BIOPRODUCTION OF LIPOPEPTIDES BY DYE DECOLOURISING BACTERIA AND ITS ROLES IN TEXTILE DYE DECOLOURISATION

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Specially dedicated to,

My parents, family, members and friends,

For all the supports, encouragement, understanding and all favours.

May Allah bless all of you.

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ABSTRACT

Textile dyes are recalcitrant molecules and contain a high level of chemicals and colour which poses a serious challenge to surrounding environments. Therefore, this study aims to determine the ability of isolated bacteria in producing a biosurfactant and investigate their synergistic action on decolourisation of textile dyes by the combination of isolated bacteria and biosurfactant. An effective dye degrading bacteria and biosurfactant-producers, *Bacillus cereus* KH1 and *Kurthia gibsonii* KH2, were isolated from textile industrial effluents and were identified and screened for biosurfactant production using haemolytic activity, oil spreading technique, drop collapse test and emulsification index. The optimisation for growth of bacteria for biosurfactant production also was measured based on effect of molasses concentration, pH, salinity and temperature. Fourier Transform Infrared Spectrum (FTIR) and Thin Layer Chromatography (TLC) analyses were carried out to detect the type of biosurfactant. The effect of different physicochemical parameters on textile effluent decolourisation was assessed within 24 hours. As results, both isolated bacteria showed β-hemolysis for haemolytic activity. For oil displacement technique, the clear zone diameter that exhibited by *Bacillus cereus* KH1 and *Kurthia gibsonii* KH2 were 61 mm and 50 mm. While, for drop collapse test, the results displayed a flat shape with a diameter of oil displacement of 30 mm for *Bacillus cereus* KH1 and 10 mm for *Kurthia gibsonii* KH2. The emulsification test (E_{24}) revealed that *Bacillus cereus* KH1 and *Kurthia gibsonii* KH2 had a higher emulsification index of 67% and 63% respectively. FTIR and TLC analyses indicated that the biosurfactant was a lipopeptide and was formed with a yield of 2.98 gL^{-1} for *Bacillus cereus* KH1 and 2 gL^{-1} for *Kurthia gibsonii* KH2. The synergistic action between isolated bacteria and lipopeptide biosurfactant resulted the maximum decolourisation levels of 87% for *Bacillus cereus* KH1 and 85% for *Kurthia gibsonii* KH2. The high attributes of these combinations and the phytotoxicity test implied that the metabolites were less toxic, making it a promising option for the biodecolourisation of textile industrial effluent.

ABSTRAK

Pewarna tekstil adalah molekul yang sukar dikendalikan dan mengandungi bahan kimia dan warna yang tinggi sehingga menimbulkan masalah yang serius bagi persekitaran sekelilingnya. Oleh itu, kajian ini bertujuan untuk menentukan keupayaan bacteria terpencil dalam menghasilkan biosurfaktan dan mengkaji tindakan sinergistik terhadap penyahwarnaan pewarna tekstil dengan gabungan bakteria terpencil dan biosurfaktan. Bakteria pengdegradasi pewarna dan penghasil biosurfaktan, *Bacillus cereus* KH1 dan *Kurthia gibsonii* KH2, telah diasingkan dari efluen industri tekstil dan telah diidentifikasi dan disaring untuk pengeluaran biosurfaktan menggunakan aktiviti hemolitik, teknik penyebaran minyak, ujian ketidakstabilan cecair oleh surfatan dan indeks pengemulsi. Pengoptimuman pertumbuhan bakteria untuk pengeluaran biosurfaktan juga diukur berdasarkan pengaruh kepekatan *molasses*, pH, kemasinan dan suhu. Analisis Fourier Tranfom Spektrum Inframerah (FTIR) dan Kromatography Lapisan Tipis (TLC) dilakukan untuk mengesan jenis biosurfaktan. Pengaruh parameter fizikokimia yang berbeza terhadap penyahwarnaan air sisa tekstil dinilai dalam masa 24 jam. Hasilnya, kedua-dua bakteria terpencil menunjukkan β-hemolisis untuk aktiviti hemolitik. Untuk teknik penyebaran minyak, diameter zon jelas yang ditunjukkan oleh *Bacillus cereus* KH1 dan *Kurthia gibsonii* KH2 ialah 61 mm dan 50 mm. Manakala, untuk ujian ketidakstabilan cecair oleh surfatan, hasil menunjukkan bentuk rata dengan diameter anjakan minyak 30 mm bagi *Bacillus cereus* KH1 dan 10 mm bagi *Kurthia gibsonii* KH2. Ujian pengemulsi (E_{24}) menunjukkan bahawa *Bacillus cereus* KH1 dan *Kurthia gibsonii* KH2 mempunyai nilai indeks pengemulsian yang lebih tinggi masing-masing 67% dan 63%. Analisis FTIR dan TLC menunjukkan bahawa biosurfaktan adalah lipopeptida dan menghasilkan 2.98 gL^{-1} biosurfaktan bagi *Bacillus cereus* KH1 dan 2 gL^{-1} bagi *Kurthia gibsonii* KH2. Tindakan sinergistik antara bakteria terpencil dan biosurfactant lipopeptida menghasilkan tahap penyahwarnaan maksimum sebanyak 87% bagi *Bacillus cereus* KH1 dan 85% bagi *Kurthia gibsonii* KH2. Kombinasi yang tinggi serta ujian fitotoksisiti menyatakan bahawa metabolit adalah kurang toksik dan menjadikannya sebagai pilihan untuk penyahwarnaan efluen industri tekstil.

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LIST OF SYMBOLS AND ABBREVIATIONS

$(NH_4)_2 SO_4$	-	Ammonium sulfate
16 rRNA	-	16S ribosomal RNA
ABTS	-	2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
ADMI		American Dye Manufacturers Institute
Al	-	Aluminium
$Al_2(SO_4)_3$	-	Aluminium sulphate
APHA	-	American Public Health Association
BOD	-	Biochemical oxygen demand
C=O	-	Carbonyl
Ca	-	Calcium
Ca^{2+}	-	Calcium ion
Cd^{2+}	-	Cadmiun ion
CFU	-	Colony forming unit
CH=	-	Methine
CMC	-	Critical micelle concentration
CO_2	-	Carbon dioxide
COD	-	Chemical oxygen demand
COOH	-	Carboxyl
Cr	-	Chromium
Cu^{2+}	-	Copper ion
DI	-	Deionized water
DNA	-	Deoxyribonucleic acid
DO	-	Dissolved oxygen
DOE	-	Department of Environment
DW	-	Distilled water
E_{24}	-	Emulsification index
Fe	-	Iron

Fe^{3+}	-	Ferric ion
FTIR	-	Fourier transform infrared spectroscopy
G6P	-	Glucose-6-phosphate
GI	-	Germination index
H^+	-	Hydrogen ion
H_2O	-	Water
HCl	-	Hydrochloric acid
K_2HPO_4	-	Potassium hydrogen phosphate
KBr	-	Potassium bromide
KCl	-	Potassium chloride
LB	-	Luria Bertani
M.W	-	Molecular weight
Mb	-	Molybdenum
Mg	-	Magnesium
MT		Metric ton
Mg^{2+}	-	Magnesium ion
$MgSO_4$	-	Magnesium sulphate
$MgSO_4.7H_2O$	-	Magnesium sulphate heptahydrate
Mn^{2+}	-	Manganese ion
N=N	-	Azo double bond
NA	-	Nutrient agar
Na^+	-	Sodium ion
Na_2SO_4	-	Sodium sulphate
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NB	-	Nutrient broth
NH_3		Amine
NH_3-N	-	Ammoniacal nitrogen
NO_2	-	Nitro
OH	-	Hydroxyl
OH^-	-	Hydroxyl radicals
Pb	-	Lead
Pb^{2+}	-	Lead ion

PCR	-	Polymerase chain reaction
PVA		Polyvinyl alcohol
RBB	-	Remazol black-B
RBBR	-	Remazole Brilliant Blue R
Sn	-	Tin
SO_3H	-	Sulfonic acid
TDS		Total dissolve solid
TSS	-	Total suspended solid
TLC	-	Thin layer chromatography
TOC	-	Total organic carbon
TSS	-	Total suspended solids
UN	-	United Nation
USEPA	-	United States Environmental Protection Agency
Zn	-	Zinc
Zn^{2+}	-	Zinc ion
^{o}C	-	Celsius

CHAPTER 1

INTRODUCTION

1.1 Background of study

The textile industry is a significant water consumer and one of the most chemically-intensive industries in the world (Toprak and Anis, 2017). It is also become the most critical environmental challenge due to its release of huge amounts of effluent containing high levels of dye (Holkar *et al*., 2016). Statistics indicated that the annual production of textile dyes exceeded 70,000 metric tons per year and 10-15% of dyes are required for the process of dyeing (Guadie *et al*., 2017; Bello *et al*., 2018; Slama *et al*., 2021). Due to the manufacturing of textile, dye pollution are produced by a series of industrial processes which include raw material, inspection, shearing, cropping, desizing, scouring, bleaching, drying, mercerising, soaping, dyeing and finishing which involves the utilisation of many chemicals (Holkar *et al*., 2016; Ramachandran *et al*., 2019). These chemicals are considered hazardous to the environment and difficult to be treat due to their complex aromatic molecular structures, synthetic origin and are stable to against light, heat and oxidising agents (Sarkar *et al*., 2017). Therefore, consequences of the effect of improper discharge of textile industrial effluents into the water body could cause eutrophication, rapid depletion of dissolved oxygen (DO) levels, disequilibrium of ecological systems, inhibition of photosynthesis and subsequently affect human health (Singh and Singh, 2017).

According to the Environmental Quality Report by the Malaysian Department of Environment (DOE), the total quantity of scheduled waste generated by the textile industry is 1,923.56 MT/year (0.08%) (Department of Environment, 2018). To control the amount of effluents discharge, all the textile industry must abide by the

Environmental Quality (Industrial Effluent) Regulation 2009 where the discharge limit for colour is 100 (ADMI) for standard A and 200 (ADMI) for standard B (Environmental Quality (Industrial Effluent) Regulation, 2009).

Currently, there are various physical, chemical and biological treatment processes that have been used to decolourise dyes from textile industries (Shanmugaraju and Rajan, 2018; Rostam and Taghizadeh, 2020). Although some of these treatments are effectively remove dyes from contaminated water but they are expensive and might generate by-products with excessive gas and sludge productions (Hayat *et al*., 2015; Rawat *et al*., 2016). In contrast to the physicochemical method, these toxic compounds can be spontaneously degraded by indigenous microorganisms through the biological method. By the biological approach for the effluent treatment, the pollutant degrading microbes can be identified and effectively used to remove the contaminants under controlled conditions while simultaneously produce a value-added product. Thus, biological are the attractive methods as they are highly efficient, do not cost much, environmentally friendly and can selectively completely degrade organic pollutants without collateral damage (Saratale *et al*., 2011).

A wide range of microorganisms including bacteria, fungi, algae and also plants have been reported to decolourise the dye in effluent treatment with the additional catalyst. However, the role of bacteria is receiving attention to be a suitable organism for the treatment for dye removal due to their capabilities to decolourise a variety of dyes efficiently under anaerobic or aerobic condition (Mahmood *et al*., 2015). Microbial decolourisation is a biological technique that complements existing technologies since it is can be used to degrade other contaminants from industrial effllluents (Saratale *et al*., 2011). From the perspective of microbial decolourisation, bacteria have the ability to decolourise which assumed to be associated with the production of a different enzyme. It is believed that a biosurfactant mechanism could promote decolourising enzymes activities by modify and increase the cell membranes permeability or dye solubility in aqueous media, which allows for easier enzyme release (Mnif *et al*., 2016). Thus, it can be a valuable approach to uncover the role of biosurfactants in the textile industrial effluent treatment process and its effect on the microbes and the enzyme mechanism involved in the decolourisation process.

Microbial derived biosurfactants could open up a new approach to the decolourisation treatment process. The presence of a biosurfactant may act as a green accelerator in the presence of a biotransformation enzyme, which hastens the

decolourisation process via the improvement of biosurfactant activity, which is a rate-limiting step during the process of decolourisation (Mnif *et al*., 2016). The formation of the micelle whereby the hydrophobic portion of the biosurfactant binds to dyes via hydrophobic interactions to form the micelle containing dyes and water. The micelle then makes contact with a highly hydrophilic cell membrane of a bacterial strain, which increases membrane porosity and the possibility of dyes entering the cell strain and then degraded by enzymatic catalysis (Kaczorek *et al*., 2018).

Therefore, the usage of biosurfactants may represent a best alternative for overcoming the toxicity of textile dye and others contaminant compounds. Although, many researchers have identified many bacteria species have potential for biosurfactant producer, however, the study on utilisation of microbial biocatalysts resulting in a synergistic action leading to maximum decolourisation of textile effluent has not been thoroughly explored. Lately, some research reported the effectiveness of decolourisation of a textile dye in the presence of microorganisms being enhanced by biosurfactant production, where the results showed that the biosurfactant that was produced can significantly contribute to the dye decolourisation treatment by improving the bioavailability and biodegradability of hydrophobic compounds through highly emulsification activity and the solubilisation of the dye (Mnif *et al*., 2015(a); Mnif *et al*., 2015(b); Mnif *et al*., 2016). Although it is accepted the effectiveness of biosurfactant in dye decolourisation, no critically reviewed has been discussed on the application of the biosurfactant produced from dye degrading bacteria. Therefore, the aim of this study is to isolate and determine the ability of isolated bacteria to decolourise textile effluent concomitantly with the biosurfactant addition that was produced by utilising the renewable substrate as nutrient sources for biosurfactant production. The biosurfactant-stimulated bacterial decolourisation approach may provide highly efficient, inexpensive and time-saving procedure in treatment of textile industrial effluents.

1.2 Problem statement

Water pollution caused by the recalcitrant textile dyes represents one of the most environmental problems (Jadhav *et al.*, 2011). In Malaysia, an increasing amount of discharged dye effluent from the textile industry becoming one of the most significant contributors to environmental problems due to its transformation of products into carcinogenic and mutagenic agents, which puts human health at risk (Islam and Mostafa, 2018; Dauda and Erkurt, 2020). Although, various current technologies, encompassing physical and chemical treatment have been applied to treat textile effluent, but these treatments do not comply with standards A and B of the Malaysian Environmental Quality (Industrial Effluent) Regulation Year 2009. These treatments noticeable produce an amount of secondary sludge which leads to the toxicity of the effluents after the treatment processes (Ogugbue and Sawidis, 2011; Hayat *et al.*, 2015; Ahmed *et al.*, 2019).

Discharged textile industrial effluents can often lead to immediate and long-term environmental damage. Furthermore. This problem is more aggravated due to the unsafe disposal methods owing to the associated higher cost of safe and proper disposal (Ferronato and Torretta, 2019). Thus, these detrimental pollutants make the development of remediation technology is essential for cleaning up polluted sites (Azubuike *et al.*, 2016). Relative to other strategies treating textile industrial effluents contamination, microbial decolourisation is recognised as a more effective, eco-friendly and inexpensive technologies (Sangwan and Dukare, 2018). Free-living bacteria in textile effluents have long been considered as one of the predominant dye degrading agents. Although there are numerous natural dye-degraders, however, the growth of them is hindered by several factors, such as the recalcitrant nature of the substrate and the limited availability of organic compounds in aqueous systems (Patowary *et al.*, 2017).

Bacterial decolourised dyes with the addition of biosurfactant also pay attention in quicken the decolourisation process via the improvement of biosurfactant activity (Mnif *et al.*, 2016). The unique characteristic of biosurfactants, such as higher biodegradability and solubility, low toxicity, tolerance to extreme conditions, ecological acceptability and eco-friendly bioprocesses makes it a viable replacement for synthetic surfactants, which are extremely toxic to living organisms and great diminishing property (Christopher *et al.*, 2019). However, limited full-scale

production of biosurfactant has been realised due to its expensive raw material, low production yield, high purification cost and facing the problem to finding the suitable substrate for the low cost of biosurfactant production (Banat *et al*., 2014). To overcome these issues, applying renewable substrates may produce a high yield of biosurfactants and provide an insight into the availability and applicability of biosurfactant stimulated bacterial for textile dyes decolourisation.

1.3 Objectives

The objectives of this study were as follows:

1. To characterise the textile industrial effluent and to isolate and determine the potential isolated bacteria from the textile industrial effluents to produce biosurfactant.
2. To produce and optimised the fermentation conditions for the biosurfactant production by isolated bacteria.
3. To determine the capability of bacterial decolourisation and degradation of textile dyes concomitantly with biosurfactant addition.
4. To assess the phytotoxicity of the untreated and treated textile industrial effluents.

1.4 Scope of study

The scope of this study was as follows:

1. Characterisation of textile industrial effluents.

   The samples of textile industrial effluents were collected from the textile industry located at Sri Gading, Batu Pahat, Johor. The sample was characterised in terms of pH, temperature and dissolved oxygen (DO) for in-situ analysis and parameters of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), colour and ammonical nitrogen (NH_3-N) were analysed ex-situ at the Environment Engineering Technology Laboratory, Faculty of Engineering Technology, Universiti Tun Hussein Onn Malaysia.

2. Isolation and determination of biosurfactant producing bacteria potential to produce biosurfactant.

   The isolated bacteria were screened to identify potential in dye decolouriser. The isolated bacteria were then examined based on morphological features according to the guideline of Bergey's Manual of Determinative Bacteriology. The gram types of bacteria were examined using the gram staining technique by microscope with oil immersion and the growth and pH profile were measured in 24 hours. The isolated bacteria also were screened for the biosurfactant producing bacteria and their physicochemical characteristics. The physicochemical characteristics of biosurfactant were measured in term of haemolytic activity, emulsification index (E_{24}), oil displacement technique and drop collapse test. The potential biosurfactant producing bacteria were identified based on the 16 rRNA sequence analysis using the PCR mediated amplification. The acclimatisation of potential bacteria was evaluated by gradually changing the concentration of the textile dye effluent and the number of potential bacteria were counted (in CFU mL^{-1}) using the colony counting method.

3. Production and characterisation of biosurfactant production.

   The optimisation of the biosurfactant was evaluated by four parameters: concentration of carbon sources, initial pH, the concentration of NaCl and temperature. The extraction and purification of biosurfactant were determined by acid precipitation and freeze-dry method respectively. The purified biosurfactant was identified and characterised using fourier transform infrared spectroscopy (FTIR) and thin-layer chromatography (TLC).

4. Decolourisation of textile industrial effluents.

   The potential of isolated biosurfactant-producing bacteria was assessed based on the effect of different dyes concentration, time, pH and lipopeptide addition in order to decolourise the textile industrial effluents.

5. Phytotoxicity test.

   The phytotoxicity test was performed to assess the toxicity of the untreated and treated textile dye effluents using germination of mung beans (*Vigna radiata*) seeds on a Petri dish.

1.5 Significant of study

The significance of this study was as follows:

1. The interest in the new utilisation of microbial biocatalyst can fully participates in green chemistry concept and can be used as a biological agent for various industrial purpose.
2. The capability of efficient biosurfactant producing bacteria offers a beneficial tool for remediation of pollutants that is unfriendly to the environment.
3. The biosurfactant that was produced had a capability to produce an extensive transformation of structural and toxicological properties of contaminants for conversion of toxic to non-toxic end products.

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VITA

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