DEGRADATION OF REMAZOL BLACK B BY *Bacillus cereus* STRAIN A FROM TEXTILE WASTEWATER

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DEGRADATION OF REMAZOL BLACK B BY *Bacillus cereus* STRAIN A FROM TEXTILE WASTEWATER

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TO my beloveds family:
Husband: Zulkefli Daud,
Children: Nursyazwani, M Nazirul Amin and M Iliya Uzair

"YOU ARE MY INSPIRATION"

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In the Name of ALLAH the Almighty and the Merciful

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ABSTRACT

Five bacterial strains designated as A, B1, B2, C and Y1 that were revived from the UTM glycerol stock were used for biodegradation study of azo dyes (SF BLACK EXA, SF RED 3BS, SFN BLUE 150 % and SF YELLOW EXF) at three different conditions; aerobic, facultative anaerobic and partial aerobic (without agitation). Screening test shows bacterium A, under facultative anaerobic condition demonstrated the highest decolourisation rate with removal rate (7.0% h^{-1}) of 0.1 gL^{-1} SF BLACK EXA was observed. Relationship of growth and decolourization analysis shows decolourisation rate of the same azo dye by bacterium A in CDM medium under facultative anaerobic and aerobic conditions were 26.445% h^{-1} and 24.56% h^{-1}, respectively. The growth rate of bacterium A was found higher in aerobic culture (2.733 mgh^{-1}) in comparison with that in facultative anaerobic culture (1.067 mgh^{-1}). In other assessment, bacterium A was applied into optimized CDM medium (glucose (1 gL^{-1}), NH_4Cl (0.5 gL^{-1}), K_2HPO_4 (7 gL^{-1}), KH_2PO_4 (2 gL^{-1}), $MgSO_4.7H_2O$ (0.1 gL^{-1}), $CaCl_2$ (0.02 gL^{-1}), RBB (0.1 gL^{-1})), pH 6.5, incubated at 35 °C, with 10 % v/v inoculum under facultative anaerobic condition. Result showed that bacteria A successfully degraded more than 80 % RBB after 20 h incubation. Degradation of RBB molecules increased up to 90 % and 30 % COD removal before attaining stable condition within 164 h incubation. Azoreductase analysis revealed that enzyme was produced intracellularly (11.45 x 10^{-3} $Umin^{-1}$) and stabled at pH 6-8 and temperature ranged from 30 ºC to 40 ºC. HPLC analysis revealed that biodegradation of RBB under combined facultative anaerobic-aerobic condition produced sulfanilic acid (R_t = 3.988 min) after 160 h incubation and two unknown metabolites (R_t = 6.071 min and 7.480 min) after 164 h incubations. The partial 16S rRNA analysis showed that bacterium A closely related to *Bacillus cereus* (99 % sequence similarity). These findings suggest that this bacterium has the capability to assist in the degradation of wastewater containing azo dyes from industry of textile-based.

ABSTRAK

Lima strain bakteria bertanda A, B1, B2, C dan Y1 yang telah dipencilkan semula daripada stok kultur gliserol digunakan untuk mengkaji biodegradasi pewarna azo (SF BLACK EXA, SF RED 3BS, SFN BLUE 150 % dan SF YELLOW EXF) dalam tiga keadaan berbeza: aerobik, fakultatif anaerobik dan separa aerobik (tanpa goncangan). Kadar penyingkiran warna tertinggi daripada ujian penyaringan bakteria A di bawah keadaan fakultatif telah diperhatikan sebanyak 7.0% j^{-1} dalam medium yang mengandungi 0.1 gL^{-1} SF BLACK EXA. Analsis perkaitan antara pertumbuhan dan penyahwarnaan menunjukkan kadar penyahwarnaan pewarna azo tersebut oleh bakteria A dalam medium CDM di bawah keadaan fakultatif anaerobik dan aerobik adalah 26.445% j^{-1} dan 24.56% j^{-1}, masing-masing. Kadar pertumbuhan bakteria dalam keadaan aerobik (2.733 mgj^{-1}) didapati lebih tinggi berbanding keadaan fakultatif anaerobik (1.067 mgj^{-1}). Dalam kajian yang lain, bakteria A diaplikasikan dalam keadaan CDM optimum (glukosa (1 gL^{-1}), NH_4Cl (0.5 gL^{-1}), K_2HPO_4 (7 gL^{-1}), KH_2PO_4 (2 gL^{-1}), $MgSO_4.7H_2O$ (0.1 gL^{-1}), $CaCl_2$ (0.02 gL^{-1}), RBB (0.1 gL^{-1}), pH 6.5, suhu eraman 35°C, 10% i/i inoculum) di bawah keadaan fakultatif anaerobik. Keputusan menunjukkan bakteria A berjaya menyingkirkan lebih daripada 80 % RBB dalam tempoh 20 jam pengeraman. Degradasi molekul RBB meningkat sehingga 90 % dan 30 % penyingkiran COD sebelum mencapai kestabilan dalam tempoh 164 jam pengeraman. Analisis azoreduktase mendapati enzim dihasilkan adalah intraselular (11.45 x 10^{-3} $Umin^{-1}$) dan stabil pada pH 6-8 serta dalam julat suhu dari 30 ºC hingga 40 ºC. Analisis HPLC menunjukkan biodegradasi pewarna azo RBB dalam fakultatif anaerobik-aerobik menghasilkan asid sulfanilik, (R_t = 3.988 min) setelah 160 jam pengeraman dan dua jenis metabolit tidak diketahui, (R_t = 6.071 min dan 7.480 min) setelah 164 jam pengeraman. Analisis separa jujukan 16S rRNA menunjukkan bakteria A berkait rapat dengan *Bacillus cereus* (99% kesamaan jujukan). Penemuan ini mencadangkan bahawa bakteria ini berkeupayaan membantu degradasi air sisa yang mengandungi pewarna azo dari industri tekstil.

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°C	-	Degree Celsius
%	-	Percentage
$\%h^{-1}$	-	percentage per hour
λ	-	Wavelength
ABS	-	Aminobenzene sulphate
3-ABS	-	3-Aminobenzene sulphate
4-ABS	-	4-aminobenzene sulphate
5-ABS	-	5-aminobenzene sulphate
ADMI	-	American Dye Manufacturer Institute
APHA	-	American Public Health Association
4-AP	-	4-Aminophenol
ANS	-	Aminonaphythyl sulphate
BC	-	Before Century
BOD	-	Biological Oxygen Demand
C.I	-	Colour Index
$CaCl_2$	-	calcium chloride
CDM	-	Chemically Defined Medium
CDW	-	cell dry weight
Cl	-	chloride
Cl^{-1}	-	ion chloride
CO_2	-	Carbon dioxide
COD	-	chemical oxygen demand
d^{-1}	-	per day
dATP	-	deoxyadenosine 5’-triphosphate
dCTP	-	deoxycytosine 5’ triphosphate
dGTP	-	deoxyguanosine 5’ triphosphate
DNA	-	deoxyribonucleic acid

dNTP	-	deoxynucleotide triphosphate
dTTP	-	deoxythymidine 5’-triphosphate
EDTA	-	ethylene diamine tetra acetic acid
Et Br	-	Ethidium bromide
FAD	-	flavin adenine dinucleotide(oxidized)
FAD^+	-	ion flavin adenine dinucleotide
$FADH_2$	-	flavin adenine dinucleotide(reduced)
Fe^{2+}	-	ion ferum
$FMNH_2$	-	flavin adenine mononucleotide (reduced)
g	-	gram
gL^{-1}	-	gram per litre
h	-	hour
h^{-1}	-	per hour
H_2O	-	hydrogen dioxide
HPLC	-	high performance liquid chromatography
K_2HPO_4	-	dipotassium hydrogen phosphate
kb	-	kilobase
KH_2PO_4	-	potassium dihydrogen phosphate
kg	-	kilogram
mg	-	milligram
mgh^{-1}	-	milligram per hour
min	-	minute
mol	-	mole
mgL^{-1}	-	milligram per litre
$MgCl_2$	-	magnesium chloride
$MgSO_4.7H_2O$	-	magnesium sulphate heptahydrate
mL	-	milliliter
mm	-	millimeter
mM	-	milimol
mV	-	miliVolt
NA	-	nutrient agar
NAD^+	-	nicotinamide adenine dinucleotide(oxidized)
NADH	-	nicotinamide adenine dinucleotide(reduced)
NADP	-	nicotinamide adenine dinucleotide phosphate

NADPH	-	nicotinamide adenine dinucleotide phosphate(reduced)
NB	-	nutrient broth
NH_4Cl	-	ammonium chloride
$(NH_4)_2SO_4$	-	ammonium sulphate
$ngmL^{-1}$	-	nanogram per liter
nm	-	nanometer
NO_{3-}	-	nitrate
OD_{600nm}	-	optical density at 600nm
PAAB	-	p-Aminoazobenzene
PCR	-	polymerase chain reaction
pH	-	potential ion hydrogen
ppm	-	part per million
Pt-Co	-	platinum cobalt
RBB	-	Remazol Black B
RM	-	redox mediator
rpm	-	rotation per minute
rRNA	-	ribosomal RNA
SO_4^{2-}	-	sulphate
TAE	-	tris-acetate buffer
Tris	-	2-hydroxymethyl-2-methyl-1,3-propanediol
TSS	-	total suspended solid
U	-	enzyme unit
UV	-	ultraviolet
UV-vis	-	ultraviolet-visible
v/v	-	volume per volume
w/v	-	weight per volume
μgmL^{-1}	-	microgram per milliliter
µL	-	microliter
ƿM	-	picomolar

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CHAPTER 1

INTRODUCTION

1.1 Background Study

Synthetic dyes are one of the main wastewater pollutants. It is estimated about 10^{9} kg of dyes are produced annually in the world, which azo dyes represent about 70% by weight (Zollinger, 1987). Azo dyes characterized by one or more azo groups (-N=N-) (Selvam *et al*., 2003) are considered as the most important group of synthetic colorants used in textile, printing, pharmaceutical, cosmetic and food industries (Beydilli and Pavlostathis, 2005). It's generally xenobiotic compounds that are very recalcitrant against biodegradation processes (Van der Zee, 2002). Azo dyes are designed to resist chemical and microbial attacks (Ramalho *et al*., 2002) and to be stable in light during material washing (Rajaguru *et al*., 2000).

As azo dyes are widely used in textile industry, its wastewater are characterised by their highly visible colour with range of 10-200 mgL^{1} (O'Neill *et al.*, 1999), high chemical oxygen demand (COD), suspended solids and alkaline pH (9-11) (Manu and Chaudhari, 2002). According to O'Neil (1999), textile processing wastewater typically containing dye in a range of 10-200 mgL^{-1} due to its disability to bind to cloth during the dyeing process. Reish (1996) also reported that over 10% of the dye used in textile processing does not bind to the fibres and is therefore released to environment. Fixation efficiency for basic dyes and reactive dyes was reported to vary around 98% and 50%, respectively (O'Neil, 1999). Thus, coloured effluent that was released into the environment is undesirable, not only because of its colour, but also many dyes and their breakdown products are toxic and/or mutagenic to living things (Chung and Cerniglia, 1992). Without adequate treatment, these dyes are stable and can remain in the environment for an extended period of time. For example the half-life of hydrolysed Reactive Blue 19 is about 46 years at pH 7 at 25°C (Hao *et al*., 2000).

Several physicochemical techniques, such as carbon absorption, ozonation, coagulation/adsorption that have been used for treating wastewater-containing dyes (Somasiri *et al*., 2006), are in general inefficient, costly and limited applicability, while sometimes producing large amount of difficult to disposed toxic waste (Sanayei *et al*., 2010). Recent researchers focus on bacteria as produce nontoxic waste (Somasiri *et al*., 2006), cheep and effective dyes degrader using anaerobic-aerobic treatment system (Dos Santos *et al*., 2005). Azo dyes are reduced by the cleavage of the azo bond in anaerobic system to formed carcinogenic aromatic amines which need to be further degraded (Haugh *et al*., 1991; Coughlin *et al*., 1997). Most azo dyes are reduced anaerobically to the corresponding amines but they are difficult to degrade aerobically (Zimmerman *et al*., 1982; Banat *et al*., 1996). Under aerobic conditions, bacteria are able to mineralise some aromatic amines (Stolz, 2001; Pearce *et al*., 2003). Rajaguru *et al*., (2000) revealed that the performance of sequential anaerobic aerobic bacterial degradation system has been shown to be efficient in the degradation of azo dyes.

The effectiveness of microbial decolourisation depends on the adaptability and the activity of selected microorganisms (Chen *et al*., 2003). Kodam *et al*., (2005) found that KMK 48 bacterium able to decolourize reactive azo dyes under aerobic conditions with high effectiveness. To date, the ability of microorganisms to carry out dye decolourisation has received much attention. Microbial decolourisation and degradation of dyes is seen as a cost effective method for removing these pollutants from the environment. Therefore in this study, specialised strains of anaerobic aerobic bacteria would be acclimatized its ability to reduce the azo groups of azo dyes to non-colored intermediates and/or even to partially mineralize them, which are safe and less toxic to the environment.

1.2 Scope and Objectives of Study

This study was focused on the optimization of azo dye degradation by selected bacteria locally isolated from acclimatized bacterial consortium used in textile wastewater treatment. Five bacterial strains coded A, B1, B2, C and Y1 were initially acclimatized in chemically defined medium containing pure azo dye. This is to ensure that the bacteria have consistent growth and activity on the dye used. The bacteria were then screened based on their ability to decolorize selected type of pure dye provided by the textile industry. Optimization is carried out under facultative anaerobic condition as the factors of temperature, types and concentrations of carbon and nitrogen sources, inoculums size, rate of agitation and dye concentration. The intermediates of azo dye biodegradation will be determined by carrying out the experiment under sequential anaerobic-aerobic condition.

This study is carried out with the specific objectives:

i. To screen and characterize potential dye degrading bacteria using four different azo dyes in chemical defined medium (CDM).
ii. To optimize physical and chemical condition for azo dye decolonization by selected bacteria in chemical defined medium.
iii. To determine enzyme localization and azoreductase activity for Remazol Black B color removal using selected bacteria.
iv. To analyze biodegradation products of Remazol Black B and its intermediate in synthetic medium using selected bacteria.

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