International Journal of Environmental Bioremediation & Biodegradation, 2013, Vol. 1, No. 2, 37-42
Available online at http://pubs.sciepub.com/ijebb/1/2/1

DOI:10.12691/ijebb-1-2-1

Microbial Decolorization of Reactive Black *by* *Pseudomonas stutzeri* ETL-79

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Received October 01, 2013; Revised October 27, 2013; Accepted November 01, 2013

Abstract Azo reductase are often associated with decolorization of non-degradation of non-degradable azo dyes via cleavage azo bonds. In this study, *Pseudomonas stutzeri* ETL-79 bacterium was used for the decolorization of Reactive Black dye. The highest activity of azoreductase was obtained during the end of log phase. Azoreductase produced intracellularly had the highest specific activity of 0.0334 U/mg compared to the culture supernatant (Extracellular), resting cell and cell debris with low enzyme activity of 0.034 U/mg, 0.010 U/mg and 0.200 U/mg respectively. The optimum assay condition for the maximum azoreductase activity were at 37°C, pH 7, Reactive Black dye concentration of 100 mg/L and NADH concentration of 0.2 Mm by using phosphate buffer as a medium for the enzyme reaction. Alternatively the azoreductase assay was also carried out using ionic liquid that may function to enhance the activity and stability of azoreductase. Results using phosphate buffer (pH-7) showed higher enzyme activity twice that of the ionic liquid besides enhancing the stability of enzyme. Under the optimum assay condition upto 93% of decolorization was achieved after 8h of incubation. In addition, growth of bacteria was also concurrently observed during decolorization of Reactive Black.

Keywords: *azoreductase, Pseudomonas stutzeri ETL-79, Reactive Black 5, decolourisation, azoreductase assay*

Cite This Article: Maulin P Shah**,** Kavita A Patel, and A M Darji, "Microbial Decolorization of Reactive Black *by Pseudomonas stutzeri* ETL-79." *International Journal of Environmental Bioremediation & Biodegradation* 1, no. 2 (2013): 37-42. doi: 10.12691/ijebb-1-2-1.

1. Introduction

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 [1]. Azo dye is being widely used as the colouring agent in textile industries because of its lower production cost and has more variations in colour compared to natural dyes. A major environmental concern in the release of coloured effluents is the high content of dye chemicals and their breakdown products may even be more toxic and/or mutagenic to aquatic life [2]. At present, the removal of colour from dye-containing water and wastewater, especially that of textile wastewaters, is a difficult task; there is no economical treatment that can effectively treat dyes. Most of them use chemical treatment which adds more pollutant such as generation of toxic sludge which requires special handling procedures prior to disposal as scheduled wastes. A good alternative treatment method for dye-containing wastewater is to bioaugment effective azo dye-degrading microorganisms capable of decolourising and degrading azo dyes. Besides reducing the amount of toxic compounds [3] in the wastewater, it is environmentally friendly and cost-effective. In this study, *Pseudomonas stutzeri* ETL-79, which was previously isolated by our researcher was used to decolourise and degrade azo reactive dye, Reactive Black using the combination of anaerobic-aerobic treatment. Azoreductases are generally responsible for reducing the azo double bond in azo dyes structures. They undergo enzymatic biotransformation to produce colourless aromatic amines which are more toxic than the parent compound. In this study, azoreductase produced by *Pseudomonas stutzeri* ETL-79 is reported to decolourise reactive azo dye, Reactive Black, which is recalcitrant towards microbial degradation. Therefore, it is important to study the characteristics of azoreductase-mediated degradation since azoreductases isolated from different microorganisms are known to have broad specificities in their enzymatic reactions [8]. This finding may further contribute to highly adaptable bacteria with good potential application for treatment of azo-containing wastewater. Hence it is of great interest to investigate factors affecting its enzyme activities such as pH, temperature, substrate concentration, NADH concentration during decolourising of Reactive Black 5. In addition, ionic liquid which may influence activity and stability of enzyme was also studied to determine if it can be used as an alternative solvent for the enzyme reaction. Azoreductase enzymes have been isolated from various types of microorganisms. These enzymes are of broad specificities and can either be tolerant or sensitive to oxygen. Their activities may be flavin-dependent or may not require flavin [4]; studies on specific characteristic of azoreductases were reported in

microorganisms such as *Enterobacter agglomerans [5]*, *Staphylococcus aureus [6]* and *Micrococcus* strain [7]. Several species of mammalian intestinal bacteria had also been reported to have azoreductase activities. Some examples of these bacteria include *Shigella [4]*, *Escherichia coli [8]*, *Enterococcus faecalis* and *Staphylococcus aureus [6]*. Nevertheless, studies of azo dye-degrading fungi have also been established in several reports that include *Trametes versicolor*, *Phanerochaete chrysosporium* and *Phlebia tremellosa* [9]. Another example of fungi was *Issatchenkia occidentalis* which was used for decolourisation of methyl orange and orange II [10]. Ionic liquids are low melting (< 100°C) salts and a new class of solvents that have been widely used for enzymatic catalysis [12]. 1- ethyl-3- methylimidazolium ethylsulfate [emim] [EtSO4] has been used throughout this study because it is one of the most promising ionic liquid used to enhance the performance enzyme in terms of its activity [13]. This ionic liquid has been proven to increase the activity of glucose oxidase and laccase enzyme [13,14]. The effect of ionic liquid on enzyme performance is largely influenced by the enzyme structure, the substrate and the water molecules associated with the enzyme [15].

2. Materials and Methods

2.1. Growth of Pseudomonas stutzeri ETL-79

The single colony of *Pseudomonas stutzeri* ETL-79 from the stock culture was transferred into 50 mL of nutrient broth and grown under aerobic conditions at 37°C for 24 h. The culture medium contained 10 mL of inoculums (v/v), 39.2 mL of Reactive Black 100 mg/L, 0.6 mL yeast extract 1.2% (v/v) and 0.2 mL glucose 0.4% (v/v). This medium was incubated at 37°C under anaerobic condition until decolourisation was observed after which the culture medium was incubated under aerobic condition in the shaking incubator at 37°C, 150 rpm. Growth was indirectly measured from the absorbance of cell suspension using a spectrophotometer at 600 nm. The decolourisation of Reactive Black was determined using American Dye Manufacturing Institute (ADMI) method and measured at 597 nm using SHIMADZU UV-1800 spectrophotometer.

2.2. Azoreductase Assay

Azoreductase assay was carried out according to the modification of method described by Moutaouakkil *et al.* (2003) [5]. The amount of Reactive Black decolourised was determined using a standard curve of the absorbance (A597) versus Reactive Black concentrations. One Unit (U) of azoreductase activity was defined as 1 µmol of Reactive Black decolourised per minute. Protein concentration of azoreductase was determined using Lowry method [16] and Bovine Serum Albumin (BSA) as the standard protein.

2.3. Dermination of Azoreductase Localisation

To determine azoreductase localisation, the experiment was carried out according to the modified of method described by Moutaouakkil *et al.* (2003) [5]. The absorbance was measured at 597 nm.

2.4. Effect of PH, Temperature, Substrate Concentration, NADH Concentration and Ionic Liquid Concentration on Activity and Stability of Azoreduactase

To determine the effect of pH on the activity of the enzyme, three different buffer systems were used for pH ranging from 3.0-9.0; the buffer systems were acetate buffer (pH 3-5), phosphate buffer (pH 6-8) and Tris-HCl (pH 9). To determine the optimum temperature, the incubation temperature was varied in the range of 25°C to 70°C. The azoreductase activity was also determined at substrate concentration ranging from 50 mg/L to 150 mg/L. In addition, the effect of NADH concentration on the enzyme activity was also determined. To investigate if ionic liquid can improve the enzyme activity and stability, 1-ethyl-3-dimethyl imidazolium ethylsulfate [emim] [EtSO4] was used instead of the phosphate buffer. The stability of azoreductase was investigated using the same method as above with an extended reaction time of 1 hour.

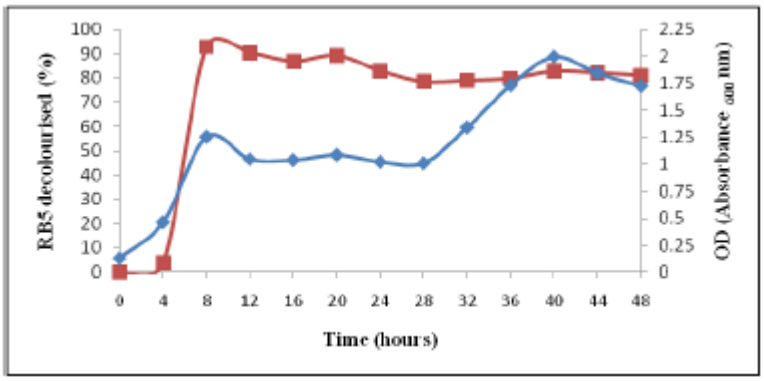

Figure 1. Decolorization of Reactive Black by *Pseduomonas stutzeri* ETL-79 under sequential anaerobic-aerobic system (■ = percentage of dye decolourised; ♦ = optical density as indirect measurement of bacterial growth)

3. Results And Discussions

Enzyme-mediated decolourisation is is an efficient method in dye containing effluent and currently is of great interest in the biological treatment of textile wastewater. From previous studies, crude and purified azoreductases were able to decolourise different textile dyes such as Reactive Black 5 [11], Methyl Red [5] and Acid Orange 7 [17], to mention a few. The rate of decolourisation for

each type of azo dye is expected to vary within minutes to hours depending on the type of azo bonds and the structural complexity of the azo dyes. Hence the presence of a bacterial azoreductase to decolourise Reactive Black makes *Pseudomonas stutzeri* ETL-79 important for textile wastewater treatment. Figure 1 below shows the decolourisation of Reactive Black under facultative anaerobic condition and subsequent aerobic process. The combined condition allowed the decolourisation process to occur during which growth was also observed as displayed by a typical growth profile of *Pseudomonas stutzeri* ETL-79 during 48 hours of incubation. It is interesting to note that exponential growth was observed during facultative anaerobic condition in contrast to what has been reported in many studies whereby the most active growth phase occurred during the aerobic condition. This may imply complete breakdown or mineralisation process of RB5 may involve azoreductase and oxido-reductases of *Pseudomonas stutzeri* ETL-79 that are induced under the facultative anaerobic condition. Figure 1 Decolourisation of Reactive Black by *Pseudomonas stutzeri* ETL-79 under sequential anaerobic-aerobic system. (■ = percentage of dye decolourised; ♦ = optical density as indirect measurement of bacterial growth) As shown in Figure 1, the maximum percentage of dye decolourised was up to 93% for Reactive Black after 8 hour of incubation which corresponded to the end of exponential growth phase. Significant increase in biomass in this phase may subsequently influence the decolourisation during anaerobic condition. In previous studies however, the decolourisation of azo dyes by *Enterobacter* sp. EC3 took about 60 to 108 hours to achieve between 78.4% to 92.6% of decolourisation [18]. Comparatively, *Pseudomonas stutzeri* ETL-79 showed higher decolourisation of Reactive Black dye within a shorter period of time. Since the bacterium was able to grow during the facultative anaerobic condition and reached its maximum growth under aerobic condition, it may be concluded that *Pseudomonas stutzeri* ETL-79 is a facultative anaerobic bacterium with the ability to grow in the presence of oxygen as well as limited amount of oxygen. Results from this study showed that decolourisation of Reactive Black was preferred under anaerobic condition since decolourisation of azo dyes in the presence of oxygen might be inhibited due to the competition between oxygen and azo bonds to be reduced by electron from electron carriers such as NADH. The localisation of azoreductase was studied in four types of cell fraction which were culture supernatant (CS), cell free extract (CFE), resting cell (RC) and cell debris (CD). These four fractions were tested for enzyme activity using azoreductase assay. The enzyme activity for each fraction was significantly lower than the fraction of cell free extract. The cell free extract showed the highest specific activity compared to the other fractions which the enzyme activity was 0.334 U/mg. The culture supernatant which represented the extracellular fraction had significantly lower enzyme activity of 0.034 U/mg. Hence, the localisation of azoreductase was probably the bacterial cytoplasm. This suggested that the bacterial membranes could act as efficient barriers for the uptake of sulfonated azo dyes. Several similar findings have been reported on azoreductase that are produced intracellularly by bacterial species such as *Bacillus* sp. Strain SF, *Lactobacillus casei* TISTR 1500 and *Bacillus* sp. B29 [11,19,20]. For the optimization of the azoreductase assay, a typical bell-shaped profile was obtained for pH optimisation as shown in Figure 2. The highest enzyme activity, 0.332 U/mL was found to be at pH 7. This finding was similar to the studies reported by Moutaouakkil *et al*. (2003) and Maier *et al.* (2004) [5,11]. The decolourisation of textile wastewater usually occurred at neutral pH or slightly alkaline pH. The extreme pH exhibited a decrease in enzyme activity due to protein denaturation at high pH and protein inactivation at low pH. This is caused by the changes of the enzyme structure especially at the active site [19]. This enzyme was found to be most stable at pH 7 with the activity of 0.301 U/mL. Furthermore, the azoreductase activity was slightly higher at the alkaline pH. Hence, this enzyme can be categorised into alkaline-tolerant enzyme. Maier *et al.* (2004) has reported that an alkali-thermostable azoreductase was successfully isolated from *Bacillus* sp. Strain SF which has significantly similar pH stability with azoreductase produced from *Pseudomonas stutzeri* ETL-79 [11]. The stability of enzyme decreased drastically with the change of ±1 pH unit. This may be due to the loss of cell viability that is caused by the conformational changes in the protein structure. Figure 3 shows the optimum temperature for azoreductase assay was 37°C. The acclimatisation of this bacterial growth in a specific medium culture has likely influenced its optimum activity during azoreductase assay. Decolourisation of Reactive Black 5 and azoreductase activity was significantly reduced at 40°C and above. This result might be due to the loss of cell viability and deactivation of the enzyme that is responsible for catalysing the reductive cleavage of azo dye at high temperature [21]. In addition, elevated temperatures could denature the enzyme. Hence, the enzyme active site would be disrupted causing the active site to undergo conformational changes. As shown in Figure 3, the azoreductase extracted from *Pseudomonas stutzeri* ETL-79 was most stable at 37°C with the azoreductase activity of 0.312 U/mL. The azoreductase was slightly stable at the temperature of 40°C and 50°C. Moutaouakkil *et al.* (2003), reported that the stability of azoreductase from *Enterobacter agglomerans* was influenced by the thermal inactivation above 40°C and further increase in temperature to 60°C reduced the enzyme stability drastically [5]. This may also resulted in the total loss of enzyme activity. Enzymatic assay was also performed by varying concentration of RB5. The optimum substrate concentration was found to be 100 mg/L with enzyme activity of 0.309 U/mL (Figure 4). At lower concentration of the Reactive Black (below 100 mg/L), increase in concentration showed proportional increase in enzyme activity. However, increase in dye concentration above 100 mg/L caused a drastic reduction of the enzyme activity. Higher concentrations of azo dyes may exert its toxic effects, affecting viability and subsequent decolourisation abilities [22]. Another plausible explanation is the saturation of enzyme binding sites. Similarly, the stability of azoreductase showed optimum azoreductase activity of 0.303 U/mL at substrate concentration of 100mg/L. Figure 5 shows the effect of NADH concentrations on azoreductase activity. NADH is an important redox mediator that supply electrons to reduce the azo bonds [11]. The maximum enzyme activity was found to be 0.319 U/mL using 0.2 mM NADH . Further increase in NADH concentration significantly

reduced the enzyme activity. This might be due to the excess consumption of NADH in oxidative phosphorylation pathway (during the aerobic process) that affects the decolourisation of azo bonds [19,21]. The azoreductase was found to be most stable at 0.2 mM and slightly less stable at 0.4 mM. The stability of this enzyme was significantly decreased with further increase of NADH concentration above 0.4 mM. This observation is in contrast to the previous observations which reported that azoreductase activity has a linear dependence with NADH concentration up to 1.5 mM and further increase of concentration did not increase the reaction velocity [11]. As an alternative medium for the enzymatic reaction, ionic liquid was used instead of phosphate buffer. This solvent has become a great interest amongst researchers due to its high thermal stability, low volatilities and low flammability [22]. In this study, the ionic liquid used was 1-ethyl-3-dimethyl imidazolium ethyl sulfate [emim] [EtSO4]. The presence of this non-aqueous solvent is required to increase the solubility of the reaction mixtures for azoreductase assay. The maximum activity of azoreductase was found to be at 70% concentration of [emim] [EtSO4] which shows the activity of 0.116 U/mL. According to Yang *et al*. (2008), the enzyme activity was altered by the repelling effect of the ionic liquid on the dynamics and conformation of the enzyme [23]. Based on the results shown in Figure 6, the activity of enzyme tested using ionic liquid was significantly lower than that of the phosphate buffer. It should be noted that most of the previous studies reported on the use of ionic liquid in the enzymatic studies of cellulose, protease and amylase. According to Tavares *et al.* (2008), high concentration of ionic liquid caused a decrease in enzyme activity besides causing the enzyme to precipitate out of solution [14]. Similar results of enzyme precipitation in ionic liquid were also observed with [emim][EtSO4] at higher concentration (75% (v/v) and above). The azoreductase was found to be most stable at 30% of ionic liquid concentration with the azoreductase activity of 0.157 U/mL. The lower concentration of ionic liquid shows slightly higher enzyme stability compared to the higher concentration of ionic liquid. This may be due to the delayed reaction between azoreductase and a lower concentration of [emim][EtSO4]. The reaction mixture becomes more saturated and azoreductase loses its activity because of excess ionic liquid. The enzyme activity decreased drastically after further increase in [emim][EtSO4] concentration above 30%. It was assumed that the high concentration of [emim][EtSO4] led to the total loss of enzyme activity. Duplissa *et al*. (2010) suggested that enzyme could maintain its activity a higher concentration of ionic liquid, if it is diluted with phosphate buffer instead of water [24]. The combination of water structuring and direct interactions between the ions and the protein could help to stabilise the enzyme structure. In addition, the enzyme activity was significantly decreased at high ionic liquid content because of the mass-transport limitation.

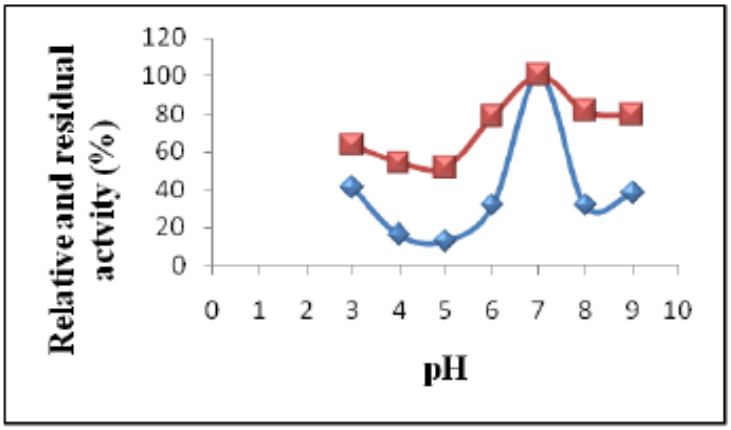

Figure 2. The effect of pH on azoreductase activity and stability

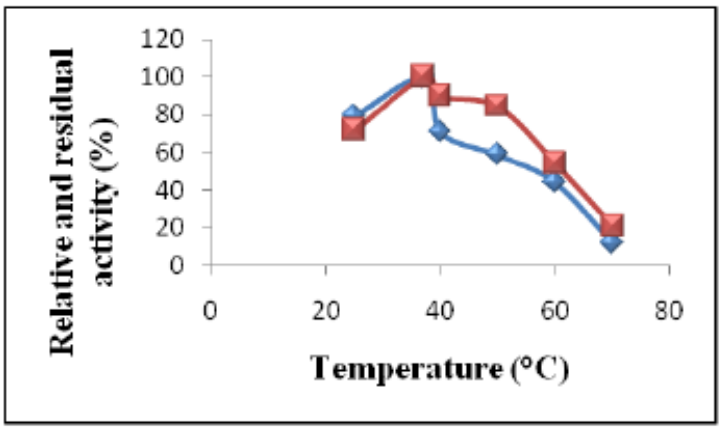

Figure 3. The effect of temperature on azoreductase activity and stability

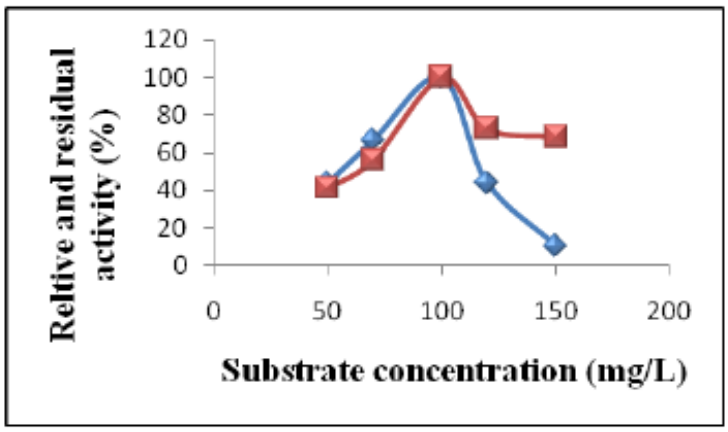

Figure 4. The effect of substrate concentration of azoreductase activity and stability

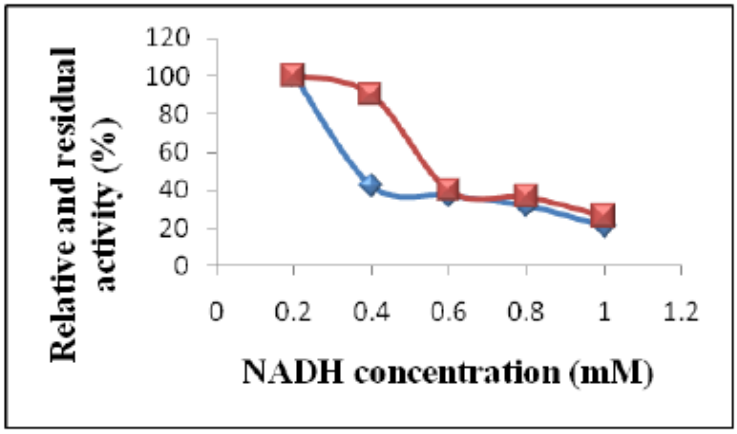

Figure 5. The effect of NADH concentration of azoreductase activity and stability

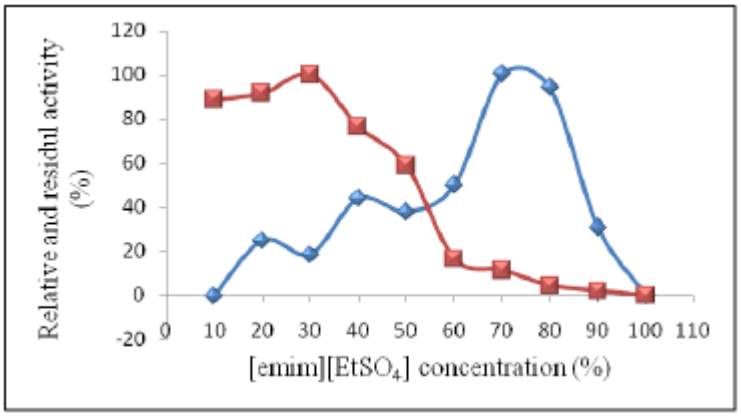

Figure 6. The effect of Ionic liquids concentration on azoreductase activity and stability (= activity; = stability)

4. Conclusion

As a conclusion, the optimum assay conditions for the maximum azoreductase activity were found to be at 37°C, pH 7, RB dye concentration of 100 mg/L and NADH concentration of 0.2 mM. Phosphate buffer was used to stabilise and minimise the changes in the reaction mixture of azoreductase assay. Results showed that ionic liquid [emim][EtSO4] cannot be used to enhance azoreductase activity. Further studies using other ionic liquid can be carried out to fully explore the potential of these organic solvents to enhance enzyme activity and stability. Decolourisation of Reactive Black was successfully achieved up to 93% under the optimized assay condition after 8h hours of incubation.

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