THE PERFORMANCE OF PHENOL BIODEGRADATION BY *Candida tropicalis* RETL-Cr1 USING BATCH AND FED-BATCH FERMENTATION TECHNIQUES

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Dedicated especially to my wife,
Nur Shiqah @Chuah Kim Hong Abdullah and
my children,
Nur Azidah, Nur Sulina and Nurul Atiqah

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ABSTRACT

Phenol is a toxic compound found in many industrial-waste effluents. A locally isolated yeast strain RETL-Cr1 from the effluent of the Exxon Mobil Oil Refinery wastewater treatment plant was investigated for phenol degradation using batch and fed-batch fermentation under aerobic condition. Based on a BLASTN search of GenBank, the complete sequences of ITS1-5.8S rDNA-ITS2 regions and portions of I8S and 28S for the purified DNA products of RETL-Cr1 shared 98% similarity with *C. tropicalis*. This yeast strain RETL-Cr1 was redesignated *C. tropicalis* RETL-Cr1 and was deposited at the GenBank under the accession number AY725426. The optimum condition for phenol degradation was at 30°C, pH 6.5 in RM in the absence of glucose. The highest phenol biodegradation efficiency in shake-flask cultures with IPC of 3mM was 100% achieving a degradation rate of 0.0257 g L^{-1} h^{-1} at μ 0.3718 h^{-1} after 14 h cultivation. Degradation of phenol was faster by 1.5-fold in bioreactor than in shake-flask whereby degradation rate was improved to 0.0395 g L^{-1} h^{-1} at μ 0.5391 h^{-1} after 10 hours of incubation. When tested at various IPC (0.0028 – 0.94 g L^{-1}), inhibition was evident at IPC levels above 5 mM (0.470 g L^{-1}). The fed-batch system in a bioreactor offered an 85 times fold degradation rate (2.3 g L^{-1} h^{-1}) over shake-flask culture (0.0257 g L^{-1} h^{-1}) and 61-fold over 2L bioreactor (0.0395 g L^{-1} h^{-1}) batch system. It was observed that kinetically phenol degradation by RETL-Cr1 was significantly high in fed-batch culture as indicated by high degradation rate (2.3 g L^{-1} h^{-1}) and substrate yield ($Y_{x/s}$ = 0.71-4.48 g g^{-1}). However, a lower product yield ($Y_{pc/s}$ = 1.6x10^{-4} – 2.1x10^{-3} g g^{-1}; $Y_{pc/x}$ = 3.5x10^{-5} – 1.4 x10^{-3} g g^{-1}; $Y_{ccMA/s}$ = 1.0x10^{-4} – 2.0x10^{-4} g g^{-1}; $Y_{ccMA/x}$ = 4.4x10^{-5} – 1.8x10^{-4} g g^{-1}) and productivity (catechol = 1.2x10^{-5} – 5.3x10^{-5} g L^{-1} h^{-1}; ccMA = 1.4x10^{-5} – 2.6 x10^{-5} g L^{-1} h^{-1}) were achieved. When catechol and ccMA were analysed to determine whether an *ortho* or *meta* pathway was taken, it was found that these two metabolites were present in low amounts. This probably indicates further degradation of the metabolites. Hence, RETL-Cr1 strain metabolizes phenol via *ortho*-cleavage pathway. The optimum condition for both phenol hydroxylase and catechol 1,2-dioxygenase were at 30°C, pH 6.5. The most distinctive feature of this yeast strain is that it has a very high tolerance limit towards phenol reaching up to 60 mM. Based on the observations, RETL-Cr1 has a good potential to be used for treatment of phenol in industrial effluent.

ABSTRAK

Fenol adalah sebatian toksik terdapat dalam pelbagai efluen sisa buangan industri. Yis tempatan strain RETL-Cr1 dipencilkan daripada efluen loji pengolahan air sisa kilang penapis minyak Exxon Mobil telah dikaji untuk pembiodegradasian fenol menggunakan fermentasi kultur kelompok dan kelompok suapan dalam keadaan aerobik. Berdasarkan pencarian pada GenBank, jujukan sepenuhnya kawasan ITS1-5.8S rDNA- ITS2 dan bahagian-bahagian 18S dan 28S produk DNA RETL-Cr1 menyumbang 98% kesamaan dengan *C. tropicalis*. Strain yis RETL-Cr1 ini telah dinamakan semula sebagai *C. tropicalis* RETL-Cr1 dan disimpan dalam GenBank di bawah nombor penambahan AY725426. Keadaan optimum bagi pembiodegradasian fenol adalah pada suhu 30°C, pH 6.5 dalam RM tanpa glukosa. Pembiodegradasian fenol dalam kultur kelompok kelalang goncangan pada kepekatan fenol permulaan 3 mM adalah 100% mencapai kadar pendegradasian 0.0257 g L^{-1} j^{-1}, = μ 0.3718 j^{-1} selepas 14 jam pengeraman. Pembiodegradasian fenol didapati 1.5 kali lebih cepat dalam kultur kelompok bioreaktor berbanding dengan kelalang goncangan dengan pencapaian 0.0395 g L^{-1} j^{-1} pada μ 0.5391 j^{-1} selepas 10 jam pengeraman. Apabila diuji pada pelbagai IPC (0.028–0.94 g L^{-1}), kesan perencatan adalah jelas apabila kepekatan fenol melebihi tahap 5 mM (0.470 g L^{-1}). Sistem suapan sesekelompok mencapai 85 kali lebih baik dengan kadar pemdegradasian 2.3 g L^{-1} j^{-1} dari sistem kelompok kelalang goncangan (0.0257 g L^{-1} j^{-1}) dan 61 kali dari 2L bioreaktor. Didapati dari segi kinetik, pembiodegradasian fenol dalam sistem suapan kelompok adalah bersignifikan tinggi seperti ditunjukkan oleh kadar degradasi (2.3 g L^{-1} h^{-1}) dan hasil substrat ($Y_{x/s}$ = 0.71-4.48 g g^{-1}) yang tinggi. Walau bagaimanapun hasil produk ($Y_{pc/s}$ = $1.6x10^{-4}$ – $2.1x10^{-3}$ g g^{-1}; $Y_{pc/x}$ = $3.5x10^{-5}$ – $1.4x10^{-3}$ g g^{-1}; $Y_{ccMA/s}$ = $1.0x10^{-4}$ – $2.0x10^{-4}$ g g^{-1}; $Y_{ccMA/x}$ = $4.4x10^{-5}$ – $1.8x10^{-4}$ g g^{-1}) dan produktiviti (katekol = $1.2x10^{-5}$ – $5.3x10^{-5}$ g L^{-1} h^{-1}; ccMA =$1.4x10^{-5}$ –$2.6x10^{-5}$ g L^{-1} h^{-1}) adalah rendah. Apabila katekol dan ccMA dianalisis untuk menentukan samada laluan *ortho* atau *meta*, didapati amaun kedua-dua metabolit ini adalah rendah. Ini menunjukkan berlakunya proses pemdegradasian terhadap kedua-dua metabolit ini. Oleh itu, strain yis RETL-Cr1 ini mendegrad fenol melalui laluan belahan *ortho*. Keadaan optimum bagi enzim fenol hidroksilase dan katekol 1,2-dioksigenase adalah pada 30°C, pH 6.5. Ciri tersendiri yis ini adalah ketolerannya yang tinggi terhadap fenol sehingga mencapai 60 mM. Berdasarkan kajian ini, RETL-Cr1 berpotensi digunakan untuk rawatan fenol dalam efluen industri.

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LIST OF SYMBOLS

α	-	alpha
ß	-	beta
γ	-	gamma
abs	-	absorbance
^{o}C	-	degrees Celsius
g	-	gram
$g\ L^{-1}$	-	gram per litre
h^{-1}	-	per hour
L	-	litre
$mg\ L^{-1}$	-	milligram per litre
mM	-	millimolar
mL	-	millilitre
nm	-	nanometer
%	-	percent
OD_{600}	-	optical density at 600
S	-	substrate concentration ($mg\ L^{-1}$ or $g\ L^{-1}$)
S_o	-	initial substrate concentration ($mg\ L^{-1}$ or $g\ L^{-1}$)
t		time (h)
T_L	-	lag period (h)
μ	-	specific growth rate (h^{-1})
$\mu g\ L^{-1}$	-	microgram per litre
µL	-	microlitre
µm	-	micrometer
% v/v	-	percentage volume per volume
% wt/v	-	percentage weight per volume
X_{max}	-	maximum biomass concentration ($gdw\ L^{-1}$)

$Y_{x/s}$	-	cell mass yield on phenol (g g $^{-1}$)
Cat_{max}	-	catechol maximum concentration (mg L^{-1} or g L^{-1})
$Y_{pc/s}$	-	catechol yield on phenol (g g $^{-1}$)
$Y_{pc/x}$	-	catechol yield on cell mass (g g $^{-1}$)
$ccMA_{max}$	-	*cis,cis*-muconic acid maximum concentration (mg L^{-1} or g L^{-1})
$Y_{ccMA/s}$	-	*cis,cis*-muconic acid yield on phenol (g g $^{-1}$)
$Y_{ccMA/x}$	-	*cis,cis*-muconic acid yield on cell mass (g g $^{-1}$)

LIST OF ABBREVIATIONS

ATCC	-	American Type Culture Collection
AGE	-	agarose gel electrophoresis
bp	-	base pairs
C1,2D	-	catechol 1,2-dioxygenase
*cc*MA	-	*cis*,*cis*-muconic acid
ccMALe	-	*cis,cis*-muconic acid lactonizing enzyme
CFU	-	colony forming unit
CIF	-	constant intermittent feeding
DNA	-	deoxyribonucleic acid
2-HMSA	-	2-hydroxymuconic semialdehyde
IPC	-	initial phenol concentration
HPLC	-	high-performance liquid chromatography
ITS	-	internal transcribed spacer
MCA	-	MacConkey agar
PCR	-	polymerase chain reaction
PH	-	phenol hydroxylase
psi	-	pounds per sq. in
rDNA	-	ribosomal deoxyribonucleic acid
RM	-	Ramsay medium
rpm	-	revolutions per minute
sp.	-	species
pH	-	hydrogen ion concentration
ppm	-	parts per million
RETL-Cr1	-	**R**amsay **E**ffluent of **T**reatment **L**agoon-**Cr**eam **1**
TCA	-	tricarboxylic acid cycle
TSI	-	triple sugar iron
UV	-	ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Environmental pollution has been considered as a side effect of industrial society. Soil, lakes, rivers, and seas are highly contaminated with different toxic compounds (Alexander, 1981). An example of such compound is phenol. Phenol is released into the environment from industrial discharges (Keith, 1976; Jungclaus *et al.*, 1978; Parkhurst *et al.*, 1979; Pfeffer, 1979) and spills (Delfino and Dube, 1976). According to Prasad and Ellis (1978), phenols and its derivatives are among the most frequently found pollutants in rivers, industrial effluents and landfill run-off waters. Hence, populations residing near waste disposal sites, landfill sites or phenol spills may be at risk for higher exposure to phenol than other populations. An example of such spill was one that occurred in June, 2001 when the Indonesian-registered oil tanker MT Endah Lestari capsized off the coast of Johore, southern Malaysia spilling 600 metric tons of phenol and large amount of diesel killing thousands of marine life in the nearby fish farming ground.

Nowadays, environmental preservation has become a key issue in a society because it is often linked to quality of life. The impacts of pollution on the environment have led to an intense scientific investigation. The removal of phenol from industrial effluents has attracted researchers from different fields (Yang and Humphrey, 1975;

Shingler, 1996). The increasing awareness on the environment in both developed and developing countries has initiated more studies of possible solutions for treating phenol.

Environmental biotechnology relies on the pollutant-degrading capacities of naturally occurring microorganisms (Liu and Suflita, 1993). It has been reported to be advantageous over physical and chemical treatments due to its relatively low cost and has less ecological impact to the environment (Head, 1998; Edington, 1994). Researchers are studying pollutant-degrading microorganisms which inhabit polluted environments (Kumaran, 1980; Kapoor *et al*., 1998; Yap *et al*., 1999; Heinaru *et al*., 2000; Komarkova *et al*., 2003; Santos and Linardi, 2004; Margesin *et al*., 2005) as well as uncontaminated environment (Bastos *et al*., 2000a; Koutny *et al*., 2003). Harnessing the potential of microbes (Ahmed, 1995; Fulthorpe and Allen, 1995; Bastos *et al*., 2000b; Ruiz- Ordaz *et al*., 2001; Vojta *et al*., 2002; Páca Jr. *et al*., 2003) to degrade phenol has been an area of considerable study to develop bioremediation approaches which has been considered as a “green option” (Singleton, 1994) for treatment of environmental contaminants.

Many researchers support the biological treatment of phenols. A number of studies with prokaryotic microorganisms have been carried out for the purpose to improve the technological processes of biodegradation. Some examples are, *Pseudomonas* sp. have demonstrated the ability to mineralize phenol (Ehrhardt and Rehm, 1989; Hinteregger *et al*., 1992; Ahmed, 1995; Chitra *et al*., 1995; Dapaah and Hill, 1992; Fulthorpe and Allen,1995; Fava *et al*., 1995; Loh and Wang, 1998), *Alcaligenes* sp. (Hill *et al*., 1996; Valenzuela *et al*., 1997), *Azotobacter* sp. (Li *et al*., 1991), *Rhodococcus* sp. (Apajalahti and Salkinoja-Salonen, 1986; Oh and Han, 1997), *Phanerochaete* sp. (Perez *et al*., 1997; Larmar *et al*., 1990), and *Cryptococcus* sp. (Mörsen and Rehm, 1987).

However, according to Katayama-Hirayama *et al*., (1994) information on degradation of phenol is limited in the yeast strains. Among the eukaryotic microorganisms, only some members of yeast genera *Candida, Rhodotorula*, and *Trichosporon* that able to metabolize phenolic compounds as a sole carbon and energy

source (Neujahr, 1990; Katayama-Hirayama *et al*. 1994; Chen *et al*., 2002). Among the *Candida* strain, *Candida tropicalis* has been the most studied in the biodegradation of phenol (Shimizu *et al.,* 1973; Kumaran, 1980; Krug *et al*., 1985; Bastos *et al*., 2000a; Chen *et al.*, 2002; Vojta *et al*., 2002; Yan *et al*., 2005). However, none of these yeast strains were isolated from Malaysian environment.

Studies on the naturally pollutant-degrading microorganisms termed as environmentally relevant microorganisms (ERM), include the isolation of bacteria from the environment, their classification and physiological characterization, molecular analysis of their degradative enzymes (Watanabe and Baker, 2000). Biodegradation of phenol by many microorganisms has been studied in order to understand the nutrient requirements, environmental physico-chemical factors, and complex biochemistry involved that may assist in bioremediation of this toxic compound.

1.2 Objectives of the study

The aim of this study is to investigate the ability of locally isolated microorganisms to degrade phenol with the specific objectives listed below:

1. To isolate, screen and identify phenol-degrading microorganisms from oil, waxy oil and petrochemical wastes.

2. To optimize and conduct kinetic analyses on the aerobic phenol biodegradation in batch and fed-batch cultures by potential strains.

3. To postulate possible metabolic pathway of phenol degradation by the microorganism of interest.

4. To identify the potential strain by a molecular mechanisms (PCR amplification of ribosomal DNA targeting the conserved regions of 5.8S, 18S and 28S using universal primers ITS1 and ITS4).

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