, perfumes and bakelite (Environment Canada, 1998a; Deichmann and Keplinger, 1981). It is also used as a feedstock in the production of other organic substances (including phenolic resins, bisphenol A, caprolactam, aniline, adipic acid, alkyl phenols, chlorophenols and other chemicals). In Canada, the manufacturing of phenolic resins (Figure 2.2) account for about 85% of phenol consumption (Environment Canada, 1998b). Most of these are used for oriented strand board production, panels, insulation, and articles and formulations such as paints, lubricants, creams, adhesives, brakes, electrical components and electrodes (SRI International, 1993).

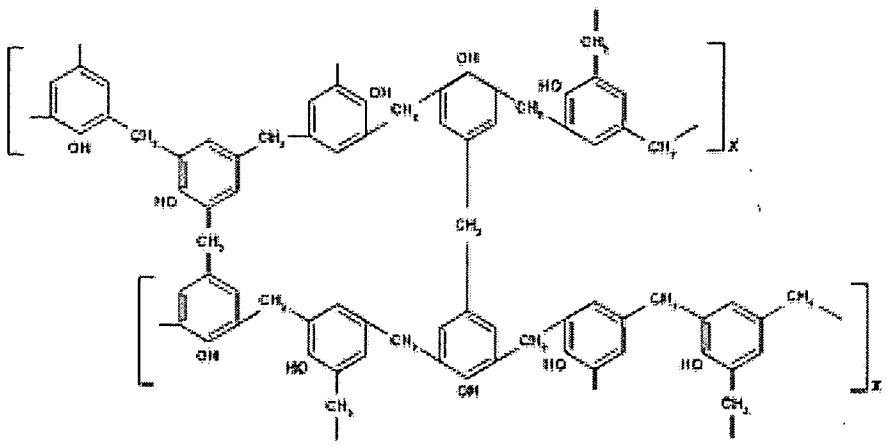


Figure 2.2: Phenolic resins.

Phenol is also an effective disinfectant against vegetative Gram-positive and Gram-negative bacteria and some fungi and viruses (Reynolds, 1989). Weak solutions of phenol have been used topically for disinfection, and a 5% solution is used as a disinfectant for excreta. Other disinfectants contain 20–50% phenol compounds, and fungicides contain 2–4.5% phenol (Gosselin *et al.*, 1984). It is also present in antibacterial preparations that are given topically or orally. In liquid formulations, it is present at about 2%, and in medicinal tablets and lozenges up to 33 mg per tablet/lozenge (Gosselin *et al.*, 1984). Phenol is commonly used as an antipruritic (relieves itching) and is used in the form of phenolated calamine lotion (1%), phenol ointment (2%) or a simple aqueous solution (0.5–1%) (Gennaro, 1990). In a number of non-prescription drugs in Canada, phenol can be found as an active ingredient including in lip balms, ointments and throat sprays or lozenges (Kealey, 1997).

2.2 An "Unfriendly-Phenol" with Its Hazardous Effects

Phenol exposure to human occurs most often through skin contact. Being a local anaesthetic, no pain is normally felt initially upon initial contact with phenol. By the time pain is felt, serious burns and absorption through skin may have occurred (Allen, 1991). Local damage to the skin includes erythema, inflammation, and necrosis. Chronic doses may result in ochronosis (yellowing of the skin) and skin eruption (WHO, 1994). The effects are worse when the application sites are bandaged (WHO, 1994). A white, brown or red discolouration of the skin may occur (Leikin and Paloucek 1996-7). Systemic intoxication can occur from absorption (WHO, 1994); with roughly 50 % of all reported cases have fatal outcomes (Horch *et al.,* 1994). The cause of death is often respiratory failure (WHO, 1994). Phenol vapors are irritating to the upper respiratory tract. Upon ingestion, phenol produces burning pain and white necrotic lesions in the mouth, esophagus and stomach, vomiting and bloody diarrhea and can also lead to death (WHO, 1994; Reynolds, 1993; Allen, 1991; Foxall *et al.,* 1989). Direct contact of phenol with the eyes can cause severe burns and permanent corneal damage which could result in blindness (WHO, 1994).

The toxic effects of phenol on terrestrial and aquatic organisms have been extensively studied. Rats exposed continuously for 15 days to a concentration of 26 ppm phenol, have shown muscle tremors, twitching and disturbances in walking rhythm and posture after 3-5 days exposure (Dalin and Kristofferson, 1974). After 15 days, severe neurological impairment has been reported as measured by decreased performance on

tilting plane test, and liver damage as observed by the elevated serum concentrations. Male rats exposed to 5000 ppm of phenol in their drinking waters for 103 weeks had a higher incidence of kidney inflammation (NCI, 1980). Jones-Price *et al.* (1983b) report that pregnant mice dosed orally with phenols exhibit decreased maternal weight gain, tremors, and increased maternal mortality; whilst the fetus shows reduced growth, decreased viability, and increased incidence of cleft palate. Increased mortality is also observed in earthworms and terrestrial plants (Environment Canada, 1995; Neuhauser and Callahan, 1990; Neuhauser *et al.*, 1986). Toxic levels of phenol could inhibit seedling emergence and nitrification in plants (Den Blanken, 1993; Beccari *et al.*, 1980). Of aquatic organisms examined such as fish and other invertebrates, freshwater algae are far less sensitive to phenol exposure in fresh water (Tisler and Zagorc-Koncan, 1995), suggesting its potential as an environmental screen from toxic contaminants.

2.3 Phenol as an "Environmentally-Unfriendly" Pollutant

The origin of phenol in the environment is from natural, man-made and endogenous sources. Phenolic substances may occur naturally in water and soil as the decomposition product of plants, vegetation and animal waste (Dobbins *et al.,* 1987). Animal and decomposition of organic wastes are the two natural sources of phenol in aquatic media. However, these are mostly in trace amounts (CCREM, 1987). Increased concentrations from natural sources may instead result from forest fires (IPCS, 1994a). The major contributor of phenolic pollutants are man-made from industrial wastes such as fossil fuel extraction, chemical, pharmaceutical, wood processing industry and pesticide manufacturing plants (Kumaran and Parachuri, 1997). Table 2.1 shows different sources of phenols and related aromatic compounds in wastewater.

Table 2.1: Sources of phenols and other related aromatic compounds in wastewater.

Sources	Significant Phenolic Compounds
Petroleum refining	Hydrocarbons (alkanes, cycloalkanes, polyaromatic hydrocarbons), benzenes, substituted benzenes, toluenes, *n*-octanes, *n*-decanes, naphthalenes, biphenyles, **phenol**, cyanide, sulphide and ammonia.
Petrochemicals	Naphthalene, hepatanes, benzenes, butadiene, C-4 alcohols, **phenol** and resorcinol.
Basic organic manufacturing chemicals	*m*-amino **phenol**, resorcinol, dinitrophenol, p-nitrophenol, trinitrophenol, benzene sulphonic acids, aniline, chlorobenzenes, toluene and resorcinol.
Coal refining	**Phenol,** catechol, *o*-, *m*-, *p*-cresols, resorcinol, hydroquinone, pyrogallol, polyaromatic hydrocarbons, pyridine, pycolines, lutidines, xylenes, toluenes, benzoic acid.
Pharmaceuticals	Toluenes, benzyl alcohols, phenyl acetic acid, chlorinated products of benzene, chloroform, ether, ethyl alcohol.
Tannery	Tannin, catechin, **phenol**, chlorophenol, nitrophenols.
Pulp and paper mills	Lignin, vanillin, vanillic acid, dehydrodivanillin, ferulic acid, cinnamic acid, synringic acid, vieratric acid, protocatechuic acid, gentisic acid, benzoic acid, guadiachols, catechol, coniferyl alcohol, dehydrodihydroconiferyl alcohol, phenyl propionic acid, **phenols** and chlorophenols.

(Source: Kumaran and Paruchuri, 1997)

Phenol has been found in surface water, ground water, soil and sediment (HazDat, 1998). It is estimated that a total of 10.6 million kg of phenol has been released into the environment from 689 large processing facilities; 4.3 million kg released into the air, (accounting for about 5% of environmental releases); 71,577 kg released into the soil from 102 large processing facilities, (accounting for about 0.7% of total environmental releases) and another 32,650 kg released into water from 230 large processing facilities, (accounting for about 0.3% of total environmental releases) (TRI, 1998). Phenol may be released into the atmosphere from storage tank vents during transport loading (Delaney and Hughes, 1979), incinerators, residential wood burning and automobile exhaust (Den Boeft *et al.,* 1984; Scow *et al.*, 1981). Phenol may be released to the soil during its manufacturing process, loading and transport when spills occur or when it leaches from hazardous waste sites and landfills (Xing *et al.*, 1994). In the exhaust gases of private cars, phenol is detected at 0.3 ppm (approximately 1.2 mg/m^3) to 1.4-2.0 ppm (5.4-7.7 mg/m^3) (Verschueren, 1983; Kuwata *et al.*, 1980). Emissions from waste incinerator plant have been reported at 0.36 ppb (Jay and Stieglitz, 1995) and from cigarette smoke, in quantities that are comparable to an average emission of 0.4 mg/cigarette (Groenen, 1978).

Generally, the data on phenol concentrations in soil at sites other than hazardous sites are lacking. This can be attributed to a rapid biodegradation rate and leaching. Phenol found in soils may be a result of continuous or consistent releases from a point source. Phenol that leaches through soil to groundwater spends at least some time in that soil as it travels to the groundwater. Phenol in groundwater can be detected mainly at or

near hazardous waste sites. Levels of phenol concentration in wastewater from selected industries are as shown in Table 2.2. The most common anthropogenic sources of phenol in natural water include coal tar (Thurman, 1985) and wastewater from manufacturing industries such as resins, plastics, fibers, adhesives, iron, steel, aluminum, leather, rubber, and influents from synthetic fuel manufacturing (Parkhurst *et al.*, 1979), paper pulp mills (Keith, 1976) and wood treatment facilities (Goerlitz *et al.*, 1985). About 3.8 kg/day of phenol is released to seawater from municipal treatment facilities (Crawford *et al.*, 1995). Other release of phenol resulting from commercial uses of phenol and phenol-containing products, include slimicides, general disinfectants (Budavari *et al.*, 1989; Hawley, 1981), medicinal preparations such as ointments, ear and nose drops, cold sore lotions, mouthwashes, gargles, toothache drops, analgesic rubs, throat lozenges (USEPA, 1980), and antiseptic lotions (Musto *et al.*, 1977).

Table 2.2: Typical levels of phenol concentration in wastewater of some selected industries.

Selected industry	Phenol concentration ($mg\ L^{-1}$)	Reference
Phenol production	3,000-4000	Godjevargova *et al.*, 2003
Pulp and paper	33.1-40	Peralta-Zamora *et al.*, 1998; Minussi *et al.*, 1998
Textile	12.3	Kunz *et al.*, 2001
Olive oil mill	3000-10,000	Klibanov *et al.*, 1983; Borja *et al.*, 1992; Hamdi, 1992; Martinez-Neito *et al.*, 1992; Knupp *et al.*, 1996; Robards and Ryan, 1998
Coal conversion plant	4-4780	Parkhurst *et al.*, 1979
Shale oil wastewater	4.5	Hawthorne and Sievers, 1984
Ash-heap water (oil shale)	500	Kahru *et al.*, 1998
Phenolic resins production	1200->10,000	Patterson, 1985; Kavitha and Palanivelu, 2004
Methyl violet and cumenephenol production	310-660	Kanekar *et al.*, 1999
Chemical specialities-manufacturing	0.01-0.30	Jungclaus *et al.*, 1978
Petroleum oil refinery	33.5	Pfeffer, 1979

(Source: Piakong, 2006)

2.4 Methods of Phenol Remediation

Different methods have been designed to remediate phenol contamination either *in situ* or *ex situ*. These could be either by physico-chemical means (Larson and Chuang, 1994) or by biological means (Fang *et al.,* 1996). Adsorption by activated carbons is the most frequently used method to remove phenol (Dabrowski *et al.,* 2005; Snoeyink *et al.,* 1969), but it can be costly. Chemical oxidation, solvent extraction or irradiation, oxidation by ozone and ion exchange (Nuhoglu and Yalcin, 2005; Annadurai *et al.,* 2002; Garcia *et al.,* 2000; Saha *et al.,* 1999) have some serious drawbacks which include the formation of hazardous by-products (secondary pollution) and high costs (Atlow *et al.,* 1984).

Interest in the use of microbial degradative ability is growing due to its environmentally-friendly nature and cost-effectiveness (Alexander, 1999; Müller and Babel, 1996). Biodegradation is defined as the biologically-catalyzed reduction in the complexity of chemical compounds into lesser or non-toxic products, the latter being preferable (Alexander, 1994). Bacteria, fungi, yeast and algae of different genera and species have been reported to be capable of degrading phenol.

2.5 Engineering Considerations in Phenolic Bioremediation

2.5.1 Aerobic or Anaerobic System

Phenol can be degraded biologically via either aerobic or anaerobic pathways. In anaerobic respiration, different inorganic electron acceptors, such as NO_3^-, SO_4^{2-}, CO_2 and Fe^{3+} are possible. A different pathway must be utilized to breakdown phenol as oxygenases will not be induced under anaerobic conditions. The carboxylation of phenol to 4-hydroxybenzoate by a denitrifying *Psedomonad* can occur and is the first step in the breakdown of phenol under anaerobic conditions (Tschech and Fuchs, 1987). Major disadvantages with anaerobic systems are the length of time required to completely degrade phenol (Wang *et al.,* 1989), and the difficulty in ensuring a system with complete lack of oxygen. Equipment costs may be increased, maintenance of culture collection is harder and process control is difficult. Tolerance to phenol is also limited in anaerobic cultures (Craik *et al.,* 1992), with a limit of only 1g L^{-1}, as compared to up to 4g L^{-1} in aerobic cultures (Betmann and Rehm, 1984). Hence, aerobic processes are generally preferred to degrade phenolic compounds (Fedorak *et al.,* 1984) due to the low costs as well as the possibility of complete mineralization of phenol (Collins and Daugulis, 1997b).

Aerobic processes require molecular oxygen for complete degradation of phenol. The initial stages in phenol catabolism involve the use of oxygenases to oxidize the substrate. Here, oxygen acts as the electron acceptor. Molecular oxygen is a reactant for

oxygenases and is incorporated into the final products. In the transfer of electrons between the electron-donor and electron-acceptor, substrates are essential for maintaining the biomass. Aromatic compounds may be converted to a few substrates such as catechol, protocatechuate or gentisate (Löcher, 1991). Figure 2.3 shows representative aromatic compounds that are converted via catechol. In the biodegradation of phenol, phenol is the primary substrate and must be made available in order to have active biomass in the biodegradation process. Once active biomass is present, any biotransformation reaction can occur, provided the microorganisms possess the necessary enzymes to catalyze the reaction (Rittmann and Sàez, 1993). Enzymes that are involved in the aerobic metabolism of aromatic compounds would define the range of substrates that can be transformed by certain metabolic pathways (Pieper and Reineke, 2000).

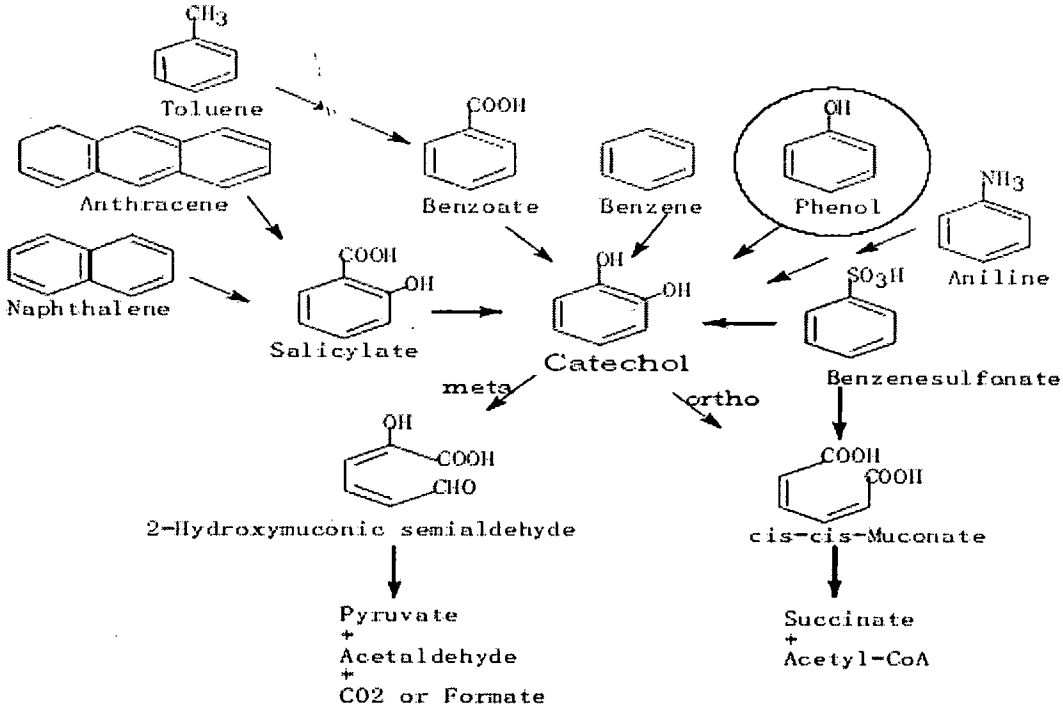


Figure 2.3: Microbial metabolism of some aromatic compounds via catechol (Adapted from Löcher, 1991).

The first step in aerobic metabolism of phenol is phenol hydroxylation to catechol by phenol hydroxylase (EC 1.14.13.7), a NADPH-dependant flavoprotein (Enroth *et al.*, 1998; Neujahr and Gaal, 1973). It incorporates one oxygen atom of molecular oxygen into the aromatic ring to form catechol as shown in Figure 2.4. Phenol hydroxylases, strictly dependent on the presence of NADPH, have been described in extracts of *Trichosporon cutaenum* (Neujahr and Gaal, 1973) and *Candida tropicalis* (Neujahr *et al.*, 1974). The second step is catalyzed by catechol 1, 2- dioxygenase (EC 1.13.11.1; *ortho*

fission) to form cis,cis-muconic acid or catechol 2, 3-dioxygenase (EC 1.13.11.2; *meta* fission) to form 2-hydroxymuconic semialdehyde. After several subsequent steps, the products are incorporated into the tricarboxylic acid (TCA) cycle as Acetyl-CoA and succinate or acetaldehyde and pyruvate (Shingler, 1996). It has been established that the aerobic degradation of phenolic compounds is metabolized by different strains through either the *ortho*-or the *meta*-cleavage pathway (Ahamad and Kunhi, 1996; Shingler, 1996; Bayly and Barbour, 1984). Table 2.3 shows phenol metabolism pathway by bacteria, fungi, yeasts and algae. It is observed that *meta* cleavage pathway is more predominant in bacteria and algae, while *ortho* cleavage pathway is distinct in fungi and yeasts.

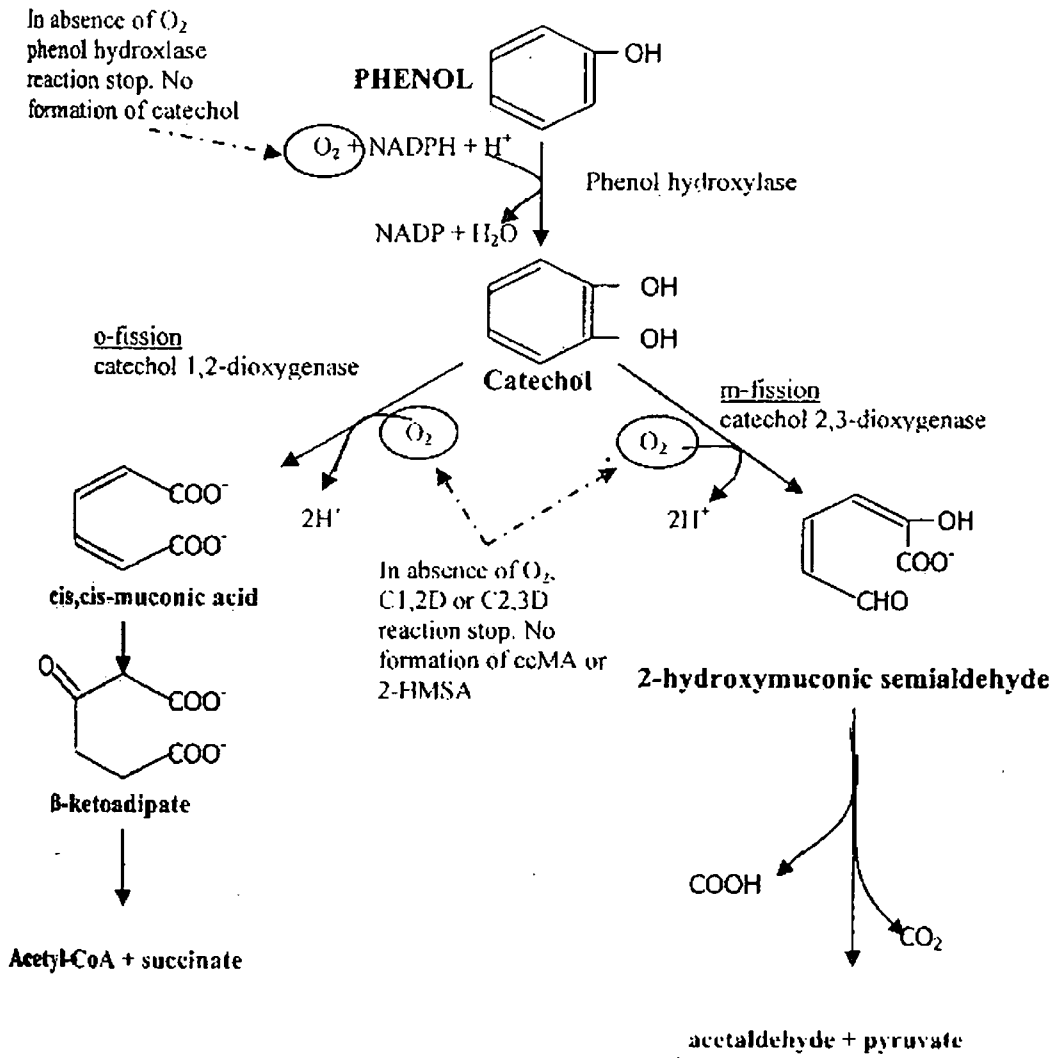


Figure 2.4: The main pathways of phenol degradation under aerobic condition (*ortho-* and *meta* fission of the benzene ring) (adapted from Krug *et al.*, 1986).
{Reaction: Phenol + O_2 + NADPH +H^+ $NADP^+$ + H_2O + catechol (Mörtberg and Neujahr, 1987); catechol + O_2 ccMA + $2H^+$ }(Ngai *et al.*, 1990).

Table 2.3: Phenol metabolism pathway of microorganism.

Microorganism	Pathway	Reference
A. Bacteria		
Acinetobacter calcoaceticus	*ortho*	Paller *et al.*, 1995
A. radioresistens	*ortho*	Pessione *et al.*, 1999 Pessione and Giunta, 1997
Alcaligenes eutrophus	*meta*	Leonard and Lindley, 1998
A. eutrophus JMP 134	*meta* and *ortho*	Müller and Babel, 1996
A. faecalis	*ortho*	Bastos *et al.*, 2000b
Bacillus strain Cro3.2	*meta*	Ali *et al.*, 1998
B. stearothermophilus	*meta* and *ortho*	Adams and Ribbons, 1988
B. thermoleovorans A2	*meta*	Milo *et al.*, 1999
B. thermoglucosidasius A7	*meta*	Duffner *et al.*, 2000
Comamonas testosteroni	*meta*	Yap *et al.*, 1999
Ochrobatrum tritici	*meta*	El-Sayed *et al.*, 2003
Pseudomonas sp.	*meta* and *ortho*	de Lipthay *et al.*, 1999
	meta	Kang and Park, 1997
P. sp. CF600	*meta*	Powlowski and Shingler, 1994
P. cepacia AC1100	*meta*	Ghadi and Sangodkar,1994 Nelson *et al.*, 1987
P. pickettii	*meta*	Kukor and Olsen, 1991
P. putida	*meta*	Hill and Robinson, 1975 Yang and Humphrey, 1975 Mörsen and Rehm, 1990
P. putida BH	*meta*	Takeo *et al.*, 1995
P. putida NCIB 10015	*meta*	Dagley and Gibson, 1965 Bayly and Dagley, 1969 Sala-Trepat *et al.*, 1972
P. putida P35X	*meta*	Ng *et al.*, 1994
P. vesicularis	*meta*	Mrozik and Łabużek, 2002
Ralstonia	*meta* and *ortho*	de Lipthay *et al.*, 1999
R. eutropha	*meta*	Leonard *et al.*, 1999
Staphylococcus sciuri	*ortho*	Mrozik and Łabużek, 2002
B. Fungi		
Penicillium (AF2, AF4, FIB9)	*ortho*	Santos and Linardi, 2004
Graphium (LE9, LA1,LA5, - (LE11, FIB4, LE6, AE2)	*ortho*	Santos and Linardi, 2004
Scedosporium apiospermum	*ortho*	Clauβen and Schmidt, 1998
C. Yeast		
Aureobasidium	*ortho*	Santos and Linardi, 2001
Candida tropicalis	*ortho*	Bastos *et al.*, 2000a
Rhodotorula	*ortho*	Santos and Linardi, 2001
R. glutinis	*ortho*	Katayama-Hirayama *et al.*, 1994
R. rubra	*ortho*	Katayama-Hirayama *et al.*, 1991
Trichosporon sp. LE3	*ortho*	Santos and Linardi, 2001
Fusarium (FE16, FE11)	*ortho*	Santos and Linardi, 2004
D. Algae		
Ochromonas danica	*meta*	Semple and Cain, 1995, Semple, 1997

(Source: Piakong, 2006)

2.5.2 Pure or Mixed Culture

Phenol degradation by pure and mixed microbial cultures have been reported (Chang *et al.*, 1998; Ruiz-Ordaz *et al.*, 1998; Collins and Daugulis, 1997a; Schroder *et al.*, 1997; Ahmed, 1995). If a mixed culture is chosen, the microbes should be highly suited to the substrate being degraded and the conditions should be optimal. Based on batch systems, 3 groups of interacting bacteria responsible for phenol degradation have been identified:- phenol metabolisers, hydrogen-utilising methanogens and acetotrophic methanogens (Satsangee and Ghosh, 1990; Wang *et al.*, 1989). In treatment tanks, bacterial species have been found dominant, but synergistic relationships may occur (Barnes and Fitzgerald, 1987). This relationship requires a balance between cell numbers and the degrading substrate and must be maintained for a successful degradation of a compound (Barnes and Fitzgerald, 1987).

For mixed culture systems, the different populations of organism makes optimization difficult as conditions that are optimal for one organism may not be the same for the other. For example, control of pH may not enhance the capabilities of the system as different optima are necessary to optimize conditions for each microorganism (Zache and Rehm, 1989). The changing conditions in the mixed culture create problems in replicating the system where slight environmental changes would enhance a different organism's growth to that previously investigated. Therefore, the basis of choice may not entirely for improved productivity alone, but also ease of operation, stability and ease of replication. There are some success stories with mixed culture. Defined mixed-culture

system using *Cryptococcus elinovii* H1 and *Pseudomonas putida* has shown complete degradation of phenol as high a concentration as 17g L^{-1} in a batch system (Mörsen and Rehm, 1990; Zache and Rehm, 1989). Compared to free cell systems, a mixed culture of bacteria and yeast could degrade 1g L^{-1} of phenol faster. It can be attributed to higher cell numbers and the action of two rings cleavages systems (both *meta* and *ortho* cleavage systems with *meta* cleavage by the bacteria and the *ortho* cleavage by the yeast).

2.5.3 Batch or Continuous Mode

Current technology for the biodegradation of toxic compounds involves the use of microorganisms in batch and continuous mode, using either suspended or immobilized cultures (Zache and Rehm, 1989; Mörsen and Rehm, 1987; Bettman and Rehm, 1984; Yang and Humprey, 1975). In batch and continuous culture systems, the use of aerobic cultures has been widely researched (Hutchinson and Robinson, 1988; Tschech and Fuchs, 1987; Molin and Nilsson, 1985; Bettman and Rehm, 1984; Hill and Robinson, 1975; Feist and Hegeman, 1969).

Most bioprocesses are based on batch reactors (Shuler and Kargi, 2002). The principal advantages of batch cultures are low contamination risk, the ability to run different successive phase in the same vessel, and close control of the genetic stability of microorganism (Scragg, 1992; Panikov, 1995). However, batch cultures can suffer great variability from one run to another. Problems such as substrate inhibition, low cell concentration, glucose effect, catabolite repression, auxotropic mutants and high viscosity

of the culture broth are common in batch processes (Shuler and Kargi, 2002). Batch culture under conditions of mineral limitation or glucose repression may be protracted and even irreversible (Panikov, 1995). Conventional batch fermentation has been used to degrade phenol, but is limited by the low initial concentrations required to prevent complete inhibition of microbial activity (Andrews, 1968).

A possible solution to extending the length of maximum growth is to maintain conditions at an optimal level, giving increased biomass growth and therefore high phenol utilization rates. This can be achieved by prolonging the exponential growth phase via the addition of fresh medium to the culture vessel (medium that is not growth-limiting nor toxin-producing) as is practiced in continuous cultures. During steady state, the cell population and the concentration of the media components reach equilibrium. The spent medium or effluent contains the same concentration of components as that in the reactor and the substrate concentration is never depleted. An optimal system would be one in which dilution rate, D, is high enough to sustain a high population of cells, but low enough to allow efficient utilization of the medium components or substrate. This offers a high process throughput of wastewater, a constant culture environment and an extended exponential cell growth phase (Lakhwala *et al.*, 1992; Autenrieth *et al.*, 1991; Bettman and Rehm, 1985).

With pure culture operated in a continuous mode, cell growth can be sustained on phenol concentration greater than 500 mg L^{-1}. This concentration can be maintained with low dilution rates, which is below 0.4 h^{-1} (Hill and Robinson, 1975; Yang and Humprey,

1975). This is important to avoid bacterial growth on reactor walls and blockage which is a major cause of shortening reactor usage time. Biomass wall-growth and retention change the dynamics of the system and causes non-Newtonian flow behavior and may destabilize steady-state condition (Erhardt and Rehm, 1989; Hill and Robinson, 1975; Yang and Humprey, 1975). Hence, two-prolonged strategies must be adopted when using pure culture in a continuous mode:

i. Phenol concentrations greater than 500 mg L^{-1} is used so as to provide a high enough carbon substrate level to enable sustainable cell growth population and avoid major fluctuations in steady state due to dynamic population.

ii. Very low dilution rate is used which is below the critical dilution rate, *D*, rate at which cell washout occurs, so that complete degradation of phenol can occur. High dilution rates will not achieve complete degradation (Erhardt and Rehm, 1989; Yang and Humprey, 1975).

2.5.4 Free or Immobilized Cells

In continuous culture, very often the critical dilution rate, D, is low and cell washout occurs early on, which reduces the efficiency of the system. Thus, immobilization is the solution which not only prevents cell wash out, but also protects the cells from direct contact with the toxic phenol concentrations. Immobilization confines a catalytically active enzyme or cell within a reactor system and prevents its entry into the mobile phase, which carries the substrate or product (Rosevear, 1984). This can be divided into;- 1) immobilized, treated cells (dead cells) being subjected to appropriate treatment before or after the immobilization, whilst the desired enzymes are in active and stable form; and 2) immobilized, living cells (cells either growing in or on gel matrices) (Fukui and Tanaka, 1982). Cell immobilization has been widely used in phenol degradation (Loh *et al.,* 2000; Aksu and Bulbul, 1999; Hannaford, 1992; Lakhwala *et al.,* 1992; Bettman and Rehm, 1985). A major feature of immobilization is the high cell density and the ease with which cells can be separated from the culture broth. Immobilized cells offer several advantages compared to free cell systems. These are:

i. Continuous or repeated-batch use of cells. With immobilization a high through-put of liquid can pass through the reactor while the cells are retained within, without cell washout. The culture liquor can be removed at the end of the batch, and the same population of cells can be reused by refilling the vessel with fresh medium. This removes the need for reculturing of cells for each new batch (Webb, 1987).

ii. High productivity, as cells are confined and the density is high in the reactor.

iii. Tolerance to increased levels of toxic substances, owing to a barrier being present between the cells and the liquid phase.

iv. Reduce reactor wall-growth and blockage. Dilutions rates can be higher than that of free cell systems as washout does not occur; and large dynamic changes in liquid loading do not cause significant disruption of the cell population, enabling stable steady states to be achieved (Anselmo and Novais, 1992; Betmann and Rehm, 1985).

There is a variety of methods available to immobilize cells including cross-linking, entrapment and encapsulation. Detailed reviews on these have been reported (Webb, 1987; Chibata *et al.,* 1986 Cheetham and Bucke, 1984). One important criterion is to have immobilization matrices with high integrity preventing the bacteria from leading out into the environment and signal transduction (Premkumar *et al.,* 2002). An ideal immobilization matrix would prevent cell flow within the matrix, functional at ambient temperatures, survive harsh wastewater conditions including contaminated water and turbidity, and allow the flow of nutrients and oxygen and analytes through the matrix along with wastes and signals out. A simple schematic of a desirable immobilization matrix is as shown in Figure 2.5. Among common immobilization matrices include alginate beads, diatomaceous earth, ionic network polymer, activated carbon, sintered glass, polyacrylamide beads and polymeric membranes (Chung *et al.,* 2003; Zhu *et al.,* 2000; Loh *et al.,* 2000; Bandyopadhyay et *al.,* 1999; Aksu and Bulbul, 1999; Karel *et al.,* 1985; Tanaka *et al.,* 1984).

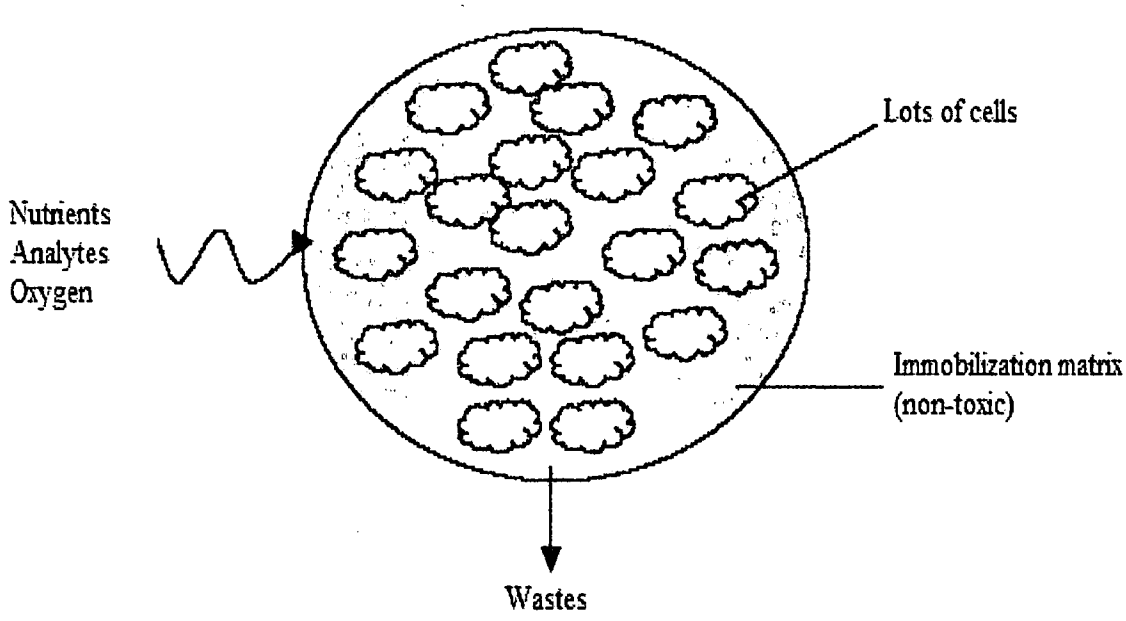


Figure 2.5: General schematic of a desirable immobilization matrix.

In the case of entrapment, the cell is merely restricted in its movements and confined to a small volume of liquid within a defined microenvironment. The gelation of alginic acid by polyvalent metal ions is one of the most important methods of cell immobilization. Alginates are often used in the food industry as a gelling compound, such as in the production of meat-like chunks in pet food. Alginate is commercially available as the sodium salt of alginic acid (sodium alginate). It is a linear polysaccharide isolated from strains of seaweed and algae. Alginate bearing weeds are typically found in temperate or cold water. Major commercial sources of alginates are *Macrocystis pyrifera*, the giant kelp from California and *Ascophyllum nodosum* from the north Atlantic. Alginates are formed by converting mannuronic and guluronic acid into their salt forms

of mannuronate (M) and guluronate (G). They are copolymers consisting of (1,4-) linked β-D-mannuronic acid and α-L-guluronic acid (Smidsrod and Skjak Braek, 1990). As shown in Figure 2.6, alginate structure may comprise of blocks of M and G, or alternating GM blocks. The type of structure is influenced by the seaweed source as well as the growing conditions of the weed. The block structure ultimately dictates the gelling properties of the alginate being produced. Alginates are available with varying molecular masses, mannuronate: guluronate ratios and distribution of units between blocks and alternating sequences. *Durvillea* and *Ascophyllum* species tend to be high in mannuronic acid and hence form softer gels whereas alginate sources such a *Laminaria hyperborea* stems tend to have a higher guluronic acid content and hence form much more rigid gels (Dara, 2004). Alginate solutions with a concentration range of 0.5-10% can be used for cell immobilization (Beshay, 2003).

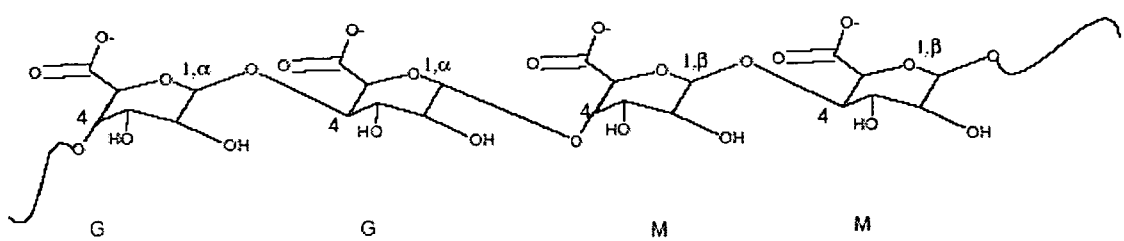


Figure 2.6: G and M blocks of the alginate structure.

Immobilization of cells via entrapment in alginate gel involves drop-wise addition of cells suspended in sodium alginate into calcium chloride solution (Kierstan and Bucke, 1977) to form stable gel where cells are entrapped. Polyvalent cations bind to the

polymer whenever there are two neighbouring guluronic acid residues. Therefore, polyvalent cations can cross-link nearby polymer chains and/or cross-link different segments of the same polymer chain. The mechanism of gelation is simply the exchange of calcium ions for sodium ions, and can be carried out under mild conditions.

$$2Na\,[alginate] + Ca^{2+} \longrightarrow Ca^{2+}\,[alginate]_2 + 2Na^{+}$$

(Soluble) (Insoluble)

The ionically linked gel structure is stable over the 0-100 °C.

2.6 Characterization and Identification of Phenol-Degrading Bacteria

Traditionally, classification of bacteria has relied entirely on phenotypic characteristics, such as cell wall type, morphology, motility, nutritional requirements and fatty acid profile. Although these characteristics certainly give useful information for bacterial taxonomy, the problem is that individual microorganisms may exchange genes with one another via horizontal gene transfer. Consequently, genes for characteristics such as the ability to grow on certain carbon source may be transferred between distantly related strains and complicate the classification.

An alternative approach to bacterial taxonomy is to study the genotype of a strain by its nucleic acids analysis. A widely used method is cloning and sequencing of the 16S rRNA genes (Olsen *et al.*, 1986; Pace *et al.*, 1986). These genes have become useful for phylogenetic analyses as they are present in all microorganisms. They are long enough to provide taxonomic information but short enough to be easily PCR-amplified. They also contain conserved regions as targets for PCR primers. The sequences of large number of 16S rRNA genes are available in public databases and novel strain can easily be compared to already studied microorganisms. Moreover, the 16S rRNA sequence can be used to trace the phylogenetic history (lineage) of an organism.

However, analysis of the 16S rRNA gene itself is not enough to assign a strain as a novel species as more data is necessary. The "gold standard" of bacterial phylogenetics is DNA: DNA hybridization between total genomic DNA of two related microorganisms. Here, the entire genomes of two strains are compared, and this method can be used to define a bacterial species. Two strains with 70% or greater DNA similarity as determined by DNA: DNA hybridization is considered to belong to the same species (Wayne *et al.*, 1987).

Since cloning and sequencing of 16S rRNA genes is technically much simpler and cheaper than DNA: DNA hybridization, analysis of 16S rRNA has its place as an initial screening method to determine the closest relative of a novel strain (Stackebrandt and Goebel, 1994). The level of 16S rRNA similarity is also an indication of whether the strain may constitute separate species and need further investigation. Comparison of 16S rRNA gene sequences with DNA: DNA hybridization data indicates that when two strains have 16S rRNA genes which are identical to less than 97.5%, they are likely to belong to different species (Stackebrandt and Goebel, 1994). Generally, a polyphasic approach to bacterial systematic identification is favored by combining phenotypic and genotypic characteristics.

CHAPTER III

PHENOTYPICAL AND MOLECULAR CHARACTERIZATION OF PHENOL-DEGRADING BACTERIA

3.1 Introduction

Phenol and its derivatives are some of major hazardous compounds in industrial wastewater (Peters *et al.*, 1997; Watanabe *et al.*, 1996). Fortunately, mother nature has her own way of dealing with these toxic compounds as phenol can be utilized by microorganisms as carbon and energy sources (van Schie and Young, 2000; Gibson *et al.*, 1990; Gibson, 1968). Biodegradation of phenol has attracted considerable attention for *in situ* or *ex situ* remediation (Shingler, 1996; Yang and Humphrey, 1975). In actual fact, bacteria play a major role in the degradation of phenol in the ecosystem: in soil (Hickman and Novak, 1989), sediments (Shimp and Young, 1987) and water (Howard, 1989). Large numbers of phenol-degrading bacteria have been isolated, characterized and identified (El-Sayed *et al.*, 2003; Begona Prieto *et al.*, 2002; Monteiro *et al.*, 2000).

Several studies have shown that most 16S ribosomal RNA (rRNA) sequences amplified directly from environmental samples are different from the sequences of comparable laboratory strains. It has been concluded that many bacteria that are predominant in the natural environment have yet to be isolated in the laboratory and that

the microbial diversity in the natural environment is much greater than the diversity of the bacteria that has been isolated (Snaidr *et al.,* 1997; Bond *et al.,* 1995; Muyzer *et al.,* 1993; Delong, 1992; Wagner *et al.,* 1993; Shmidt *et al.,* 1991; Giovannoni *et al.,* 1990; Ward *et al.,* 1990). Ribosomal RNA (rRNA) approach has been used to detect microbial populations and to describe the structures of microbial communities in various environments without isolating the component microorganisms (Olsen *et al.,* 1986; Pace *et al.,* 1986). One important aspect of microbial ecology study is functional dissection of microbial communities based on structural information obtained by the 16S rRNA approach. An analysis of a population shift accompanied by a change in the function of community, yields information useful for identifying functionally dominant populations (Borneman and Triplett, 1997; Watanabe and Hino, 1996; Bond *et al.,* 1995). However, information concerning the function (activity) of each population can never be obtained by this approach. Hence, pure-culture experiments may still be indispensable for detailed analysis of the functions of each population and that isolation of the functionally dominant populations in a microbial community remains important.

The potential of native, Malaysian bacteria in the degradation of phenol has not been comprehensively explored. Yet, the state of phenolic-based contamination in waste water and is alarming in Malaysia. National Guideline for Raw Drinking Water Quality has stated that the permissible level for phenolic compounds is 0.002 mg/L. Based on the report by the Department of Environment (DOE) in 2005, phenol levels exceeding permissible level has been recorded at solid landfill (39 %), urban/suburban (33 %), agricultural areas (29 %), industrial areas (26 %), ex-mining areas (17 %) and rural areas

(10 %). The objectives of this study are therefore to isolate, characterize and identify phenol-degrading bacteria from Malaysian soil.

3.2 Materials and Methods

3.2.1 Phenotypical and Biochemical Characterization of Phenol-Degrading Bacteria

3.2.1.1 Bacterial Isolates

The bacterial strain was provided by Bioremediation Laboratory, Universiti Putra Malaysia. It was isolated from soil contaminated with petrol wastes from a car workshop in Serdang, Selangor. The strain was initially assigned Isolate Serdang 1. The morphology of the bacteria was observed by Gram staining and microscopy. Important features such as the shape, elevation and pattern of colonies were observed and recorded.

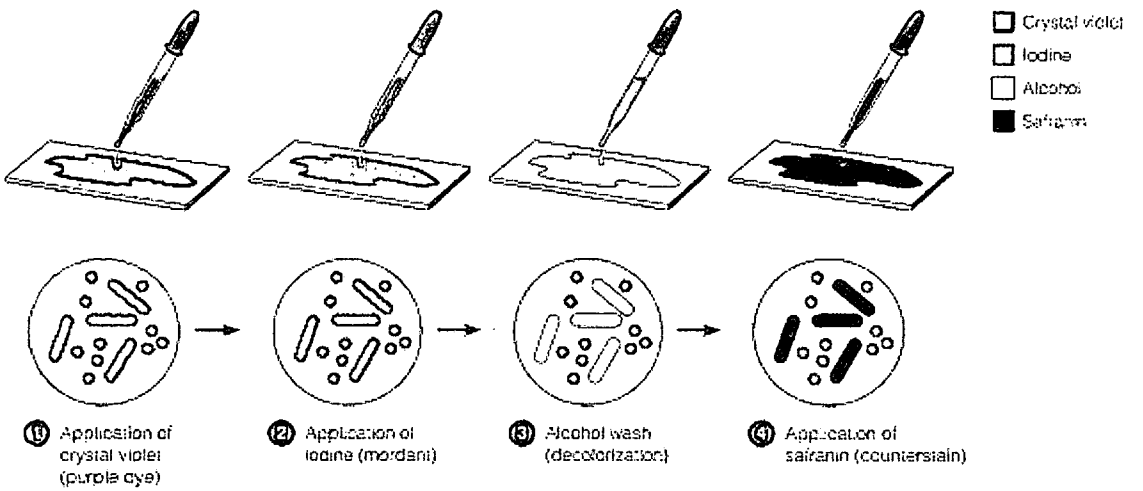
Microbial isolation strategies usually involve a period of enrichment in liquid culture, followed by separation of organisms in or on solid media where they are allowed to grow as colonies (Hardman *et al.,* 1993). Bacteria capable of utilizing phenol as a sole carbon source was isolated by enrichment in mineral salts medium containing 500 mg/L phenol as carbon source. The mineral medium constituents were as follows (g/L): 1.0 $(NH_4)_2SO_4$ (R&M Chemical, China), 0.25 $MgSO_4.7H_20$ (Merck, Germany), 0.25 KH_2PO_4 (Merck, Germany), 0.07 $CaCl_2.2H_2O$ (Merck, Germany), 0.1 yeast extract, 0.5 phenol (Hamburg Chemical, Germany) (Ali *et al.,* 1998). The isolation of microbial strains was done by plating technique as prescribed in APHA 9215 (APHA, 1989). This enables observation and enumeration of colonies. A series of dilutions was made and selected, transferred to the petri dishes and then the molten phenol agar medium was poured in.

When the agar solidified, the plate was incubated for 48 hours at room temperature. Colonies would grow within as well as on the surface of the agar plate. Single colony was plated out and bacterial colonies obtained. In order to obtain pure cultures, a small portion of an individual colony was picked up using sterile inoculating loop and then streaked out on fresh agar plate. In this way, mixed culture can be separated into single colonies and grown up individually as culture stocks.

3.2.1.2 Gram Staining Method

Gram staining is an important differential staining procedure widely used in bacteriology. It is developed empirically by Christian Gram (1884) to differentiate bacteria in tissue sections. Bacteria are subdivided by their reaction to this stain into those which retain it, termed Gram positive, and those which are decolorized, termed Gram negative. A suspension of microorganisms was transferred to a glass microscope slide and allowed to dry. The slide was then quickly passed through a flame to affix the cells to the slide. The smear was applied with crystal violet dye (Fluka, Switzerland) as the primary stain, which stained both Gram-positive and Gram-negative cells blue-purple (Figure 3.1). After 1 min, the slide was washed with distilled water for 5 sec. The specimen should appear blue-purple when observed with the naked eye as the dye entered the cytoplasm of both types of cells. The slide was then flooded with Gram's iodine (Ajax Chemical, Australia), a mordant (a substance that increases the affinity of the primary stain for the bacterial cells). After 1 min, the slide was rinsed with distilled water for 5 sec. When iodine (the mordant) was applied, it formed large crystals with the dye,

and were large enough to escape through the cell wall. At this point, the specimen should remain blue-purple in color (Figure 3.1). The smear was then discolored by 95% ethyl alcohol (Hamburg Chemical, Germany). This step should be taken cautiously as too much decolorizer could result in a false Gram (-) result. Likewise, not using enough decolorizer may yield a false Gram (+) results. The application of alcohol dehydrates the peptidoglycan of Gram-positive cells to make it more impermeable to the cystal violet-iodine. The effect on Gram-negative cells is quite different; alcohol dissolves the outer membrane of Gram-negative cells and even leaves small holes in the thin peptidoglycan layer through which crystal violet-iodine diffuse. The slide was rinsed with distilled water for 5 sec, before counterstained with safranin (Ajax Chemical, Australia) for 45 seconds, which stains those bacteria that were decolorized in the previous step so that they can be easily seen (Figure 3.1). Again, the slide was rinsed with distilled water for 5 sec to remove any excess dye. The slide was blotted with bibulous paper and examined under the light microscope (Olympus) at 100X magnification with an oil immersion. Gram-positive bacteria should retain the crystal violet-iodine complex and appear purple when viewed under the light microscope. Gram-negative bacteria would be the color of the counterstain (pink). The morphology of the cell was also observed; whether it was a rod or a coccus, and whether it was in pairs or in the form of chain.



(Source: Tortora *et al.*, 2004)

Figure 3.1: Gram staining procedures.

3.2.1.3 Oxidase Test

Oxidase test was conducted to test for the presence of cytochrome C oxidase, a component of electron transport chain. A separate and well-grown colony from a culture plate of 24 hours old was taken up with an inoculating loop. The colony was spread on to a reaction zone of an oxidase strip (Medvet, South Australia) and the colour change was observed for up to 5 sec. A deep blue/violet color indicates positive reaction, and a no color change indicates no reaction.

3.2.1.4 Catalase Test

Catalase test was carried out to check the presence of catalase enzyme. The method was as prescribed by Cappuccino and Sherman (2005). Culture streak of 24 hours old was applied with three or four drops of 3% (v/v) hydrogen peroxide to the entire

culture surface to check for the presence or absence of bubbling or foaming. Bubble formation indicates catalase presence.

3.2.1.5 Biolog™ Identification System

Biolog™ system uses a 96 well microplate having different carbon source in each well (Biolog, 2002). The microplates are specific for Gram positive and Gram negative isolates. This system identifies bacteria on the basis of the exchange of electrons produced during the respiration of an organism, which causes a subsequent tetrazolium-based color change from clear to purple. Purple wells indicate positive growth, and wells that remain colorless indicate negative growth. The test inoculum was prepared from overnight culture of bacterial Isolate Serdang 1 on nutrient agar plate. The bacterial colonies were picked using sterile cotton swab and resuspended into inoculation fluid (0.4% (v/v) NaCl, 0.03% (v/v) Pluronic F-68, and 0.02% (v/v) Gellan Gum). The bacterial suspension was adjusted to 61% turbidity using the Biolog™ Turbidimeter (Biolog, Inc) according to the manufacturer's recommendation. One hundred and fifty µl of the suspension was dispensed into all wells of the Biolog™ ID Microplates using the 8-channel micropipettor. The ID Microplate was incubated at room temperature for 16 to 24 hours. To analyze the sample, the ID Microplate was placed in the Biolog™ Microstation reader without tilting the plate. Identification was carried out using the pre-loaded ID Database on a computer which identifies bacteria to the species level.

3.2.2 Molecular Characterization of Phenol-Degrading Bacteria

3.2.2.1 Genomic DNA Extraction

Cells from overnight broth cultures were harvested from 1.5 ml culture by centrifugation (Eppendorf, North America) at 13,000 rpm for 2 minutes and the supernatant was discarded. Genomic DNA was extracted from the harvested cells by alkaline lysis using Wizard® Genomic DNA Purification Kit™ (Promega, USA) according to the manufacturer's procedure. In order to lyse the cells, 600 µl cell lysis solutions was added and mixed gently by repeated pipeting, followed by incubation at 80°C for 5 minutes and cooling to room temperature. Then, 3 µl RNase solutions was added and mixed, followed by incubation at 37°C for 5 minutes. For protein precipitation, 200 µl protein precipitation solutions was added, vortexed and the tube was cooled on ice for 5 minutes. The solution was then centrifuged at 13,000 rpm for 3 minutes. The supernatant was transferred to a clean tube containing 600 µl of room temperature isopropanol and mixed. The solution was centrifuged at 13,000 rpm for 2 minutes and the supernatant discarded. Six hundred microliters of room temperature 70% ethanol was added and mixed. The solution was centrifuged at 13,000 rpm for 2 minutes. The ethanol was aspirated and the pellet was air-dried for 15 minutes. The DNA pellet obtained at the end of the protocol was used as a template in PCR. The DNA pellet was stored at 4°C.

3.2.2.2 DNA Quantification and Purity

The concentration and purity of the extracted DNA was determined by a spectrophotometer (Beckman, USA) according to the method described by Sambrook *et al.,* (1989). Spectrophotometric readings were taken using the wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the DNA concentration in the sample, where an optical density (OD) of 1 corresponds to approximately 50 µg of double stranded DNA per ml of DNA sample. Therefore, the net absorbance at 260 nm was multiplied by 50; the result obtained was again multiplied by dilution factor to get the concentration of DNA in µg/ml. The ratio of the reading at 260 nm to 280 nm provides an estimate for the purity of the DNA. A pure DNA sample has a ratio of 1.7 to 2.0.

3.2.2.3 Amplification of Genomic DNA by Polymerase Chain Reaction (PCR)

PCR was performed by incubating the samples at three temperatures in a thermal cycler (MJ Research Inc., USA) corresponding to three steps in an amplification cycle-denaturation, annealing and extension in a cycle of amplification. To amplify the 16s rRNA region, PCR was performed using the 16s universal primer (Turenne *et al.,* 2002). Forward PCR primers (5'-aga-gtt-tga-tcc-tgg-ctc-ag-3') and reverse primers (5'-aag-gag-gtg-atc-cag-ccg-ca-3') synthesized by First Base Laboratories Sdn Bhd, Malaysia, were used for the amplification of the 16s rRNA region. This forward and reverse primers flank the 16s rRNA coding region of bacteria.

For the PCR mixture; 1 µl of DNA template was mixed with 2 µl of *Taq* polymerase in NH_4SO_4 buffer (MBI Fermentas, Lithuania), 2 µl of 20 mM $MgCl_2$ (MBI Fermentas, Lithuania), 0.5 µl of 10 mM dinucleotide triphospate (dNTP) (MBI Fermentas, Lithuania), 0.5 µl of 00 µM forward primer (First Base, Malaysia), 0.5 µl of 100 µM reverse primer (First Base, Malaysia), 43 µl of deionized water and 0.5 µl of *Taq* polymerase (MBI Fermentas, Lithuania) to a total volume of 50 µl. The PCR reaction mixture was dispensed into a 0.5 ml thin-walled PCR tube. The samples were then placed in the thermal cycler (MJ Research Inc., USA) for amplification. The double stranded DNA was predenatured by heating at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min. The primers were allowed to anneal to their complementary sequence by cooling at 56.5°C for 2 min, followed by heating to 72°C for 2 min to extend the annealed primers. Following these cycles, a prolonged extension at 72°C for 10 min was used (Sambrook *et al.,* 1989). Negative control reactions without template DNA were carried out simultaneously and for the negative control, ultra pure water was used as a substitute for DNA template.

3.2.2.4 Detection of PCR Products

DNA amplified was separated using agarose gel electrophoresis. A 1.0% agarose gel was prepared in a 250 ml conical flask by melting 0.3 g of agarose in 30 ml of 1X TBE electrophoresis buffer (0.1 mM Tris/HCL, 0.1 mM boric acid, 0.002 mM EDTA, pH 8.3) (Sambrook *et al.*, 1989). The agarose gel at 50-70°C was poured into the gel tray and the comb was inserted. After the gel has hardened completely, the comb was remove

carefully and the gel placed in the electrophoresis chamber. The chamber was then filled with 1X TBE electrophoresis buffer. Seven microliters of amplified PCR products were pre-mixed with 1 µl of 6X loading dye (MBI Fermentas, Lithuania) on a piece of parafilm and loaded into the gel well. Gene Ruler^{TM} 1kb DNA marker (MBI Fermentas, Lithuania) was used as a standard.

The DNA was electrophoresed at 70 V supplied from a Power Supply EPS 600 (Pharmacia, Biotech) for 1 hour and 30 min. After electrophoresis, the gel was removed from the tray, stained with 0.5 µg/ml ethidium bromide (as described in Appendix A) for 15 min and then destained with distilled water. The bands on the gel were visualized on an ultraviolet light transilluminator and photographed using the Bio Imaging System in Gene Snap Program (Syngene,UK).

3.2.2.5 Purification of Desired PCR Product

The QIAquick Gel Extraction (QIAGEN, Germany) was used to extract and purify the expected DNA fragment from the agarose gel, according to the manufacturer's instruction. The DNA fragment band was excised from the agarose gel with a clean, sharp scalpel. The gel slice in a colorless tube was then weighed and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg~100 µl). The tube was incubated at 50°C for 10 min and vortexed every 2-3 min to dissolve the gel. After the gel slice has dissolved completely, a QIAquick spin column was placed in a provided 2 ml collection tube. To bind DNA, the sample was applied to the QIAquick column. Then, the solution was

centrifuged at 13 000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. In order to remove all traces of agarose, 0.5 ml of Buffer QG was added to QIAquick column and the solution was centrifuged at 13 000 rpm for 1 min. To washout, 0.75 ml of Buffer PE was added into the QIAquick column. Then, the solution was centrifuged at 13 000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same collection tube and the QIAquick column was centrifuged for an additional 1 min at 13 000 rpm to remove residual ethanol from Buffer PE. The QIAquick column was then transferred into a new microcentrifuge tube and 50 µl of Buffer EB (10 Mm Tris.Cl, pH 8.5) was added to the center of QIAquick column membrane to elute the DNA out and incubated for 1 min. The solution was centrifuged at 13 000 rpm for 1 min at room temperature.

3.2.2.6 Plasmid Vector

TOPO TA Cloning® provides a highly efficient, one step cloning strategy for direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector (Figure 3.2a). It was supplied in linear form with single 3'-thymidine (T) overhanged at both ends of the TA Cloning vector and topoisomerase 1 enzyme covalently bound to the vector. The plasmid vector (pCR® 2.1-TOPO) was as shown in Figure 3.2b.

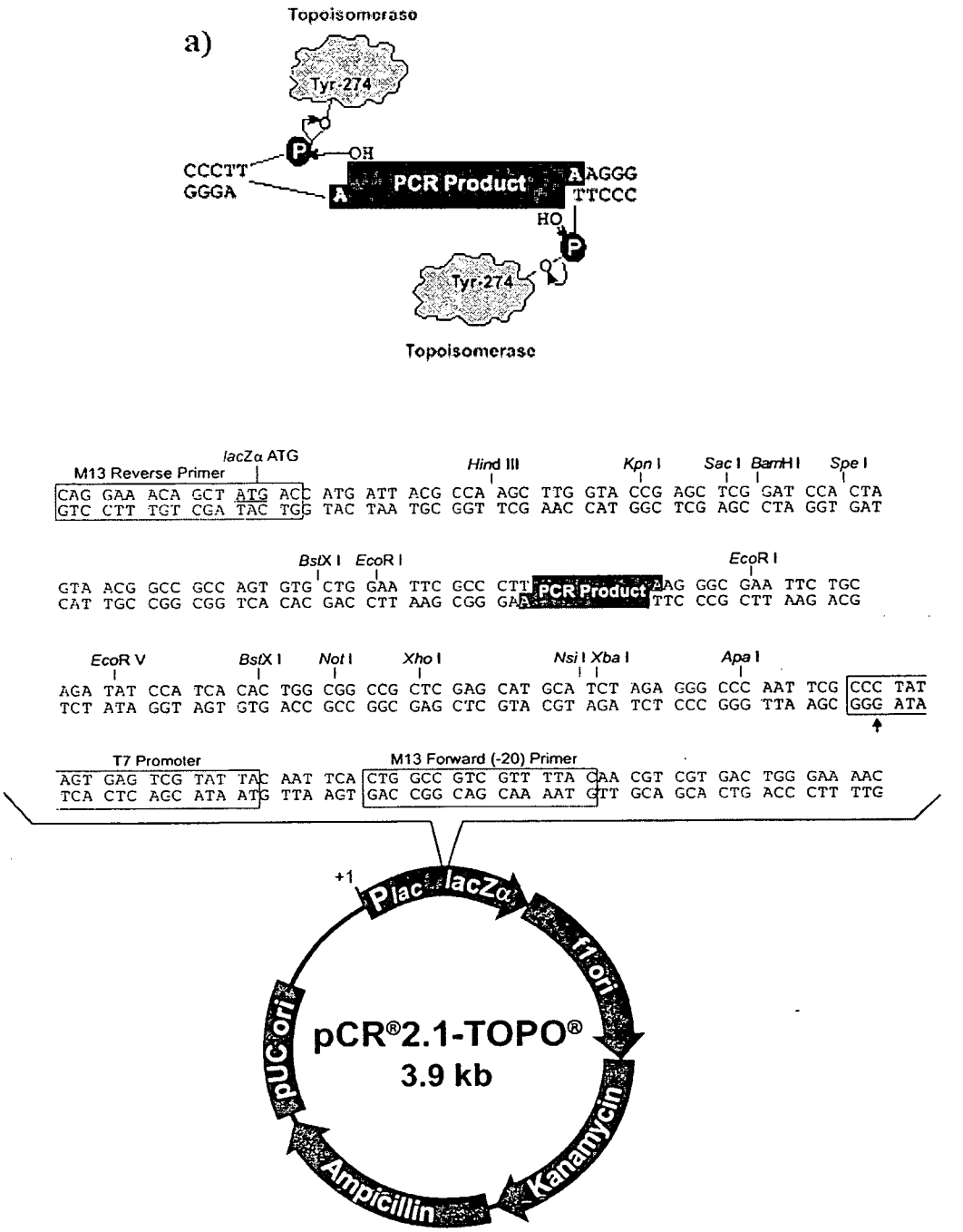


Figure 3.2: a) TOPO TA Cloning® and b) Map of pCR® 2.1-TOPO.

3.2.2.7 TOPO Cloning Ligation Reaction

Six microliters of TOPO Cloning reaction mixture was prepared as shown in Table 3.1. The solution was mixed gently and incubated for 5 min at room temperature to prevent the reduction of transformation and cloning efficiency.

Table 3.1: TOPO cloning reaction mixture.

Reagents	Quantity
Fresh PCR product	4 µl
Salt solution	1 µl
pCR® 2.1-TOPO vector	1 µl
Final volume	6 µl

3.2.2.8 TOP 10 One Shot Chemical Transformation

In transformation reaction, the procedure was performed according to the manufacturer's instruction, 20 µl chemical competent cells of *E.coli* TOP 10 One Shot (Invitrogen, USA) were thawed on ice. Six microliters of TOPO Cloning ligation reaction mixture was added to a vial containing *E.coli* TOP 10 One Shot cells and mixed. The mixture was incubated on ice for 30 min. The cells were heat shocked for 1 min at 42°C without shaking. The tube was then incubated on ice for 2 min. Two hundred and fifty microliters SOC medium was added to the tube at room temperature before capping tightly and shaken horizontally at 200 rpm, 37°C for 1 hour. While the cells were left growing, X-gal

(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (4mg/ml) was added to the LB (Luria-Bertani) agar plates containing 50μg/ml ampicillin prepared earlier. The preparation of X-gal, LB and ampicillin was as described in Appendix A. The X-gal was spread immediately and evenly on the plate with a glass spreader. After 1 hour incubation, 50 to 100 μl of the transformed bacteria was pipetted out and dispensed onto the centre of the plate. The cells were spread evenly on the plate with a glass spreader. The plates were incubated at 37°C for 16 hours. After incubation, blue and white colonies were observed.

3.2.2.9 Plasmid Extraction

Plasmid extraction was performed according to the protocol as described by GeneJET™ Plasmid Miniprep Kit (MBI Fermentas, Lithuania). Overnight culture of plasmid colonies was transferred into microcentrifuge tubes and centrifuged at 13 000 rpm for 1 min at room temperature and the supernatant discarded. The pellet was resuspended in 250 μl cell resuspension solution. Then, 250 μl cell lysis solutions was added and mixed gently by inverting the capped tube six times. Three hundred and fifty microliters of neutralization solution was then added and mixed by inverting the tube for six times. The solution was centrifuged at 13 000 rpm for 15 min. Cleared lysate was transferred to the supplied GeneJET™ spin column by decanting or pipetting. Then, the solution was centrifuged at 13 000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. Five hundred microliters column wash solution containing ethanol was added into the GeneJET™ spin column.

The solution was centrifuged at 13 000 rpm for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. The washing procedure was repeated twice. The GeneJET^{TM} spin column was then transferred into a new microcentrifuge tube and 50 µl of elution buffer was added to the center of GeneJET^{TM} spin column membrane to elute the plasmid DNA and incubated for 2 min. The solution was centrifuged at 13 000 rpm for 2 min at room temperature. The plasmid DNA was stored at -20°C until use. The concentration and purity of the extracted plasmid was determined as described in section 3.2.2.2.

3.2.2.10 Analysis of Positive Clones by Restriction Endonuclease (RE) Analysis

The presence of the inserted gene in the plasmid was confirmed by digesting the extracted plasmid with restriction endonucleases- BamHI and XhoI (MBI Fermentas, Lithuania). The restriction digestion reaction was prepared as in Table 3.2.

Table 3.2: The restriction digestion reaction.

Reagents	Quantity
Sterile deionized water	1 µl
Restriction endonuclease Buffer	2 µl
Restriction endonuclease (BamHI)	1 µl
Restriction endonuclease (XhoI)	1 µl
Recombinant plasmid	5 µl
Final volume	10 µl

The solution was mixed gently by repeated pipeting, followed by incubation at 37°C for 2 hours. The digested recombinant plasmids were analyzed by agarose gel electrophoresis. Seven microliters of the digested recombinant plasmids were mixed with 1 µl of 6X loading dye and then loaded on to 1% (w/v) agarose gel. Five microliters of Gene Ruler™ 1kb DNA marker (MBI Fermentas, Lithuania) was used to estimate the molecular weight of the DNA bands. The sample was electrophoresed in 1X TBE electrophoresis buffer at 70 V supplied from a Power Supply EPS 600 (Pharmacia, Biotech) for 1 hour and 30 min. After electrophoresis, the gel was removed, stained with 0.5 µg/ml ethidium bromide for 15 min and then destained with distilled water. The bands on the gel were visualized on an ultraviolet light transilluminator and photographed by BioImaging System in Gene Snap Program (Syngene,UK).

3.2.2.11 Automated DNA Sequencing

The isolated plasmid DNA was subjected to automated sequencing on both strands using forward and reverse universal primers. The sequencing was carried out by First Base Laboratories Sdn Bhd, Malaysia. ABI PRISM BIGDYE™ and Amersham Pharmacia Biotech DYEnamic ET Terminator Chemistry were used for the sequencing reactions. The ABI PRISM® 377-96 DNA Sequencer automatically analyses DNA molecules labeled with multiple fluorescent dyes by the BIGDYE™ or DYEnamic ET Terminator sequencing reactions. The samples were loaded onto vertical 5% Long Ranger DNA (BioWhittaker Molecular Applications) sequencing gels.

3.2.2.12 DNA Sequence Homology Analysis

The homology of the PCR amplified DNA fragment was analyzed and compared with Genbank database using the BLASTN 2.0.11 programs of the National Center for Biotechnology Information, which can be accessed through the website http://www.ncbi.nlm.nih.gov/Genbank/

3.2.2.13 Phylogenetic Analysis

A multiple alignment of 22 16S rRNA gene sequences which closely matches Phenol-degrading strain was retrieved from GeneBank and was aligned using clustal-W (Higgins *et al.*, 1994) with the PHYLIP (phylogeny inference package) output option. The aligment was visually checked for any obvious mis-alignments. Alignment positions with gaps were excluded from the calculations. A phylogenetic tree was constructed using PHYLIP, version 3.573 (J.Q. Felsenstein, PHYLIP-phylogeny inference package, version 3.573, Department of Genetics, University of Washington, Seattle (http://evolution.genetics.washington.edu/phylip.html), with *Bacillus* strain as the out group in the cladogram.

3.3 Results and Discussions

3.3.1 Morphological and Physiological Characterization of Phenol-Degrading Bacteria

The colonies of bacteria on agar medium were observed as creamy colonies, round in shape with either smooth or irregular edges as shown in Plate 3.1.

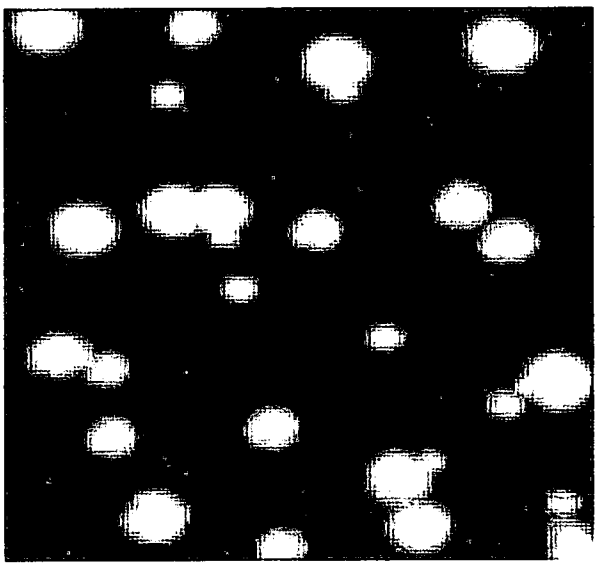


Plate 3.1: Colonies formed on agar medium in a Petri dish after 24 hours of incubation period at room temperature.

Microscopic examination under light microscope with 100X magnification showed that the cells were cocci in shape and formed chains of 3-4 bacterial cells per chain (Plate 3.2). The pink-red colour indicated that the isolates were Gram-negative. Different kinds of bacteria react differently to the Gram staining because structural differences in their cell walls. Chemically, all bacterial cell walls are composed of a macromolecular network called peptidoglycan (also known as *murein*), which is present

either alone or in combination with other substances. Peptidoglycan consists of a repeating disaccharide attached by polypeptides to form a lattice that surrounds and protects the entire cell. The disaccharide portion is made up of monosaccharides called N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The structural formulas for NAG and NAM are shown in Figure 3.3.

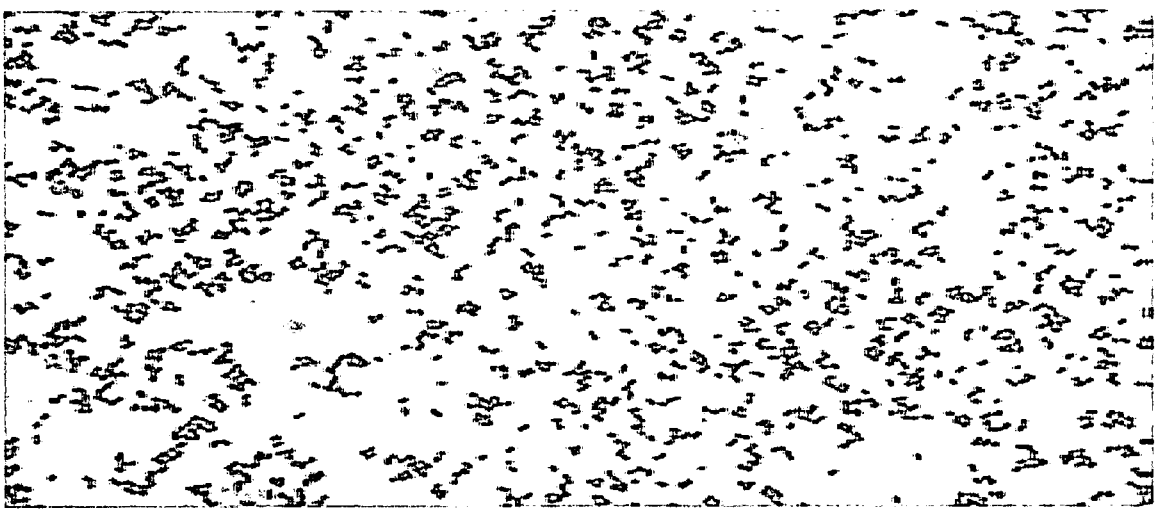


Plate 3.2: Micrograph of bacteria Isolate Serdang 1 in pink-red colour.

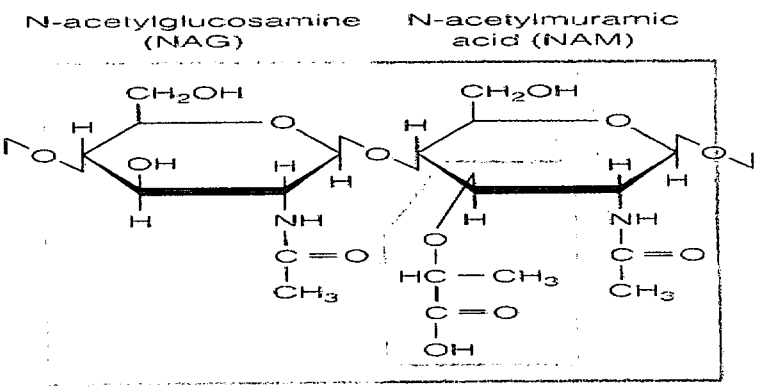


Figure 3.3: N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) joined as in peptidoglycan. The light purple areas show the differences between the two molecules. The linkage between them is called a β–1,4 linkage.
(Source: Tortora *et al.*, 2004)

In most Gram-positive bacteria, the cell wall consists of many layers of peptidoglycan, forming a thick, rigid structure. By contrast, Gram-negative cell walls contain only a thin layer of peptidoglycan and an outer membrane (Figure 3.4). When crystal violet and iodine were applied to both Gram-positive and Gram-negative cells, crystal violet and then iodine readily enter the cells. Inside the cells, the crystal violet and iodine combine to form CV-I. This complex is larger than the crystal violet molecule that entered the cells, and, because of its size, it cannot be washed out of the intact peptidoglycan layer of Gram-positive cells by alcohol. Consequently, gram-positive cells retain the color of the crystal violet dye. In Gram-negative cells, however, the alcohol wash disrupts the outer lipopolysaccharide layer, and the CV-I complex is washed out through the thin layer of peptidoglycan. As a result, gram-negative cells are colorless until counterstained with safranin, after which they are pink-red.

The outer membrane of Gram-negative cell wall consists of lipoproteins, phospholipids and lipopolysaccharides (LPS). The lipid portion of LPS, called lipid A, is the endotoxin. Toxins are poisonous substances produced by certain microorganisms, and can be divided into two, based on their position relative to microbial cell: exotoxins and endotoxins. Exotoxins are produced inside some bacteria as part of their growth and metabolism and are secreted by the bacterium into the surrounding medium or released following lysis. Endotoxins are released when Gram-negative bacteria die and their cell walls undergo lysis. Representative microorganisms that produce endotoxin are *Salmonella typhi* (the causative agent of typhoid fever), *Proteus* spp. (frequently the

causative agents of urinary tract infections) and *Neisseria meningitidis* (the causative agent of meningococcal meningitis (Tortora *et al.*, 2004).

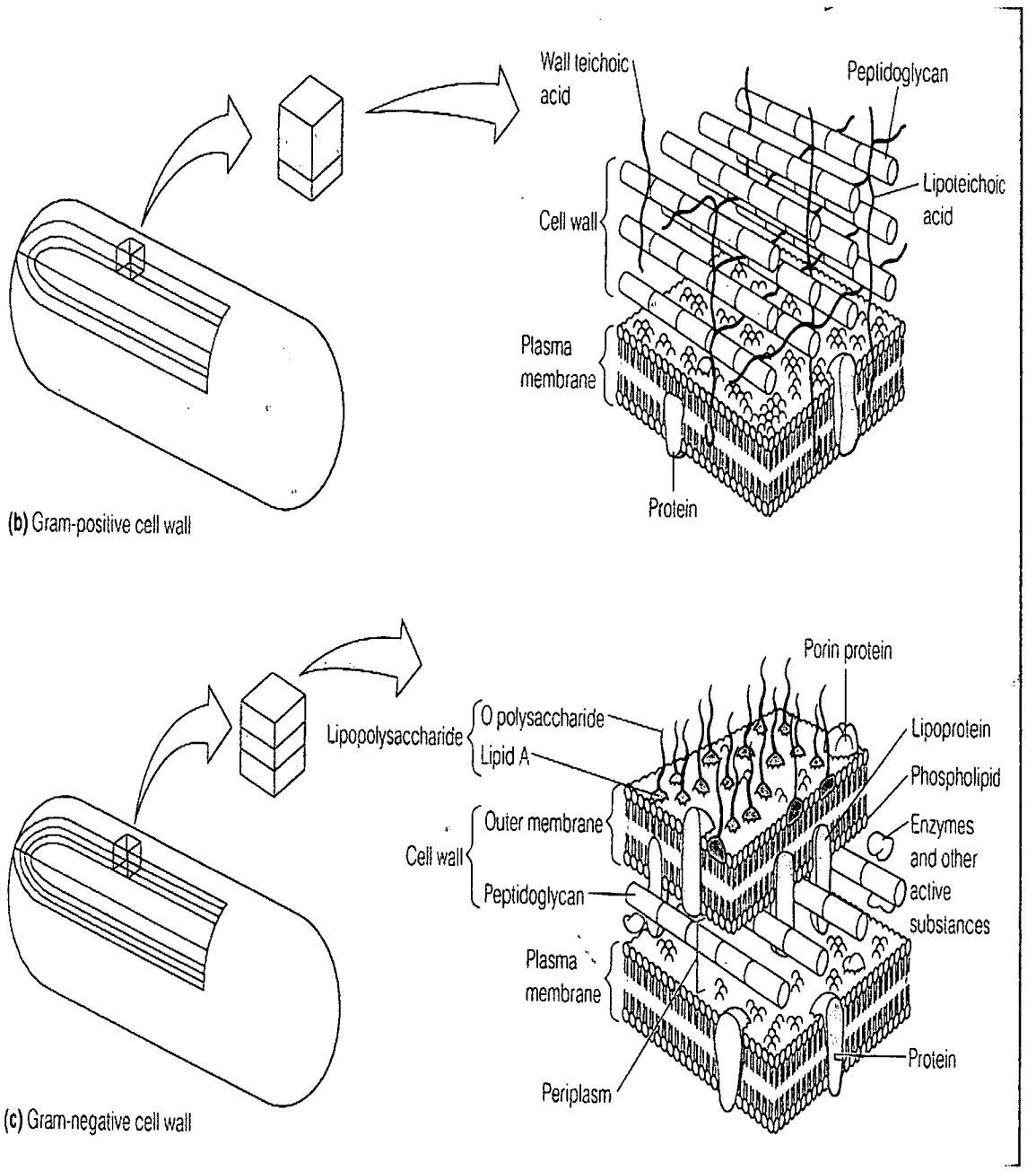


Figure 3.4: The difference between Gram-positive and Gram-negative bacteria. (Source: Tortora *et al.*, 2004)

In the oxidase test, oxidase strip is impregnated with colorless tetramethyl-p-phenylene-diamine dihydrochloride, a redox dye, for the detection of bacterial cytochrome c oxidase enzyme. If the bacteria has cytochrome c oxidase, it can oxidize the reagent (remove the electrons), turning it blue. The result with oxidase test strip suggests that the isolates do not produce cytochrome c oxidase as no color changes on the oxidase test strip was observed (Plate 3.3). It means that it lacks cytochrome c oxidase but not that it lacks an electron transport chain. Oxidase enzymes play a vital role in the operation of the electron transport system during aerobic respiration. Cytochrome c oxidase is an enzyme found in many electron transport chains, including those of eukaryotes. Cytochrome c oxidase catalyzes the oxidation of a reduced cytochrome by molecular oxygen (O_2), resulting in the formation of H_2O_2 or H_2O (Wilson and Miles, 1964a; Steel, 1961). The oxidase test aids in differentiation among members of the genera *Pseudomonas*, which are oxidase positive, and Enterobacteriacea, which are oxidase negative. *E.coli* is a great example as it belongs to the family of Enterobacteriacea and an oxidase negative, but it does have an electron transport chain that contains other cytochromes in its cytoplasmic membrane (Cappuccino and Sherman, 2005).

The catalase test involves adding hydrogen peroxide to the culture sample or agar slant. If the sample produces catalase, hydrogen peroxide will be converted and oxygen evolved. The formation of bubbles in this test may suggest the evolution of oxygen and the presence of catalase in the isolates (Plate 3.4). During aerobic respiration, aerobes, facultative anaerobes and microaerophiles produce hydrogen peroxide and in some cases,

an extremely toxic superoxide. Microorganisms utilize aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. Accumulation of hydrogen peroxide could kill the organism unless they can be enzymatically degraded. Organisms capable of producing catalase rapidly degrade hydrogen peroxide as illustrated below:

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \uparrow$$

Hydrogen Peroxide — Water — Free Oxygen

Aerobic organism that lack catalase can degrade especially toxic superoxide using the enzyme superoxide dismutase; the end product of a superoxide dismutase is H_2O_2, which is less toxic to the bacterial cells than are the superoxides. The inability of strict anaerobes to synthesize catalase, peroxidase or superoxide dismutase may explain why oxygen is poisonous to anaerobic microorganisms (Cappuccino and Sherman, 2005).

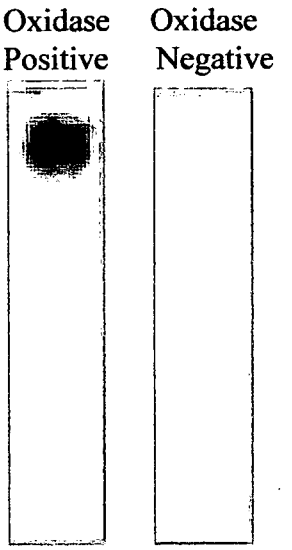


Plate 3.3: Negative result on oxidase test strip.

Plate 3.4: Bubbles formation to indicate the presence of catalase.

Table 3.3 summarizes the results for preliminary morphological and physiological characterization of Isolate Serdang 1. Based on these, there are several candidates that could possibly be Isolate Serdang 1:- *Neisseria* spp., *Moraxella* spp. and *Acinetobacter* spp.

Table 3.3: Preliminary morphological and physiological characterization of Isolate Serdang 1.

Source	Morphology	Gram Staining	Oxidase Test	Catalase Test	Possible Bacterial Strains
Soil contaminated with petrol waste, Serdang, Selangor	Colour: Cream Configuration:Round Margin: Smooth Elevation: Convex Shape: Cocci	Negative	Negative	Positive	*Neisseria* spp. *Moraxella* spp. *Acinetobacter* spp.

The physiological characteristics of Isolate Serdang 1 were further determined by Biolog ™ Identification System. The Biolog ™ system is a useful tool as it can identify isolate to the species level and show the metabolic capabilities of the isolate. The system tests the ability of microorganisms to oxidize a panel of 95 different carbon sources as shown in Table 3.4. Based on the results, Isolate Serdang 1 showed similar characteristics to *Acinetobacter baumannii* with 99% probability (Figure 3.5). The identification was accepted as correct if the similarity index of the genus and species name was 0.75 or greater at 4 hours; or 0.5 or greater at 24 hours. Sample identities were accepted as correct if the sample organism is listed in the system database and if the assigned identity

matched the genus and species of the reference ATCC strain (Klingler *et al.*, 1992). Isolate Serdang 1 produced similarity index of 0.712 at 24 hours.

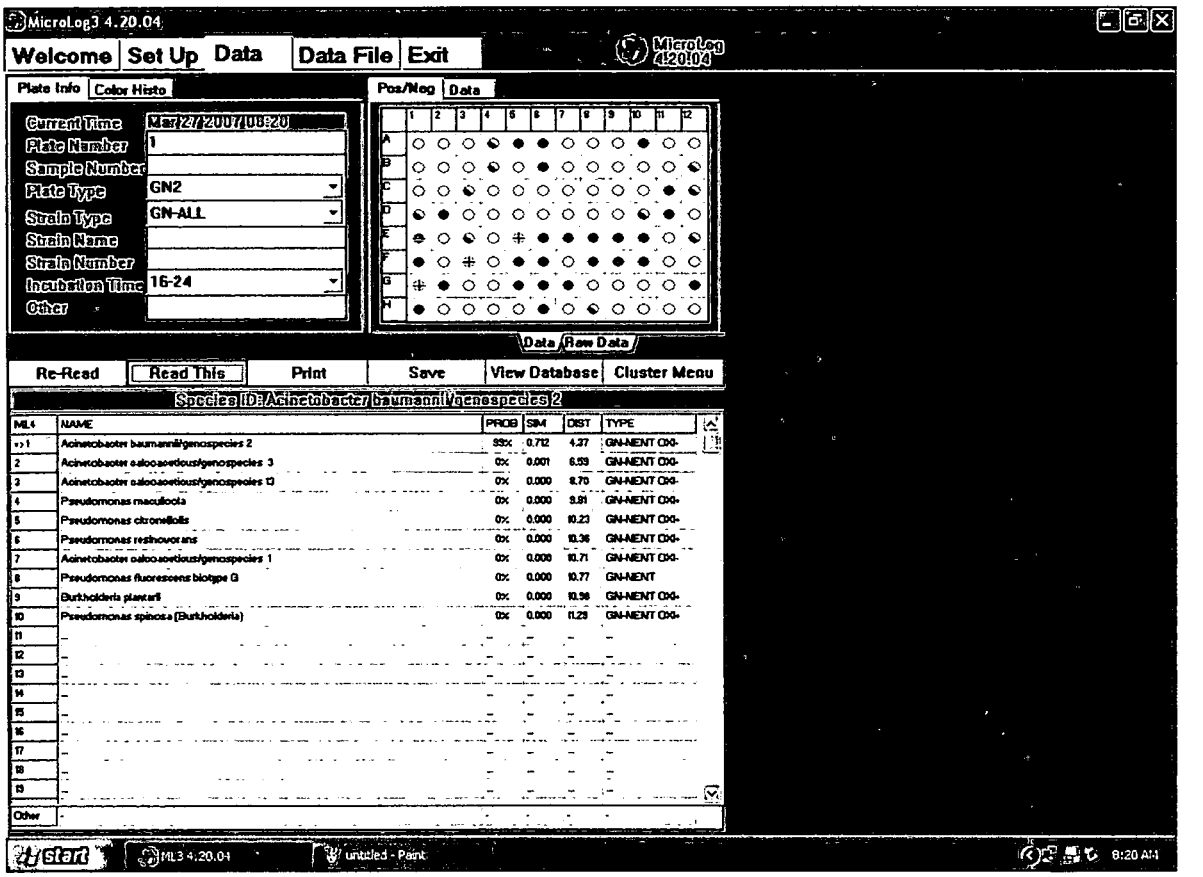


Figure 3.5: Isolate Serdang 1 showed similar characteristics to *Acinetobacter baumannii* with 99% probability.

Table 3.4: Growth of Isolate Serdang 1 on various carbon sources in Biolog ID microplates.

Carbon Sources	Growth (After 24 hours incubation)
Water (control)	-
α-Cyclodextrin	-
Dextrin	-
Glycogen	+/-
Tween 40	+
Tween 80	+
N-Acetyl-D-Galactosamine	-
N-Acetyl-D-Glucosamine	-
Adonitol	-
L-Arabinose	-
D-Arabitol	-
D-Cellobiose	-
i-Erythritol	-
D-Fructose	-
L-Fructose	-
D-Galactose	+/-
Gentiobiose	-
α-D-Glucose	+
m-Inositol	-
α-D-Lactose	-
Lactulose	-
Maltose	-
D-Mannitol	-
D-Mannose	+/-
D-Melibiose	-
β-Methyl-D-Glucoside	-
D-Psicose	+/-
D-Raffinose	-
L-Rhamnose	-
D-Sorbitol	-
Sucrose	-
D-Trehalose	-
Turanose	-
Xylitol	-
Pyruvic Acid Methyl Ester	+
Succinic Acid-Mono-Methyl-Ester	+/-
Acetic Acid	+/-
Cis-Aconistic Acid	+
Citric Acid	-
Formic Acid	-
D-Galactonic Acid Lactone	-
D-Galacturonic Acid	-
D-Gluconic Acid	-
D-Glucosaminic Acid	-
D-Glucuronic Acid	-
α-Hydroxy butyric Acid	+/-
β-Hydroxy butyric Acid	+
γ-Hydroxy butyric Acid	-
p-Hydroxy Penylacetic Acid	+
Itaconic Acid	-
α-Keto Butyric Acid	+/-
α-Keto Glutaric Acid	-
α-Keto Valeric Acid	+
D,L-Lactic Acid	+
Malonic Acid	+
Propionic Acid	+
Quinic Acid	+
D-Saccharic Acid	+
Sebacic Acid	-
Succinic Acid	+
Bromosuccinic Acid	+

Succinamic Acid	-
Glucuronamide	+
L-Alaninamide	-
D-Alanine	+
L-Alanine	+
L-Alanyl-Glycine	-
L-Asparagine	+
L-Aspartic Acid	+
L-Glutamic Acid	+
Glycyl-L-Aspartic Acid	-
Glycyl-L-Glutamic Acid	-
L-Histidine	+
Hydroxyl-L-Proline	+
L-Leucine	-
L-Ornithine	-
L-Phenylalanine	+
L-Proline	+
L-Pyroglutamic Acid	+
D-Serine	-
L-Serine	-
L-Threonine	-
D,L-Carnitine	-
γ-Amino Butyric Acid	+
Urocanic Acid	+
Inosine	-
Uridine	-
Thymidine	-
Phenylethyl-amine	-
Putrecine	+
2-Aminoethanol	-
2, 3-Butanediol	+/-
Glycerol	-
D,L-α-Glycerol Phosphate	-
α-D-Glucose-1-Phosphate	-
D-Glucose-6-Phosphate	-

Note:
+ = positive reaction
- = negative reaction
-/+ = borderline

3.3.2 Molecular Characterization of Phenol Degrading Bacteria

3.3.2.1 DNA Purity and PCR Products

Total cell DNA will often be required as a source of material from which to obtain genes to be cloned. As bacterial cell is enclosed in a cytoplasmic membrane and surrounded by a cell wall, and with Gram-negative species, a second outer membrane may envelop the cell wall; the barriers will have to be disrupted first to release the cell components. In this case, nucleic lysis solution would attack the cell wall and disrupt the cell membrane. RNase solutions added could eventually remove RNA. To deprotenize a cell extract, protein precipitation solution added would precipitate protein but leaving the DNA out in aqueous solution to be pipetted out. The use of ethanol precipitation is to precipitate the DNA and increase DNA concentration. The intensity of absorbance of the DNA solution at wavelength 260 nm and 280 nm is used as a measure of DNA purity. DNA absorbs UV light at 260 nm and protein absorbs UV light at 280 nm. Pure sample of DNA has the 260/280 ratio at 1.7 to 2.0 and is relatively free from protein contamination. In this study, the 260/280 ratio at 1.7 was recorded, suggesting that pure DNA was obtained.

Based on gel electrophoresis analysis, ~1.5 kbp band was seen as shown in Figure 3.6. This corresponds to the 16s rRNA region of the complete genomic DNA.

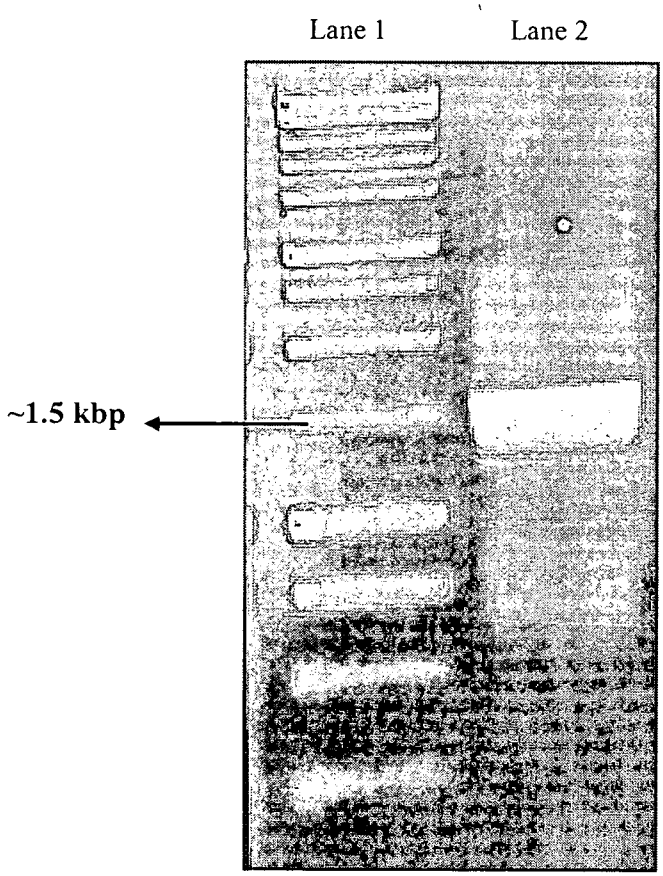


Figure 3.6: Detection of PCR product. Lane 1: DNA ladder marker. Lane 2: PCR product ~1.5 kbp.

3.3.2.2 Gene Cloning, DNA Sequencing and Phylogenetic Analysis

The desired PCR product has been purified in order to remove primer dimers, nucleotides and extraneous bands. The next step was gene cloning and the construction of the recombinant DNA molecule. The step involved the joining together or ligation of the vector molecule (pCR 2.1® –TOPO TA Cloning vector) and the DNA to be cloned. In this study, the recombinant DNA molecules were transformed into *E.coli* TOP 10 One Shot (competent cell) which would then grow and divide to produce clones. The movement of DNA into competent cells (cells which are in a physiological state to take up large DNA molecules from donor) is stimulated by briefly raising the temperature to 42°C for 1 min. When *E. coli* was subjected to 42 °C temperature, a set of genes are expressed which aid the bacteria to survive at such temperatures. These sets of genes are called the heat shock genes. The heat shock step is necessary for the uptake of DNA. It loosens up the membrane structure and the lipids in the membrane become more fluid which makes the entire membrane less stable (Brown, 1998). Unstable membrane could be detrimental to the cell, which explains the need to return to an ice bath immediately after the heat shock. Above 42°C, the ability of bacteria to take up DNA becomes reduced, and at extreme temperatures the bacteria will die. To help bacterial cells recovering from the heat shock, cells were briefly incubated with non-selective growth media (S.O.C medium). As the cells recovered, plasmid genes were expressed, including those that enable the production of daughter plasmids which will segregate with the dividing bacterial cells.

Plating on to a selective medium enables transformants to be distinguished from non-transformants. This is to determine which of the transformed colonies comprise of cells that contain recombinant DNA molecules, and which one contains self-ligated vector molecules. The blue-white screening was carried out in selecting a clone (Plate 3.5). The plasmid vector contains a gene (*amp*^{R}) coding for resistance to the antibiotic ampicillin. The host bacterium will not be able to grow on the test medium, containing ampicillin, unless the vector has transferred the ampicillin-resistance gene. The plasmid vector also contains a second gene coding for the enzyme β-galactosidase (*lacZ*) (Figure 3.2b). Cloning with pCR 2.1® –TOPO TA Cloning vector involves insertional inactivation of the *lacZ'* gene where recombinants could be identified because of their inability to synthesize β-galactosidase. β-galactosidase is one of a series of enzymes involved in the breakdown of lactose to glucose and galactose. It is normally coded by the gene *lacZ*, which resides on the *E.coli* chromosome. Some strains of *E.coli* have a modified *lacZ* gene, one that lacks the segment referred to as *lacZ'* which codes for the α –peptide portion of β–galactosidase. These mutants can synthesize the enzyme only when they harbour a plasmid, such as pCR 2.1®, that carries the missing *lacZ'* segment of the gene (Tortora *et al.*, 2004). A cloning experiment with pCR 2.1® involves selection of transformants on ampicillin agar, followed by screening for β–galactosidase activity to identify recombinants. Cells that harbour a normal pCR 2.1® are *amp*^{R} and able to synthesize β–galactosidase; recombinants are also *amp*^{R} but unable to make β–galactosidase. Screening for β–galactosidase presence or absence is easier than assaying for lactose being splitted into glucose and galactose, a slightly different reaction catalyzed by the enzyme is tested for. This involves a lactose analogue called X-gal

(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which can be broken down by β–galactosidase to a product that is colored deep blue. If X-gal (plus an inducer of the enzyme such as isopropylthiogalactoside, IPTG) is added to the agar, along with ampicillin, then non-recombinant colonies, the cells of which synthesizing β–galactosidase, will be coloured blue, whilst recombinats with a disrupted *lacZ'* gene and unable to make β–galactosidase, will be white.

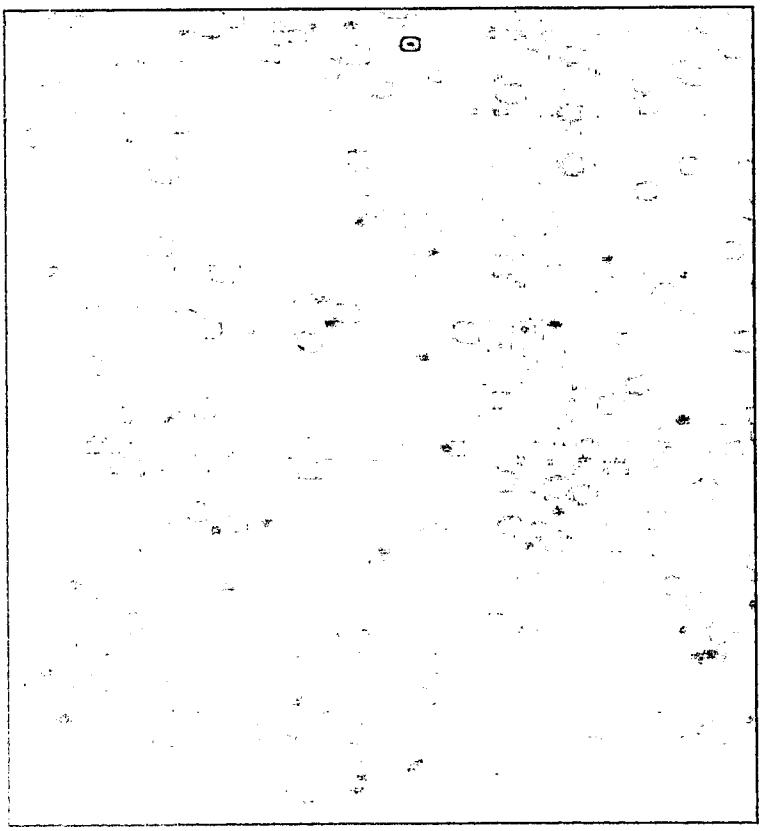


Plate 3.5: Blue-white screening.

For analyzing positive clones (white colonies), Restriction Endonuclease (RE) analysis were carried out. The recombinant plasmids were isolated and digested with BamHI and XhoI which produced two bands, the vector (~3.9 kbp) and the insert

(~1.5 kbp) as shown in Figure 3.7. This analysis confirmed that the clone carrying the gene as an insert, has been obtained.

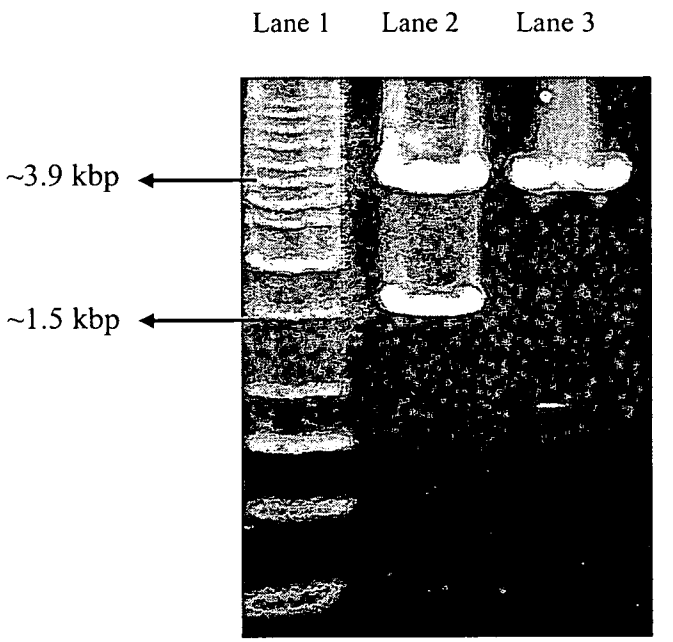


Figure 3.7: Restriction Endonuclease (RE) analysis of the extracted plasmid of positive transformant colony. Lane 1: DNA ladder marker; Lane 2: White colony: Vector (~3.9 kbp) with released PCR products (~1.5 kbp) Lane 3: Blue colony: Vector (~3.9 kbp) without released PCR products.

The vector acts as a vehicle that transports the gene into a host cell. The genetic and physical map of pCR 2.1®–TOPO TA Cloning vector gives an indication of why this plasmid has been such a popular cloning vector (Figure 3.2b). The first useful feature of pCR 2.1® –TOPO TA Cloning vector is its size. A cloning vector ought to be less than 10kb in size, to avoid problems such as DNA breakdown during purification. The pCR 2.1® –TOPO TA Cloning vector is 3.9kb, which means that not only can the vector itself be purified with ease, the recombinant DNA molecules can also be constructed with it. The second feature is that either ampicillin or kanamycin resistance gene can be used for selectable marker for cells containing the plasmid, or each marker gene includes unique restriction sites that can be used in cloning experiments. A third advantage is that it has a reasonably high copy number. *Taq* polymerase has a nontemplate-dependent terminal transferase activity, which tends to add an additional nucleotide, usually an adenosine (A) to the end of each strand that it synthesizes. This means that a double-stranded PCR product is not blunt-ended, and instead most 3' termini have a single nucleotide overhang. The overhangs could be removed by treatment with an exonuclease enzyme, resulting in PCR products with true blunt ends. However, this is not a popular approach as it is difficult to prevent exonucleases from becoming overactive and causing further damage to the ends of the molecules. One solution is to use a cloning vector which carries thymidine (T) overhangs and which can therefore be ligated to a PCR product. The plasmid vector (pCR 2.1®–TOPO TA Cloning vector) is supplied in linear form with 3'-thymidine (T) overhangs and topoisomerase I enzyme covalently bound to the vector. This allows PCR inserts to ligate efficiently with the vector.

The PCR product was sequenced using M13 forward and reverse universal primers on both strands and the complete sequence revealed that this gene consists of 1501 nucleotides (Figure 3.8). Based on a BLASTN search of GenBank, the complete sequences of these bacteria shares 98% similarity with *Acinetobacter baumannii*. This is also confirmed by a neighbour-joining phylogenetic tree (Figure 3.9) based on the alignment of 16S rRNA gene sequence of Isolate Serdang 1 with 16S rRNA sequence of the 22 described *Acinetobacter* type strains available in GenBank databases and rooted by using *Bacillus*. Species names are followed by the accession numbers of their 16s rDNA sequences. The numbers at branching points refer to bootstrap values, based on 1000 resamplings. The accession number for each bacterium is indicated in brackets. From earlier results as shown in Table 3.3 and confirmed by Biolog ^{TM} Identification System (Table 3.4), the sequence alignment and phylogenetic tree analysis, the Isolate Serdang 1 has been redesignated *Acinetobacter baumannii* Serdang1 and the complete sequence has been submitted to GeneBank database under the accession number EF525671.

```
   1 agagtttgat cctggctcag attgaacgct ggcggcaggc ttaacacatg caagtcgagc
  61 gggggaaggt agcttgctac cggacctagc ggcggacggg tgagtaatgc ttaggaatct
 121 gcctattagt gggggacaac atctcgaaag ggatgctaat accgcatacg tcctacggga
 181 gaaagcaggg gatcttcgga ccttgcgcta atagatgagc ctaagtcgga ttagctagtt
 241 ggtggggtaa aggcctacca aggcgacgat ctgtagcggg tctgagagga tgatccgcca
 301 cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga atattggaca
 361 atgggggaa ccctgatcca gccatgccgc gtgtgtgaag aaggccttat ggttgtaaag
 421 cactttaagc gaggaggagg ctactttagt taatacctag agatagtgga cgttactcgc
 481 agaataagca ccggctaact ctgtgccagc agccgcggta atacagaggg tgcgagcgtt
 541 aatcggattt actgggcgta aagcgtgcgt aggcggctta ttaagtcgga tgtgaaatcc
 601 ccgagcttaa cttgggaatt gcattcgata ctggtgagct agagtatggg agaggatggt
 661 anatttccag gtgtagcggt gaaatgcgta aagatctgga ggaataccga tggcgaggca
 721 gcatctgcta atacngacgc tgangtacga aagcatgggg aggcaacagg attagatacc
 781 ctggtagtcc atgcgtaaac gatgtctact agccgttggg cctttgaggc tttagtggcg
 841 cagctaacgc gataagtaga ccgcctgggg agtacggtcg caagactaaa actcaaatga
 901 attgacgggg gcccgcacaa gcggtggagc atgtggttta attcgatgca acgcgaagaa
 961 cttacctggc cttgacatac tagaaacttt ccagagatgg attggtgcct tcgggaatct
1021 agatacaggt gctgcatggc tgtcgtcagc tcgtgtcgtg agatgttggg ttaagtcccg
1081 caacgagcgc aacccttttc cttacttgcc agcatttcgg atgggaactt taaggatact
1141 gccagtgaca aactggagga aggcggggac gacgtcaagt catcatggcc cttacggcca
1201 gggctacaca cgtgctacaa tggtcggtac aaagggttgc tacacagcga tgtgatgcta
1261 atctcaaaaa gccgatcgta gtccggattg gagtctgcaa ctcgactcca tgaagtcgga
1321 atcgctagta atcgcggatc agaatgccgc ggtgaatacg ttcccgggcc ttgtacacac
1381 cgcccgtcac accatgggag tttgttgcac cagaagtagc tagcctaact gcaaagaggg
1441 cggttaccac ggtgtggccg atgaccgggg tgaagtcgta acaaggtagc cgtaggggaa
1501 cctgcggctg gatcacctcc ttaa
```

Figure 3.8: Complete sequence of 16s rRNA *Acinetobacter baumannii* Serdang1.

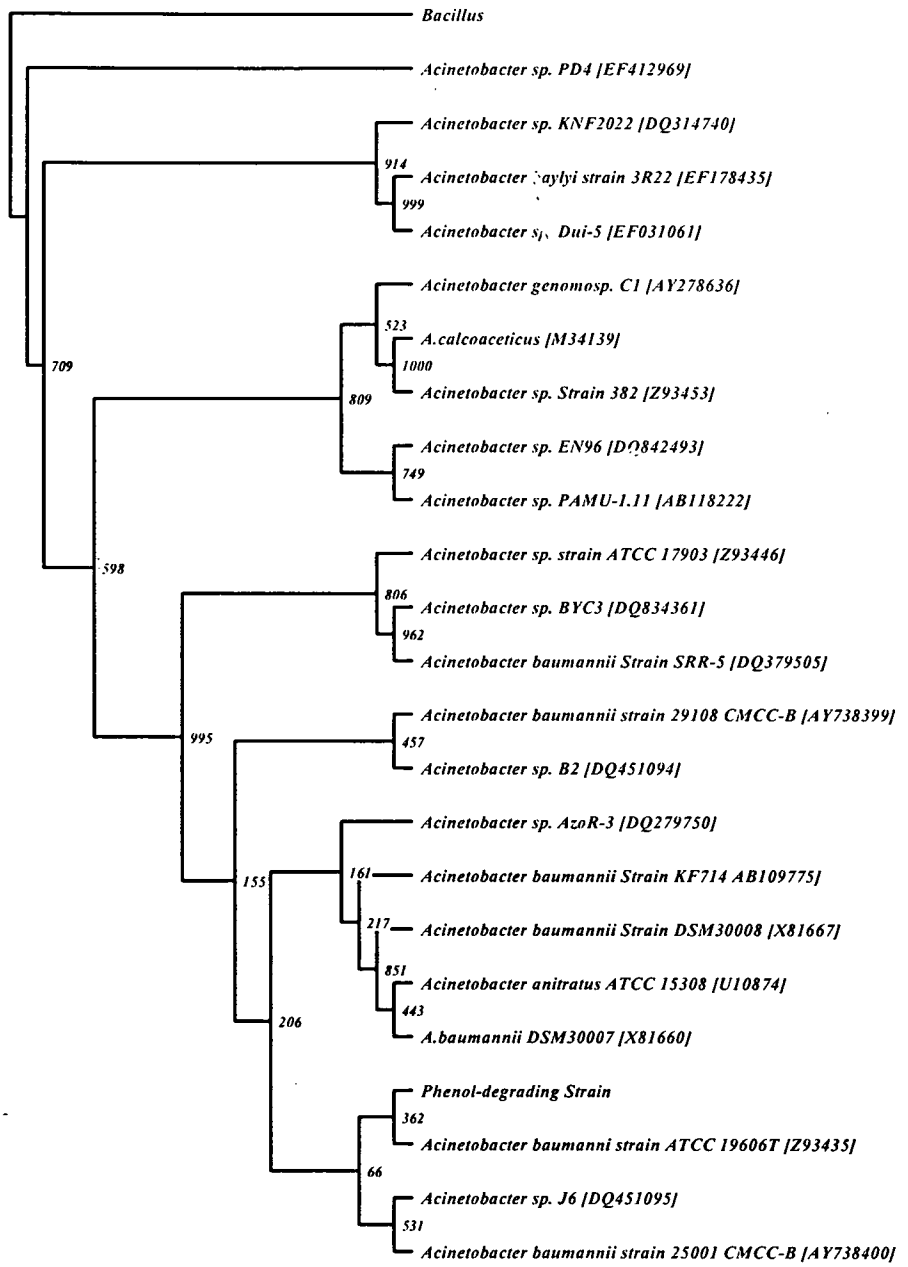


Figure 3.9: Neighbour-joining method cladogram showing phylogenetic relationship between Phenol-Degrading Strain and other related reference microorganisms based on the 16s rDNA gene sequence analysis.

3.3.2.3 *Acinetobacter* spp. and Their Potentials

Acinetobacter spp. are ubiquitous and can be found in water, soil and living organisms. These are Gram-negative bacteria, oxidase-negative, non-motile, strictly aerobic and tend to be paired cocci, rather than monoflagellate rods. They can use various carbon sources for growth and can be cultured on relatively simple media (Juni, 1954). Based on cell shape, absence of flagella, G+C content of DNA and nutritional properties, these organisms are initially classified in the genus *Moraxella* but now known more as *Acinetobacter* (Baumann *et al.*, 1968). The basis for the present classification of *Acinetobacter* was laid in 1986 by Bouvet and Grimont, with the description of 12 DNA-DNA hybridization groups (genomic species) within the genus. This scheme was extended with 11 additional genomic species in the period between 1986-1999 (Vaneechoutte *et al.*, 1999; Gerner-Smidt and Tjernberg, 1993; Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989). Eight genomic species are given names (*A. calcoaceticus*, *A. baumannii*, *A. haemolyticus*, *A. junii*, *A. johnsonii*, *A. lwoffii*, *A. radioresistens, and 'A. venetianus'*); while the others are designated by numbers. Strains *A. calcoaceticus*, *A. baumannii*, the unnamed groups 3 and 13TU are genetically closely related and difficult to separate phenotypically. They are therefore sometimes unified in the so-called *A. calcoaceticus-A. baumannii* (*ACB*) complex (Gerner-Smidt *et al.*, 1991).

Acinetobacter species have attracted growing interest in both environmental and biotechnological applications. They are known to be involved in the biodegradation of a number of different pollutants and in the extra- and intracellular production of a number of economically valuable products (Abd-El-Haleem, 2003). Several strains of

Acinetobacter, can use phenol as a sole energy and carbon source (Briganti *et al.*, 1997; Chibata and Tosa, 1981). Recently, out of twelve bacterial phenol-degrader isolated from different Egyptian ecosystems, four are closely related to *Acinetobacter* (Abd-El-Haleem et al., 2002a). Various *Acinetobacter* strains are also capable of degrading other xenobiotic compounds such as toluene (Zilli *et al.*, 2001), 4-hydroxybenzoate (Allende *et al.*, 2000), 2-chloro-N-isopropylacetanilide (Martin *et al.*, 1999), 4-hydroxymandelic and 4-hydroxy-3-methoxymandelic acids (Rusansky *et al.*, 1987), benzoic and p-hydroxybenzoic (Delneri *et al.*, 1995), 4-chlorobenzoate (Adriaens and Focht, 1991) and 3-chlorobenzoic acid (Zaitsev and Baskunov, 1985). It has also been reported that certain *Acinetobacter* strains can utilize biphenyls including chlorinated biphenyls (Adriaens and Focht, 1991; Furukawa and Chakrabarty, 1982). Singer *et al.* (1985) observed that out of 36 pure isomers of polychlorinated biphenyls examined, 33 have been metabolized by *Acinetobacter* sp. strain P6. Some *Acinetobacter* spp. isolated from mixed cultures has also proven to be proficient at complete mineralization of monohalogenated biphenyls (Shields *et al.*, 1985). Others have reported degradation of lignin (Buchan *et al.*, 2001; Mak *et al.*, 1990; Crawford, 1975); amino acids (Kahng *et al.*, 2002; Kim *et al.*, 2001); oil degradation (Rusansky *et al.*, 1987) and heavy metals removal (Francisco *et al.*, 2002; Boswell *et al.*, 2001) by *Acinetobacter* strains.

Beside remediation, several strains of *Acinetobacter* produce extracellular polysaccharides with sizes up to several million Daltons. These polysaccharides can consist of D-galactose, D-2-acetamido-2-deoxy-D-glucose, 3-(L-2-hydroxypropionamido)-3,6-dideoxy-D-galactose (Haseley *et al.*, 1997), rhamnose, 3-deoxy-3-(D-3-hydroxybutyramido)-D-quino-vose, S-(+)-2-(4'-Isobutylphenyl)propionic acid or

lipopolysaccharide (Kunii *et al.,* 2001; Haseley *et al.,* 1997; Yamamoto *et al.,* 1990). Some are able to grow on ethanol and synthesize exopolysaccharides called ethapolan (Johri *et al.,* 2002; Pirog *et al.,* 2002; Pyroh *et al.,* 2002) while several strains are known to accumulate wax esters, polyhydroxyalkalonic acids and cyanophycin (Krehenbrink *et al.,* 2002; Pirog *et al.,* 2002; Vinogradov *et al.,* 2002; Spiekermann *et al.,* 1999). Various types of this biopolymer are widely used in the manufacture of fine chemicals such as cosmetics, candles, printing inks, lubricants, and coating. A vast number of *Acinetobacter* lipases have wide range of potential applications in the hydrolysis, esterification, and transesterification of triglycerides, and in the chiral selective synthesis of esters (Li *et al.* 2000, 2001; Chen *et al.,* 1999). In addition, *Acinetobacter* strains have been reported to synthesize a wide variety of bioemulsifiers (Rosenberg and Ron, 1998). Bioemulsifiers, which contain both hydrophobic and hydrophilic groups, are used widely in food, agrochemical, cosmetic, and pharmaceutical industries. Among the so-called "bioemulsans," the most studied are those produced by *A. calcoaceticus* RAG-1 (Rosenberg and Ron, 1998), *A. calcoaceticus* BD4 (Kaplan *et al.,* 1987), and *A. radioresistens* KA53 (Navon-Venezia *et al.,* 1995).

Recently, Abd-El-Haleem *et al.* (2002b) have constructed a bioluminescent reporter strain, *Acinetobacter* sp. DF4 for the detection of phenol, by inserting the phenol 3-hydroxyacyl-CoA-dehydrogenase (*3-hcd*) promoter upstream of the bioluminescence genes *luxCDABE.* When it is introduced into the chromosome of *Acinetobacter* sp. DF4, the resulting strain, produces a sensitive bioluminescence response to phenol

concentrations ranging from 5 to 100 ppm. With such specificity, *Acinetobacter* strains are prime candidates for whole-cell bioreporter monitoring of phenol.

3.4 Conclusions

A new indigenous phenol-degrading bacteria strain from Malaysian soil had been successfully isolated, characterized and identified. Based on a BLASTN search of GenBank, the complete sequences of bacteria shared 98% similarity with *Acinetobacter baumannii*. This is confirmed by Biolog ™ Identification System, sequence alignment and phylogenetic tree analysis. The Isolate Serdang 1 has been redesignated *Acinetobacter baumannii* Serdang1 and the complete sequence submitted to GeneBank database under the accession number EF525671.

CHAPTER IV

IMPORTANT BATCH OPERATIONAL PARAMETERS FOR PHENOL BIODEGRADATION BY *Acinetobacter baumannii* SERDANG 1

4.1 Introduction

Biodegradation of organic substrates provide microorganisms with energy and building materials for growth of new cells, cell maintenance and co-metabolism of other less degradable substances (Cornelissen and Sijm, 1993). Phenol can be degraded by a wide variety of microorganisms. *Acinetobacter* species have been attracting considerable attention in environmental applications due to their versatility.

In phenol-contaminated sites, toxicity studies have shown that bacteria can adapt to low phenol concentrations, but the overall biodegradative capability appears to be reduced with increasing phenol concentration (Dean-Ross, 1989). At toxic level, phenol inhibits microbial growth (Chung *et al.,* 1998; Ahmed, 1995; Fava *et al.,* 1995; Zaisev, 1995) and causes cell-lysis (Ruiz-Ordaz *et al.,* 1998). For continued viability, it is necessary to construct a barrier between microorganisms and the toxic phenol concentration (Chung *et al.,* 1998). Cell immobilization technology is studied for its potential to improve fermentation processes and bioremediation (Abd-El-Haleem *et al.,* 2003; Beshay *et al.,* 2002). Immobilization of cells can not only potentially reduce the inhibitory effect of phenol, there is also a possibility that the immobilized cells can be reused continuously (Chung *et al.,* 2005). On the negative side, the increase in cell mass

when cells proliferate may result in the interior of the support becoming crowded with the cells leading to breakage of the support from the inside in extreme cases (Mattiasson, 1983). However, for practical wastewater treatment with immobilized microorganisms, an outgrowth of cells is not important as the ultimate aim is for remediation of toxic pollutants (Bettmann and Rehm, 1984; Annadurai *et al.*, 2000).

Immobilization via cell entrapment within calcium alginate is a simple and fast technique. Cells are combined with an alginate solution, extruded drop wise into a stirred solution of calcium chloride where it gels to form solid beads. Calcium alginate gels form rapidly in mild conditions and provide suitable media for the entrapment of whole microbial cells (Beshay, 2003). Moreover, alginate is cheap and readily available. As fermentation parameters may affect the reproducibility of the system and phenol degradation, the effect of important parameters on phenol degradation by immobilized and free cell system should be determined and optimized.

The objectives of this study were therefore:

1. To determine important parameters for phenol biodegradation in a batch system.
2. To compare the activities of phenol degradation by Ca-alginate immobilized and freely suspended cells.
3. To investigate the reusability of immobilized cells for continued use in bioremediation processes.

4.2 Materials and Methods

4.2.1 Culture Media

The medium used in all experiments was as described by Ali *et al.*, (1998) as follows (g/l): 1.0 $(NH_4)_2SO_4$ (R&M Chemical, China), 0.25 $MgSO_4.7H_20$ (Merck, Germany), 0.25 KH_2PO_4 (Merck, Germany), 0.07 $CaCl_2.2H_2O$ (Merck, Germany), 0.1 yeast extract, 0.5 phenol (Hamburg Chemical, Germany). Phenol solution was filter sterilized using 0.2 µm syringe filter (Sartorius, Germany) and added to the sterilized medium at the beginning of the experiment. For the preparation of phenol solid medium in plates, 20 g/l of agar was added.

4.2.2 Bacterial Stock and Working Culture

Well-defined colonies on the basis of morphological characteristics of all pure bacterial isolates were transferred to a phenol medium slant and preserved at 4°C in refrigerator for future experimental use. Working pure culture of *Acinetobacter baumannii* Serdang 1 was transferred on fortnightly basis onto phenol agar medium and preserved at 4°C in refrigerator for ongoing experiments. For culture library collection, pure culture of *Acinetobacter baumannii* Serdang 1 was transferred into Eppendorf tubes containing 20% (v/v) glycerol and preserved at -20°C for future experimental use.

Working culture of *Acinetobacter baumannii* Serdang 1 was streaked on phenol solid medium. The plates were incubated at 30°C for 48 hours. Single colony was transferred into a universal bottle containing 5 ml liquid phenol medium at 500 mg/l phenol concentrations. Cultures were incubated at 30°C for 48 hours at 100 rpm. One percent of bacterial suspension (absorbency for concentration A_{600}= 1.00) was inoculated into a 250 ml Erlenmeyer flask (Pyrex) containing 50 ml liquid phenol medium and incubated on a rotary shaker (100 rpm) at 30°C. The cultures were used as inoculum for experiments after leaving overnight (~15 hours).

4.2.3 Cell Immobilization

For the preparation of calcium alginate beads, sodium alginate was first dissolved in boiling water and autoclaved (Hirayama, USA) at 121°C for 15 minutes. In this study, the source of sodium alginate (BDH Chemical, Australia) was from *Laminaria hyperborea* stems, which are rich in guluronic acid and forms much more rigid gels. The bacterial liquid cultures were aseptically centrifuged (Selecta, Spain) at room temperature at 7000 rpm for 5 minutes and the supernatant discarded. The pellet was re-suspended in sodium alginate at 35 mg wet weight for 1 ml sodium alginate solution. The mixture was then extruded drop wise by gravity into a sterile calcium chloride solution using sterile syringe (12 ml) from a height of about 15 cm. The alginate droplets subsequently gelled, upon contacting with calcium chloride solution to form uniformly-sized spheres. The beads were kept in calcium chloride solution at 30°C for 2 hours to complete gel formation. This way, insoluble and stable immobilized beads were obtained

(Keweloh *et al.*, 1989). Beads were kept overnight at 4°C before being harvested by filtration. Finally, beads were washed with sterile distilled water to remove excess calcium ions and free cells (Beshay, 2003).

4.2.4 Experimental Design

The experiments were divided into 3 parts:

1. Optimization of immobilization protocols.
2. Comparison of phenol-degrading activities between freely-suspended and immobilized cells.
3. Reusability of immobilized cells.

4.2.4.1 Optimization of Immobilization Protocols

4.2.4.1.1 Effect of Composition of Gelling Components

Immobilization effectiveness was tested by varying sodium alginate solution between 2-5% (w/v), with cell suspension added to sterile calcium chloride solution at 2-5% (w/v). The effects of polymer compositions were determined with respect to percentage of phenol removal. Samples were taken out at fixed time intervals, every 24 hours and analyzed for phenol concentration.

4.2.4.1.2 Effect of Initial Cell Loading (ICL)

The effect of initial cell loading was tested by varying the number of beads from 100 to 600 beads/flask using 3% (w/v) of both sodium alginate and calcium chloride. The effectiveness was determined with respect to phenol removal.

4.2.4.1.3 Effect of Bead Sizes

The effect of bead sizes at 2, 3, 4, 5 and 6 mm were determined with respect to phenol removal.

4.2.4.2 Comparison of Phenol-Degrading Activities Between Freely-suspended and Immobilized Cells

Comparison of phenol-degrading activities between immobilized and free cells were carried out using equivalent initial biomass concentrations 180 mg wet weight for each. Batch cultures were cultivated for 5 days with 50 ml of liquid phenol medium at 500 mg/l phenol concentrations, in 250 ml Erlenmeyer flask. Cultures were incubated on a rotary shaker at 100 rpm at 30°C. One milliliter of samples were taken out every 24 hours and analyzed for phenol concentration.

Control 1 experiments involved the use of the same medium minus the bacterial cells and the Ca-alginate beads to evaluate the possible degree of phenol removal as a result of volatilization. Control 2 experiments involved the use of sterile Ca-alginate beads in the same medium to check on the possibility of phenol being adsorbed onto the immobilizing

agent. All experiments were carried out in triplicates. Results were shown as the average of triplicate data with standard error.

4.2.4.2.1 Effect of pH

For optimum microbial activity in the environment, the preferred range of pH is between pH 6 to 8 (McLelland, 1996). In this study, liquid phenol medium at pH 6, 6.5, 7, 7.5 and 8 were prepared. pH was adjusted using 0.1 M HCL and 0.1 M NaOH.

4.2.4.2.2 Effect of Temperature

The culture temperature was tested at 10, 25, 30, 35 40, 50 and 60°C.

4.2.4.2.3 Effect of Nitrogen Sources

Eight nitrogen sources were tested individually for the ability to support growth with phenol as a carbon source. These were L-leucine ($(CH_3)_2CHCH_2CH(NH_2)COOH$), ammonium chloride ($NH_4Cl$), ammonium sulphate ($(NH_4)_2SO_4$), ammonium format ($HCOONH_4$), sodium nitrate ($NaNO_3$), glycine ($NH_2CH_2COOH$) and urea ($NH_2CONH_2$). The level of nitrogen source was prepared at 1g/l in place of ammonium sulphate initially formulated in the medium. Another study was carried out on the effects of ammonium sulphate concentration on cell growth with enhanced phenol degradation. The ranges of concentrations tested were 0 to 2.5 g/l. The basic pH used was 7.5 at 30°C.

4.2.4.2.3 Effect of Initial Phenol Concentrations

Liquid phenol medium was prepared by varying phenol concentration from 100 mg/l to 3000 mg/l. The optimum conditions would be the level that was non-toxic to the bacteria and with highest percentage of phenol removal.

4.2.4.3 Reusability of Immobilized Cells

After 5 days of culture period for the first cycle, the spent medium was removed by filtering out aseptically the alginate beads containing the cells. The beads were washed and used in the next cycle of biodegradation with initial phenol concentration of 500 mg/l in each cycle. Effects of succinic acid as a potential precursor to speed up phenol removal rate were tested at 10, 100, 1000 mg/L. Succinic acid together with Acetyl-CoA and the product from phenol degradation via *ortho*-fission pathway before entering the TCA cycle (Figure 2.4).

4.2.5 Analyses

4.2.5.1 Bacterial Growth Determination

Bacterial growth population was determined using a serial dilution technique to enumerate the colony-forming unit (CFU). One hundred micro liter of bacterial suspensions was transferred aseptically to Eppendorf test tube containing 900 µl sterile

saline (0.9%), which has 10 times dilution factors (10^{-1}). The bacterial suspensions were continuously transferred to each Eppendorf tube, which has dilution factors of 100 times (10^{-2}) until 10,000,000 times (10^{-7}) or more and vortexed. The suspensions (100µl) were spread onto phenol media agar and mixed by rotation. The plates were incubated for 48 hours at 30°C and the resulting colonies were counted.

In a batch culture, the exponential increase in cell density after inoculation is measured as a function of time and analyzed to obtained the specific growth rate (μ), for that substrate concentration (Yoong *et al.,* 2004; Yoong and Edgehill, 1993). The specific growth rate (μ) was measured from the slope of the cell density curve by delineating the log growth phase, represented by the equation below.

$$\mu = \frac{\text{(log number of cells at time b – log number of cells at time a)}}{\text{time b – time a}}$$

To calculate the number of generations a culture has undergone, cell numbers must be converted to logarithms. The log of 2 (0.301) is used because one cell divides into two.

$$\text{No. of generations} = \frac{\text{(log number of cells at time b – log number of cells at time a)}}{0.301}$$

4.2.5.2 Phenol Concentration Determination

Colorimetric method based on 4-aminoantipyrine (4-AAP) was used to determine phenol concentrations using phenol solution as a standard (APHA, 1998). Sample was centrifuged at 7000 rpm for 5 minutes to get the supernatant. Each sample was added with a buffer solution until it reached pH 10. Test tubes were added with 200 µl of 4-aminoantipyrine (4-AAP) solution and 200 µl of potassium ferric cyanide in three replicates. After 15 minutes, the absorbance was read at 510 nm using spectrophotometer. The amount of phenol concentrations was estimated from the standard curve shown in Appendix. This method determines phenol and *ortho-* and *meta*-substituted phenols. Phenolic materials react with 4-AAP in the presence of potassium ferric cyanide at pH 10 to form a stable reddish-brown antipyrine dye. The reaction is as shown in Figure 4.1. The intensity of color produced is a function of the concentration of phenolic material. The percentage of phenol removal and phenol degradation rate was calculated as follows:

$$\% \text{ Phenol Removal} = \frac{(\text{Initial Phenol Concentration} - \text{Final Phenol Concentration}) \times 100}{\text{Initial Phenol Concentration}}$$

$$\text{Phenol degradation rate} = \frac{\text{Amount of phenol consumed}}{\text{Time required for consumption of phenol}}$$

The time required for total consumption of phenol was calculated by subtracting the phenol degradation lag period from the total time required for the consumption of phenol.

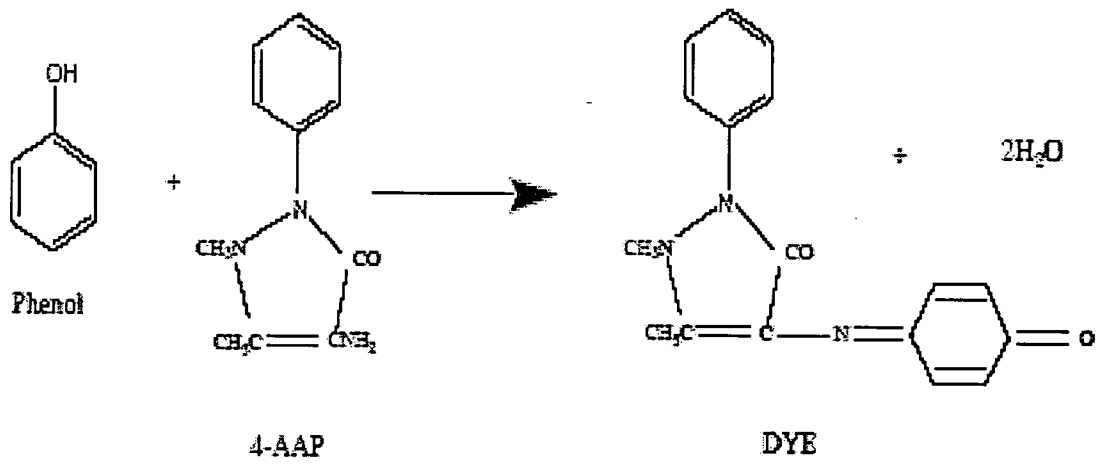


Figure 4.1: Phenol reacts with 4- aminoantipyrine (4-AAP) in alkaline solution in the presence of ferric cyanide to produce a red product.

4.3 Results and Discussions

4.3.1 Kinetics of Microbial Cell Growth and Phenol Degradation

The batch kinetic profile shows the variation of phenol and cell density versus time as depicted in Figure 4.2. A decrease in phenol concentration was concomitant with an increase in cell growth showing that phenol is being assimilated for energy and growth. The culture medium became cloudy as shown in Figure 4.3. There was no lag phase suggesting that phenol is readily degradable. This observation has also been reported by Buswell (1975) and Annadurai *et al.* (2002). The profile shows that there were two phases in the utilization of phenol. In the first phase, (0-5 days) when cells begin to divide and enter a logarithmic phase, the degradation rate was lower at 48 mg L^{-1} day^{-1}; and the second phase when cells were in stationary phase (5-7 days), the degradation rate was 130 mg L^{-1} day^{-1}.

The maximum cell density on day 5 was 8.9 log CFU/ml which corresponds to 66 x 10^{-7} CFU/ml. The cell growth rate was 0.560 day^{-1} with the generation time of 3 hours/generation. The generation time is the time taken per generation. In this case number of generations was calculated to be 2 generations. In this preliminary study, we showed that the time needed to complete the phenol degradation at 500 mg/L initial concentration was 7 days. Beshay *et al.* (2002) have reported that the time to completely degrade phenol at 500 mg/L for *Acinetobacter* sp. strain W-17 is 5 days. This difference shows the need for further improvement in our process.

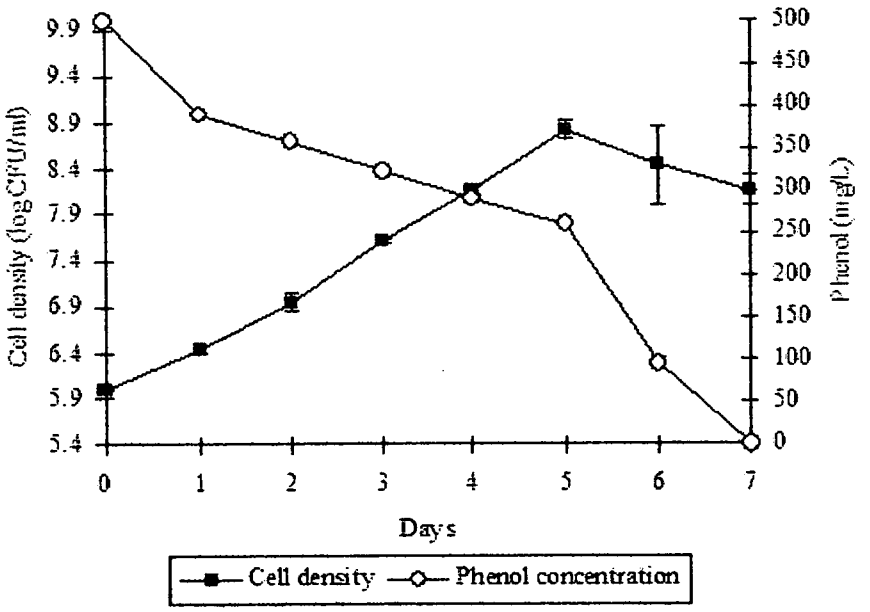


Figure 4.2: Cell growth profile of *Acinetobacter baumannii* Serdang 1 at basic conditions; 30°C, pH 7, 100 rpm on orbital shaker in basic medium with 500 mg/L phenol.

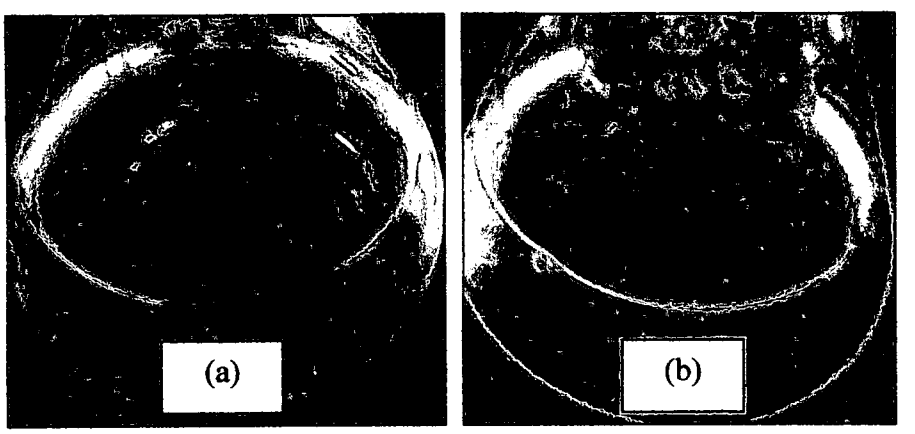


Figure 4.3: Culture medium without (a) and with (b) the growth of bacteria.

4.3.2 Cell Immobilization

In designing an immobilized cell system to degrade phenol, it would seem that optimizing the bead environment and the reaction environment will be of prime importance if degradation is to be enhanced. In an immobilized system, the physical structure of the immobilizing material can inhibit degradation, due to restriction on oxygen and nutrient flow (Lakhwala *et al.,* 1992). The structural strength of immobilizing material can be improved by changing the composition of gelling components. In our preliminary study with immobilization of cells (Figure 4.4), a total of $57x10^5$ CFU/ml/bead was obtained.

It is important to achieve acceptable number of cells/bead as insufficient number could lead to the following problems:

1. Zonation of the microenvironment within the bead may alter intraparticle growth, metabolism and product formation (Doherty *et al.,* 1995; McLoughin, 1994).
2. Low cell numbers at the center will generate unproductive regions and influence particle density (McLoughin, 1994).
3. In the event of too high number of cells, accumulation of cells on the bead surface may rupture the gel surface, resulting in outgrowth and leakage (McLoughin, 1994).
4. Non-homogenous distribution of cells can cause insufficient diffusivity of solutes (Doherty *et al.,* 1995).

Figure 4.4: Immobilized cells.

4.3.3 Effects of Composition of Gelling Components, Initial Cell Loading (ICL) and Bead Sizes

The concentration of both sodium alginate and calcium chloride solutions were varied from 2% to 5% (w/v) in an aqueous medium. Figure 4.5a shows that phenol biodegradation at 3% (w/v) composition of both sodium alginate and calcium chloride was the highest at 77.2% removal. This was achieved by retaining ICL at 300 beads/flask and bead size of 2 mm. When the numbers of beads were varied from 100 to 600 beads/flask using 3% (w/v) composition and the bead size of 2 mm (Figure 4.5b), the highest phenol removal at 83.5% was achieved at 400 beads/flask. Bead size of 2 mm was optimum for phenol removal of 81.5% as shown by Figure 4.5c when gelling composition was retained at 3% (w/v) and ICL at 400 beads/flask.

These results suggest two important conclusions:

1. more and larger beads do not necessarily translate into optimum bioremediation;
2. the major limiting factor could be the encapsulating matrix.

Entrapment of cells involves the building of a matrix around the population of cells. The matrix structure determines the reaction capabilities of the cell population. This is because binding strength is represented by the pore size, which affects the diffusion of substrates or products, and could possibly promote or inhibit the production or breakdown of products. Too small a pore size only allow, low molecular weight substrates and products to diffuse through; while too large a pore size may result in cell leakage (Chibata *et al.,* 1986; Cheetham and Bucke, 1984). The pore size may also be reduced into a closely-linked, stronger matrix with subsequent effects but this again could

result in a molecules diffusing in and out. This can be seen in Figure 4.5a where an increase in the concentration of both sodium alginate and calcium chloride solutions beyond 3% (w/v) reduced the effectiveness for phenol biodegradation. This is in agreement with a study on immobilized *Pseudomonas putida* MTCC 1194 where phenol removal was reduced from 99% to 92% and 85% when gelling components was increased to 4- 5% (w/v) (Bandyopadhyay *et al.,* 1999).

With greater number of beads per flask, and larger bead size, one could expect an increase in the total surface area, which eventually could facilitate the mass transfer to/from the Ca-alginate beads. Instead more beads per flask and larger bead size only result in decreased phenol biodegradation (Figure 4.5b). A significant problem with immobilized cells appears to be the diffusion of oxygen and nutrients through the gel matrix (Doherty *et al.,* 1995; Lakhwala *et al.,* 1992; McLoughin, 1994). At median ICL (400beads/flask) and correct bead size, there may be no oxygen/nutrient diffusion limitation to the cells. The liquid layer surrounding the beads could facilitate easy transport of oxygen/nutrients to the cells and optimum number of cells keeps oxygen/nutrient demand manageable. However, diffusion becomes increasingly difficult as size of the bead increases, inhibiting the transfer of oxygen and nutrients through the gel matrix (Doherty *et al.,* 1995). With increasing cell numbers in the bead and with more number of beads per flask, oxygen is consumed faster than it can diffuse. There will be greater competition for oxygen (Gosmann and Rehm, 1986) and the nutrient becomes limiting as the nutrient/bead ratio decreases (Beshay, 2003).

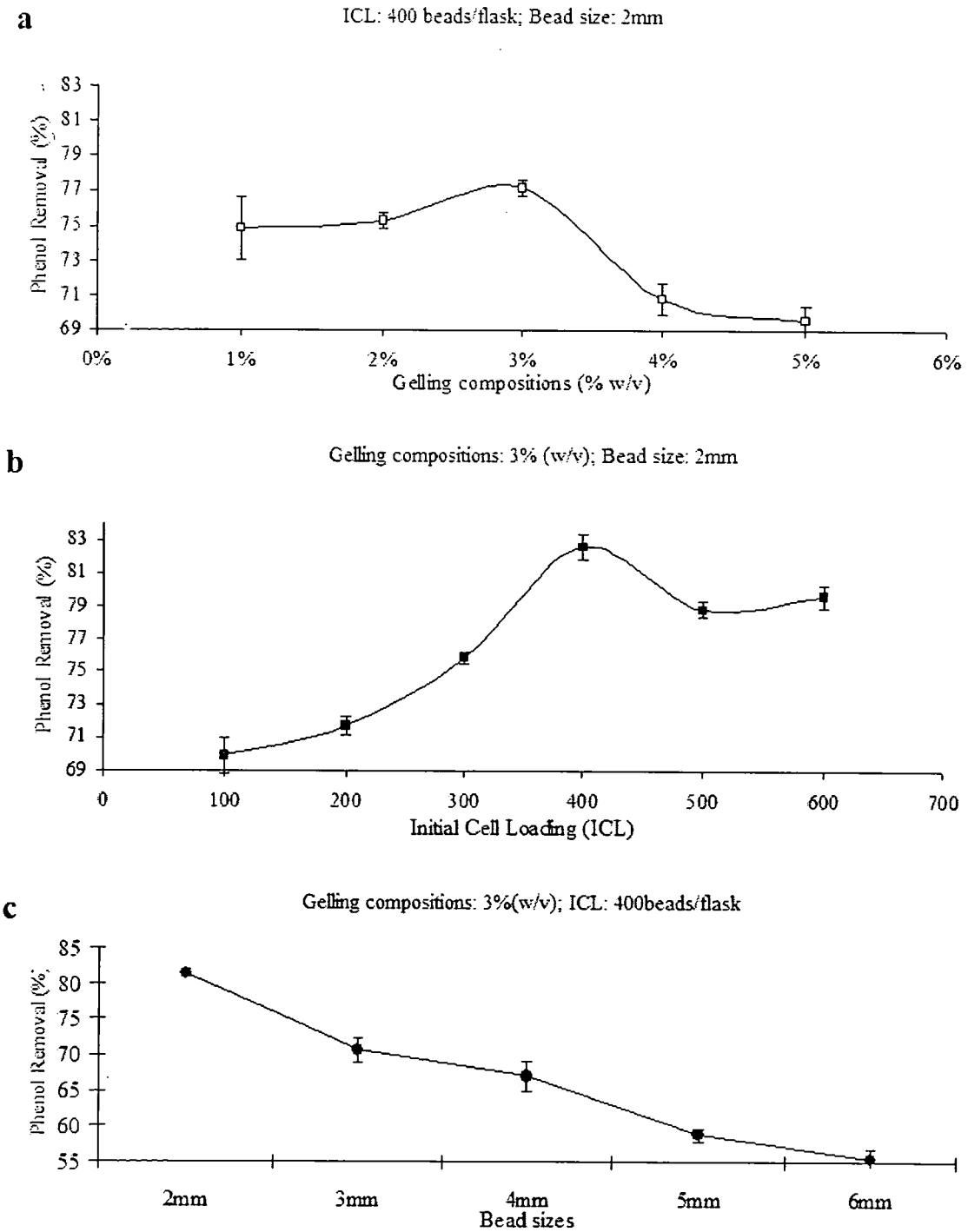


Figure 4.5: The effects of (a) gelling compositions, (b) initial cell loading (ICL), (c) bead sizes; on effectiveness of immobilized cells for phenol removal at basic conditions.

4.3.4 Comparison of Activities between Freely-suspended and Immobilized Cells

4.3.4.1 Effect of Initial pH and Temperature

The optimal pH for free cell systems may not be the same as that for an immobilized cell system. Immobilization increases cell protection against environmental conditions. In addition, alginate gel matrices are negatively charged, so that the pH of the surrounding environment influences the diffusion of substrates and the excretion of products (McLoughin, 1994). As shown in Figure 4.6a, the effects of pH on free and immobilized cells were studied by varying pH between 6 to 8. Immobilized cells achieved higher phenol removal than free cells at all pH range with the highest removal almost 100% at pH 7.5 as compared to 71.7% for free cells. This optimum pH was similar to that of *Acinetobacter radioresistens* as reported by Divari *et al.,* (2003). Results from control experiments suggest that volatilization and immobilizing agent were not the causes of these phenol removals.

This study clearly shows that alteration of pH can affect the amount of phenol utilized. As the environment becomes increasingly basic (from pH 6-7.5) with the addition of OH^- group, there could be a pH gradient that drives phenolic compounds to leave the environment and diffuse into the gel matrices. Alternatively, more positive ions or H^+ diffuse out or have the tendency to neutralize the negative charges on the matrices, which subsequently attract the phenols onto the gel surface and into the cellular

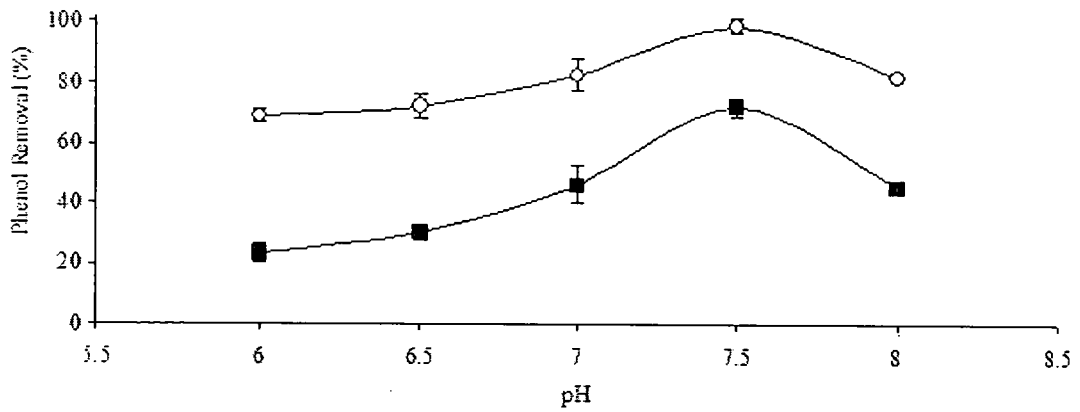
environment. As the pH moves to pH 8, the negatively charged environment could possibly repel the negatively charged gel matrices.

pH affects the activity of enzymes and therefore the microbial growth rate. Variations in pH of the medium may change the ionic form of the active site and change the activity of the enzyme and the reaction rate (Shuler and Kargi, 2002). This provides explanation as to why an enzyme activity is only active over a certain pH range. pH also affects the microorganism's cellular functions, cell membrane and protein transport (Weidemeier *et al.,* 1994). In addition, the solubility of a compound at different pH values will also determine the rate of substrate degradation (Singleton, 1994). The effects of temperature on phenol removal for free and immobilized cells were studied at optimal pH 7.5 (Figure 4.6b). The profile shows that there is a minimum temperature where activity does not occur; an optimum temperature at which activity is the best; and a maximum temperature at which activity ceases. This corresponds well with optimum temperature for cell growth. As the temperature of a reaction increases passing the maximum temperature for growth, lethal effects become apparent. The optimal temperature was determined at 30°C for free and immobilized cells, with phenol removal of 69.9% and 99.8%, respectively. As with pH effect, immobilized cells again showed better performance than free cells. The immobilized cells in principle could tolerate more drastic changes in pH and temperature than do the free cells (Dursun and Tepe, 2005). This tolerance enhancement may be due to the formation of cell colonies in the immobilization matrices as the close cell-to-cell contact affects the dynamics of the cell

membrane and increases the tolerance limit (Chung *et al.*, 2003; Zhu *et al.*, 2000; Keweloh and Heipieper, 1989).

The optimum physiological temperature of 30°C for phenol degradation has been quoted for a number of mesophilic bacteria, fungi and yeast. Some examples of bacteria are *Bacillus* sp. (Buswell, 1975), *Pseudomonas* sp. (Reardon *et al.*, 2000; Zilli *et al.*, 1996; Allsop *et al.*, 1993); fungi such as *Graphium* and *Fusarium* sp. (Santos and Linardi, 2004); and yeast such as *Candida tropicalis* (Yan *et al.*, 2005; Shimizu *et al.*, 1973) and *Trichosporon cutaneum* (Yang and Humphrey, 1975). Our study has confirmed that mesophilic temperature between 30-37°C was suitable for phenol degradation. Temperature affects the rate of enzyme reaction. The movement of molecules is slower at lower temperature, as there is not enough energy to spark a chemical reaction. Thus, in this study, temperature below 30°C may not be sufficient to drive the reaction forward. However, as temperature hits above 40°C, the enzymatic reaction starts to decline. The decline of microbial activity beyond the optimum temperature could be due to enzyme denaturation (Shuler and Kargi, 2002; Suthersen, 1999; Rochkind *et al.*, 1986). The decrease in activity could be a result of protein or catalytic site denaturation (Skopes, 1994), which in turn result in the loss of the enzyme three-dimensional structure. The unfolding of enzyme due to denaturation may have broken bonds in the enzyme structure crucial for maintaining its shape and activity. Continued exposure to high temperature may subsequently denature membrane lipids, resulting in cell death (Gaudy and Gaudy, 1988).

a



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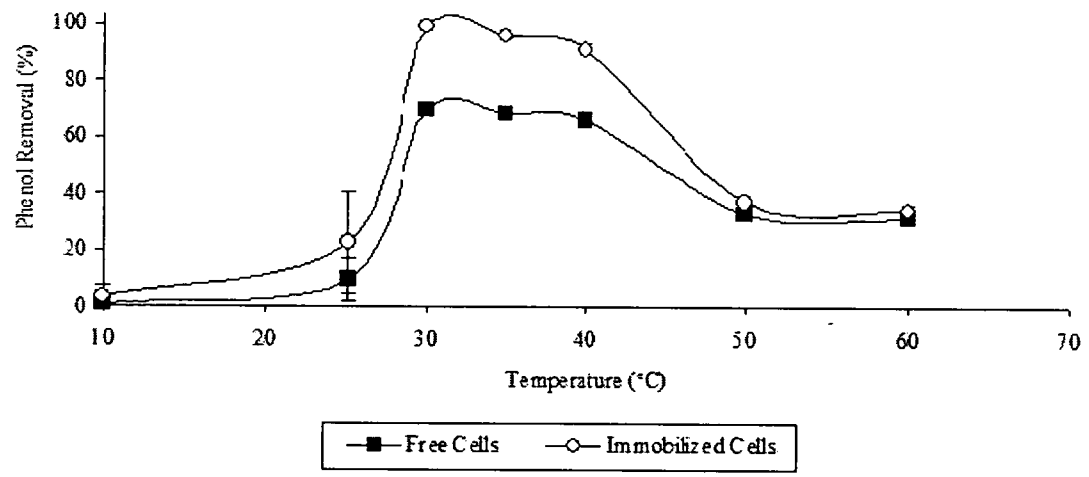


Figure 4.6: The effect of (a) pH at 30°C; and (b) temperature at pH 7.5; on phenol removal.

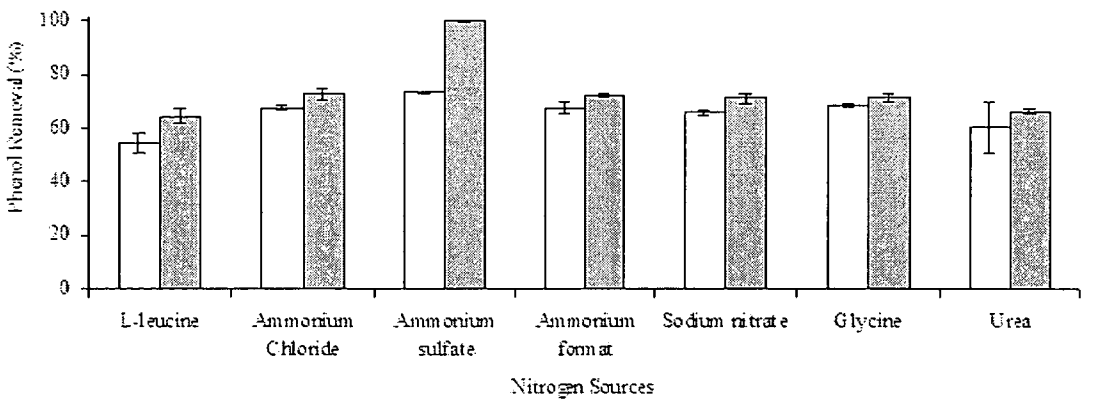
4.3.4.2 Effect of Nitrogen

Nitrogen is an important nutrient component for microorganism as it is the building block of DNA, RNA and protein (Wu and Li, 2002). In many natural environments, the amounts of usable nitrogen sources are insufficient to support optimal growth of bacteria. Different types of nitrogen sources were tested in this study at 1g/l concentration- L-leucine ($C_6H_{13}NO_2$), ammonium chloride (NH_4Cl), ammonium sulphate ($(NH_4)_2SO_4$), ammonium format ($HCOONH_4$), sodium nitrate ($NaNO_3$), glycine (NH_2CH_2COOH) and urea (NH_2CONH_2). As shown in Figure 4.7a, most nitrogen sources showed phenol removal in the range of 50-70%, and no significant difference was observed between free and immobilized cells. However, immobilized cells on ammonium sulphate showed almost 100% removals. In terms of nitrogen contents as shown in Table 4.1, apart from ammonium sulphate, it appears that level of nitrogen is not an important factor to affect phenol removal as all sources achieve almost comparable performance despite varying nitrogen level. The effect of ammonium sulphate concentration was further studied.

It is interesting to note that in the absence of ammonium sulphate; almost 20% and 40% of phenol removal was achieved with free and immobilized cells respectively (Figure 4.7b) as compared to 80-100% removal at 1 g/L ammonium sulphate. As nitrogen content was increased to 2.5 g/L, there is a possibility of nitrogen toxicity, as phenol removal was progressively reduced to 60%. This study confirms the need for nitrogen source to be available for effective phenol removal and 1 g/L concentration of

ammonium sulphate is optimum. However, in the absence of nitrogen sources, there is a possibility that the bacteria fix the atmospheric nitrogen as a result of respiratory nitrate reaction. Species known to be capable of reactions include *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, *Agrobacterium*, *Rhodopseudomonas* and *Pseudomonas* species (van Schie and Young, 2000). Respiratory denitrification provides ATP that can drive the energy-intensive process of nitrogen fixation (Tiedje, 1988).

a



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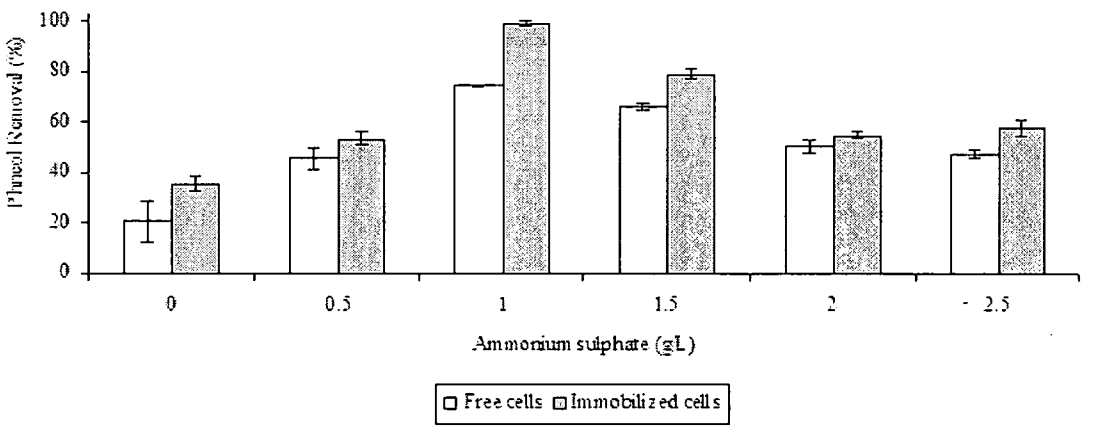


Figure 4.7: The effects of a) nitrogen sources and b) ammonium sulphate by free and immobilized cells.

Table 4.1: At 1g/L, nitrogen content in mM:

Nitrogen sources	Nitrogen content (mM)
L-leucine ($C_6H_{13}NO_2$)	106
Ammonium chloride (NH_4Cl)	262
Ammonium sulphate ($(NH_4)_2SO_4$)	212
Ammonium format ($HCOONH_4$)	222
Sodium nitrate ($NaNO_3$)	329
Glycine (NH_2CH_2COOH)	186
Urea (NH_2CONH_2)	466

4.3.4.3 Effect of Initial Phenol Concentrations

Substrate concentration is another important factor to determine biodegradation efficiency, as a concentration below threshold level is too low to support growth and maintenance and will not be degraded (Boethling and Alexander, 1979). On the other hand, too high a concentration may be toxic and reduce the biodegradation rates (Cornelissen and Sijm, 1993; Alexander, 1985). The effect of initial phenol concentrations from 100 mg/l to 2000 mg/l by free and immobilized cells, on the degree of biodegradation were studied as shown in Figure 4.8a-e. At 100 mg/l phenol, (Figure 4.8a) phenol removal achieved with the free cells at 6.25 mg l^{-1} h^{-1} was faster than immobilized cells at 6.70 mg l^{-1} h^{-1}. In fact, immobilized cells even experienced the phenol degradation lag period of 12 hours. Free cells could tolerate phenol at 100 mg/l phenol whilst internal mass transfer of phenol or oxygen could be the limiting factor in immobilized cells (Dursun and Tepe, 2005).

The shift in performance could be seen, as phenol concentration was increased to 200 mg/l and higher. At 200 mg/l phenol, whilst both systems experienced the same phenol degradation lag period of about 8 hours, the degradation rate of immobilized cells was almost 1.3-fold higher than free cells. The difference was even more glaring for 400 mg/l phenol and higher. Phenol degradation lag period was not observed with immobilized system at 400 mg/l phenol and higher. The degradation rate was negligible at 2000 mg/l phenol for free cells, whilst the degradation was completed within 12 days by immobilized cells. Freely suspended cells are not able to tolerate phenol toxicity at

such high levels as also observed by many researchers (Chung *et al.*, 2005; Erhan *et al.*, 2002; Hao *et al.*, 2002; Bandyopadhyay *et al.*, 1998; Ruiz-Ordaz *et al.*, 1998; Wang *et al.*, 1996).

Before a microbial cell can commence active metabolism of a substrate, they have to adjust to their surrounding environment (Bailey and Ollis, 1986). Phenol degradation lag period was commonly observed in the course of phenol degradation. This has also been reported in other studies irrespective of microbial strain and conditions (free, immobilized or engineered) and is likely a function of other variables such as pH, temperature and phenol concentration as a substrate, inoculum size, electron acceptors, adaptation of bioparticles, and physiological state of cells (Prieto *et al.*, 2002; Baek *et al.*, 2001; González *et al.*, 2001a; Tarighian *et al.*, 2001; Tibbles and Baecker, 1989a). As shown in Table 4.2 for free cells, phenol degradation rate increased initially with phenol concentration but eventually decreased after phenol concentration reached 400 mg/L. This could suggest that phenol inhibits *Acinetobacter baumannii* Serdang 1 at concentration level higher than 400 mg/L. Free cells of *Acinetobacter baumannii* Serdang 1 had degradation efficiency of only between 1.4 - 42% when initial phenol concentration was at 1500-2000 mg/L.

Besides being a substrate, phenol could act as an inhibitor termed "self-inhibition" in which high concentration of a substrate inhibits its own degradation (Sàez and Rittmann, 1991; Godrej and Sherrard, 1988; Mörtberg and Neujahr, 1987; Neujahr and Kjellén, 1978). Self-inhibitory substrates not only inhibit their own

enzyme-catalyzed transformation but also probably hinder energy and electron flows at several locations. Substrate inhibition is also a characteristic of phenol metabolism being a toxic substrate for different microorganisms at different concentration levels (Santos and Linardi, 2004). For instance, *Pseudomonas putida* cannot tolerate phenol toxicity at high concentrations of between 800-1000 mg/l (Chung *et al.,* 2003). Level above 1500 mg/l has resulted in lower efficiency of *Candida tropicalis* free cells to degrade phenol (Chen *et al,* 2002). Increasing phenol concentrations could decrease the overall phenol biodegradation of bacteria (Dean-Ross, 1989). However, immobilization has clearly protected the cells from toxic effect. We have successfully developed an immobilization system in this study that could biodegrade phenol at as high as 2000 mg/l initial concentration. This could pave the way for continuous use of immobilized cells for *in situ* or *ex situ* remediation.

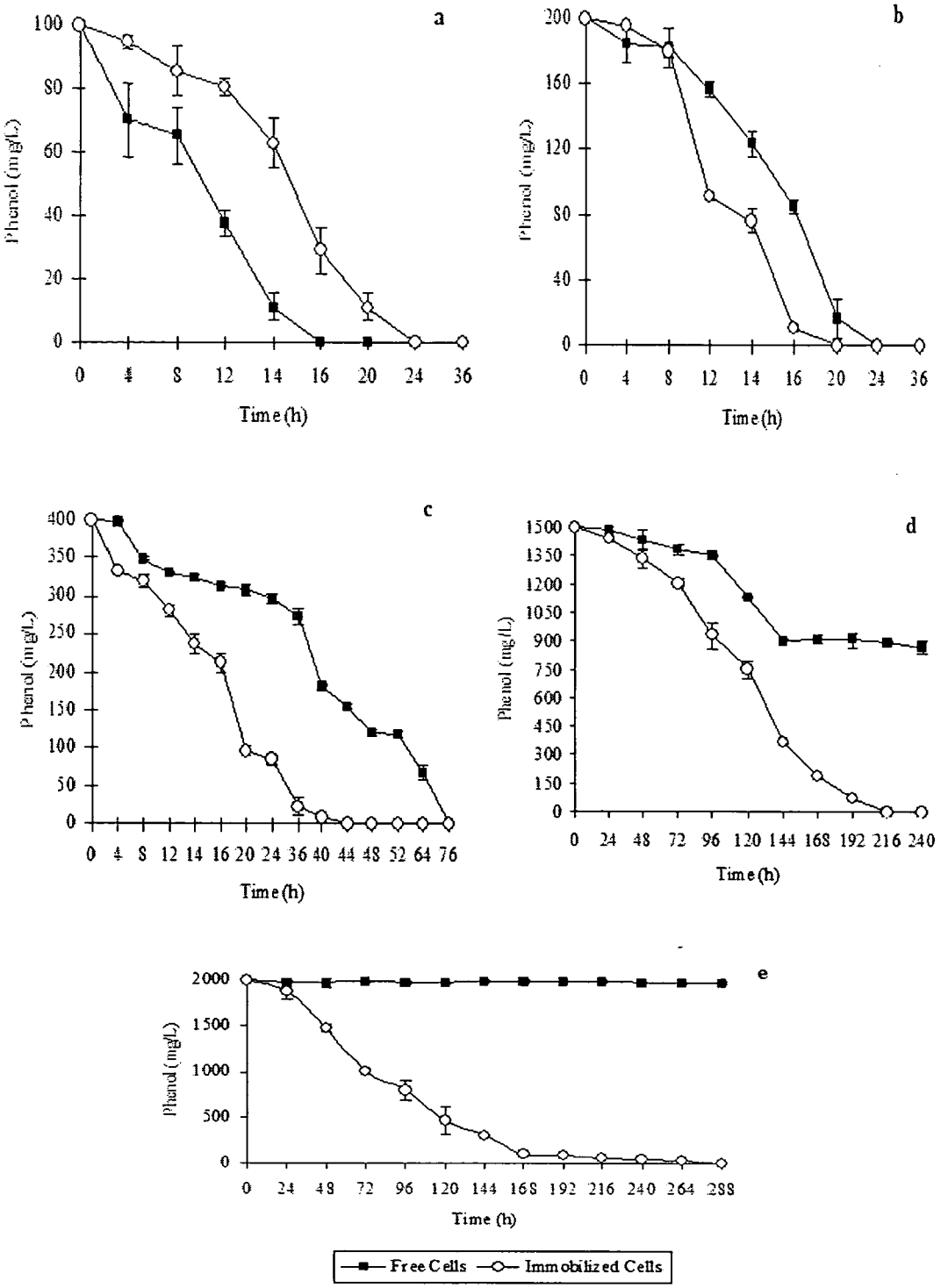


Figure 4.8: The effects of phenol concentration (mg/L): a) 100, b) 200, c) 400, d) 1500 and e) 2000 on phenol removal by free and immobilized cells.

Table 4.2: The effect of initial phenol concentration on phenol degradation by *Acinetobacter baumannii* Serdang 1 at 30°C, pH 7.5 in shake flask.

Kinetic parameters/Performance	Initial phenol concentration (mg/l)									
	100		200		400		1500		2000	
	I	F	I	F	I	F	I	F	I	F
Rate of phenol degradation (mg l^{-1} h^{-1})	6.70	6.25	15.0	11.4	9.09	7.00	6.25	4.37	6.94	0.09
Rate of phenol degradation of beads (mg l^{-1} h^{-1} ml^{-1})	1.34	-	3	-	1.82	-	1.25	-	1.39	-
Incubation time (h)	24	16	20	24	44	64	240	240	288	288
Biodegradation time (h)	12	16	12	16	44	44	240	144	288	288
Phenol degradation lag period (h)	12	0	8	8	0	20	0	96	0	0
Residual phenol (mg l^{-1})	0	0	0	0	0	0	0	870.5	0	1971.9
Phenol removal (%)	100	100	100	100	100	100	100	42	100	1.4

* I : Immobilized cells
  F: Free cells

4.3.5 Reusability of Immobilized *Acinetobacter baumannii* Serdang 1 Cells for Phenol Biodegradation

Degradation experiments were carried out to test the reusability of immobilized cells. As shown in Figure 4.9, at 500 mg/l initial phenol concentration, immobilized cells of *Acinetobacter baumannii* Serdang 1 could be reused up to 5 cycles with 5 days duration per cycle, achieving phenol-degrading activity of 97-100%. The 6^{th} cycle showed the degrading activity was reduced to only 80%. Our study has successfully shown the positive aspects of immobilized cells for continued use in phenol removal. This observation may have a positive impact on the economics should the strategy be applied at large scale or at contaminated site. This may involve further improvement on the delivery at the contaminated site which would include issues such as ease of application, robustness and speed of remediation.

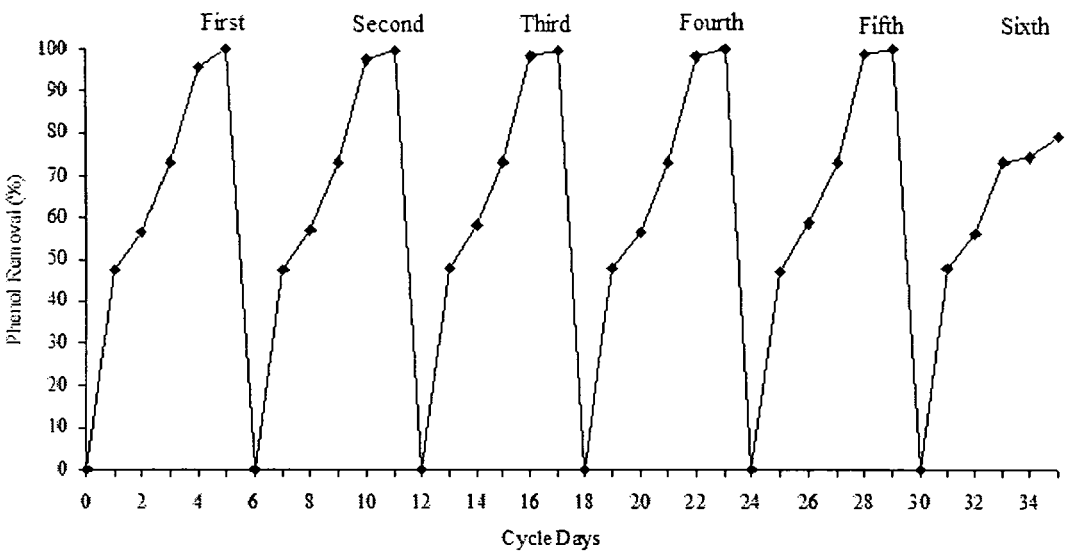


Figure 4.9: Repeated usage of immobilized *Acinetobacter baumannii* Serdang 1.

4.3.5.1 Effect of Succinic Acid

Several investigators have suggested that *Acinetobacter* sp. degrade phenol by the *ortho* cleavage pathway (Pessione *et al.*, 1999; Paller *et al.*, 1995; Doten *et al.*, 1987). The following experiments were initiated to investigate whether succinic acid could be use as a precursor to enhance the rate of phenol removal and thus reduce especially the removal time when use repeatedly in cycles. Succinic acid was selected as one of the product from phenol metabolism before the pathway enters the TCA cycle. As shown in Figure 4.10, at the concentration range tested (10-1000 mg/l), phenol removal efficiency was greatly reduced. At these concentrations, there could be a competition for substrate, as both succinic acid and phenol can be metabolized by the cells (Table 3.4). This may suggest the preference for succinic acid instead of phenol. As the main objective is to remove phenol, future studies should consider lowering the concentration to below 10 mg/l, just enough to trigger metabolic reaction and hopefully assist in speeding up the metabolic rate of phenol.

In nature microorganisms grow mostly in a medium supplemented with additional substrates (Harder and Dijkhuizen, 1982). Hence, growth could be manipulated by addition of two or more nutrients simultaneously (Egli, 1991; Rutgers *et al.*, 1990). In general, microbial degradation of a compound in a mixture can be strongly influenced by other compounds present in the mixture (Egli, 1995). If a microbial population is grown on mixed substrates present in the medium, the microbes consume only one, or both the substrates. Consequently, several utilization patterns can be observed. In a mixed

substrates, individual substrates can have a synergistic, antagonistic, or no effect on one another, resulting in a growth rate that is higher, lower, or the same than if the substrates were present individually (Egli, 1995; Saéz and Rittmann, 1993; Meyer *et al.*, 1984).

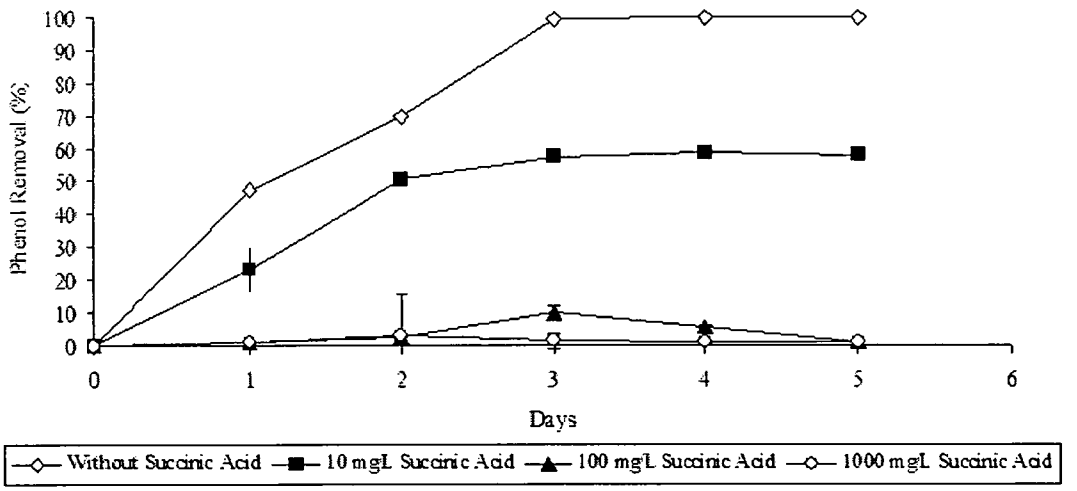


Figure 4.10: The effects of succinic acid.

4.4 Conclusions

The optimal conditions for immobilization of cells were found to be: 3% (w/v) of both sodium alginate and calcium chloride solution; 2 mm bead diameter and initial cell loading (ICL); 400 beads/flask. Phenol degradation rate were affected by both temperature and pH. The optimal condition for growth and phenol degradation was at 30°C and pH 7.5, respectively for both free and immobilized cells. The best nitrogen source for both free and immobilized cells was ammonium sulphate at 1 g/L concentration. Repeated uses of immobilized cells suggest that they could be used as much as five cycles without loss of activity. The immobilized cell system we developed was able to degrade a higher throughput of effluent containing phenol more effectively than a free cell system. Initial phenol concentration as high as 2000 mg/L has been successfully removed in 12 days at 6.94 mg l^{-1} h^{-1} which was as effective as working on 100 mg/L phenol concentration.

CHAPTER V

CONTINUOUS PHENOL BIODEGRADATION BY IMMOBILIZED *Acinetobacter baumannii* SERDANG 1

5.1 Introduction

In most wastewater treatment plants, treatment is normally operated in a continuous mode. It is therefore of great importance to investigate phenol biodegradation under continuous operation. Continuous culture is a system in which a well mixed culture is continuously supplied with fresh nutrients and the volume of the culture is kept constant by continuous removal of the culture liquid at the same flow rate as the feeding rate of fresh nutrient. The main advantage of a continuous process for the degradation of toxic materials over a batch process is the ease of automation and control. This could translate into high increase in throughput, more consistent rate of treatment, and improvement in the economics of the process (Sheeja and Murugesan, 2002).

Considerable attention has been given towards using immobilized cells in continuous reactors for the degradation of stable organic compounds in the industrial effluents. The main advantage is they provide high cell density in the reactor, even beyond washout conditions, plus increased resistance to the detrimental effects of toxic shock loadings (Worden and Donaldson, 1987). Immobilized cells in various reactor configurations have been reported such as packed bed reactors (Ehlers and Rose, 2005;

Hsien and Lim, 2005; Illiuta and Larachi, 2005; Tziotzios *et al.,* 2005; Prieto *et al.,* 2002; Sheeja and Murugesan, 2002); and fluidized bed reactors (Vinod and Reddy, 2003; Ensuncho *et al.,* 2005; González *et al.,* 2001; Hecht *et al.,* 2000; Fan *et al.,* 1990; Livingstone and Chase, 1989; Worden and Donaldson, 1987).

The objectives of this study were therefore:

1. To develop a reactor module for continuous degradation of phenols in the form of packed-bed reactor using *Acinetobacter baumannii* Serdang 1-alginate beads.
2. To study the effects of the operating parameters such as flow rate, bed height and initial phenol concentrations on phenol degradation rate in an up-flow mode of operation.

5.2 Materials and Method

5.2.1 Culture Media

The culture media used was as described in Section 4.2.1.

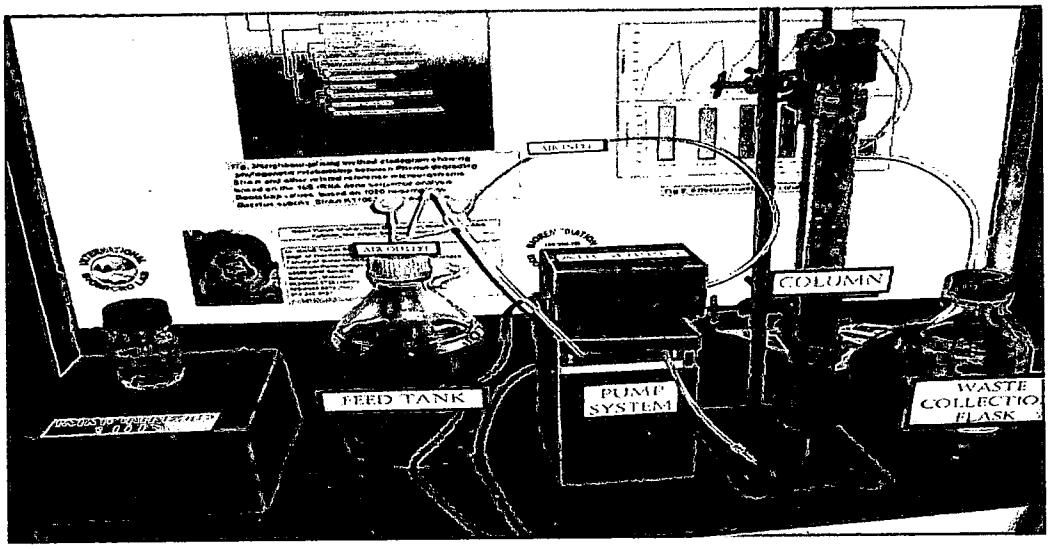
5.2.2 Cell Immobilization

The immobilized cells were prepared following the procedures outlined in Section 4.2.3.

5.2.3 Packed-Bed Bioreactor

The up-flow, continuous mode of phenol biodegradation was carried out in a packed-bed bioreactor. The set up was as shown in Figure 5.1. Packed-bed glass column (Pharmacia, USA) of 2.5 cm diameter and 40 cm height was used as a reactor column. The column was packed to a required bed height with *Acinetobacter baumannii* Serdang 1 immobilized in alginate beads (2 mm bead size). A known concentration of synthetic aqueous phenolic solution and all other nutrients in concentrations as indicated in Section 4.2.1, were pumped into the inlet at the bottom of the reactor, using a peristaltic pump (Perista Pump, Germany) at a desired flow rate. Influent feed consisting of phenol solution and growth medium (pH 7.5) was pre-aerated with air at 0.1ml/min. Sterile air filtered through a sterile 0.2 μm cellulose acetate filter (Sartorius, Germany), was supplied to provide the dissolved oxygen necessary for the bacteria. The reactor outlet

was connected at the top of the reactor. The experiments were carried out at room temperature and the pH was not controlled. The entire closed system consisting of the packed column, feed vessel, waste vessel, and connecting tubing and filter vents were sterilized by autoclaving. Samples were collected from the outlet for the determination of residual phenol at fixed intervals during start-up until steady state was attained. Steady state conditions were considered 'attained' when phenol concentration in the effluent remained constant for a period of 10 h. The experiments were repeated for different conditions by changing the liquid flow rates, bed heights and initial phenol concentrations.



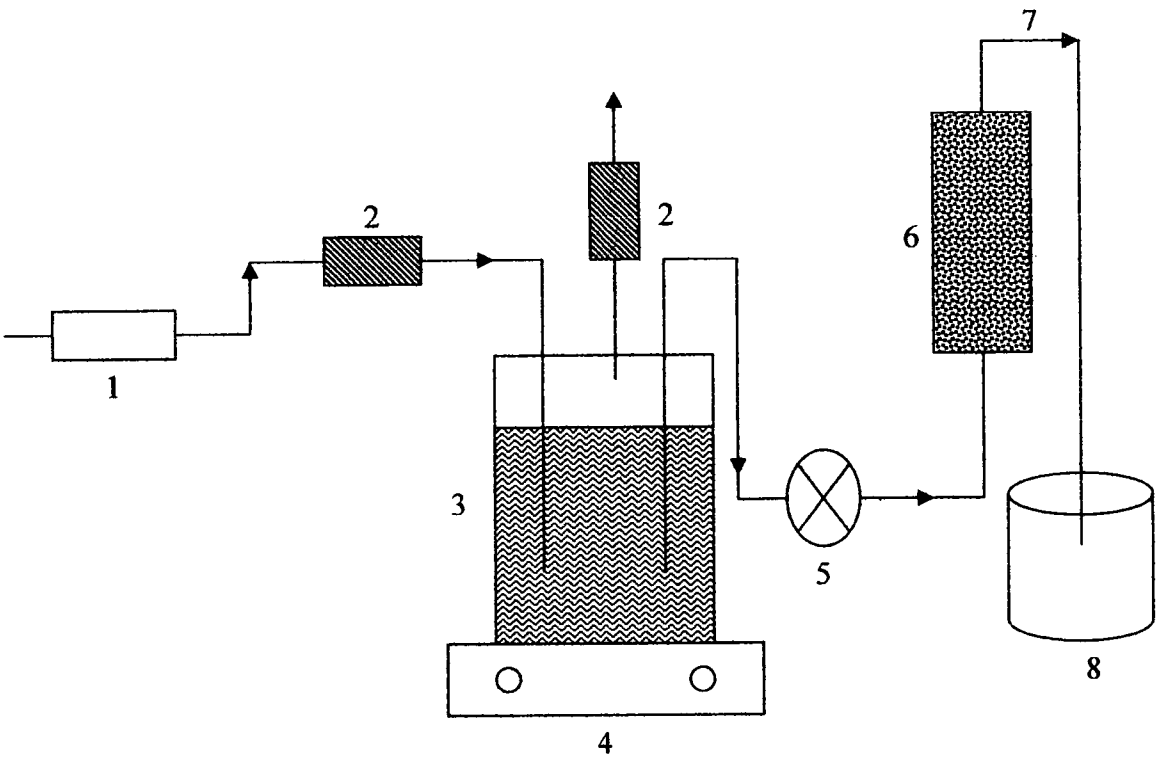


Figure 5.1: Continuous system: (1) air pump; (2) air filters; (3) feed reservoir; (4) magnetic stirrer; (5) peristaltic pump; (6) reactor; (7) sampling/effluent; (8) waste collection flask.

5.2.3.1 Effect of Flow Rates

The effects of feed flow rates at 1.5, 2, 2.5, 3 and 4 ml/min were tested on phenol biodegradation rate. Samples were taken out at fixed time intervals and analyzed for phenol concentration.

5.2.3.2 Effect of Height to Diameter Ratio (Bed Height/ Column Diameter)

The effects of height to diameter ratio of the packing 15.2 (= 38cm/2.5cm), 14 (= 35cm/2.5cm), 12 (= 30cm/2.5cm), 11.2 (= 28cm/2.5cm) and 10 (25cm/2.5cm) were tested. Samples were taken out at fixed time intervals and analyzed for phenol concentration.

5.2.3.3 Effect of Initial Phenol Concentrations

The effects of initial phenol concentrations at 200, 400, 500 and 1500 mg/L were tested. Samples were taken out at fixed time intervals and analyzed for phenol concentration.

5.2.4 Phenol Concentration Determination

Phenol was determined by the spectrophotometric method using 4-aminoantipyrine as the coloring agent. The procedures were as presented in Section 4.2.5.2.

5.3 Results and Discussions

5.3.1 Effects of Flow Rate

The effect of flow rate during start up of the bioreactor on the effluent phenol concentration is shown in Figure 5.2. This was achieved by retaining the height to diameter ratio at 11.2 (=28/2.5 cm), at 200 mg/L initial phenol concentration. The results in Figure 5.2a indicate that the rate of phenol removal was not significantly different as the flow rate was increased. Phenol removal of 71% could be achieved at low flow rate of 1.5 ml/min, while about 65% removal was achieved at high flow rate of 4 ml/min. While the difference was not significant, the time taken to reach steady state however increased with increase in flow rate (Figure 5.2c). At 1.5 ml/min flow rate, the phenol concentration in the reactor decreased significantly within the first 8 hours, whilst at 4 ml/min, it took 20 hours to achieve almost the same level of removal. It is possible that with up-flow mode of influent, low flow rate allows longer residence time for the immobilized cells to make contact with the substrate. Hence, the shorter time to achieve steady state. During steady state, the cell population is at a dynamic equilibrium within the calcium alginate bead. The cells utilize phenol at the level at which the cell populations could grow and be maintained. With increase in flow rate, the time of contact between the cells and substrate (residence time in the reactor) is reduced. This could have resulted in reduced cell growth, a decrease in percentage of phenol removal and longer time taken to achieve a steady state (Vidya Shetty *et al.,* 2007).

The effects of flow rate are important for two reasons. First, as the flow rate is increased, the dissolved oxygen carried through per pass from the reservoir to the reactor increases, and this may increase the reaction rates. Secondly, the mass transfer resistance (solid/liquid) across the biofilm is reduced, and this may further increase the reaction rates (Lakhwala *et al.,* 1992). Our study, on the other hand, show that phenol degradation rate at 1.5 ml/min flow rate is 0.177 mg L^{-1} h^{-1} mL^{-1} of beads (as shown in Table 5.1), which was almost three times the degradation rate achieved at 4 ml/min flow rate. Theoretically, at low flow rate, diffusion of oxygen is not such a problem because cell numbers are lower (Lakhwala *et al.,* 1992; Gosmann and Rehm, 1986). However, as the flow rate increases, as does the available phenol, the cell numbers should increase. With increasing cell numbers in the bead, it is possible that oxygen is consumed faster than it can diffuse into the beads, and so cells have to compete for oxygen (Lakhwala *et al.,* 1992; Gosmann and Rehm, 1986). As a result, the rate of removal of phenol in our study may be slowed down with high flow rate. When oxygen can no longer be transferred across the matrix, degradation ceased. Whilst 1.5 ml/min flow rate may be the lowest flow rate, it appears to be high enough to eliminate the external mass transfer resistance. Plus, the cells may already reach the state of substrate saturation, and with a fixed number of beads, increase in flow rate does not improve the rate of substrate removal. This saturation has been suggested, can be relieved when the total population size is increased such as by using more beads (Mordocco *et al.,* 1999).

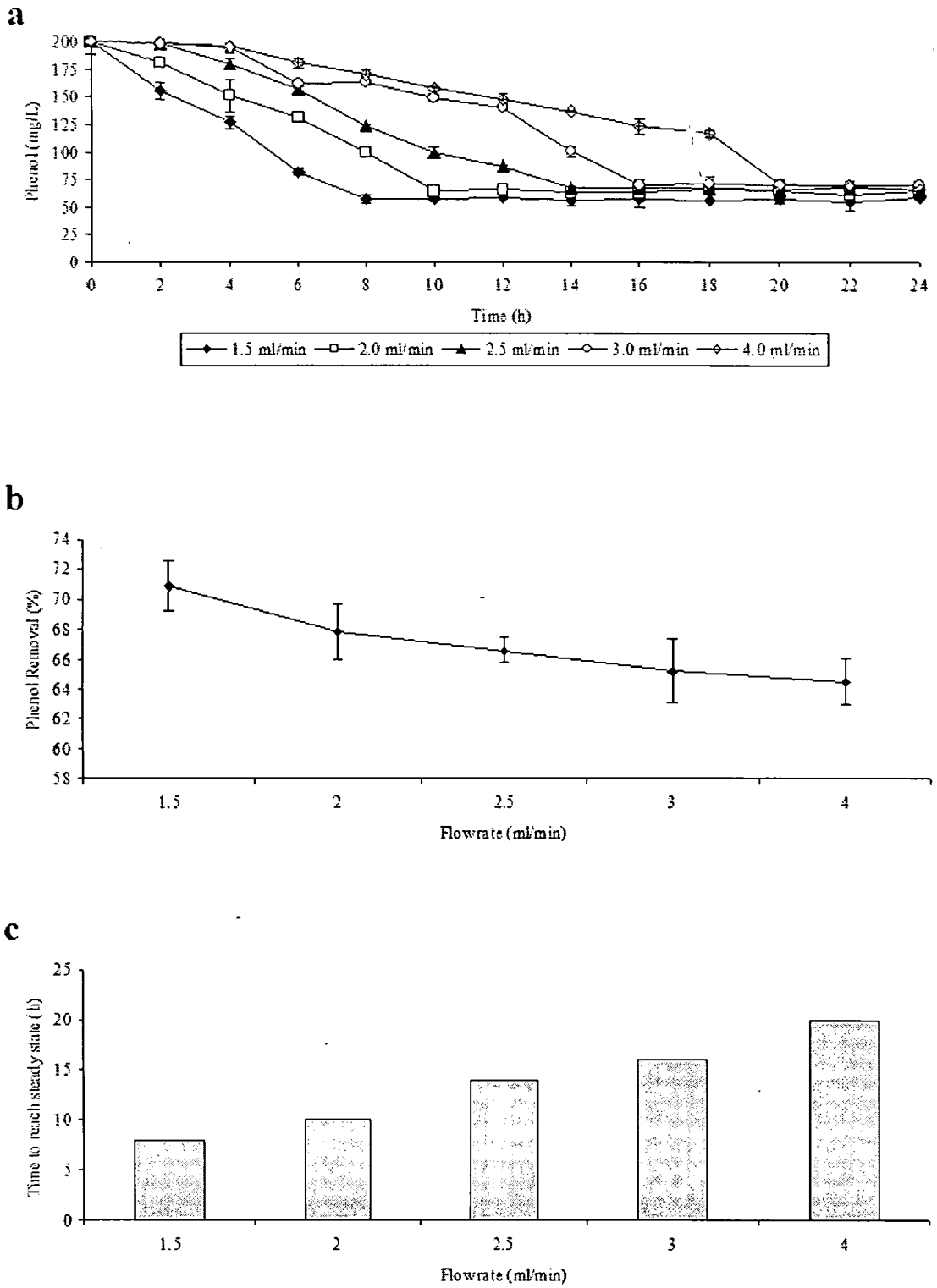


Figure 5.2: The effects of flow rate on (a) effluent phenol concentration, (b) percentage removal of phenol at steady state, (c) time taken to reach steady state.

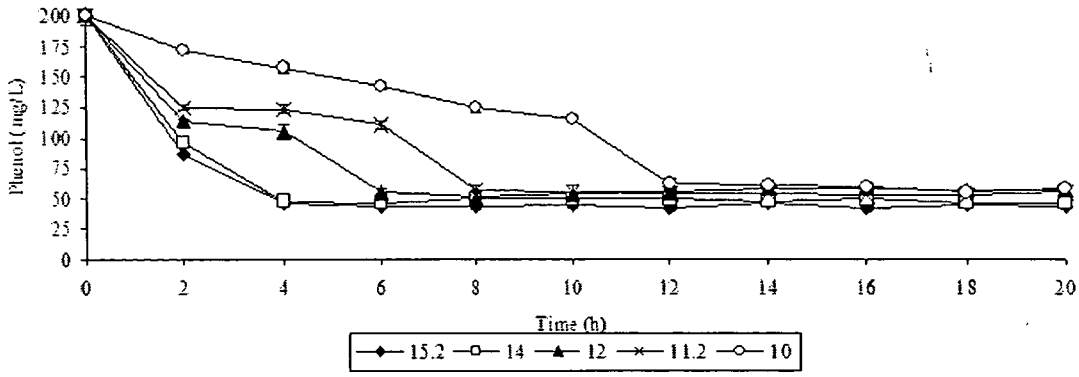
Table 5.1: The effect of flow rate.

Kinetic parameters/Performance	Flow rate (ml/min)				
	1.5	2.0	2.5	3.0	4.0
Rate of phenol degradation (mg l^{-1} h^{-1})	17.7	13.6	9.51	8.15	6.46
Rate of phenol degradation of beads (mg l^{-1} h^{-1} ml^{-1})	0.177	0.136	0.095	0.082	0.065
Time to reach steady state (h)	8	10	14	16	20
Residual phenol at steady state (mg l^{-1})	~58	~64	~66	~69	~70
Phenol removal (%)	70.8	67.8	66.6	65.2	64.5

5.3.2 Effects of Bed Height to Diameter Ratio

The effects of bed heights to diameter ratio (H/D_c) on phenol degradation, in an immobilized continuous culture system are shown in Figure 5.3. This was achieved by retaining the flow rate at 1.5 ml/min and at 200 mg/l initial phenol concentration. The results in Figure 5.3a and 5.3b suggest that as the H/D_c was increased, the rate of phenol degradation rate and the percentage of phenol removal was increased. The highest H/D_c ratio of 15.2 showed the highest phenol removal at 76.7 %. In addition, the time taken to reach steady state was three times shorter than that achieved with H/D_c ratio of 10; and the rate of phenol degradation at 0.284 mg L^{-1} h^{-1} mL^{-1} of beads was 2-fold that the latter (Table 5.2). With an increase in H/D_c ratio (at constant D_c= 2.5 cm), there are greater number of beads and cells available to degrade phenol (Mordocco *et al.*, 1999). Hence, the better performance observed with H/D_c of 15.2.

a



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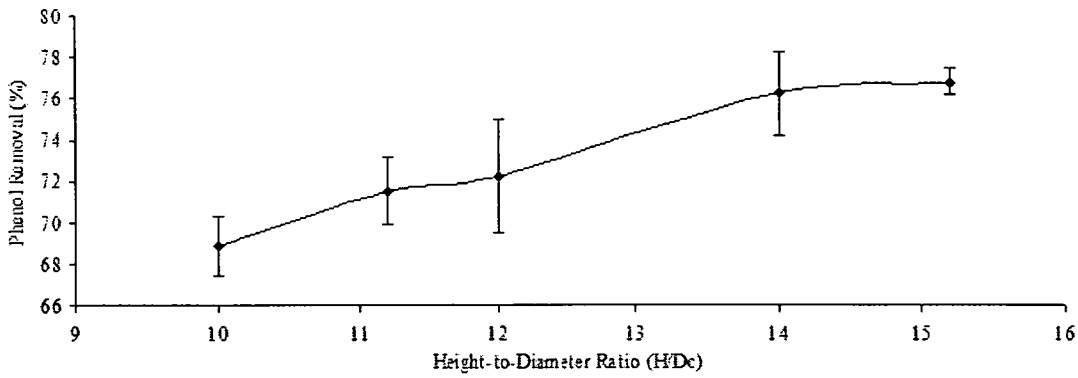

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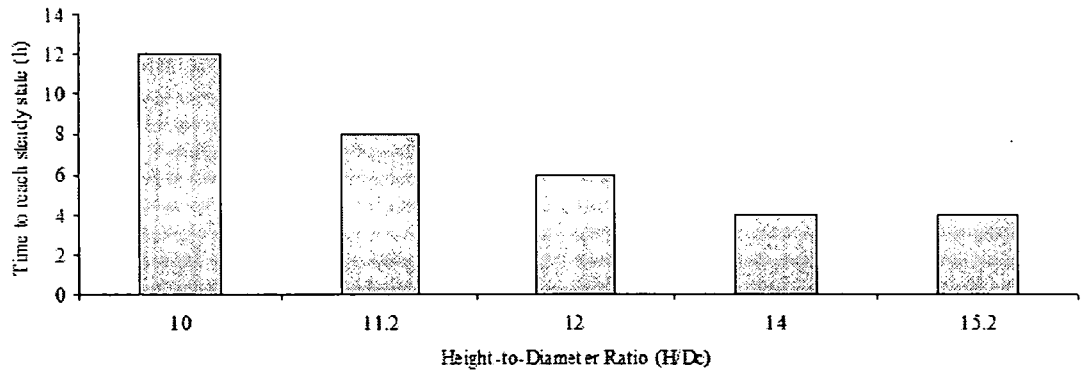


Figure 5.3: The effects of height-to-diameter ratio (H/D_c) (a) on phenol degradation, (b) steady state percentage removal of phenol, (c) time taken to reach steady state.

Table 5.2: The effect of height-to-diameter ratio (H/D_c)

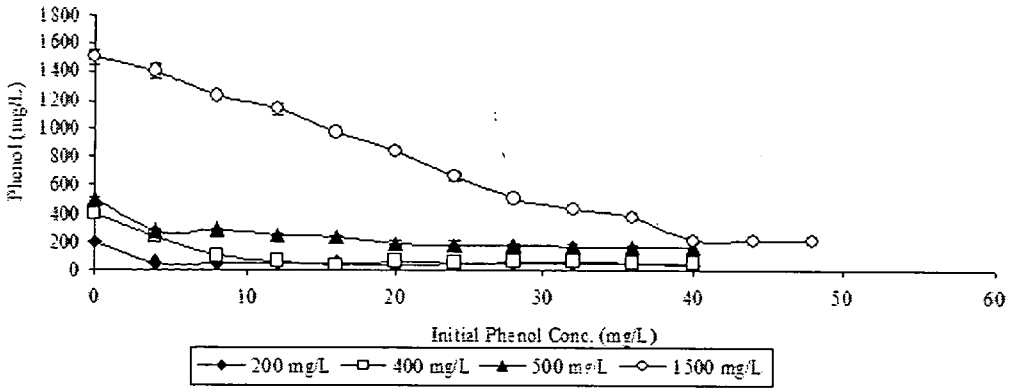
Kinetic parameters/Performance	Height of Packing (H/D_c)				
	10	11.2	12	14	15.2
Rate of phenol degradation (mg l^{-1} h^{-1})	11.5	17.9	24.1	38.1	38.4
Rate of phenol degradation of beads (mg l^{-1} h^{-1} ml^{-1})	0.128	0.179	0.230	0.305	0.284
Time to reach steady state (h)	12	8	6	4	4
Residual phenol at steady state (mg l^{-1})	~62	~52	~56	~48	~46
Phenol removal (%)	68.9	71.5	72.2	76.2	76.7

5.3.3 Effects of Initial Phenol Concentrations

Figure 5.4 shows that the percentage of phenol removal was not significantly different in the range of 68-76% as the influent phenol concentration was increased from 200 to 1500 mg/L, at the same flow rate of 1.5 ml/min and the same H/D_c ratio of 15.2. Although the time taken to reach steady state for 1500 mg/L at 40 hours was eight times longer than at 200 mg/L concentration, the degradation rate of phenol was almost equivalent (Table 5.3). The interesting observation is that the bulk concentrations prevailing in the reactor at steady state in all were lower than the inhibitory concentrations. The effect of inhibition was observed at phenol concentration above 400 mg/L (Table 4.2), because the degradation rate for both immobilized and free cells were reduced at concentration higher than 400 mg/L.

The percentage of removal also depends on the ratio between the phenol degradation rate and the volumetric phenol-loading rate (Vidya Shetty *et al.,* 2007). As observed in Figure 5.2, 5.3 and 5.4, although the phenol degradation rate may differ significantly, the percentage of phenol removal remained in the range of 65-76% (Table 5.1, 5.2 and 5.3). This shows that the major parameter to assess efficiency of phenol removal in a continuous system is the time taken to achieve steady state. The most important factors in this study are the influent flow rate and height-to-diameter ratio, when the D_c remains constant.

a



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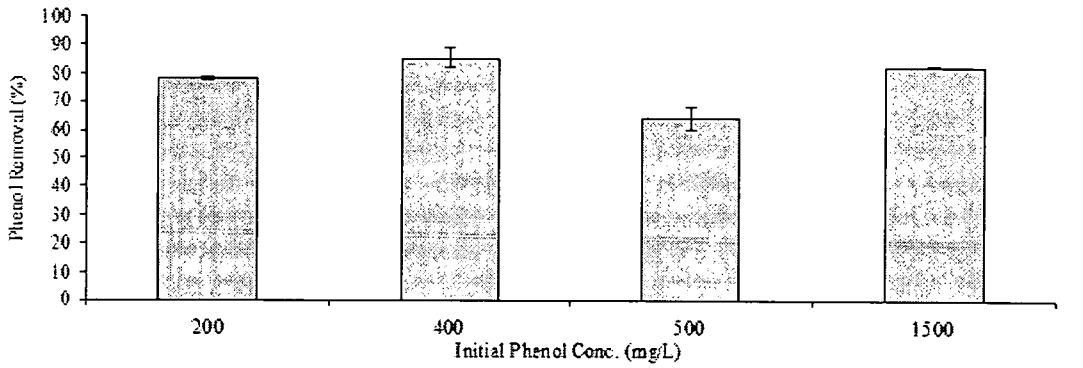

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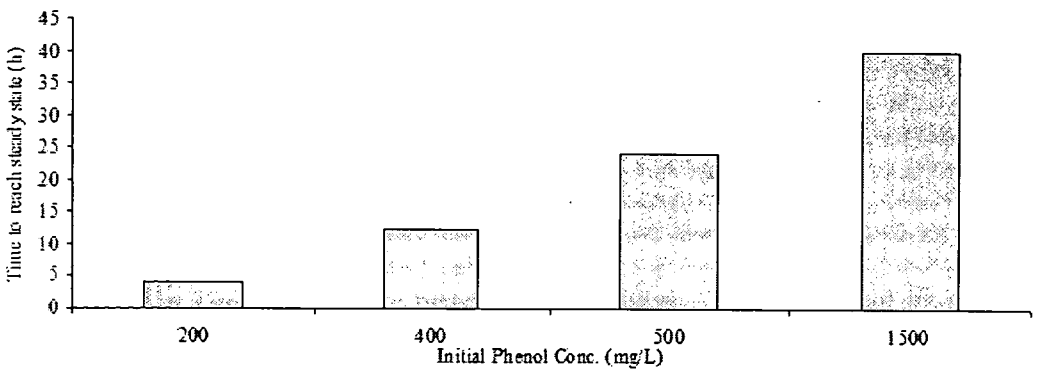

Figure 5.4: The effects of initial phenol concentration (a) on phenol degradation rate, (b) steady state percentage removal of phenol, (c) time taken to reach steady state.

Table 5.3: The effect of initial phenol concentration.

Kinetic parameters/Performance	Initial Phenol Conc. (mg/L)			
	200	400	500	1500
Rate of phenol degradation (mg l^{-1} h^{-1})	38.9	28.5	13.4	32.4
Rate of phenol degradation of beads (mg l^{-1} h^{-1} ml^{-1})	0.289	0.211	0.1	0.240
Time to reach steady state (h)	4	12	24	40
Residual phenol at steady state (mg l^{-1})	~44	~58	~178	~204
Phenol removal (%)	77.9	85.4	64.3	82.5

5.4 Conclusion

With the packed-bed bioreactor consisting of immobilized *Acinetobacter baumannii* Serdang 1 cells, about 76.7% degradation of 200 mg/L phenol was achieved at 1.5ml/min flow rate and bed height of 15.2. Although the phenol degradation rate may differ significantly, the percentage of phenol removal remained in the range of 65-76%. The major parameter to assess efficiency of phenol removal in a continuous system in this study is the time taken to achieve steady state. The most important factors affecting the performance were the influent flow rate and height-to-diameter ratio, when the D_c remained constant.

CONCLUSIONS AND FUTURE RESEARCH

A new indigenous phenol-degrading bacteria strain from Malaysian soil had been successfully isolated, characterized and identified. Based on a BLASTN search of GenBank, the complete sequences of isolate *Serdang 1* shared 98% similarity with *Acinetobacter baumannii* which is confirmed by Biolog ^{TM} Identification System, sequence alignment and phylogenetic tree analysis, the isolate *Serdang 1* has been redesignated *Acinetobacter baumannii* Serdang1 and the complete sequence submitted to GeneBank database under the accession number EF525671.

The optimal conditions for immobilization of cells were found to be: 3% (w/v) of both sodium alginate and calcium chloride solution; 2 mm bead diameter and initial cell loading (ICL); 400 beads/flask. Phenol degradation rate were affected by both temperature and pH. The optimal conditions for growth and phenol degradation were 30°C and pH 7.5, for both free and immobilized cells. The best nitrogen source for both free and immobilized cells was ammonium sulphate at 1 g/L concentration. Repeated use of immobilized cells suggests that they could be used as much as five cycles without loss of activity. The immobilized cell system we developed was able to degrade a higher throughput of effluent containing phenol more effectively than a free cell system. Initial phenol concentration as high as 2000 mg/L has been successfully removed in 12 days at 6.04 $mgl^{-1}h^{-1}$ which was as effective as working on 100 mg/L phenol concentration.

With the packed-bed bioreactor consisting of immobilized *Acinetobacter baumanii* Serdang 1 cells, the percentage of phenol removal in continuous mode was in the range of 65-76% at 200 mg/L initial phenol. Low flow rate of influent at 1.5ml/min flow rate and bed height of 15.2 reached the steady state faster than higher flow rate though the percentage of phenol removal was not significantly different. Based on 200 mg/L phenol influent, the continuous system was better than the batch system in terms higher biodegradation rate of 38.9 mg L^{-1} h^{-1} almost 3-fold higher than batch system. Although the phenol degradation rate may differ significantly, the major parameter to assess efficiency of phenol removal in a continuous system in this study is the time taken to achieve steady state. The most important factors affecting the performance were the influent flow rate and height-to-diameter ratio, when the D_c remained constant.

Based on the present experimental results, it can be concluded that *Acinetobacter baumannii* Serdang 1 has high potential to be applied for the bioremediation of phenol-contaminated sites. The data thus generated may be used for designing a treatment pilot plant for phenolic effluents wherein continuous removal or collection can be achieved on larger scale. Isolation, identification and characterization of *Acinetobacter baumannii* Serdang 1 genes and enzymes such as phenol hydroxylase and catechol 1, 2 dioxygenase and muconate cycloisomerase responsible for the degradation of phenol, must be made. Further studies on biodegradative capability of *Acinetobacter baumannii* Serdang 1 towards other recalcitrant aromatic hydrocarbons, and developing a cell-based biosensor using *Acinetobacter baumannii* Serdang 1 that could detect phenol, are two important

areas that must be developed so that an integrated approach in bioremediation strategies will be a reality.

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Appendix A

X-gal Stock Solution

1. To prepare a 40mg/ml stock solution, dissolve 400 mg X-gal in 10 ml dimethyl-formamide.
2. Protect from light by storing in a brown bottle at -20°C.

Luria Agar (GIBCOBRL 500g)

1. For 1 liter, dissolove 37 grams of Luria agar powder in 950 ml distilled water.
2. Adjust the pH of the medium to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle at 121°C for 15 minutes. Allow the medium cool to ~55°C, add antibiotic and pour into plates.
4. Let harden, then invert and store at 4°C, in the dark.
5. To add X-gal and IPTG to the plate, warm the plate to 37°C. Pipette 40µl of the 40 mg/ml X-gal stock solution and 40µl of 100 mM IPTG onto the plate, spread evenly, and let dry 15 minutes. Protect plates from light.

Ampicillin stock (50µg/ml)

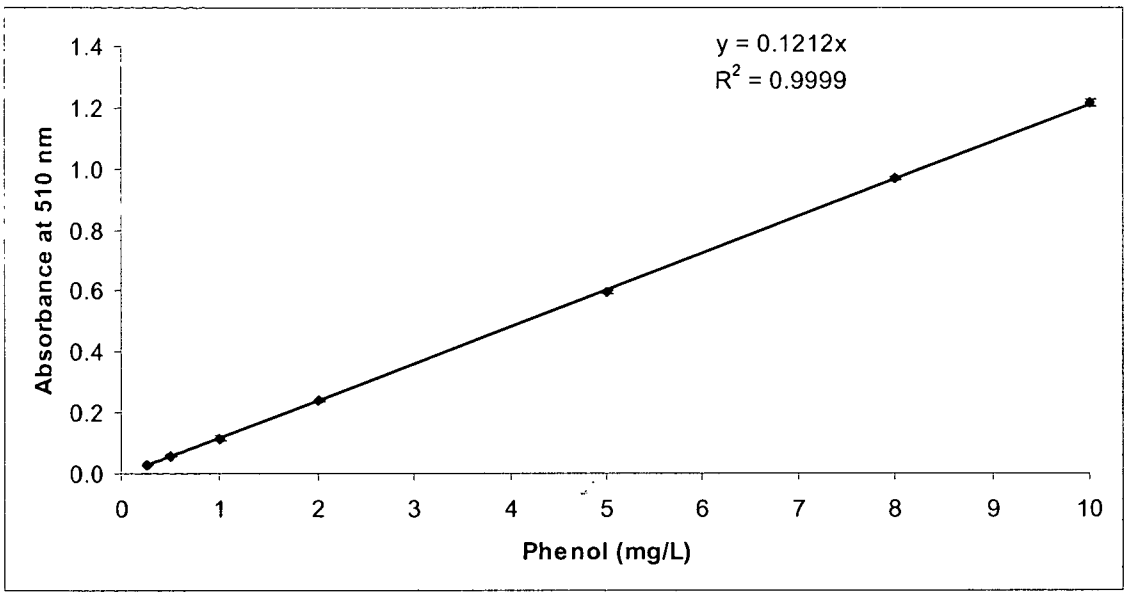
1. To prepare 50µg/ml stock, dissolve 0.5 g of ampicillin, sodium salt in 10 ml of deionized water.
2. Filter-sterilize and store at -20°C.

Ethidium Bromide (0.5µl/ml)

Ethidium bromide	0.2 ml
Distilled water	400 ml

Note: Stir on magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer to a dark bottle and store at room temperature.

Appendix B


y = 0.1212x
R2 = 0.9999
Absorbance at 510 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
7
8
9
10
Phenol (mg/L)

BIODATA OF THE AUTHOR

The author of this thesis is Zailatul Hani binti Mohamad Yadzir (IC No. 831022-07-5028) from Perak. She was born on the 22th of October 1983. Her family consists of five sisters of which she is the eldest. She started her primary education in Sekolah Rendah Kebangsaan Pengakalan TLDM, Lumut, before continuing her secondary studies in MARA Junior Science College (MRSM) Terendak, Melaka. In 2002, she was accepted into a matriculation in Universiti Putra Malaysia, before pusuing her degree in the same university. She graduated in 2005 with the degree of Bachelor of Science in Biotechnology Second Class Upper Honours. She received an award from Sartorius for academic excellence in 2005. In July 2005, she was awarded a scholarship by Universiti Teknologi Petronas to pursue her Masters of Science Degree in Environmental Engineering. She has been awarded silver medal for a poster presentation entitled "Biodegradation of Phenol Using the Xenoclean-Phenol® System" in the Malaysia Technology Expo (MTE) held on 29-31 March 2007 Putra World Trade Centre, Kuala Lumpur, Malaysia.