DECOLOURISATION AND DEGRADATION OF ACID ORANGE 7 USING AN ACCLIMATISED BAC-ZS MIXED BACTERIAL CULTURE

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…. For my parents; Bay Nguang Hong and Chai Nyuk Mui
.... For my lovely husband Khoo Kiat Siong
…. For my sisters; Wendy, Elaine and Caroline

You brought colours into my life

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"*The roots of all goodness lie in the soil of appreciation for goodness*" – Dalai Lama

ABSTRACT

Formation of dark coloured auto-oxidation compounds had resulted in reduced efficiency of the sequential anaerobic-aerobic treatment system to decolourise sulphonated azo dyes. In view of this, a monosulphonated azo dye, Acid Orange 7 (AO7) was selected as a model dye to study the decolourisation of AO7 and its auto-oxidation compounds by a mixed bacterial culture, BAC-ZS. It consisted of three bacteria namely *Brevibacillus panacihumi* strain ZB1, *Lysinibacillus fusiformis* strain ZB2 and *Enterococcus faecalis* strain ZL. The decolourisation and degradation process was performed using the sequential facultative anaerobic-aerobic system. Optimisation of the co-substrate showed that the combination of glucose (5 g/L) and yeast extract (3 g/L) was the best co-substrate for decolourisation; 98% of AO7 colour was removed within 2 h of facultative anaerobic phase. When the decolourised solution was further treated under the aerobic phase, auto-oxidation reaction resulted in heavy browning effect after 24 h of agitation. The browning effect had drastically decreased the decolourisation to 72%. However, continuous agitation up to 48 h successfully decolourised the auto-oxidation compounds as indicated by the increase in decolourisation up to 90%. Consequently, the decolourisation was accompanied by 73% decrease in Chemical Oxygen Demands (COD) and an increase of 94% of bacteria concentration (absorbance at 600 nm). It was also found that the initial pH 6.6 of AO7 solution dropped to pH 4.5 during facultative anaerobic decolourisation and increased to pH 7.7 at the end of aerobic treatment. The degradation of AO7 dye was determined and confirmed using the UV-Vis spectrophotometry and FTIR analysis. In addition, the formation of auto-oxidation compounds, 1,2-naphtholquinone and 1,4-benzoquinone were detected and monitored using HPLC analysis. Further phytotoxicity tests using *Cucumis sativus* confirmed detoxification of the final treated solution by BAC-ZS. Quantification of BAC-ZS using real-time polymerase chain reaction (RT-PCR) showed *E. faecalis* strain ZL was the dominant bacteria in the acclimatised BAC-ZS and throughout the AO7 treatment process. The annotatation of the draft genome of each bacteria revealed presence of genes coding for the azoreductases, dioxygenases and monooxygenases which played important roles in degradation and mineralisation of AO7 dye. In conclusion, the acclimatised BAC-ZS mixed bacterial culture has good potential to be used in the biological treatment of textile effluent.

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ABSTRAK

Pembentukan sebatian gelap auto-oxidasi mengakibatkan penurunan efikasi sistem rawatan berjujuk anaerobik-aerobik untuk menyahwarna pewarna sulfur azo. Oleh itu, pewarna monosulfur azo, Acid Orange 7 (AO7) digunakan sebagai pewarna model dalam kajian untuk menghasilkan campuran kultur bakteria yang berkeupayaan menyahwarnakan AO7 dan juga sebatian auto-oxidasinya. Tiga jenis bakteria; *Brevibacillus panacihumi* strain ZB1, *Lysinibacillus fusiformis* strain ZB2 dan *Enterococcus faecalis* strain ZL digunakan untuk membentuk kultur bacteria campuran, BAC-ZS. Proses penyahwarnaan dan degradasi AO7 adalah berpandukan sistem berjujukan fakultatif anaerobik-aerobik. Substrak optimasi menunjukkan gabungan glukosa (5 g/L) dengan ekstrak yis (3 g/L) adalah yang terbaik dan 98% warna AO7 dinyahwarnakan dalam tempoh 2 jam fakultatif anaerobik. Lanjutan rawatan ke fasa aerobik akibatkan oxidasi larutan dalam tempoh 24 jam. Pemerangan larutan akibatkan penurunan drastik peratusan penyahwarnaan (72%). Akan tetapi, pengoncangan berterusan selepas 48 jam berjaya menyahwarnakan sebatian auto-oxidasi berikutan peningkatan peratusan penyahwarnaan (90%). Proses penyahwarnaan diikuti penurunan Keperluan Oksigen Kimia (COD) sebanyak 73% dan peningkatan konsentrasi bakteria sebanyak 94% berdasarkan ketumpatan optik (Abs_{600nm}).. Bacaan pH larutan AO7 (pH 6.6) menurun ke pH 4.5 semasa penyahwarnaan fakultatif anaerobik dan meningkat ke pH 7.7 pada peringkat akhir rawatan aerobik. Degradasi AO7 ditentukan berdasarkan spektroskopi UV-Vis dan analisi FTIR. Malahan, pembentukan sebatian auto-oxidasi; 1,2-naphtholquinone dan 1,4-benzoquinone dikesan menggunakan analisi HPLC. Ujian fitotoksisitas menggunakan *Cucumis sativus* membuktikan larutan AO7 telah dinyahtoksikan selepas rawatan dengan BAC-ZS. Quantifikasi BAC-ZS menggunakan real-time polymerase chain reaction (RT-PCR) menunjukkan *E. faecalis* strain ZL adalah bakteria dominan dalam BAC-ZS dan kekal sebagai spesies dominan sepanjang proses degradasi AO7. Anotasi draf genom bakteria menunjukkan kehadiran gen-gen pengekodan enzim azoreductase, dioxygenase dan monooxygenase yang penting untuk mineralisasi AO7. Kesimpulannya, kultur campuran BAC-ZS mempunyai potensi yang baik untuk digunakan dalam rawatan biologi kumbahan textil.

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LIST OF SYMBOLS

% v/v	-	percentage volume per volume
% w/v	-	percentage weight per volume
°C	-	celcius
µl	-	microlitre
µM	-	micromolar
∞	-	infinity
Ct	-	threshold cycle
Da	-	dalton
g	-	gram
g/L	-	gram per litre
Kb	-	kilo base
kPa	-	kilopascal
L	-	litre
mg/L	-	milligram per litre
min	-	minutes
ml	-	millilitre
mM	-	milimolar
mm	-	milimeter
ng	-	nanogram
ng/L	-	nanogram per litre
nm	-	nanometer
pg	-	picogram
pM	-	picamolar
ppm	-	part per million
rpm	-	rotation per minute

sec	-	seconds
Tm	-	melting temperature
V	-	volt

LIST OF ABBREVIATIONS

2-ABS	-	2-aminobenzenesulfonic acid
4-ABS	-	4-aminobenzenesulfonic acid
AO7	-	Acid Orange 7
BLASTn	-	Basic Local Alignment Search Tool - nucleotide
BLASTp	-	Basic Local Alignment Search Tool - protein
bp	-	basepair
COD	-	Chemical Oxygen Demand
dH_2O	-	distilled water
DNS	-	3,5-Dinitrosalicylic acid
EC	-	Enzyme code
EDTA	-	Ethylenediaminetetraacetic acid
FADH	-	Flavin adenine dinucleotide
FISH	-	Fluorescence *in situ* hybridisation
FMN	-	Flavin mononucleotide
FTIR	-	Fourier transform infrared spectroscopy
GO	-	gene ontology
GST	-	gluthione S-transferase
HPLC	-	High-performance liquid chromatography
KEGG	-	Kyoto Encyclopedia of Genes and Genomes
NADH	-	Nicotinamide adenine dinucleotide
NADH-DCIP	-	Nicotinamide adenine dinucleotide-2,6 dichlorophenol indophenol
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NaOH	-	Sodium hydroxide
NCBI	-	National Center for Biotechnology Information

OD	-	Optical density
OFAT	-	One-Factor-A-Time
PCR-DGGE	-	Polymerase chain reaction-denaturing gradient gel electrophoresis
PCR-SSCP	-	Polymerase chain reaction-single stranded conformation polymorphism
QRT-PCR	-	Quantitative real time polymerase chain reaction
Rnase	-	Ribonuclease
rRNA	-	Ribosomal ribonucleic acid
SD	-	Standard deviation
SMILES	-	Simplified Molecular-Input Line-Entry System
SOD	-	Superoxide dismutase
TAE	-	Tris-acetate-EDTA
UM-BDD	-	University of Minnesota-biocatalysis and biodegradation database
UV	-	Ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 Background of the problem

Synthetic dyes such as azo dye are extensively used in the food, pharmaceutical, textile, cosmetics and paper industries because of their colour varieties and fastness in production as compared to natural dyes (Chang and Lin, 2001; Carneiro *et al*., 2007). Among the total synthetic dyes produced annually, around 60 – 70% consist of azo dyes (Mc Mullan, 2001). Azo dyes that are characterised by double bonds (-N=N-) are the largest and most versatile class of dyes and are the most common chromophore in azo dyes (Stolz, 2001). Most of the azo dyes released into environment originate from the textile and the dyestuff manufacturing industries (Carliell *et al.*, 1995). Pearce *et al.* (2003) explained during dye processing approach, about 2 % of dyes are directly dicharged into aqueus effluent. Additionally, an estimation of 2 – 50% of various applied dyes can be lost in the effluent during textile colouring processes (Easton, 1995).

The azo dyes that dissolve in water can cause water stream to become highly coloured even at low concentrations. Dye concentration as low as 0.005 ppm is visible in clear river water (Banat *et al.*, 1996) whereas according to O'Neill *et al.*

(1999), textile processing wastewater usually contains between 10 – 200 mg/L dye concentration. Once the intense coloured wastewater flow into the river as seen in Figure 1.1, the colour will influence the absorbance of light entering the water thus, greatly reduces photosynthesis of aquatic flora (Slokar *et al.*, 1998).

> JOHOR

The ever-changing colours of a river

Raban showing the colour of the water in the Sungai Perling tributary. — NST picture by Roslan Khamis

JOHOR BARU: The water in the Sungai Perling tributary here changes from scarlet to blue and on some days, it is tinted gold.

But to residents of Taman Johor Indah, it is a common occurrence.

"The water looks like *teh tarik* sometimes, but on most days it is dark red," said Mahmood Samiran, 41, a resident of the Seri Indah flats nearby.

Mahmood, who highlighted the matter to the *New Straits Times* yesterday, said he had reported this to the state Department of Environment (DOE) twice.

"I was told that the matter was not under the jurisdiction of the DOE by one of the officers," he added.

"Numerous 'Love Your River' campaigns have been held but when we want to show our love, the relevant authorities tell us they cannot do anything about it," said Mahmood, who together with a friend, Raban Daing Pelalau, 51, have tried to trace the source of the pollution by heading upstream.

Raban Daing, a taxi driver, said they suspected a cottage industry in Taman Johor may be the culprit.

"However, we would like the DOE to check on the factory and maybe collect water samples before the coloured water pollutes the Tebrau Straits, which Sungai Perling flows into," he added.

The DOE could not be reached for comment.

Figure 1.1: A newspaper article from News Strait Times shows one of the polluted rivers in Malaysia due to textile wastewater

Kodam *et al.* (2006) mentioned most of the azo dyes are either inert or non-toxic, but become toxic, mutagenic and carcinogenic upon their biotransformation. The azoreductases in intestinal bacteria, liver cells and skin surfaces micro flora would reduce azo bonds in azo dyes to colourless aromatic amines (Xu *et al.*, 2007). Several researchers have done studies related to risk assessment of aromatic amines and they have proven carcinogenicity of aromatic amines to the human body (Hildenbrand *et al.*, 1999).

Considering both environmental pollution and serious health-risk factors caused by azo dyes, finding alternative ways to treat synthetic dyes are mandatory particularly for small scale textile industries. This is because in reality, the textile effluents were directly channelled into the main streams of water resources without proper treatment system (Moosvi *et al.*, 2005). This is a major environmental issue as most of the textile dyeing and processing industries are located in developing countries whereby rivers are the main source of drinking water and daily activities. In India for instance, an average mill discharges is about 15 milion litres of contaminated effluent per day that causes chronic and acute toxicity (Dave *et al.*, 2009).

While in Malaysia, there are more than 200 textiles factories with majority consisting of small scale textile industries. Textile industry is the major source of wastewater and accounts for 22 % of the total volume of industrial wastewater produced in Malaysia (Idris *et al.*, 2007). Most of these textile industries use conventional treatment methods to treat the textile effluents. The conventional methods are effective in removing the fiber and to reduce COD reading of the wastewater however, it is not effective to treat the colour of the wastewater as the presence of sulpho and azo groups in the azo dyes, make the dyes xenobiotic and recalcitrant to oxidative biodegradation (Killa *et al.*, 1983). As a result, the treated textiles wastewater remains highly coloured as seen in Figure 1.2.

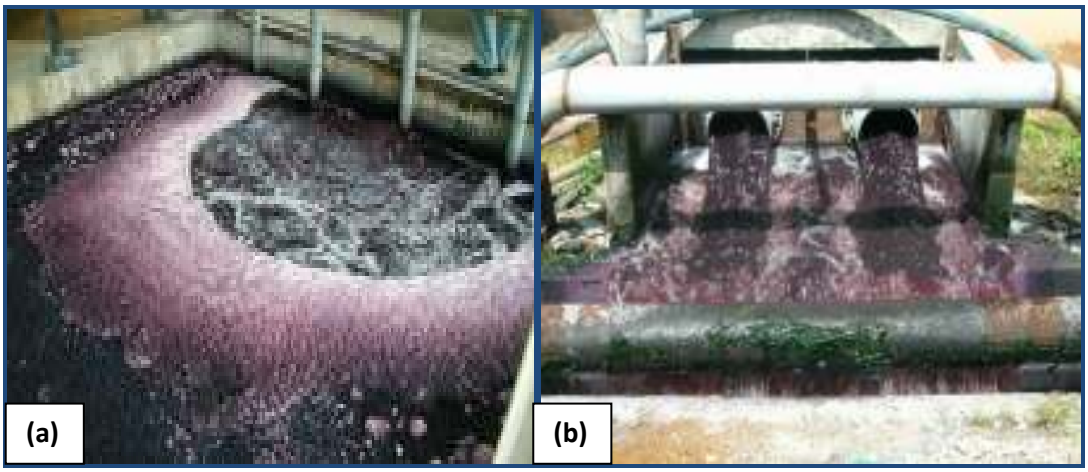

Figure 1.2: Pictures taken in one of the local textiles factories in Malaysia. (a) Textiles wastewater in treatment tank. (b) Treated textiles wastewater discarded to drain

1.2 Problem statement

Several physical and chemical techniques were tested to be effective in dye removal such as physical adsorption using activated carbon (Choi *et al.*, 2008), ozonation (Gharbani *et al.*, 2008), electrolytic treatment using a three dimensional electrode reactor (Xu *et al.*, 2008) as well as Fenton-like reaction (Rahmani *et al.*, 2010). The use of physico-chemical methods however, have their shortcomings due to excess amount of chemical usage, excessive sludge generation with obvious disposal problems, costly plant requirements or operating expenses and lack of effective colour reduction, particularly for sulfonated azo dyes (Banat *et al.*,1996).

The bioremediation using effective microbial to decolourise and degrade dyes used in textile industry is an environmental-friendly and cost-effective method compared to the physical and chemical decomposition processes. The successful application of bioremediation processes are very dependent on the microorganisms

exploited in the systems. In recent years, attention has been focused on fundamental work in revealing the existence of wide range of microorganisms capable of decolourising a wide range of dyes (Moosvi *et al.*, 2005, 2007; Dafale *et al.*, 2008; Telke at al., 2010). Practically, it has been reported that mixed cultures are useful in decolourisation of synthetic dyes, as some microbial consortia can achieve the biodegradation tasks that no individual pure strain can undertake successfully (Nigam *et al.*, 1996).

Among the biological methods that have been introduced, sequential anaerobic-aerobic treatment system was considered the most reliable method to treat textile wastewater that consists of various dyes (Khan and Banarjee, 2010). Successful application of mixed bacteria cultures in decolourising textile dyes in the sequential anaerobic-aerobic treatment system had been well reported (O'neill *et al.*, 200; Oh *et al.*, 2004; Ong *et al.*, 2005). Although being a reliable system, this method still has a major drawback; it is not effective in degrading sulphonated azo dyes. The sulphonated azo dyes could be decolourised under anaerobic phase however, once in contact with air under aerobic phase, the colourless aromatic compounds are fast auto-oxidised to form dark and recalcitrant by-products (Supaka *et al.*, 2004; Mendez-Paz *et al.* 2005, 2005a). The formation of dark solution is non-reversible (Coughlin *et al.*, 2002; Wang *et al.*, 2012).

Therefore, developing a mixed bacterial culture with ability to decolourise sulphonated azo dyes as well as to overcome formation of dark solution during auto-oxidation process in a sequential anerobic-aerobic treatment system is essential. For this reason, three objectives were outlined for depth investigation of the mixed bacteria culture as an alternative approach for decolourisation and degradation of sulphonated textile dyes.

1.3 Objectives of study

(1) To isolate and develop a mixed bacterial culture for decolourisation of azo dye
(2) To evaluate the efficiency of acclimatised mixed bacterial culture for dye degradation using sequential facultative anaerobic-aerobic treatment system
(3) To monitor population dynamics of acclimatised mixed bacterial culture using real-time polymerase chain reaction (RT-PCR).

1.4 Scope of the study

The present study was focused on the development of a mixed bacterial culture with potential use for the decolourisation of textile dyes in a sequential anaerobic-aerobic system. Acid Orange 7(AO7) has wide application in textiles, cosmetics and leather tanning industries. Being a monosulphonated dye, degradation of AO7 in a sequential anaerobic-aerobic treatment system would form dark browning solution at the end of treatment therefore; this dye was used as the model azo dye in this study. The mixed bacterial culture was formed using two bacteria screened and isolated from textile wastewater together with *E. faecalis* strain ZL that earlier has been proven to decolourise the AO7 dye. The mixed bacterial culture was acclimatised in AO7 decolourised solution prior used for treatment and named as BAC-ZS. Optimisation of co-substrate was investigated to increase the decolourisation efficiency. The decolourisation and degradation of AO7 was performed in sequential facultative anaerobic-aerobic system. In general, the degradation performances were observed based on the colour removal, the COD reduction, pH changes and indirect measurement of bacteria concentration during the treatment process. The efficiency of the BAC-ZS to mineralise AO7 was tested by investigating the dye solution before and after treatment using HPLC and FTIR analyses as well as the phytotoxicity test. Further, species-specific primers were

designed and used in determining the population dynamic of BAC-ZS during the dye treatment process via RT-PCR.

solution. The use of microorganisms to degrade polymerised compounds has not been reported before and is worthy for further investigation to elucidate degradation pathway associated with the BAC-ZS mixed bacterial culture.

ii. The potential application of BAC-ZS mixed culture can be extended to include real textile waste water using the sequential anaerobic-aerobic bioreactor.

iii. With the advance of bioinformatics, the information derived from the annotated draft genomes of each bacterium can be further exploited to treat other types of pollutants.

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