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Localization of dye degrading enzymes in *Xanthomonas campestris* MTCC 10, 108

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Abstract

Direct Red 28 is a carcinogenic direct diazo dye used for the coloration of paper products. It is recalcitrant and is mostly found in effluents of paper factories. Bacteria in consortia and monocultures those capable of decolorizing Direct Red 28 were isolated previously. The culture *Xanthomonas campestris* MTCC10, 108 was found able to decolorize dye consortia of Direct Red 28, Amido Black, Reactive Black, Reactive Blue, Reactive Red concentration of 20 mg/l each, thus making final concentration approximately to 100 mg/l. It was observed that the rate of decolorization by *Xanthomonas campestris* MTCC10, was varied when incubated under optimum environmental conditions. Dye degradation occurred in the supernatant of sonicated cells, indicating that the dye degrading enzyme was located intracellularly. In present study the active component responsible for decolorization. Direct Red 28 was found as azoreductase rather than laccase and peroxidases enzymes. The optimum concentration of NADH was 0.10 mM and 250 µg of enzyme resulted reduction of 100 µg/ml (highest) Direct Red 28. Based on these results, the optimal enzyme assay conditions were 100µg/ml Direct Red 28, 0.1mM NADH and 250 µg/ml enzyme in 1 ml assay mixture.

Keywords: *Azoreductase, Decolorization, Direct Red 28, Laccase, NADH, Xanthomonas campestris*

Introduction

Azo dyes are characterized by the presence of one or more R_1-N=N-R_2 bonds and is widely used in the paper, textile, plastic, pharmaceutical, food, cosmetic, enamels and drug industries (Collier *et al*., 1993; Dillon *et al*., 1994; Levine, 1991). The ability of micro-organisms to degrade textile azo dyes has been studied extensively in both aerobic and anaerobic processes (Banat *et al*., 1996; Pearce *et al*., 2003). More and more recalcitrant dyes are manufactured with the hope of improving the delivery of color onto fabric, at the expense of becoming increasingly difficult to bioremediate. This has created a need to investigate and understand the actual mechanisms behind the biodegradation of textile waste water. Several enzymes from fungi and bacteria have been identified and used in the breakdown of azo dyes.

Azo dye metabolites are produced after being reductively cleaved at the –N=N– position and are considered toxic aromatic amines. For example, the metabolism of the azo dye Direct Red 28 yields benzidine derivatives, a potential carcinogen (Cerniglia *et al*.,1982). In addition, *in vivo* and *in vitro* experiments supported the toxicity of these metabolites (Morgan *et al*., 1984; Chung, 1983*)*.

Sulphonated azo dyes are the largest and most versatile class of dyes. During textile processing, large amounts of dyestuff directly lost to the wastewater which ultimately finds its way into the environment. The discharge of such effluents from textile industries can result in serious environmental damages. Bioremediation is seen to be an attractive method for the treatment of textile effluent due to its low cost and environmental friendly nature (Banat *et al*., 1996*)*. The sulphonic acid groups that are introduced to increase the water solubility of the dye and azo group confer resistance to microbial attack and make them recalcitrant to oxidative decolorization (Nachiyar and Rajkumar, 2003). Microorganisms are efficiently used for the bioremediation due to their natural catalytic activities. Enzymatic treatments have less impact on the ecosystem as they present no risk of biological contamination. The efficiency of enzymatic reactions in textile processing has been recognized for many years and increasingly

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gained importance as biocatalysts in textile wet processing. Number of different redox enzymes has been noted for their stability to transform a wide variety of toxic pollutants. Redox enzymes may encounter field of application not only in bioremediation of polluted environments, but also in the development of novel clean technologies to avoid or diminish the environmental contamination. The textile dyes are able to undergo extensive oxidation coupling reactions mediated by the naturally occurring biocatalysts such as peroxidases, laccases and azoreductase (Huang *et al.*, 2002).

Laccase, Lignin peroxidase has ability to oxidize large number of aromatic compounds including highly polluting and recalcitrant compounds such as azo dyes. The enzyme produced by bacteria that is involved in reducing azo dyes is called azoreductase and the properties of the azoreductase vary from species to species.

Numerous enzymes for the pharmaceuticals and cosmetic industries are currently isolated by multi stage processes such as precipitation, dialysis, followed by several column chromatographic steps. In the present study, enzyme responsible for Direct Red 28 decolorization and location of active component (azoreductase) in *Xanthomonas campestris* cells was determined and optimization of enzyme assay conditions.

Materials and Method

Test for the Location of Azo Dye Degrading Enzyme

Extracellular Location

The decolorized culture broth of monocultures was centrifuged at 3,500 rpm for 10 minutes at 4°C. The supernatant was collected and sterilized using 2 µm sterile syringe filter. The sterile supernatant was incubated at 30 °C with 100 µg/ml Direct Red 28 and was observed for decolorization.

Intracellular Location

Cells from 2 l cultures were harvested by centrifugation at 3,500 rpm for 10 minutes, It was then washed three times with 50 mM sodium phosphate buffer (pH 6.0) and then suspended in 100 ml of the same buffer. Lysozyme and DNase I were added at final concentrations of 1 mgml $^{-1}$ and 10 µgml^{-1} respectively and the sample was incubated at 30 °C for 20 minutes (Punj and John, 2008). The sample was cooled by sonication (30 s, 70% output, 16×) using a Bandelin Sonopuls sonifier. Cellular debris and unbroken cells were removed by centrifugation at 8,000 rpm for 45 min at 4 °C. The supernatant thus obtained constitutes the crude bacterial extract (soluble protein fraction). Protein was determined by the method of Bradford using bovine serum albumin as a standard.

Enzyme screening assays

Laccase assay

Laccase enzymes have been widely shown to catalyze the degradation of azo dyes through a one step electron oxidation using molecular oxygen as a terminal electron acceptor (Stolz, 2001). In light of this, it became necessary to investigate the potential of terminal electron acceptor to replace the oxygen under anaerobic conditions. Laccase activity was determined using a modified protocol from Zarvazina *et al.* (2004), which used 2, 2´- azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) as a substrate. The reaction mixture contained ABTS (2 ml) dissolved in 50mM sodium phosphate buffer, (pH 6.0). The reaction was started by adding 1 ml of sample and was monitored at 30 °C for 3 minutes. The change in absorbance was monitored spectrophotometrically at 436 nm. One unit of activity was regarded as the amount of enzyme capable of converting 1µmole ABTS per min per ml.

Lignin peroxidase assay

Lignin peroxidase activity was measured by recording the increase in absorbance at 310 nm at 30°C due to the oxidation of veratryl alcohol (VA) to veratraldehyde (VAD) (Have *et al.*, 1997). The total volume of reaction mixture was 2 ml that contained 1 ml of sample and 1 ml of VA (2 mM) dissolved in 50 mM sodium phosphate buffer (pH 6.0). The reaction was started by adding 100 µl of 0.5 mM H_2O_2 and was monitored for over 10 minutes. One unit of activity was regarded as the amount of enzyme capable of converting 1µmole VA per min per ml.

Azoreductase assay

Azoreductase activity was assayed by the method of Zimmermann *et al.* (1982) using Direct Red 28 as dye substrate. The activity of azoreductase was determined spectrophotometrically at room temperature, using a UV/Visible spectrophotometer. In general, enzyme preparation was added to 50 mM sodium phosphate buffer (pH 6.0) containing 100 µM NADH (Sigma), 5 µM Direct Red 28 and 100 µl enzyme solutions to the total volume of the reaction mixture was 1.0 ml.

The reaction mixture was pre-incubated for 5 minutes followed by the addition of NADH and was observed the decrease in absorbance at 497 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1µmol of dye/min. All experiments and assays were carried out in triplicate. Protein concentration was measured as per standard methods of (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Optimization of Enzyme assays

All enzyme assays were carried out in a quartz cuvette with a total reaction volume of 1ml. The activity of the enzyme was assayed by measuring the decrease in the optical density for the azo dye at 497 nm for Direct Red 28 with a UV-visible spectrophotometer. The 1ml reaction mixture contained 50mM sodium phosphate buffer (pH 6.0), different concentrations of the azo dye Direct red 28 (60 to 140 µg/ml), different concentrations of NADH (0.05 mM, 0.1 mM, 0.15 mM) and at three different concentrations of the enzyme (100 µg, 250 µg , 500 µg) were used for the assays. Enzyme denatured by boiling and the addition of few drops of HCl was used as a control for all enzyme assay experiments and in second part of experiment under varying enzyme concentrations with optimum concentrations of NADH and dye was conducted to optimize the concentration of enzyme (Macwana, 2007). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the decolorization of 1 µM of azo dye per minute. Enzyme reactions were carried out under static conditions at room temperature and the reactions were initiated with the addition of NADH. All reactions were done in triplicates. A time course experiment was carried out for 2 minutes and readings were acquired every second.

Results and Discussion

Location of Azo Dye Degrading Enzyme

In this study, it was observed that dye degradation occurred in supernatant of the sonicated cells of the culture incubated with Direct Red 28. In the case of the filter sterilized culture broth with Direct Red 28, no decolorization occurred even after 10 days of incubation, indicating that the enzyme responsible for the reduction of Direct Red 28 is located intracellular (Stolz, 2001). Enzyme activity was expressed as a relative percentage in order to allow comparison of the azoreductase activity of the different cell fractions. The sonicated cell supernatant fraction was considered as 100% because it exhibited the highest activity of the enzyme. The disruption of the cells by sonication resulted in approximately 3-fold increase in azoreductase activity of the cell free extract. This indicates that there was release of intra-cellular azoreductase that did not have access to the substrate when the cell was intact, and this together with the periplasmic azoreductase gave the activity.

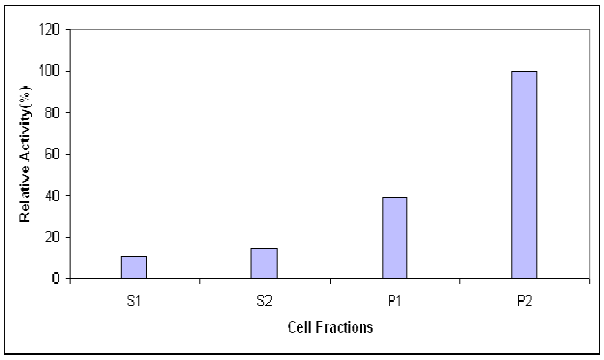

Figure 1: Relative Activity of azoreductase in different cell fractions before and after cell disruption by sonication, S_1=Supernatant before sonication, S_2 = Supernatant after sonication, P_1 = Pellet before sonication, P_2 = Pellet after sonication

Under anaerobic conditions, it has been reported that azoreductase require reducing equivalents to provide the electrons for the reductive cleavage of the dyes which act as the terminal electron acceptors. In our current experiment, NADH was used as a redox mediator since previous work indicated that azoreductase enzymes strictly require NADPH to provide hydrogen and electrons required for reductive cleavage of the azo bond (Zimmermann *et al.*; 1982, Stolz, 2001).

Screening for dye degrading enzymes

Once the ability of *Xanthomonas campestris* MTCC 10,108 to grow in the presence of a Direct Red 28 azo dye had been demonstrated, the next step was to identify the enzymes responsible for the reductive cleavage of the azo bond. Lignin modifying enzymes (laccase, lignin and manganese peroxidase) were investigated in this experiment with the aim of finding a substitute enzyme that would operate under partial anaerobic conditions. Unfortunately, but expectedly the activities for the laccase and the peroxidases were 0.171 and 0.147 Uml^{-1} respectively which were significantly lower than that for azoreductase activity as shown in Fig.2

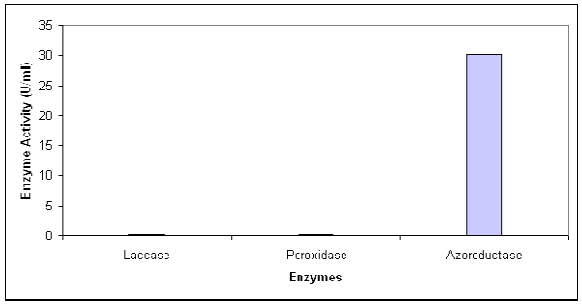

Figure 2: Identification of enzymes that can degrade azo dyes produced in *Xanthomonas campestris* cells

Azoreductase assays resulted in an activity of 30.20 Uml^{-1} which was significantly high in comparison to that of Laccase and Peroxidase activity. These enzymes have been successfully isolated and purified under aerobic conditions using *Pseudomonas* strains K22 and KF46 (Zimmermann *et al.*, 1982)

Optimum Enzyme Assay Conditions

To test azoreductase activity, three experimental approaches were used and the result obtained was at the concentration of 1.435 mM Direct Red 28 the percent relative activity was high. The significant activity was observed at 0.1 mM NADH and 250g enzyme concentration as shown in Figure 3, 4, 5. The result was almost similar at the enzyme concentration 250 µg and 500 µg. Hence, 250 µg was the optimum enzyme concentration at which further assay were conducted where as in case of NADH there was increase in enzyme activity to certain extent and afterwards there was no significant change observed in enzyme activity so the optimum concentration of NADH was 0.1mM To determine if the enzyme was responsible for the decrease in dye concentration, a few drops of HCl (2 µl, 36 %) was added, followed by boiling for 30 minutes. When the reaction was carried out in the presence of denatured enzyme, there was no reduction of Direct Red 28 demonstrating azoreductase activity of the enzyme. In addition different dye and cofactor NADH concentrations were tested with the same enzyme concentrations and a similar conclusion resulted. Interestingly, the different dye and NADH concentrations caused some change in the reduction of the concentration of Direct Red 28, suggesting some enzyme conditions were not optimal. Based on these results, the optimal enzyme condition was 150 µM dye and 0.15 mM NADH.

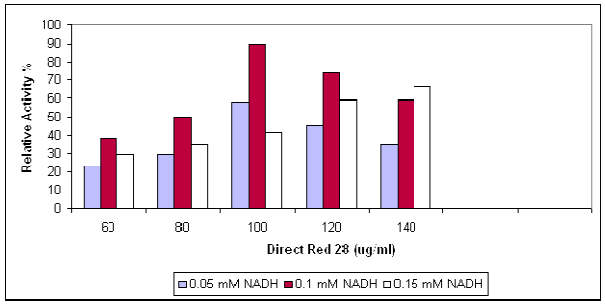

Figure 3: Rate of Decolorization at 100 µg enzyme concentration and different concentration of NADH and Direct Red 28

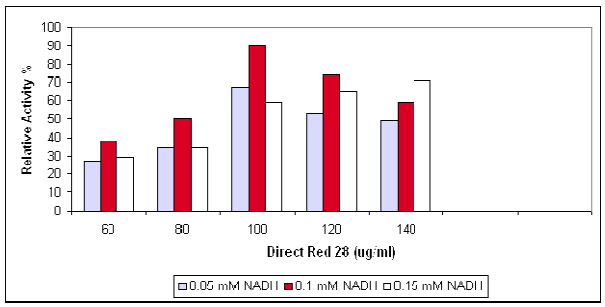

Figure 4: Rate of Decolorization at 250 µg enzyme concentration and different concentration of NADH and Direct Red 28

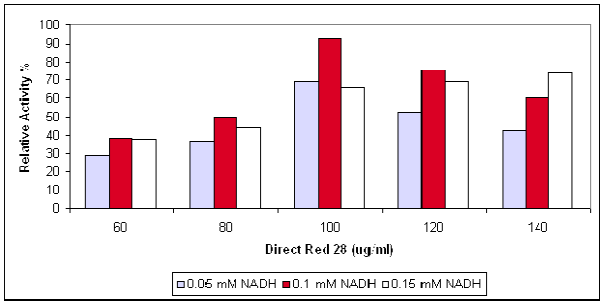

Figure 5: Rate of decolorization at 500 µg enzyme concentration and different concentration of NADH and Direct Red 28

Reduction of Direct Red 28 under optimal conditions

Based on the pH and temperature experiments, a time point measurement of the complete reduction of Direct Red 28 was taken. Using three different concentrations (100 µg, 250 µg and 500 µg) of enzyme, 0.1 mM NADH and 100 µg/ml of Direct Red 28 incubated at 30 ºC, it took approximately 14 minutes to reduce most of the dye (Table-1 and 2). The varied and incomplete reduction of the dye with three different enzyme concentrations suggested that Direct Red 28 concentration is limiting or inhibitory factor.

Table-1: Mean residual dye reduction of the concentration of the azo dye Direct Red 28(100 µg/ml) with three concentrations of the enzyme and differentconcentrations of NADH (0.01-0.2mM)

Enzyme Conc.	Direct Red 28 (100 µg/ml)				
	NADH				
	0.01 (mM)	0.05 (mM)	0.1 (mM)	0.15 (mM)	0.20 (mM)
100 (µg)	60.76±0.053	56.48±0.020	34.57±0.011	50.31±0.043	47.65±0.022
250 (µg)	46.76±0.040	32.17±0.047	11.60±0.079	38.9± 0.039	40.14±0.032
500 (µg)	41.62±0.020	26.35±0.022	9.68±0.007	15.32±0.056	19.19±0.020

Table 2: Mean residual dye reduction of the concentration of the azo dye Direct Red 28(120 µg/ml) with three concentrations of the enzyme and different concentrations of NADH (0.01-0.2 mM).

Enzyme Conc.	Direct Red 28 (100 µg/ml)				
	NADH				
	0.01 (mM)	0.05 (mM)	0.1 (mM)	0.15 (mM)	0.2 (mM)
100 (µg)	88.18±0.053	79.48±0.020	54.47±0.043	68.21±0.043	71.92±0.011
250 (µg)	49.76±0.002	34.67±0.016	23.41±0.005	12.70±0.003	19.46±0.011
500 (µg)	55.83±0.040	51.28±0.047	17.71±0.079	26.32±0.039	28.74±0.031

Under optimum conditions Direct Red 28 (100µg/ml), 0.1 mM NADH and 250 µg enzyme concentration. The reduction in the concentration of dye was observed as shown in Figure 6 after 14 minutes of reaction. Azo dyes are used extensively in many industries. These azo dyes have been shown to be reductively cleaved by a wide range of microorganisms. Bacteria, both aerobic and anaerobic from different environment possess the ability to reduce azo dyes.

The study concluded that *Xanthomonas campestris* MTCC 10, 108 possessed the ability to reduce mono and diazo sulfonated dyes Direct Red 28 with no inhibitory effect on the growth of the bacteria. This indicates that the enzyme azoreductase is functionally expressed in *Xanthomonas campestris* MTCC 10, 108. When the supernatants were tested for activity, no activity was observed which showed that the enzyme is not extracellular but an intracellular protein and azoreductase from *Xanthomonas campestris* cells was released by sonication.

In conclusion, we have shown that the *Xanthomonas campestris* possess azo reductase enzyme rather than Laccase and Peroxidase enzymes.The enzyme azoreductase was capable of reducing the water soluble diazo dye Direct red 28

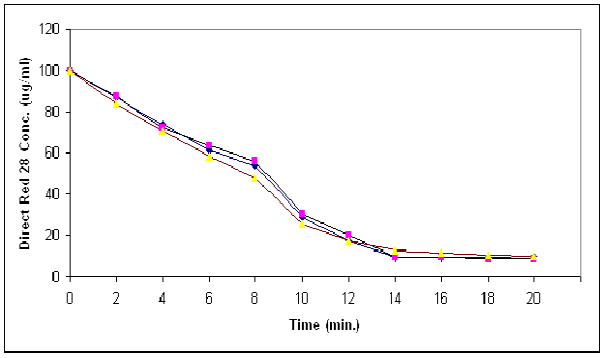

Figure 6: Time course reduction of Direct Red 28 (100 µg/ml) using optimal conditions for the enzyme. The optimal conditions were 0.1mM NADH, pH 6, 30°C (Experiments were done in triplicates).

Direct in the presence of the cofactor NADH. The reduction of the dye by the enzyme was not linear. This may include several factors. These factors may include the NADH and enzyme concentration which may affect enzyme activity. The optimal conditions for enzyme activity were found to be 30 °C, pH of 6.0, 0.1 mM NADH and 250 µg enzyme concentration.

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