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MICROBIAL DECOLORIZATION OF TRIAZO DYE, DIRECT BLUE 71 BY MIXED BACTERIAL CULTURE ISOLATED FROM MALAYSIAN SOIL

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By

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Polluted wastewater from textile dyeing industrial sectors has causes severe effect towards human, plant and marine creatures and the treatment process is truly challenging. In this study, a mixed bacterial culture was isolated from Malaysian soil, screened and identified to successfully decolorize Triazo-bond Direct Blue 71 dye and act as the sole source of carbon and nitrogen. The free cells were also immobilized for better decolorization at higher concentration of dye by using sodium alginate. Response surface methodology (RSM) and Artificial Neural Network (ANN) were used to optimize the decolorization efficiency for both free and immobilized cells. Significant effect on DB71 dye decolorization percentage by free cells is denoted by the experimental variables of dye concentration, yeast extract, and pH. The optimum conditions for dye decolorization by immobilized mixed culture were determined by four variables which were dye concentration, alginate concentration, number of beads and beads size. GCMS and FTIR analysis were used to characterize the metabolites after the decolorization. Other than that, kinetics modelling study of DB71 dye decolorization allowed the estimation of decolorization rate of free and immobilized cells. Major bacterial group found from the metagenomics analysis consist of *Acinetobacter* (30%), *Comamonas* (11%), Aeromonadaceae (10%), *Pseudomonas* (10%), *Flavobacterium* (8%), Porphyromonadaceae (6%), and Enterobacteriaceae (4%). Proteobacteria (78.61%), then Bacteroidetes (14.48%) and Firmicutes (3.08%) were among the richest phylum in the mixed bacterial culture. The optimum condition for free cells predicted by RSM is at 150 mg/L of dye concentration, 3 g/L of yeast extract and pH of 6.645. ANN predicted the optimum condition at 150 mg/L, 2.9 g/L of yeast extract and pH of 6.7. Higher prediction and accuracy in the fitness was found in ANN model as proved by R^2 and AAD values of 0.99 and 0.04 subsequently fitness compared to the RSM. ANN model for immobilized cells offered a better prediction than RSM with R^2 of 0.99. The ANN model predict

the decolorization by immobilized cell is optimum at 200 mg/L, 0.966 % of alginate concentration, 50 number of beads and 0.599 cm of beads size. Moreover, the result from GCMS and FTIR analysis of the metabolites from the decolorization of dye shows that the reduction of dye caused the absence of the untreated sample and emergence of new peaks in the treated sample in FTIR spectrum. In addition, GCMS result from the treated sample shows no toxic secondary metabolites were formed.

Luong model predicted the rate of decolorization by free cell at 10 $\%hr^{-1}$ by using the kinetic modelling and dye concentration at 159.5 mg/L completely inhibited the decolorization based on the S_m value. Aiba model predicted the rate of decolorization by immobilized cell is at 4.645 $\%hr^{-1}$. The use of mixed bacterial culture was found to be efficient for the decolorization of DB71 dye in this study. The optimization of immobilized cell by using RSM and ANN using sodium alginate resulted to better decolorization of dye at higher concentration which is up to 200 mg/L. Moreover, the effect of metal ions towards the decolorization shows that gel beads through immobilization were able to protect against toxic substance. It is reflected by a great tolerance result towards metal ions over free cells during DB71 dye decolorization where occurrence of metal ions may disrupt the decolorization process. The decolorization of Direct Blue 71 dye by immobilized cell was still higher than 90 % even with the presence of 1 mg/L of mercury, nickel, copper, lead, arsenic, chromium, cadmium and silver in the solution and slight decrease of decolorization was observed for both free and immobilized cell compared to the control samples. In conclusion, all the objectives of this study were achieved accordingly.

Environmental pollution caused by the released of industrial effluent containing dye has been affecting the water quality in Malaysia. Biological practice using microorganisms provides a complete degradation with no secondary pollutant besides the cost effective advantage compared to the physical and chemical wastewater treatment. There are few reported works on azo dye decolorization by mixed bacterial culture without the aid of carbon and nitrogen source. Therefore, this study found and optimized a potent mixed bacterial culture that could degrade Direct Blue 71 dye in facultative anaerobic condition and no added carbon and nitrogen sources are needed to completely decolorize the dye with no introduction of secondary toxic metabolites based on the metabolites analysis result.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Master Sains

DEKOLORASI MIKROB UNTUK PEWARNA “TRIAZO”, “DIRECT BLUE 71” OLEH KULTUR BAKTERIA CAMPURAN YANG DIASINGKAN DARIPADA TANAH MALAYSIA

Oleh

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Air buangan yang tercemar dari sektor industri pencelupan tekstil menyebabkan kesan yang teruk terhadap makhluk hidup manusia, tumbuhan dan laut serta proses rawatannya sangat mencabar. Dalam kajian ini, kultur bakteria campuran diasingkan dari tanah Malaysia, disaring dan dikenal pasti untuk berjaya menyahwarna pewarna Triazo Direct Blue 71 (DB71) dan bertindak sebagai satu-satunya sumber karbon dan nitrogen. Sel bebas juga telah menjadi tidak bergerak untuk penyahwarnaan yang lebih baik pada kepekatan pewarna yang kebih tinggi dengan menggunakan natrium alginat. Response Surface Methodology (RSM) dan Artificial Neural Network (ANN) digunakan untuk mengoptimumkan kecekapan dekolorasi. Kesan yang signifikan terhdadap peratusan dekolorasi pewarna DB71 adalah daripada pemboleh ubah eksperimen kepekatan pewarna, ekstrak ragi, dan pH. Keadaan optimum untuk penyahwarnaan pewarna oleh kultur campuran tidak bergerak ditentukan oleh empat pemboleh ubah iaitu kepekatan pewarna, kepekatan alginat, bilangan manik dan ukuran manik. Analisis GCMS dan FTIR digunakan untuk mencirikan metabolit setelah penyahwarnaan. Selain itu, memodelkan kajian kinetik dekolorisasi pewarna DB71 memungkinkan pengiraan kadar dekolorasi sel bebas dan tidak bergerak. Kumpulan bakteria utama yang didapati dari analisis metagenomik terdiri daripada *Acinetobacter* (30%), *Comamonas* (11%), Aeromonadaceae (10%), *Pseudomonas* (10%), *Flavobacterium* (8%), Porphyromonadaceae (6%), dan Enterobacteriaceae (4%). Proteobacteria (78.61%), kemudian Bacteroidetes (14.48%) dan Firmicutes (3.08%) adalah antara filum yang terkaya di dalam bakteria campuran itu. Keadaan optimum untuk sel bebas yang diramalkan oleh RSM ialah kepekatan pewarna 150 mg/L, 3 g/L ekstrak ragi dan pH 6.645. ANN meramalkan keadaan optimum pada 150 mg/L, 2.9 g/L ekstrak ragi dan pH 6.7. Model ANN mempunyai ramalan dan ketepatan yang lebih tinggi dalam kecekapan berbanding dengan model RSM seperti yang dibuktikan oleh nilai

R^2 dan AAD yang masing-masingnya adalah 0.99 dan 0.04 oleh model ANN. Model ANN untuk sel tidak bergerak memberikan ramalan yang lebih baik daripada RSM dengan R^2 0.99. ANN meramalkan dekolorasi oleh sel tidak bergerak adalah optimum pada 200 mg/L, kepekatan alginate 0.966%, 50 bilangan manik gel dan ukuran manik gel 0.599 cm. Lebih-lebih lagi, hasil analisis metabolit dari dekolorisasi pewarna daripada GCMS dan FTIR menunjukkan bahawa dekolorasi pewarna menyebabkan kemunculan puncak baru pada sampel yang telah dirawat dan ketiadaan puncak itu pada sampel yang tidak dirawat di dalam spektrum FTIR. Sebagai tambahan, hasil GCMS dari sampel yang dirawat menunjukkan tidak ada metabolit sekunder beracun yang terbentuk.

Model Luong meramalkan kadar dekolorasi oleh sel bebas pada 10% jam^{-1} dan kepekatan pewarna pada 159.5 mg/L adalah penghalang dekolorasi berdasarkan nilai S_m. Model Aiba meramalkan kadar dekolorasi oleh sel yang tidak bergerak adalah pada 4.645% jam^{-1}. Hasil ini menunjukkan dekolorasi pewarna DB71 oleh kultur bakteria campuran yang efisien. Pengoptimuman sel yang tidak bergerak dengan menggunakan RSM dan ANN menggunakan natrium alginat menghasilkan penyahwarnaan pewarna yang lebih baik pada kepekatan yang lebih tinggi sehingga 200 mg/L. Lebih-lebih lagi, kesan ion logam terhadap dekolorasi menunjukkan bahawa manik gel melalui imobilisasi dapat melindungi daripada bahan toksik. Ini dicerminkan oleh hasil toleransi yang tinggi terhadap ion logam berbanding sel bebas semasa penyahwarnaan pewarna DB71 kerana ion logam boleh mengganggu proses dekolorisasi. Dekolorasi pewarna DB71 oleh sel yang tidak bergerak adalah masih lebih tinggi daripada 90% walaupun dengan adanya 1 mg/L merkuri, nikel, tembaga, plumbum, arsenic, kromium, cadmium dan perak dalam larutan dan sedikit penurunan dekolorisasi diperhatikan untuk sel bebas dan tidak bergerak berbanding dengan sampel kawalan. Kesimpulannya, semua objektif kajian ini dicapai dengan sewajarnya.

Pencemaran alam sekitar yang disebabkan oleh pelepasan bahan buangan industri yang mengandungi pewarna telah mempengaruhi kualiti air di Malaysia. Amalan biologi menggunakan mikroorganisma memberikan degradasi lengkap tanpa pencemaran sekunder selain kelebihan kos efektif berbanding dengan rawatan air sisa fizikal dan kimia. Terdapat hanya sedikit kajian yang dilaporkan mengenai dekolorasi pewarna azo oleh kultur bakteria campuran tanpa bantuan sumber karbon dan nitrogen. Oleh itu, kajian ini mendapatkan dan mengoptimumkan kultur bakteria campuran yang kuat yang dapat menurunkan pewarna Direct Blue 71 dalam keadaan anaerob fakultatif dan tidak memerlukan sumber karbon dan nitrogen untuk dekolorasi sepenuhnya pewarna tanpa pengenalan metabolit toksik sekunder berdasarkan hasil analisis metabolit.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

DB71	Direct Blue 71
RSM	Response Surface Methodology
ANN	Artificial Neural Network
AAD	Absolute Average Deviation
PDW	Printing and Dyeing Wastewater
MSM	Minimal Salt Media
DNA	Deoxyribonucleic acid
ANOVA	Analysis of Variance
GPS	Global Positioning System
MNFF	Multilayer Normal Feed Forward
BBP	Batch Back Propagation
RMSE	Root Mean Square Error
3D	3 Dimensional
NADH	electron carrier
R^2	coefficient of determination
rpm	revolutions per minute
yi, exp	experimental responses
yi, cal	measured responses
p	number of experimental runs
n	number of the experimental data
% w/v	percentage weight per volume
p-value	probability value
Tanh	hyperbolic tangent
GCMS	Gas Chromatography Mass Spectrometry

FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
K_2HPO_4	Dipotassium phosphate
KBr	Potassium bromide
KH_2PO_4	Potassium dihydrogen phosphate
As	Arsenic
Pb	Lead
Zn	Zinc
Cr	Chromium
Cd	Cadmium
Co	Cobalt
Cu	Copper
Ni	Nickel
Ag	Silver
Hg	Mercury
DNA	Deoxyribonucleic acid
$CaCl_2$	Calcium chloride
Mn	Manganese
$MnSO_4.H_2O$	Manganese sulphate
MSM	Minimal salt media
Na_2SO_4	Sodium sulphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaM_0O_4	Sodium molybdate
$(NH_4)_2SO_4$	Ammonium sulphate

mg/L	milligram per liter
SEM	Scanning Electron Microscope
w/v	weight per volume

CHAPTER 1

INTRODUCTION

A massive amount of dye effluent was produced by large textile industries during the application of dye for the making of their end product. Approximately two-thirds of the total quantity of dye were constituted to textile effluent. The high concentration of color and a huge variability form of dyes were the issues in handling the textile effluent properly. The coloration process ultimately shed 10% of the dye and was discharged right away into the aqueous effluent for about 2% (K. Singh & Arora, 2011). Moreover, approximately 70% of azo dye that consists of one or more -N=N- double bond was involved in dye formation (Xie et al., 2016).

No exact figure on the production of dyes in the world was found in a year, however, about 1000 000 tons of dyes has been produced as reported from previous study (Gupta, 2009). Batik industry in Malaysia has been the source of economic growth which become the ninth in Asia region for biggest producer of textile fiber in 2008 and fifteenth in worldwide. This industry contribute to 2.3% of total exports of manufactured goods in 2011, therefore, contributed much on the decrease of environmental quality especially in East Coast of Peninsular Malaysia (Tiong, 2015).

Polluted rivers has been increasing through the year of 2006 until 2010 due to the manufacturing industry especially textile industries that contributed about 22% of total industrial wastewater in Malaysia especially in Pulau Pinang, Selangor and Johor states (Pang & Abdullah, 2013). This is due to the huge amount of textile finishing plants located in this states. Malaysia has no centralized treatment system and individual factory needs to treat their own wastewater, however, some of them still failed to meet the discharge quality standards based on the Environmental Quality Act 1974 due to high treatment cost and lack of awareness among small and medium scale factories. Textile waste in Malaysia contributed to four percent of total solid waste in 2013, approximately two million kilogrammes of textile waste per day due to the fast fashion industry . Direct Blue 71 has been extensively used for textile dyeing and 10 to 50 percent of synthetic dye residue end up in the local waterways which remain untreated, became the contributor for water pollution in Malaysia(Tiong, 2015) .

The root causes of smelly water, turbidity and awful appearance are the colloidal matter and oily impurities that present along with the dye. Consequently, penetration of sunlight was being obstructed and eventually interrupted the photosynthesis process (Muthu, 2014). The major concern involving the effect of textile waste towards marine life is the deficiency of dissolved oxygen and ultimately, the self-purification process of water was being halted (Kant, 2012). Besides that, clogged pores of soil was observed as fields were filled with these effluents and causing the dropped in soil efficiency. This is due to the penetration of roots being stagnated as a result of solidified soil. The quality of drinking water that is unfit for human uptake was coming from the corrosion of sewerage tubes due to the wastewater that flows in the drain (Kant, 2012).

Recalcitrant residual leads to environmental pollution that are complex and hazardous due to a lot of water and chemicals used for the procedures in staining for textile industry (Dasgupta et al., 2015). The textile effluent needs specified treatment mainly because dyes, along with chemicals, pigments and high chemical oxygen demand were used in coloration (Lokesh & Sivakiran, 2014). Therefore, the discovery of advanced methods should be done to attenuate the impact of discarding the textile dyes within industrial effluents and causing environmental damage.

Adsorption, flocculation, and photochemical oxidation were some great physical and chemical alternatives for the decolorization of textile wastewater. Previous studies that involved the method of adsorption (Bulut et al., 2007), ultrasound (Tauber et al., 2008), ozonation (Turhan & Turgut, 2009) and Fenton's oxidation(Ertugay & Acar, 2017) have been utilized to deal with Direct Blue 71 dye. However, the primary cons of this solution were the enormous operating expenditure and the introduced of secondary pollutants. These drawbacks of physical and chemical methods can be solved through biological practice that provides easy procedures and economical practices besides that it presently was prevalent in dye treatment method (Schütte et al., 2008). Research on microorganisms were being intensively studied despite the use of physical and chemical methods (Kumaran & Dharani, 2011) such as the utilization of fungi and bacteria for the treatment of dye.

Presently, anaerobic conditions for azo dye degradation were favoured by many of the isolated bacteria (García-Montaño et al., 2008). However, the capability of bacteria was affected by the functional group of the azo dye that created a complex structure. Food chain was badly affected as anoxic-tolerant aromatic amines build up as a result of azo dye decolorization was found to be carcinogenic, toxic and (Dos Santos et al., 2006; Işık & Sponza, 2008). Thereby, discovering the potent azo dye decolorizing bacteria was significant. Complete degradation rather than simply the removal of azo dye colour is the critical element in azo dye removal from the environment (Mohana et al., 2008).

Novel dye decolorizing mixed bacterial culture will be isolated and identified in this study. The significant parameters which will affect the decolorization of DB71 dye will be determined by using Response surface methodology (RSM) and Artificial Neural Network (ANN) along with the optimizing of the decolorization process by free and immobilized mixed culture.

To satisfy the above problem statement, the following objectives are conducted:

1. To isolate, screen and identify using Metagenomic Analysis of the best mixed bacterial culture from Malaysian soils which is able to decolorize Direct Blue 71 Dye
2. To optimize the decolorization of Direct Blue 71 Dye by the chosen mixed bacterial culture using Response Surface Methodology (RSM) and Artificial Neural Network (ANN).
3. To enhance the docolorization of Direct Blue 71 dye through immobilization using sodium alginate and characterize the metabolites from decolorization using Gas Chromatography Mass Spectrometry (GCMS) and Fourier Transform Infrared (FTIR)

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