

**KINETIC STUDY OF CHLOROBENZENE DEGRADATION BY ISOLATED
MICROBES FROM WASTEWATER**

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KINETIC STUDY OF CHLOROBENZENE DEGRADATION BY
ISOLATED MICROBES FROM WASTEWATER

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*To my beloved mother Siti Khalijah Hj Ahmad and not forgetting my family Hasbul
Khairi, Khairul Helmi, and Mohd Akmal who gave me the inspiration and
encouragement in completing my thesis*

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ABSTRACT

The performance of microbial consortia from wastewater to degrade chlorobenzene (CB) was investigated. The consortia were initially exposed to high CB concentration (i.e. 0.2mg/L) for seven months in order to isolate the most dominant survivor(s). The two survivors were known as 'Yellow Colony' or YC, and 'White Colony' or WC. In batch culture, the maximum specific CB degradation rate, or Q_s (g CB degraded/g cell per hour) of WC, YC, mixture of WC and YC, were compared. The mixture of WC and YC gave three times greater Q_s than individual WC and YC, combined. This synergistic effect has never been reported so far. Study in a continuous culture indicated that nitrogen-enriched feed has resulted in greater critical dilution rate, D_c (i.e. 0.11h^{-1}) than the unsupplemented one (i.e. 0.08h^{-1}). This proved that the nitrogen limiting could not be ignored. It was also discovered that a short term (i.e. two days) adaptation of the consortia on CB prior to the degradation test in continuous cultures, as employed in some published works, was insufficient to produce significant result in this study. Data in batch study revealed that high aeration and temperature close to ambient (versus 37°C) doubled the microbial growth in CB degradation. The batch study also showed that the CB degradation rate obeyed the first order kinetic. However, no degradation was witnessed below 0.0006 mg/L of CB. Below this threshold level, CB was almost undetectable by microbes. The outcomes of this study have not only proved the potential of employing microbes from wastewater to solve chlorobenzene contamination problem, but also provided useful parameter estimates for future up scaling works, or on site trial.

ABSTRAK

Potensi bagi konsortium mikrob dari air sisa buangan dalam penguraian klorobenzena (CB) telah dikaji. Mikroorganisma ini pada mulanya didedahkan pada kepekatan CB yang tinggi (iaitu 0.2mg/L) selama tujuh bulan untuk memencilkan species yang paling dominan. Dua spesies yang dipencilkan dipanggil 'Koloni Kuning' atau YC, dan 'Koloni Putih' atau WC. Di dalam kajian kultur sekelompok, kadar maksima degradasi spesifik CB atau Q_s (g CB/g sel per jam) bagi WC, YC, dan campuran WC dan YC telah dibandingkan. Hasil menunjukkan campuran WC dan YC memberikan tiga kali ganda nilai Q_s berbanding hasil gabungan individu WC dan YC. Kesan sinergistik ini belum pernah dilaporkan setakat ini. Kajian dalam sistem selanjara pula menunjukkan kultur yang dibekalkan dengan nitrogen menghasilkan kadar kritikal pencairan, D_c ($0.11h^{-1}$) yang lebih tinggi berbanding kultur tanpa bekalan nitrogen ($0.08h^{-1}$). Ini membuktikan kadar penghadan substrat tidak boleh diabaikan. Didapati juga pengadaptasian konsortium mikrob kepada CB dalam jangkamasa pendek (dua hari) sebelum ujian penguraian dalam kultur selanjara sebagaimana yang telah diaplikasikan oleh beberapa kajian literatur, tidak memberikan keputusan yang signifikan dalam kajian ini. Data dari kultur sekelompok pula menunjukkan kesan pengudaraan yang tinggi dan suhu yang menghampiri persekitaran (berbanding $37^{\circ}C$) mampu melipatgandakan pertumbuhan mikrob dalam penguraian CB. Kajian sekelompok juga menunjukkan kadar penguraian CB mematuhi hukum kinetik pertama. Walaubagaimanapun, tiada penguraian yang berlaku pada kepekatan dibawah paras 0.0006 mg/L. Pada bawah tahap ambang, CB hampir tidak dapat dikesan oleh mikrob. Hasil kajian ini bukan sahaja membuktikan kemampuan pengaplikasian mikrob dari air sisa buangan dalam menangani masalah pencemaran CB, malah mencadangkan parameter-parameter aplikasi yang berguna bagi tugas menskala-naik atau percubaan di tapak industri.

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LIST OF SYMBOLS

\bar{s}	-	The steady state residual substrate concentration
+ve	-	Positive
Ca	-	Calcium
CO_2	-	Carbon dioxide
Cu	-	Cuprum
D	-	Dilution rate
D_c	-	Critical dilution rate
F	-	Flow
H_2	-	Hydrogen
H_2O	-	Water
K_s	-	Substrate utilization constant
O_2	-	Oxygen
Q_s	-	Maximum CB specific degradation rate
S	-	Substrate
S_R	-	Substrate remaining in the culture
V	-	Volume
-ve	-	Negative
$Y_{x/s}$	-	Cell yield
μ_g	-	Specific growth rate
μ_{max}	-	Maximum specific growth rate

LIST OF ABBREVIATIONS

ACGIH	-	American Conference of Governmental Industrial Hygienists
ATSDR	-	Agency for Toxic Substance and Disease Registry
AU	-	Absorption Unit
BOD	-	Biochemical Oxygen Demand
CAA	-	Clean Air Act
CAS	-	Chemical Abstracts Service
CB	-	Chlorobenzene
CEPA	-	Canadian Environmental Protection Act
CERCLA	-	Comprehensive Environmental Response
CF	-	Chloroform
CICAD	-	Concise International Chemical Assessment Document 60
COD	-	Chemical Oxygen Demand
CT	-	Carbon tetrachloride
DCB	-	Dichlorobenzene
DDT	-	dichlorodiphenyltrichloroethane
DNA	-	Deoxyribonucleic acid
DOE	-	Department of Environment
DOT/UN/N A/IMCO	-	Department of Transportation/United Nations/North America/International Maritime Dangerous Goods Code
ECD	-	Electron capture detector
EIA	-	Environmental Impact

EPA	-	Environmental Protection Agency
FFW	-	Microbes from Fresh Wastewater
FID	-	Flame ionization detector
GC	-	Gas chromatography
HAA	-	Halogenated Alkanoic Acids
HCl	-	Acid hydrochloric
HMRCI	-	Hazardous Materials Control Research Institute
HPLC	-	High performance liquid chromatography
HRGC	-	High resolution gas chromatography
HSD	-	Halide specific detector
HSDB	-	Hazardous Substance Data Bank
IPCS	-	International Programme on Chemical Safety
MCL	-	Maximum Contaminant Level
MCLG	-	Maximum Contaminat Level Goals
MS	-	Mass spectrometric detector
NCI	-	National Cancer Institute
NIOSH	-	National Institute for Occupational Safety and Health
OD	-	Optical density
OEHHA	-	Office of Environmental Health Hazard Assessment
OHM/TADS	-	Oil and Hazardous Materials / Technical Assistance Data System
OSHA	-	Occupational Safety and Health Administration
PCB	-	Polychlorinated biphenyls
p-CBs	-	p-chlorobiphenyls
PCP	-	Petachlorophenol
PHG	-	Public Health Goal
PVC	-	Polyvinyl chloride
RAAG	-	Remediation Alternative Assessment Group
RNA	-	Ribonucleic acid
RTECS	-	Registry of Toxic Effects of Chemical Substances
SPME	-	Solid Phase Micro-Extraction
TCDD	-	Tetrachlorodibenzo-p-dioxin

TCE	-	Tetrachloroethylene
TDS	-	Total Dissolved Solid
TLV	-	Threshold Limit Values
TOC	-	Total Organic Carbon
TRI	-	Toxics Release Inventory
TSS	-	Total Suspended Solid
TWA	-	Time Weighted Average
US	-	United State
USA	-	United State of America
UV	-	Ultra ungu
UV/vis	-	Ultra ungu visible
VC	-	Vinyl chloride
WC	-	White Colony
WHO	-	World Health Organization
WYC	-	Mixture of YC and WC
YC	-	Yellow Colony

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Among the numerous chemical substances that enter the environment with wastewater and exhaust, a great number are benzene derivatives and nonpolar aromatics. Halogenated aromatic compounds such as chlorobenzenes (CBs) are major of concern due to its affects on human healths. The extensive use of CBs over the past few decades as organic solvents, insecticides, degreasers and deodorants, and their use as intermediates in the synthesis of chemicals such as rubber processing, antioxidants, dyes, agricultural products, and pharmaceuticals, has led to a widespread release of these xenobiotic compounds into the environment (EPA, 1980; Harris *et al.*, 1985). The usage coupled with accidental spills and through routine industrial waste disposal practices have resulted in ultimate contamination of the environment, where these pollutants tend to persist (Fathepure *et al.*, 1988). These lipophilic compounds have been found in a wide range of environmental media including soils (Ding *et al.*, 1992), groundwaters (Boyd *et al.*, 1997), sewage sludge (Rogers *et al.*, 1989a; Wang *et al.*, 1992), marine and lake sediments (Masunaga *et al.*, 1991; Lee and Fange, 1997), and open water columns (Rogers *et al.*, 1989b; Harper *et al.*, 1992). They are also known as important river contaminants especially found in United Kingdom (Meharg *et al.*, 2000).

Monochlorobenzene or chlorobenzene (CB) that is currently being targeted by bioremediation because of its resistances (Eweis *et al.*, 1998) was identified as priority pollutant by the U.S. Environmental Protection Agency (EPA, 1980). CB concentrations in surface waters are generally in the ng/L to µg/L range, with maximum concentrations up to 0.2 mg/L in area close to industrial sources (CICAD, 2004). Water samples from Scheldt estuary in Netherlands confirmed that CB levels ranging from 5 to 31.5 ng/L (Huybrechts *et al.*, 2000). In industrial wastewaters, it may be higher and vary according to the nature of the processes used. The observed levels in many surface waters and groundwaters were too low to cause immediate acute toxicity to mammals, birds and aquatic organisms, but little information exists about the long-term exposure and bioaccumulation of CB (Schraa *et al.*, 1986). CB in high concentration causes a wide variety of effects towards human ranging from immunological disorders to adverse effects on the liver, kidney, thyroid, and lung (Rapp and Timmis, 1999). Additionally, its persistency leads to enhance the transferability in the food chain. In spite of these consequences, the destruction of this pollutant was emphasized in many researches and executed under safety conditions in order to protect human and environment from the hazardous effects.

In Malaysia, presence of CB toxic in environmental mostly from the industrial activities. However, the concentration of CB found is not as critical as in other countries that have fast expanding economy in industrial and agricultural sector. Study by Soh and Abdullah (2005) when determining of volatile organic compounds (VOCs) using Solid Phase Micro-Extraction (SPME) illustrates that CB existed in drinking water samples within the range of 1.06 to 2.95 µg/L. Meanwhile, when examining the trends and prospects of environmental pollution, Abdullah (1995) revealed that organic pollution loaded in Malaysia waterways since 1960's with pollution from agro-based industries accounted for approximately 90% of the industrial pollution load. Organic solvents are among of the toxic and hazardous wastes that are defined in a schedule listing 107 categories of wastes under the Environmental Quality (Schedule Wastes) Regulations 1989. Furthermore, Malaysia industry effluents have been estimated to amount to nearly 380 000 cubic per year, comprising both organic and inorganic materials of varying chemical composition as well as aromatic compound such as CB.

1.2 Research Background

Biological method or bioremediation has become increasingly important rather than chemical and physical processes. Bioremediation is an application of biological process principle to the treatment of groundwater, soil and sludge contaminated with hazardous chemicals. The responsibility of microorganisms for CB removal from the environment *via* enzymatically catalysed reactions appears to be very important because of its perceived low cost, simplicity and its low adverse effect on the environment (Cookson, 1995). There are numerous applications of bioremediation treatment technologies, but the most commonly used includes bioaugmentation, biofilters, biostimulation, bioreactors, bioventing, composting, and landfarming (Baker and Herson, 1994).

Bioremediation techniques based on aerobic degradation reactions have been proposed as promising treatments for industrial effluents contaminated by CB because they have the potential to transform this contaminant into non toxic end products using economical growth materials (Wilson and Wilson, 1985; Fogel *et al.*, 1986; and McCarty, 1991). Moreover, CB as a less chlorinated benzene congener is amenable to aerobic degradation (Reineke and Knackmuss, 1984; de Bont *et al.*, 1986; Schraa *et al.*, 1986; Spain and Nishino, 1987). The aerobic CB degradation, which *via* oxidative dechlorination was usually initiated by dioxygenative hydroxylation, then leading to the formation of catechols. Finally, it undergoes the ring fission and subsequent mineralization to carbon dioxide and water. CB biodegradation under anaerobic condition has also been reported (Bittkau *et al.*, 2004), although it occurs at a slower rate than aerobic biodegradation. The resistances to naturally biodegradation of CB caused of low aqueous solubilities, high octanol-water partition coefficients, and both deactivation and steric hindrance due to the number and position of chlorine on the aromatic ring (Reineke and Gibson, 1984).

A wide variety of microorganisms could utilize CB as carbon and energy source, which have been reported by previous workers include de Bont *et al.*, 1986; Schraa *et al.*, 1986; Spain and Nishino, 1987; Pettigrew *et al.*, 199; Haigler *et al.*,

1992; Keener and Arp, 1994; Van der Meer *et al.*, 1997; Beil *et al.*, 1997; Fairlee *et al.*, 1997; Meckenstock *et al.*, 1998; and Kiernicka *et al.*, 1999. It has been found that different bacterial strains, mostly Gram-negative bacteria such as *Pseudomonas* sp., *Alcaligenes* sp., and *Xantobacter* sp., were individually able to use CB as growth substrate. However, very few Gram-positive bacteria, mainly rhodococci, have been described as having this capability (Zaitsev *et al.*, 1995). Reineke and Knackmuss (1984) clarified the biodegradation pathways of CB that have been thoroughly studied in pure cultures of bacteria, which has been isolated from the mixture of soil and sewage by chemostat enrichment. Besides, the indigenous microbial communities especially from the CB contaminated sites were also capable to degrade CB as cited by Aelion *et al.*, 1987; Nishino *et al.*, 1994; Kao and Presser, 1999; and Balcke *et al.*, 2004.

The ability of microorganisms to degrade CB was believed to closely depend on their long-term adaptation to the contaminated habitat (Van der Meer *et al.*, 1998). As a result, many studies have been directly elucidating the biochemical mechanisms for CB that are broken down by pure cultures from the CB contaminated sites. Hence, the use of microbes from wastewater to degrade CB is scarce in current investigation. Wastewater from residential or industrial activities comprises various compounds or organic matters from a variety of sources. Thus, the potential of these indigenous microbial populations to alleviate the CB pollution problems should be exploited. This study aimed on investigating the kinetic of microbial isolates from residential wastewater to degrade chlorobenzene (CB) in both batch and continuous modes. Investigations would be focused on the isolation approach; comparison of the specific chlorobenzene degradation rate of the identified isolates and their combinations; and the behaviour of CB degradation at different CB levels.

1.3 Objectives and Scopes of Study

The objectives of this research are:

- 1) To screen and isolate the microorganisms from local wastewater that capable to biodegrade chlorobenzene.
- 2) To study the kinetics of chlorobenzene degradation by isolated microbes.
- 3) To evaluate the CB biodegradation in batch and continuous cultures.

With the intention of achieving the objectives of this study, there were some scopes that should be comprised as follows;

- 1) Propagation and purification of the microbes by using the streak plate technique
 - i) isolate the microbes from fresh wastewater
 - ii) isolate the microbes after seven months CB adaptation at CB concentration of 0.2m g/L
 - iii) identify the dominant strains by morphological observation, staining method and biochemical tests
- 2) Evaluation of the potential of the microbes to degrade CB in batch culture condition by comparing such inoculums
 - i) pure culture (as individual)
 - ii) mixed pure cultures (as consortia)
 - iii) fresh wastewater (as indigenous communities)
- 3) Study the degradative capability of microbes in continuous bioreactor as following emphasizes
 - i) supplemented with nitrogen source (5.0g/L yeast extract)
 - ii) short acclimatization (two days) with CB prior to degradation treatment
- 4) Investigation of the environmental factors that enhanced the degradation of CB by microbes in batch mode
 - i) aeration level – between high and low aerobic condition
 - ii) temperature – compare the ambient (27°C) and temperature 37°C
- 5). Examine the behavior of CB degradation at different initial CB levels, i.e. 0.0 (control), 0.0006, 0.0553, 0.1659, and 0.3317 mg/L.

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