CHARACTERISATION OF AZOREDUCTASE PRODUCED BY *BREVIBACILLUS PANACIHUMI* DURING THE DECOLOURISATION OF REACTIVE BLACK 5

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Specially dedicated to

My beloved parents, Mohd Ramlan bin Ramle and Azmah binti Yahya,

and family members,

My supportive supervisor and co-supervisor, Assoc. Prof. Dr. Zaharah Ibrahim and

Dr. Haryati Jamaluddin, lecturers and all my friends.

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I sincerely hope this project will be of benefit and serves as future reference to those keen on doing research in decolourisation of textile effluents and enzymatic studies on azo dye-degrading enzyme.

ABSTRACT

Azoreductase plays an important role in the decolourisation of azo dyes by cleaving the azo bonds in azo dye structure. In view of this, *Brevibacillus panacihumi*, azo dye-degrading bacteria, was used for decolourisation of Reactive Black 5 (RB5) dye. Decolourisation of RB5 was carried out by growing the bacteria culture in RB5 dye solution (100 mg/L) at pH 9, supplemented with glucose 0.4 % (v/v) and yeast extract 1.2 % (v/v) and incubated at 37 °C under sequential anaerobic-aerobic condition. Azoreductase was produced during which the enzyme with the highest activity obtained during the end of log phase. Since the azoreductase activity related to the decolourisation of RB5 in anaerobic condition, the cells were harvested during this condition. Then, to determine whether the enzyme produced is found intracellular or extracellular, the cells was collected via centrifugation and the cell pellet was disrupted using sonication technique, and Lowry assay was used to determine the protein concentration. Azoreductase was found to be produced intracellularly as the cell free extract has the highest specific activity of 0.334 U/mg compared to the culture supernatant (extracellular), resting cell and cell debris which has significantly lower enzyme activity of 0.034 U/mg, 0.010 U/mg and 0.200 U/mg, respectively. The optimum assay conditions for the maximum azoreductase activity were at 37 °C, RB5 dye concentration of 100 mg/L and NADH concentration of 0.2 mM. In addition, the optimum pH and Ionic liquids [emim][$EtSO_4$] concentration was pH 7 and 70 %, respectively. Phosphate buffer, pH 7 showed a higher enzyme activity compared to the Ionic liquids as a stabiliser in azoreductase assay. Decolourisation of RB5 by azoreductase under the optimum assay conditions occured up to 93 % at 8th hour of incubation was successfully achieved.

ABSTRAK

Enzim azoreduktase memainkan peranan yang penting dalam proses penyahwarnaan pewarna azo dengan memutuskan ikatan azo dalam struktur pewarna azo. Oleh yang demikian, *Brevibacillus panacihumi*, bakteria yang berfungsi untuk mendegradasi pewarna azo telah diperkenalkan bagi tujuan penyahwarnaan Reactive Black 5 (RB5). Proses penyahwarnaan RB5 telah dijalankan dengan mengembangbiakkan kultur bakteria di dalam medium yang terdiri daripada pewarna azo RB5 (100 mg/L) pada pH 9, glukosa 0.4 % (v/v) dan ekstrak yis 1.2 % (v/v) and dieramkan pada suhu 37 °C di dalam persekitaran anaerobik-aerobik. Enzim azoreduktase telah dihasilkan ketika enzim mempunyai aktiviti yang paling tinggi iaitu yang telah terhasil pada penghujung fasa log. Oleh kerana aktiviti enzim azoreduktase berkait rapat dengan proses penyahwarnaa RB5 di dalam persekitaran anaerobik, sel telah diekstrak pada waktu tersebut. Kemudian, untuk mengenalpasti sama ada enzim ini telah dihasilkan secara intrasel atau ekstrasel, sel telah dikumpulkan melalui proses pengemparan dan sel pelet telah dipecahkan melalui teknik pemecahan sel dan analisis Lowry telah digunakan bagi menentukan jumlah protein. Enzim azoreduktase telah dikenalpasti dihasilkan secara intrasel kerana sel ekstrak mempunyai spesifik aktiviti enzim yang paling tinggi iaitu 0.334 U/mg berbanding dengan cecair kultur (ekstrasel), sel rehat dan serpihan sel yang mempunyai aktiviti enzim yang rendah iaitu 0.034 U/mg, 0.010 U/mg dan 0.200 U/mg. Aktiviti analisis yang optimum bagi menghasilkan aktiviti enzim azoreduktase yang maksimum telah dikenalpasti pada 37 °C, pewarna azo RB5 100 mg/L dan kepekatan NADH 0.2 mM. Di samping itu, pH dan kepekatan cecair ion [emim][$EtSO_4$] yang optimum ialah pH 7 dan 70 %. Penimbal fosfat, pH 7 telah menunjukkan aktiviti enzim dua kali ganda lebih tinggi daripada cecair ion sebagai penstabil di dalam analisis enzim azoreduktase. Penyahwarnaan RB5 dengan menggunakan enzim azoreduktase di dalam persekitaran analisis yang optimum yang terhasil sehingga 93 % penyahwarnaan pada jam kelapan inkubasi telah berjaya dijalankan.

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LIST OF ABBREVIATIONS

BSA	-	Bovine Serum Albumin
CBB	-	Coomasie brilliant blue
[emim][$EtSO_4$]	-	1-Ethyl-3-methylimidazolium ethylsulfate
et al.	-	and others
M	-	Molarity
mM	-	Milimolar
MW	-	Molecular Weight
NADH	-	Nicotinamide adenine dinucleotide
NB	-	Nutrient Broth
nm	-	Nanometer
pH	-	Logarithm of the hydrogen ion concentration
rpm	-	Rotation per minute
Tris-HCL	-	Tris Hydrochloric acid
Units/mg	-	Units per milligram
U/ml	-	Units per mililitre
v/v	-	Volume over volume
w/v	-	Weight over volume
%	-	Percent
°C	-	Degree Celcius
µg	-	Microgram
µL	-	Microlitre
µM	-	Micromolar

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Water pollution has become a major concern to the society since the past few decades. Approximately 280,000 tonnes of dyes has been discarded to the environment annually (Jin *et al*., 2007). The major concern of wastewater containing azo dyes is the pollution of toxic heavy metals such as Fe, Zn, Cu, Pb, and toxic compounds such as biocides (Jadhav *et al*., 2010). One of the advantages of using azo dye-degrading microorganisms to decolourise azo dyes is that it requires a lower processing cost. It also reduces the amount of toxic compounds contained in wastewater effluent through the mineralisation process (Forgacs *et al*., 2004). Azoreductase enzyme is the enzyme that is responsible in catalyzing the reductive cleavage of azo bond and led to the colour removal of azo dyes. Therefore, it is important to study the possible azo dye-degrading enzymes, the microorganisms that are responsible in producing such enzymes and the factors that may affect the activity of the enzymes.

All azo-dye degrading microorganisms are producing azoreductase enzyme that has the ability to cleave the azo bond of synthetic azo dyes. The biodegradation of wastewater containing azo dyes involves either anaerobic system, aerobic system or sequential anaerobic-aerobic system. The reduction of azo dyes produces aromatic amine products that are harmful to the human and aquatic life than the parent compound. Therefore, sequential anaerobic-aerobic or two-stage system has been of great interest as it has the capability of decolourising the azo dyes into colourless aromatic amines and

further oxidizes it into less toxic and more stable compounds. Azo dye reduction occurs preferentially under anaerobic condition. Ramalho *et al*. (2004) has observed a faster decolourisation rate of azo dyes at low oxygen concentration.

Azoreductase enzyme has been isolated and identified from various species of microorganisms. These enzymes are either oxygen insensitive or sensitive in the environment. Azoreductase from different sources of microorganisms would have different enzyme properties such as they can be categorised as flavin-dependent, flavin-independent and many others (Ghosh *et al.*, 1992). Therefore, several studies have been done on microorganisms which have the ability to produce azoreductase enzyme to determine their specific characterisitics such as *Pseudomonas* KF46 (Zimmermann *et al*., 1982), *Enterobacter agglomerans* (Moutaouakkil *et al*., 2003), *Staphylococcus aureus* (Chen *et al*., 2005), *Micrococcus* strain (Olukanni *et al*., 2009). Fungi also has the ability to produce azoreductase, one such example is using *Issatchenkia occidentalis* which is used for decolourisation of methyl orange and orange II (Ramalho *et al*., 2004). In some studies, mixed bacterial culture is more preferable than the pure bacterial culture as it has higher co-metabolic activities within a microbial community. However, the ability of pure bacterial culture in biodegradation of azo dyes producing azoreductase is much easier to be observed and studied in terms of its specific activity.

1.2 Problem of Statement

Azoreductase is responsible for reducing the azo double bond in azo dyes structures by enzymatic biotransformation step to produce colourless amine products and reduce them to a more stable product (Zimmermann *et al*., 1982). However, azoreductase isolated from different microorganisms varies in their enzymatic activities (Nakanishi *et al.*, 2001). Therefore, there is a need to study the characteristics of azoreductase-mediated biodegradation in terms of various environmental effects. Therefore, further studies on the characterisation of azoreductase in terms of its activity and stability should be done in order to obtain the maximum production and enzyme activity of azoreductase for the purpose of biological textile wastewater treatment. A higher specific enzyme activity of azoreductase was expected in azo dyes decolourisation with the used of pure bacterial culture. This is because the results may not be affected by other properties of unknown microorganisms or mixed bacterial cultures.

1.3 Research Objectives

There are 2 main objectives of this study:-

1. To optimise the decolourisation of Reactive Black 5 using azoreductase produced by *Brevibacillus panacihumi* under sequential anaerobic-aerobic condition.

2. To optimise the azoreductase assay conditions; pH, temperature, substrate concentration, NADH concentration and Ionic liquids concentration.

1.4 Scopes of Research

This project is mainly focused on the localisation and characterisation of crude azoreductase produced by azo dye-degrading bacteria using pure culture of *Brevibacillus panacihumi*. The localisation of azoreductase was first determined in order to obtain the crude enzyme extracts with the highest azoreductase activity. Lowry method was used to determine the protein concentration. The effects of pH, temperature, substrate concentration, NADH concentration and Ionic liquids concentration on crude azoreductase activity and stability were determined using azoreductase assay.

1.5 Research Significance

Textile industries have contributed about 73 to 167 m^3 of the wastewater per tonne of product and accounted for 22% of the total volume of industrial wastewater produced in Malaysia (Idris *et al.*, 2007). Thus, the biological method has been introduced to overcome the problems of conventional method that produces high sludge contents (Lucas and Peres, 2009). The enzyme involved in the biodegradation of azo dyes is mainly azoreductase. Azoreductase enzyme has been proven to have highly stable physiochemical properties. Therefore, azoreductase has been widely investigated and characterised in order to obtain the highest enzyme activity with a higher capability of azo dyes removal. Some aerobic bacteria have the ability to reduce the azo bond of synthetic azo dye by oxygen-insensitive or using aerobic azoreductase (Mazumdar *et al*., 1999). In addition, some anaerobic bacteria also have the ability to produce different forms of azoreductase (Horikoshi, 1999). This may contribute to a better biodegradation of azo dyes to be used for biological treatment of industrial wastewater containing azo dyes (Ooi *et al.*, 2007).

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