ISOLATION AND CHARACTERIZATION OF A MIXED BACTERIAL CONSORTIUM ISOLATED FROM THE JURU RIVER, MALAYSIA IN DECOLOURISING AZO DYE (REACTIVE RED 120)

By

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Faculty : Biotechnology and Biomolecular Sciences

The application of microorganisms in Reactive Red 120 (RR120) remediation has gained significant attention. Textile effluents containing RR120 is known for its carcinogenicity and mutagenicity. The major issue in the biodegradation of RR120 by microorganisms is it is very difficult to isolate microorganisms able to utilise the dye as a carbon source as this can completely mineralized the dye. This study investigates and compares the role of methods and media used in obtaining bacterial consortia capable of decolourising RR120 as the sole carbon source, which is extremely rare to find. Three RR120-decolourising consortia were isolated from contaminated water samples from the Juru River, Malaysia. Only consortium JR3 was able to decolourise RR120 as a sole carbon source compared to the rest of the consortium. Based on 16S rRNA gene sequence analysis and biochemical test, consortium JR3 consists of *Pseudomonas aeruginosa* strain MM01, *Enterobacter* sp. strain MM05 and *Serratia marcescens* strain MM06. It was found that a mix of the bacterial consortium JR3 was able to decolourise RR120 much faster compared to single strains of MM01, MM05 and MM06.

Initially, consortium JR3 was able to decolourise 42.5% of 50 ppm RR120 within 24 h of incubation. Using one-factor-at-time optimisation processes, the consortium JR3 was enhanced to decolourise 88.2% of 50 ppm RR120 within 24 h. The result illustrates that yeast extract at 0.7 g/L, ammonium sulphate at 0.75 g/L, phosphate buffer with pH 8, the temperature at 35°C and RR120 concentration at 200 ppm as the optimum decolourisation conditions required by consortium JR3. Meanwhile, based on statistical optimisation using Response surface methodology (RSM), ammonium sulphate 0.645 g/L, pH 8.293, 200.1 ppm of RR120, temp 34.53°C results in a decolourisation rate of 93.34% at 48 h. Discriminatory statistical analysis in modelling studies illustrates that the best

primary model was the modified Gompertz model, while the secondary model was best suited by Aiba.

The ability of consortium JR3 to decolourise RR120 under the presence of heavy metals such as silver, arsenic, cadmium, chromium, copper, mercury, lead, and zinc were also investigated in this study. It was found that chromium had the least effect on RR120 decolourisation followed by zinc and lead. Meanwhile, 1 ppm mercury has the highest inhibitory effect on consortium JR3, therefore, reducing decolourisation of 200 ppm RR120 by 32.5%. The consortium was able to tolerate up to 10 ppm of chromium and 1 ppm of mercury. The decolourised RR120 product showed less inhibition effect on *Vigna radiata*'s seed germination compared to the parent compound, suggesting that RR120 toxicity has been reduced. RR120 at the lowest concentration of 25 ppm reduced seed germination by 17.7%, shoot length by 1.13 cm and root length by 1.43 cm. The decolourised product of 25 ppm RR120 illustrated no significant difference ($p>0.05$) to control.

In conclusion, the consortium JR3 has the potential to be used in the management of RR120 contamination in the environment. Consortium JR3 was not only able to decolourise at high concentrations of 500 ppm RR120, but the end product is safer compared to the parent compound alone in *Vigna radiata* toxicity studies

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Doktor Falsafah

PENGASINGAN DAN PENYAHWARNAAN PEWARNA REAKTIF MERAH 120 OLEH KONSORTIUM BAKTERI YANG BERCAMPURAN DARI SUNGAI JURU, MALAYSIA

Oleh

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Penggunaan pewarna amat penting dalam mana-mana organisasi, terutamanya di dalam industri yang melibatkan penghasilan makanan dan pakaian. Disebalik pengguna dalam industri pembuatan, pembuangan limbah yang tidak dirawat ke sungai dan saluran air menyebabkan kebimbangan terhadap kesihatan kita akibat daripada kesan toksik, karsinogenisiti dan mutagenisiti pewarna ini. Oleh itu, pemerhatian harus wajar diberikan kepada isu, terutamanya semasa merawat sisa-sisa air pewarna. Kebolehan mikroorganisma untuk menyahtosikkan pewarna telah lama diketahui dan penggunaan mikroorganisma ini di dalam teknologi berasakan bioremediasi amat dialu-alukan kerana penglibatan kos yang murah. Objektif utama kajian ini adalah untuk memencilkan bakteria yang mempunyai kemampuan menggunakan pewarna Reaktif Merah 120 (RR120) dan mengoptimumkan keadaan biodegradasinya. Tiga konsortia kebolehan menggunakan RR120 berjaya dipencilkan daripada tempat tercemar di Sungai Juru, Malaysia. Konsortia ini dinamakan sebagai konsortium JR1, JR2 dan JR3 berdasarkan kaedah. Hanya konsortium JR3 mempunyai keupayaan untuk menggunakan RR120 sebagai sumber karbon dalam proses percambahan mikroorganisma. Berdasarkan gen 16S rDNA dan ujian biokimia, konsortium JR3 terdiri dari *Pseudomonas aeruginosa* strain MM01, *Enterobacter* sp. strain MM05 dan *Serratia marcescens* strain MM06. Didapati bahawa campuran ketiga-tiga strain ini mampu menguraikan RR120 dengan kadar yang baik dan cepat berbanding dengan strain tunggal.

Faktor-faktor yang mempengaruhi penyahwarnaan RR120 seperti kepekatan ekstrak ragi, jenis dan kepekatan ekstrak ragi, penimbal pH yang bertindih, suhu dan kepekatan RR120 dioptimumkan menggunakan satu faktor pada satu masa (OFAT) dan kaedah tindak balas permukaan (RSM). Asalnya, konsortium JR3 berupaya menyahwarnakan 50 bahagian per juta (bpj) RR120 sebanyak 42.5%

dalam masa 24 jam selepas inkubasi. Dengan menggunakan proses pengoptimumkan ini, kebolehan konsortium JR3 menyahwarna 50 bpj RR120 dapat ditingkatkan kepada 88.2%. Melalui proses OFAT, kepekatan amonium sulfat dalam 0.7 g/L, pH 8, suhu 35°C dan kepekatan RR120 dalam 200 bpj, merupakan keadaan penguraian RR120 paling optimum untuk konsortia JR3. Manakala, berdasarkan pengoptimuman RSM, kepekatan amonium sulfat dalam 0.645 g/L, pH 8.293, 200.1 ppm RR120 dan suhu kadar 34.53°C dapat meningkatkan kadar penguraian RR120 kepada 93.34% dalam massa 24 jam.

Penggunaan model sekunder menunjukkan model Aiba adalah yang terbaik. Keupayaan konsortium JR3 untuk menguraikan RR120 dengan kehadiran logam berat seperti perak, arsenik, kadmium, kromium, tembaga, merkuri, plumbum dan zink juga telah dikaji dalam kajian ini. Didapati bahawa 1 bahagian per juta (bpj) kromium mempunyai pengaruh paling sedikit terhadap penguraian RR120 dan diikuti zink dan plumbum. Manakala, merkuri mempunyai kesan rencatan yang tertinggi pada konsortium JR3 dengan mengurangkan penguraian RR120 sebanyak 32.5%. Tambahan pula, konsortium JR3 adalah toleran sehingga 10 bpj kromium dan 0.1 bpj merkuri.

Ketoksikan pewarna RR120 dan produk metabolit biodegradasi disiasat menggunakan biji kacang hijau (*Vigna radiata*). Produk biodegradasi menunjukkan kesan penghambatan yang lebih rendah terhadap percambahan biji *Vigna radiata* berbanding pewarna RR120. Ini menunjukkan bahawa produk metabolit yang terhasil semasa penyahwarnaan RR120 oleh konsortium RR120 adalah selamat. Kepekatan 25 bpj RR120 telah mengurangkan kadar pencambahan biji *Vigna radiata* sebanyak 17.7%, panjang pucuk sebanyak 1.13 cm and panjang akar sebanyak 1.43 cm. Produk metabolit penguraian 25 ppm RR120 menunjukkan tiada perbezaan ketara ($p>0.05$) dengan kawalan. Kesimpulannya, penemuan mikroorganisma yang dapat menggunakan pewarna akan menjadi kunci bioremediasi yang penting dalam mengawal tahap pencemaran pewarna dalam sumber air. Konsortium ini bukan sahaja dapat menguraikan pewarna RR120, tetapi produk metabolit yang terhasil adalah lebih selamat berbanding sebatian asal. Toleransi consortium JR3 terhadap logam berat merupakan nilai tambahan dalam aplikasi bioremediasi.

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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 of Doctor of Philosophy of Science. The members of the Supervisory Committee were as follows:

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%	Percent
<	Less than
>	Greater than
Abs	Absorbance
AMPA	Aminomethylphosphonic acid
ANOVA	The analysis of variance
CCD	Central composite design
cm	Centimeter
Co	Cobalt
Cr	Chromium
Cu	Copper
Da	Dalton
dH_2O	Distilled water
EDTA	Ethylene diamine tetra acetic acid
et al	and others
EC	Half maximal effective concentration
EPSPS	5-enolpyruvyl-shikimate-3-phosphate-synthase
Fe	Iron
FFD	Fractional factorial design
G	Gram
h	Hour
IPA	Isopropyl amine
Hg	Mercury
kb	Kilo base

kDa	Kilo Dalton
kg	Kilogram
L	Liter
LC	Lethal concentration
M	Molar
min	Min
mL	Milliliter
MSM	Minimal salt medium
MW	Molecular weight
NA	Nutrient agar
N.A.	Not available
ºC	Degree Celsius
OD	Optical density
OFAT	One-factor-at-a-time
POEA	Polyethoxylated tallow amine
PB	Plackett-Burman
RPM	Revolutions per min
RSM	Response surface method
RR120	Reactive Red 120
RR10	Reactive Red 10
RB 15	Reactive Black 15
Mo	Molybedum
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
µL	Microliter

µm	Micrometer
µM	Micromolar

CHAPTER 1

INTRODUCTION

There may be a preamble at the beginning of a chapter. The purpose may be to introduce the themes of the main headings. Azo dyes account for 70% of the 9.9 million tons of industrial dye used annually with a global turnover valued at USD 30.42 billion (Balapure et al., 2015; Gürses et al., 2016). The continual demand for dyes and pigments causes an increase in the supply rate to about 3.5% per annum (Pang & Abdullah, 2013). Most of the dyes synthesised contain azo compounds and are predominantly used in textile, paper, food, printing, cosmetic and leather industries (Benkhaya et al., 2020). These azo dyes are extensity used in fabric manufacturing as dyes are low in cost, ease of preparation, fastness, versatility and intensity of the colours (Rawat et al., 2016). Certain azo dyes contain chemical groups which have a high affinity for metal ions (Hussain et al., 2016). Chromium and copper are the most common metals used in these dyes, as metal ions provide better binding with fibre, improving the resistance of the dye to washing (Benkhaya et al., 2020; Xu et al., 2018; Bhatia et al., 2017). These enhanced properties provide a high degree of chemical, biological, and photocatalytic stability. Nevertheless, their resistance to breakdown for long a period of time, exposure to sunlight, detergents, water and microorganism results in poor degradation in the environment (Solís et al., 2012).

Pollution and poisoning by azo dyes still happen to this day. With the ever-increasing cancer numbers, dye plays important role in contributing it as breakdown product produces toxic amines and benzenes (Oliveira et al., 2016; Bharagava et al., 2018; Joshi & Katti, 2018; Igiri et al., 2018). These toxic metabolites easily get into us by consuming water and fish exposed to dye wastes. Discharge of untreated textile waste into nearby streams and rivers can cause anoxic conditions that are lethal to the aquatic organisms (Balakrishnan et al., 2016). Particularly, 10-15% of reactive dyes are measured to be hazardous xenobiotic concentrations in water (Selvaraj et. al. 2020). For example, the contamination of disperse orange 37 has been reported in the Khniss river with a concentration of 6.438 µg/L, while disperse Red 1 and Yellow 3 were detected at 3.873 µg/L and 1895 µg/L, respectively, in the Hamdoun river, Tunisia (Methneni et. al. 2021). Meanwhile, Ito et. al. (2016) reported a gradual decrease of 254 mg-dye day−1 g-volatile total solids to 120 mg-dye day−1 g-volatile total solids at the Yabagawa river over a 5-month period even after the dye factory was closed illustrating that dye breakdown is a slow process.

Therefore, chemical treatment of the effluents is often employed to treat the waste. These include various physical and chemical methods that have been used for the removal of azo dyes from wastewater (Khouni et al., 2011). However, only big industries such as Adidas, Nike, Polo etc. utilises this approach as it involves a huge cost. Small scaled manufacturers tend to dispose of it away without any treatment (Ayed et al., 2010).

Chemical processes such as oxidative process, H_2O_2-Fe(II) salts (Fenton's reagent), ozonation, photochemical, cucurbituril, and electrochemical destruction are examples used for decolourisation purposes in aqueous solution (Crini & Lichtfouse, 2019) although they are largely ineffective to remediate dye in soil. Granting this method is highly effective, faster, and most importantly able to be done on large scale, the drawback of this mechanism is quite distressing (Crini & Lichtfouse, 2019). Some of these processes are dye-specific making mixed dyes eluents takes several processes to completely decolourise them. Besides that, the breaking of dye's chemical structure results in releasing more toxic compounds (Oliveira et al., 2016; Bharagava et al., 2018; Joshi & Katti, 2018; Igiri et al., 2018).

Traditional biological procedures combined with physio-chemical treatment processes are capable to achieve better decolourisation results. Since chemical methods are generally costly with limited applicability, which are difficult to dispose of, combination with biological processes has received increasing interest owning to their cost-effectiveness, ability to produce less sludge, and environmental benignity (de Lorenzo, 2018; Shah, 2019). Soil bioremediation is usually a relatively cost-effective method, especially if the principal contaminant is inorganic. Estimated costs range from RM 124 – RM 3121 per cubic yard of treated soil contaminated by one type of azo dye, and subsequently, the cost increases based on the number of mix dyes present at the site (Goswami et al., 2000; Arora, 2018; Yadav et al., 2018; Ni et al., 2018). Bioremediation of wastewater/effluents is also economical if the main contaminant is free from organic matter. Costs can range from RM 137 – RM 832 per 1000 gallons of treated water (Goswami et al., 2000; Arora, 2018; Yadav et al., 2018; Ni et al., 2018). Therefore, developing a practical bioprocess for treating dye-containing wastewater is of great significance. The effectiveness of microbial decolourisation depends on the adaptability and the effectiveness of selected microorganisms (Ghatak & Das, 2018; Yan et al., 2018; Eskandari et al., 2019; Mishra & Maiti, 2019a; Franca et al., 2020).

For over a century, microbial decolourisation of azo dyes is an unsolved puzzle among scientists. Previously, in microbial azo dye decolourisation, the focus was centred towards isolating bacteria or consortium having higher azo dye tolerance level needed as a tool for bioremediation (Chen et al., 1999; Pointing & Vrijmoed, 2000; Chagas & Durrant, 2001; Yoo et al., 2001; Işik & Sponza, 2003; Song et al., 2003). To achieve this, various additional carbon sources and higher concentrations of co-substrate were introduced. During the last ten years, attention has shifted towards isolating microorganisms with the ability to decolourise azo dyes with complete mineralisation ability (Masarbo et al., 2018; Meerbergen et al., 2018; Mishra & Maiti, 2018; Eskandari et al., 2019; Parmar & Shukla, 2019). The focus changed is mainly because of degraded metabolites of azo dye were found to be carcinogenic and mutagenic (Shen et al., 2015; Yan et al., 2018; Kumar et al., 2019). However, to isolate such a decolouriser able to consume azo dye as the sole carbon source remained a big challenge to this date. Therefore, further understanding of the decolourisation mechanism and kinetics of azo dye decolourisation through various optimisation processes will help in solving the phenomenon of a better azo dye decolourisation.

Consequently, it will then become a significant step towards the effective's translation of laboratory results into field practice.

1.1 Statement of the problems and significance of the study

Dye pollution is a global issue. Countries such as China (Wu et al., 2019), Germany (Oliveira et al., 2018), India (Pandey et al., 2016), and Pakistan (Daud et al., 2017) has increasingly reported in dye contamination issues. During textile dyeing, about 10 to 60% of Reactive Red 120 is expected to be lost, resulting in large amounts of colored wastewater (Reddy & Osborne, 2020). It is estimated that an average of 10% to 15% Reactive Red 120 used in the fabrication of textile materials is discharged into the environment each year around the world (Swarnkumar & Osborne, 2020). Anvi et al. (2019) reported the outflow concentration of the Reactive Red dye from the final clarifier of the textile factory in India was at 45 ppm. Meanwhile, Chitra et al. (2018) reported Reactive Red discharged from the dye houses ranged from 10 to 250 ppm.

Dye pollution in local rivers has been reported countless of times in Malaysia (Ramakreshnan et al., 2020; Buhari & Ismail, 2016). Out of the 473 rivers monitored in Malaysia in the year 2017 alone, 244 (52%) of them are clean, 186 (39%) are slightly contaminated, and 43 (9%) are polluted (Afroz et al. 2017). In these polluted rivers, ammonical nitrogen, biochemical oxygen demand (BOD) and suspended solids (SS) continued to be significant, where high BOD has been attributed to inadequate sewage or effluent treatment from agriculture and manufacturing industries (Zin et al. 2018). Most of these contaminations are contributed by small scale manufactures whereby the cost of treating the effluents becomes the main hurdle for them. In the Juru River, it has been reported that 48 mg/L of Reactive Red 120 is found in the river basin and the concentrations vary depending on the seasons (Buhari et al., 2016). Meanwhile, in Kelantan, up to 215 mg/L of Reactive Red dyes can be found on average in textiles sludges, which is commonly used in Batik manufacturing (Razali et al. 2020, Buttiyapan et al., 2016, Yacoob et al., 2016). Thus, cost-effective remediation is much needed to address this issue.

Various azo dye decolourisation using bacteria such as Reactive Black 5 (Eskandari et al., 2019), Black 5 (Kumar et al., 2019), Methyl Orange (Masarbo et al., 2018), Acid Red (Franca et al., 2020), Metanil Yellow (Li, et al., 2020), Yellow G (Guo et al., 2020b) and Green 9 (Das & Mishra, 2017) has been reported before. Azo dyes are organic compounds containing an azo group (-N=N-), but some dyes have two (diazo), three (triazo) or more (Benkhaya et al., 2020). Reduction of the azo group (N=N linkage) leads to the production of aromatic amine compounds which are more toxic and are also known carcinogens and mutagenic agents in humans (Zahran et al., 2019). The severity of the toxic compounds produced during azo reduction depends on the presence of the number of azo linkages (Rasool et al., 2016). The higher the number of azo linkages, the more toxic amines and benzene is produced. Furthermore, diazo dyes such as Reactive Red 120 are resistant to bacteria degradation due

to the strong chemical bond, and the resulting metabolites are toxic towards bacteria (Bhatia et al., 2017). Due to these challenges, Reactive Red 120 is less studied compared to mono azo dyes which are easier to be broken down by microorganisms (Jamee & Siddique, 2019; Singh & Singh, 2017).To date, only *Acinetobacter, Bacillus*, and P*seudomonas* species are reported able to decolourise RR120 and requires co-substrate to initiate decolourisation (Reddy & Osborne, 2020; Hafeez et al., 2018, Anwar et al., 2014). The exploration in isolating bacteria capable of degrading RR120 without any co-substrate is still going.

Over the past decades, many microorganisms that are capable of degrading azo dyes, including bacteria (Mishra et al., 2019ab; Ghatak & Das, 2018), fungi (Krishnamoorthy et al., 2018; El-Rahim et al., 2017), actinomycetes (Chittal et al., 2019), and algae (El-Sheekh et al., 2018) have been identified. Most of the azo dyes are reduced anaerobically to the corresponding amines with cleavage of azo bonds by bacterial azoreductase, but they are difficult to be degraded aerobically (Jha et al., 2016). Most importantly, these microorganisms were only able to utilise dyes as their nitrogen source (Bhatia et al., 2017). The utilisation of dyes as nitrogen source leads to the production of toxic metabolites although decolourisation is achieved (Sandhya, 2010). The complete degradation process only occurs when the dyes are utilised as a carbon source (Zahran et al., 2019; Misal & Gawai, 2018). Since only a few bacterial species are reported capable of decolourising Reactive Red 120, utilising the dye as a source of energy is extremely a slow process and it would require a longer period to achieve a complete decolourisation even at the low concentrations of 25 ppm (Gao et al., 2018b; Bhatia et al., 2017; Singh et al., 2015). Therefore, this study is aimed to isolate RR120 decolourising bacterium as a sole carbon source along with optimising decolourisation using one-factor-at-a-time (OFAT) and response surface methodology (RSM). Apart from that, the kinetics of decolourisation on RR120 tolerance level need to be carried as to date, no data is available for RR120 azo dye type.

Even though decolourisation happens, total mineralisation might not occur (Zahran et al., 2019; Rawat et al., 2018; Krishnan et al., 2017). Due to this risk, most research opted to report the ability of isolated strains able to decolourise various azo dyes, however, the metabolite part usually goes unaccounted for. This dilemma has been going for the past few decades, leading to various optimisations for decolourisation of Reactive Red 120. The textile industry is the main contributor in producing the effluent wastewater containing Reactive Red 120 due to the more consumption of water for its different wet processing operations. These textile effluents are high in colour, suspended solids (SS), biochemical oxygen demand (BOD), total organic carbon (TOC), chemical oxygen demand (COD), temperature, pH, turbidity and toxicity (Aghasadeghi et al., 2018). Hence, to study the effect of these conditions, several parameters such as nitrogen concentrations, pH, temperature and Reactive Red 120 concentrations were investigated on their role in improving the decolourisation rate.

Besides that, in the case of azo dye decolourisation, the ability of the isolated strains able to decolourise the dye in the presence of heavy metals has been less studied before (Cui et al., 2020; Li, et al., 2020; Zhuang et al., 2020; Krishnamoorthy et al., 2018; Cao et al., 2019; Meerbergen et al., 2018). In the application of bioremediation, these strains will be exposed to various cocktails of chemicals, mostly consisting of heavy metals such as chromium, zinc, copper, cadmium, iron, and mercury as part of the manufacturing downline (Reddy & Osborne, 2020; Noreen et al., 2017). Hence, there is the need to isolate and identify bacteria not only able to decolourise azo dye as the sole carbon source, but also able to tolerate various concentrations and types of heavy metals. The work in this thesis is aimed at isolating such a decolouriser.

1.2 Hypothesis

Reactive Red 120 bacterium isolated from industrial effluent polluted sites containing an average of 25 ppm Reactive Red 120 can exhibit a better decolourisation ability with the need of less co-substrate for total mineralisation of resulting metabolite; meanwhile, optimisation through OFAT and RSM further increases decolourisation efficiency and limitation of bacteria ability can be analysed through kinetic and heavy metal studies.

1.3 Objectives

Based on the statements above, the present study aims at isolating a native consortium from the textile polluted environment with the potential of utilising Reactive Red 120 as the sole carbon source that can be utilised as ex-situ treatment in a controlled environment. With this in mind, the objectives of the study are as follow:

1. To isolate for Reactive Red 120-utilising bacterium or consortium from a polluted river in Malaysia as the sole carbon source,

2. To determine the role of medium composition and yeast extract in aiding Reactive Red 120 decolouration.

3. To determine the optimal nutritional and physical conditions of the bacterium/consortium for a maximum Reactive Red 120 decolourisation using OFAT and RSM approach.

4. To investigate the primary and secondary modelling of the kinetics process for Reactive Red 120 decolourisation in the dye-degrading bacterium/consortium.

5. To determine the effect of heavy metals on Reactive Red 120 decolourisation,

6. To assess the toxicity level of Reactive Red 120 degraded metabolites on Vigna radiata seed germination, shoot and root length.

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