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ENHANCED APPLICATION OF CRUDE THERMOTOLERANT LACCASE FROM *ACINETOBACTER LWOFFII* IN DECOLOURIZATION OF METHYL ORANGE (AZO DYE)

Folasade M. Olajuyigbe

Enzyme Biotechnology and Environmental Health Unit, Department of Biochemistry, Federal University of Technology, Akure 340252, Nigeria

E-mail address: folajuyi@futa.edu.ng

ABSTRACT

Discharge of synthetic dye effluents from industries into inland and coastal waters is a global environmental problem. Hence, intensive research has been focused on decolourization of wastewater by environmentally friendly biological treatments. Bacterial laccases are currently gaining increasing attention for their potential in dye decolourization and bioremediation. In this study, process parameters were optimized to achieve efficient decolourization of a selected azo dye, methyl orange, using crude thermotolerant laccase from *Acinetobacter lwoffii.* Optimum decolourization of azo dye was obtained at pH 4.0 and 50°C with crude laccase from *A. lwoffii* after 1 h of incubation. Agitation speed for maximum decolourization was 190 rpm and 100% decolourization of methyl orange was achieved in the presence of Cu^{2+} while 80% decolourization was obtained in the presence of Fe^{2+} and Ca^{2+}, respectively. Results show that crude laccase from *A. lwoffii* is efficient for dye decolourization under optimized conditions with potentials for application in the treatment of textile effluents and other dye-containing wastewater.

Keywords: *Acinetobacter lwoffii,* Laccase, Azo dye, Methyl orange, Decolourization.

INTRODUCTION

Synthetic dyes coming from industrial effluents are recalcitrant compounds that impair the life of aquatic organisms due to their toxicity, and inhibit photosynthesis in watercourses (Gioia *et al*., 2018). There are varieties of dyes based on the structure of the chromophore such as azo, anthraquinone, indigoid, phthalocyanine, sulphurous, nitro and nitroso (Gürses *et al*., 2016). The discharge of highly coloured synthetic dye effluents from textile industries into inland and coastal waters is a global environmental problem of growing concern. Azo dyes remain the most important group of synthetic dyes used in commercial applications with textile, pulp and paper industries producing large amounts of water with high azo dye contents (Singh *et al*., 2015). Unfortunately, most of these dyes escape conventional wastewater treatment processes and persist in the environment as a result of their high stability against light, temperature, water, detergents, chemicals, and microbial attack (Couto, 2009).

Discharge of these dyes into the environment has various toxic, carcinogenic, mutagenic and teratogenic impacts. In fact, their ability to absorb sunlight in water, affects the photosynthetic activity of algae and other aquatic organisms with serious influence on food chain (Guari *et al*., 2015). The ever-increasing challenge facing discharge of dyes into water bodies is that

the public perception of water quality is greatly influenced by the colour of water, hence, removal of wastewater colour is crucial (Gürses *et al.*, 2016). The treatment of azo dye-containing wastewaters still remains and presents a serious ecological and technical challenge (Arora, 2014).

Reports have shown that different physicochemical remediation techniques such as membrane filtration, coagulation and chemical flocculation have been applied to reduce levels of dye pollution in aquatic environments (Goia *et al.*, 2018). It is however disheartening that the efficacy of all the conventional approaches is limited due to high operating costs, generation of sludge, production of toxic by-products, and high requirements of chemicals and energy (Vikrant *et al.*, 2018). Presently, bioremediation, based on biodegradation of pollutants by microorganisms and isolated enzymes has attracted much interest due to reduction of sludge production and environmental friendliness (Ajaz *et al.*, 2019; Jonstrup *et al.*, 2011).

Laccases (EC 1.10.3.2) are polyphenol oxidases which belong to the family of blue multi-copper proteins containing copper atoms in their catalytic center, hence, the enzymes are referred to as multicopper oxidases (Baldrian, 2006). Laccases have found wide applications in bioremediation, textile, biofuel and other industries (Olajuyigbe *et al.*, 2019; Mate and Alcalde, 2017). They are known for their natural ability to degrade lignin, a highly complex non-phenolic polymer, which also affords them the potential capacity to decolourize a wide variety of dyes (Yang *et al.,* 2009). The decolourization ability of laccase has opened up new prospects for the development of biotechnological processes aimed at degrading complex polymers such as azo dyes and other synthetic dyes. This study focused on applying a previously characterized crude thermo-tolerant laccase (Olajuyigbe and Akinpelu, 2015) from *A. lwoffii* in decolourization of methyl orange.

MATERIALS AND METHODS

Microorganism

The microorganism used for this study was *A. lwoffii*, a non-fermentative gram-negative bacterium. This was obtained from the culture collection of Enzyme and Microbial Technology Laboratory, Department of Biochemistry, Federal University of Technology, Akure, Nigeria.

Materials

Sodium hydroxide, 2,2'azino-di-[3~ethyl benzothiazoline-6-sulphonic acid] (ABTS), glycine, sodium acetate, copper chloride, mercury chloride, manganese chloride, calcium chloride, iron (II) chloride, hydrochloric acid, sodium citrate and Azo dye (methyl orange) were all products of Sigma-Aldrich, Germany. Tris-(hydroxymethyl)-aminomethane, yeast extract, beef extract were products of Scharlau Chemie S.A. All other reagents used were of analytical grade.

Assay of laccase activity

Laccase activity was determined according to a modified method of Han *et al.* (2005). This was done by monitoring spectrophotometrically the change in absorbance at 420 nm (A_{420}) related to the rate of oxidation of 1 mM 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS) in 50 mM Na-acetate buffer (pH 3.8). Assays were performed in 1 mL cuvettes at room temperature with 750 µL ABTS and 250 µL of enzyme extract. The enzyme activity was calculated using equation 1:

$$\text{Laccase Activity (U/mL)} = \frac{\text{Change in Absorbance x Total volume of mixture}}{\text{Total time x Extinction coefficient x Volume of enzyme}} \quad (1)$$

Decolourization assay

Methyl orange (0.18 mM) was prepared in 50 mM sodium acetate buffer (pH 3.8). Dye decolourization assays were carried out spectrophotometrically at 470 nm using the modified method of Pardo *et al.* (2013). Briefly, decolourization of dye was measured by decrease

in absorbance at 470 nm. Assays were performed under respective parameters in a reaction mixture containing 1 mL methyl orange solution and 0.25 mL crude enzyme. Percentage decolourization of methyl orange was thereafter determined following the spectrophotometric method described by Ponraj *et al*. (2011) using equation 2:

$$\% \text{ Decolourization} = \frac{\text{Initial Absorbance - Final Absorbance}}{\text{Initial Absorbance}} \times 100 \qquad (2)$$

Influence of Process Parameters on Methyl Orange Decolourization

Effect of pH on methyl orange decolourization

Methyl orange (0.18 mM) was prepared in different buffers with pH range of 3.0 - 11.0. The buffers used were 50 mM glycine-HCl (pH 3.0), 50 mM sodium acetate (pH 4.0 - 6.0), Tris - HCl (pH 7.0 - 8.0) and glycine-NaOH (pH 9.0 - 11.0). The dye-buffer mixture was used for decolourization assay at different pH. Reaction mixture was composed of 0.25 mL crude enzyme and 1 mL dye-buffer mixture. Decolourization of methyl orange by crude laccase was monitored at 20 min intervals over 1 h at 470 nm. The percentage decolourization was then determined.

Effect of temperature on methyl orange decolourization

Effect of temperature on methyl orange decolourization was determined by incubating reaction mixture of enzyme and dye solution in glass test tubes at temperature range of 30 – 80ºC for 1 h at 20 min intervals. Decolourization assay was done according to the standard assay procedure earlier described and the percentage decolourization was determined.

Effect of agitation speed on methyl orange decolourization

Effect of agitation speed on methyl orange decolourization was determined by incubating reaction mixture of crude laccase and methyl orange solution at 25ºC in a shaking incubator at varying speed (150 - 240 rpm). The decolourization was monitored for 1 h at 20 min intervals. Dye decolourization assay was carried out following the spectrophotometric method earlier described, and percentage decolourization was determined.

Effect of metal ions and EDTA on methyl orange decolourization

Some metallic chlorides were used to determine the effect of divalent metal ions on methyl orange decolourization. Metal ions used for the study were Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, $Cu^{2+}$and Hg^{2+}. Effect of 5 mM ethylenediaminetetraacetic acid (EDTA) on dye decolourization was also examined. Five millimolar (5 mM) of each metallic chloride was prepared in 50 mM sodium acetate buffer (pH 3.8). Each metallic chloride solution (500 µL) was added to 500 µL crude enzyme solution with gentle mixing at 25°C. Decolourization assay was done by adding 0.25 mL enzyme-metal ion preparation to 1 mL 0.18 mM methyl orange and both were gently mixed. Dye decolourization was thereafter monitored for 1 h at 20 min intervals. Result was compared with control, which contained 0.25 mL crude laccase in 50 mM sodium acetate buffer (pH 3.8) and 1 mL 0.18 mM methyl orange. Absorbance was read at 470 nm, and percentage decolourization was determined.

RESULTS AND DISCUSSION

Effect of pH on methyl orange decolourization

Figure 1 showed that pH has a major effect on the efficiency of dye decolourization by laccase from *A. lwoffii*. Maximum decolourization efficiency of laccase was at pH 4.0. This demonstrates specificity in characteristics of the enzyme as earlier reported by Olajuyigbe and Akinpelu (2015) showing that the optimum pH of laccase from *A. lwoffii* is 4.0. The relative decolourization efficiency of laccase from *A. lwoffii* was 80.95% at pH 3.0. There was steady decrease in decolourization efficiency at pH

above 4.0 with 72.28%, 50%, 43.59%, 40.48%, 34.39%, 30.36% and 16.75% at pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, respectively. It is highly remarkable that laccase from *A. lwoffii* decolourized azo dye maximally at pH 4.0 which was the optimum pH at which the enzyme exhibited highest activity (2.4 U/mL). This shows that the medium pH has great effect on the efficiency of dye decolorization which is dependent on enzyme activity at a particular pH (Hashem *et al*., 2018). Drastic decline in percentage decolourization of methyl orange towards alkaline pH with about 34% at pH 9.0 and 17% at pH 11.0 suggests low stability of *A. lwoffii* laccase in the alkaline pH range. Tagushi *et al*., (2018) in their study had also reported decolourization of recalcitrant dyes by a multicopper oxidase produced by I*odidimonas* sp. with optimum decolourization between pH 5.0 and 6.0. In contrast, some authors have reported optimal decolourization of dyes by laccases at neutral and alkaline pH (Dube *et al*., 2008, Brander *et al*., 2014, Kaira *et al*., 2015).

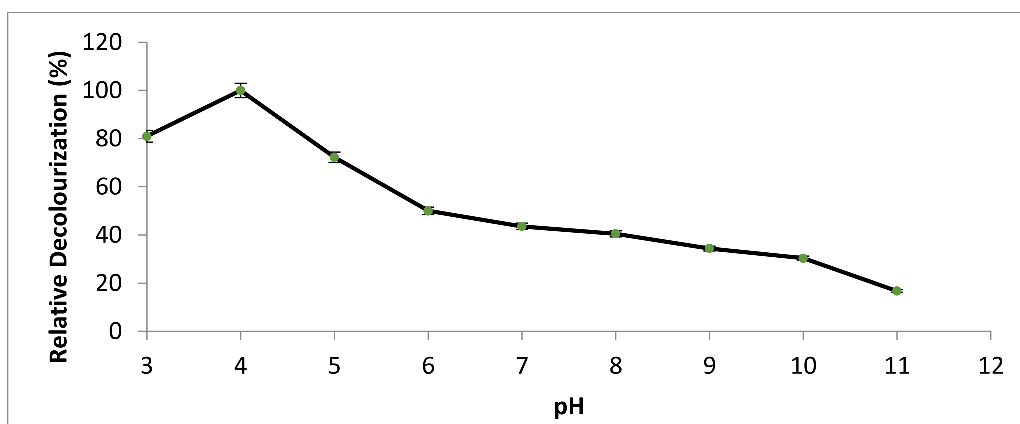

Figure 1: Relative effect of pH on decolourization of methyl orange by crude laccase from *A. lwoffii* (Error bars represent Mean ± Standard Deviation)

Effect of temperature on methyl orange decolourization

The effect of temperature was examined by incubating the reaction mixture at temperatures between 30°C and 80°C. The initial reaction rate increased with rise in temperature up to 50°C (Figure 2). Decolourization decreased as the temperature was increased further; an indication of enzyme deactivation or loss of activity at temperatures higher than 50°C. Similar result was reported on recombinant CotA laccase from *Escherichia coli* BL21 (DE3) that showed optimum dye decolourization at 50°C (Zhang *et al*., 2012). However, optimum decolourization of Congo red by spore bound laccase from *B. subtilis* WD23 was at 37°C (Zhao *et al.*, 2011).

Effect of agitation speed on methyl orange decolourization

Methyl orange decolourization by crude laccase from *A. lwoffii* increased with increase in agitation speed from 150 rpm reaching maximum decolourization at 190 rpm implying the role of dissolved oxygen in promoting laccase activity. It was however surprising that a sharp decline of 51% in decolourization efficiency of enzyme was obtained by increasing the agitation speed to 200 rpm. Crude laccase from *A. lwoffii* lost about 90% decolourization efficiency at 240 rpm (Figure 3). This strongly suggests that the agitation speed of decolourization mixture had effect on *A. lwoffii* laccase activity. It is speculated that agitation speed above 190 rpm disrupts the conformational integrity of the laccase under study thereby

inactivating the enzyme and reducing its decolourization efficiency. This corroborates extensive reports on the crucial role that protein conformation plays in the catalytic efficiency of an enzyme (Secundo, 2013). Hence, agitation is an important process parameter that should be considered during decolourization of dyes. Agitation had earlier been reported to increase percentage dye decolorization in the work done by Selvakumar *et al*. (2013) up to 200 rpm.

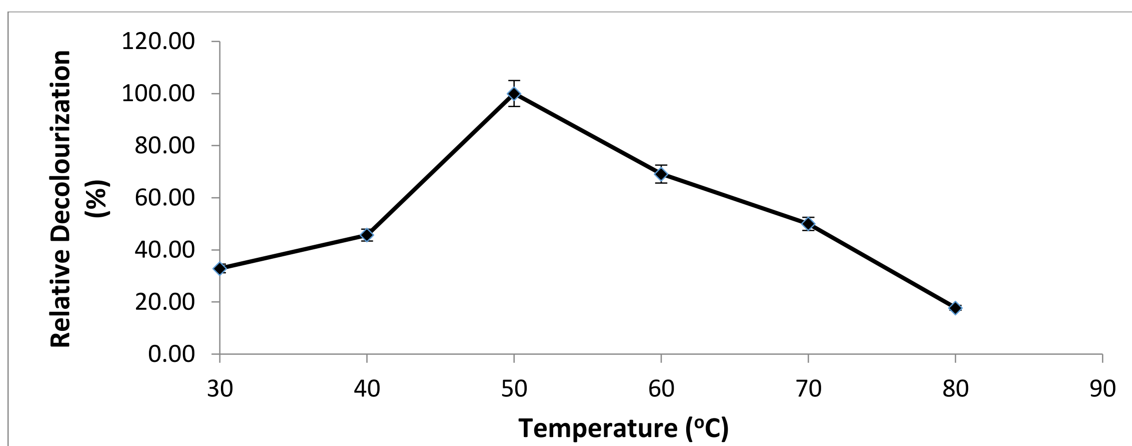

Figure 2: Relative effect of temperature on decolourization of methyl orange by crude laccase from *A. lwoffii* (Error bars represent Mean ± Standard Deviation)

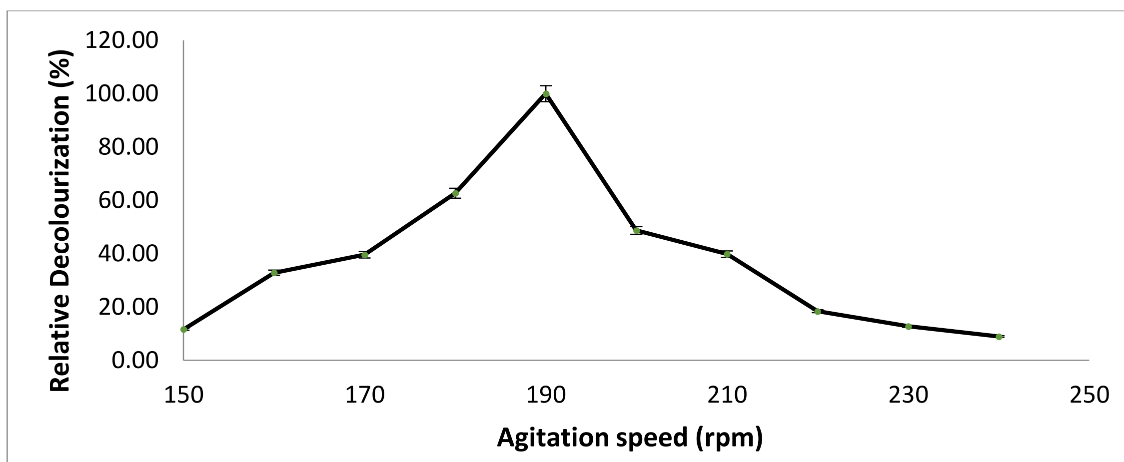

Figure 3: Relative effect of agitation speed on decolourization of methyl orange by crude laccase from *A. lwoffii* (Error bars represent Mean ± Standard Deviation)

Effect of metal ions on methyl orange decolourization

The effect of metal ions on decolourization of methyl orange by *A. lwoffi* laccase (Figure 4) revealed that 55% decolourization was achieved by crude laccase in the absence of metal ion (control). Cu^{2+} enhanced complete decolourization of methyl orange after 1 h of incubation which represents 100% decolourization efficiency. Similar activation of laccases by Cu^{2+} had been reported (Haibo *et al*., 2009). Decolourization was also enhanced in the presence of Fe^{2+} and Ca^{2+} with 80% decolourization efficiency recorded after 1 h incubation. Mn^{2+}, Mg^{2+}, Hg^{2+} and EDTA strongly inhibited decolourization of methyl orange by crude laccase from *A. lwoffii* with percentage decolourization ranging from 7% to 15%. Laccases can be inhibited by metal ions such as Fe^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+} and

Ag^+ (Zavarzina *et al.*, 2004). Fe^{2+} may interrupt the electron transport system of laccase and substrate conversion (Kim and Nicell, 2006). Interestingly, in this study, 80% decolourization efficiency was obtained in the presence of Fe^{2+} after 1 h incubation. It is surprising that despite many reports on bacterial laccases, no study has been carried out on the effect of metal ions on decolourization of dye by any bacterial laccase.

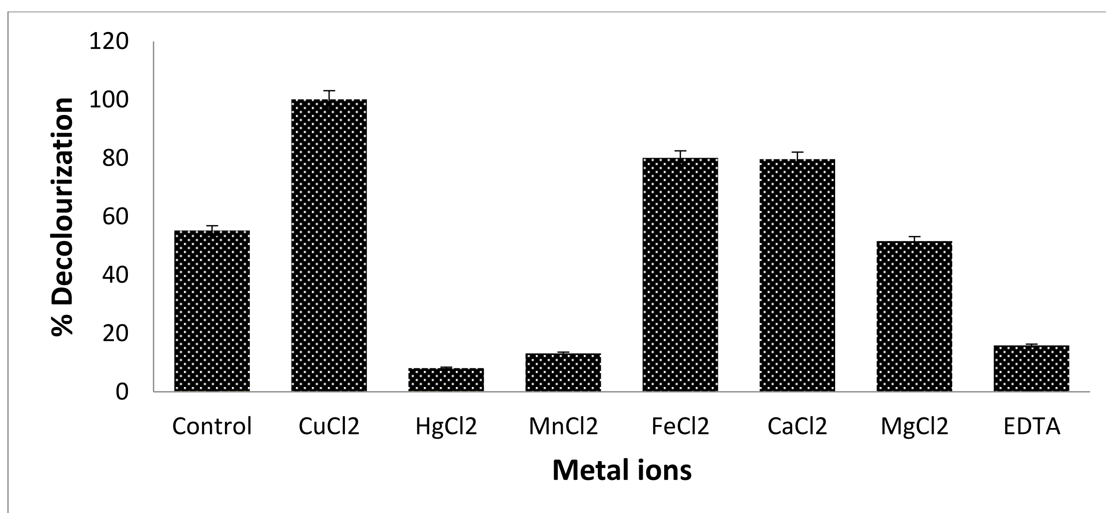

Figure 4: Effect of metal ions on decolourization of methyl orange by crude laccase from *A. lwoffii* (Error bars represent Mean ± Standard Deviation)

CONCLUSION

Findings from this study show that crude thermotolerant laccase from *A. lwoffii* decolourized methyl orange completely at pH 4.0 and 50°C with agitation speed of 190 rpm, in the presence of Cu^{2+}. Results indicate that dye decolourization by crude laccase was largely influenced by process parameters. The high decolourization efficiency exhibited by the crude thermo-tolerant laccase from *A. lwoffii* indicates that the enzyme has potentials for application in the effective treatment of azo dye-containing wastewaters and bioremediation.

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