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CALCIUM ALGINATE ENTRAPMENT OF *ASPERGILLUS NIDULANS* IMPP-0785 LACCASE FOR ENHANCED ENZYME CATALYTIC ABILITY, THERMOST ABILITY AND DYE-DECOLORIZATION

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Abstract

Production of laccase using a solid-state culture of *Aspergillus nidulans* IMPP-0785 was optimized using barley bran. *Aspergillus nidulans* IMPP-0785 laccase optimum activity was observed with filtrate (29.15 U/g) as compared to supernatant and unfiltered. Free laccase and supernatant gave best results at 30.24 and 25.95 U/g, respectively for 20 min incubation time. The entrapped spores of *A. nidulans* IMPP-0785 showed maximal activity (16.12 U/g) at incubation time for 60 min. Immobilized laccase resulted optimal activity (29.24 U/g) for 25 min of incubation. Enzyme showed higher thermo stability of 34.28 U/g when incubated for 35 min.

Introduction

Laccases are common enzyme in nature. The first laccase was reported by Wesenberg *et al*. 2003) from *Rhusvernicifera* (Basidiomycetes) and white rot fungi laccase was derived (Viswanath *et al*. 2008). Laccases depend on Cu altos in their characteristic electronic paramagnetic resonance (EPR) signals (Bento *et al*. 2006). Laccases have been reported to have several applications including bioleaching (Arias *et al*. 2003), removal of phenolics, xenobiotics, and other aromatic compounds (Duran and Esposito 2000).

They can perform functions of pharmaceutical (Mehdi *et al*. 2012), dye-degradation (Nyanhongo *et al*. 2002) and detoxification of industrial dyes. These enzymes are used for pulp delignification, pesticide or insecticide degradation, organic synthesis, waste detoxification, textile dye transformation, food technological uses, biosensor and analytical applications. These enzymes have significant HIV-1 reverse transcriptase activity (Harris *et al*. 2004).

Over the last two decades use of laccase has been explored for bioremediation of xenobiotics such as endocrine disrupting compounds (EDCs) for bleaching in pulp and paper industry, decolonization of azo dyes in textile industry (Sinirlioglu *et al*. 2013). They dominate the dyestuff market with share of about 70%, used for enzyme immobilization on solid materials and gels, low stability and productivity and high production costs, adsorption, entrapment of enzymes (Gupta and Suhas 2009). Fermentation types are; Submerged fermentation (SmF) and Solid state fermentation (SSF) (Elisashvili *et al*. 2008).The physical methods involve adsorption, entrapment of enzymes in insoluble polymeric gels (polymeric entrapment) or in micelles (encapsulation) (Duran *et al*. 2002).

Materials and Methods

Aspergillus nidulans IMPP-0785 was isolated and identified in the Department of Botany, University of Gujarat. The fungal strain was stored on barley bran extract medium with 15 g agar

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per liter at 4°C and homogenous suspension was put in another test tube. Haemocytometer was used for spore count and found to be 2.15×10^7 CFU/ml. One hundred g of dried agar was added in 500 ml cotton-plugged Erlenmeyer flasks with 100 ml of 0.1 N HCl and autoclaved at 15 lbs/in^2 pressure (121ºC) for 15 min to attain the predesigned moisture content. Contents were mixed thoroughly by keeping the flask in a shaking incubator at 200 rpm for 1 hr and centrifuged at 3,000 rpm for 20 min. Laccase activity was determined by following method enzyme extract (1 ml), 0.5 ml of 50 mM sodium acetate buffer (pH 5) and 0.1 ml of 1µM PDC were taken in a glass cuvette.

$$\text{Laccase activity (U/g)} = \frac{A_{470\,nm} \times DF \times V_1}{\text{Substrate (g)}} \quad \frac{V_2}{\Delta T}$$

where, A_{470nm} is value of absorbance, DF (dilution factor), V_1 (volume of reaction mixture in glass cuvette), V_2 (volume of extract added) and ΔT (time of incubation).

Spores of *A. nidulans* IMPP-0785 were immobilized by taking 3% of sodium alginate solution, for 30 min at 45°C in darkness at 620 nm (Sinirlioglu *et al.* 2013).

$$\text{Dye decolorization (\%)} = \frac{\text{(Initial absorbance – final absorbance)}}{\text{Initial absorbance}} \times 100$$

Treatment effects were compared by the protected least difference method (Spss-21, version-4, USA). Significance difference among the replicates has been presented as DMRT in the form of probability (p) value.

Results and Discussion

Two batch cultures were run parallel from which batch-1 diluted with 200 ml distilled water and batch-2 with 400 ml distilled water to find out dilution effect on laccase production of *A. nidulans* IMPP-0785 (Fig. 1). The less activity (7.65 U/g) was observed with supernatant of batch-2. A significant ($p \leq 0.05$) higher activity (29.15 and 27.46 U/g) was obtained with batch-1 and batch-2 filtrate, respectively.

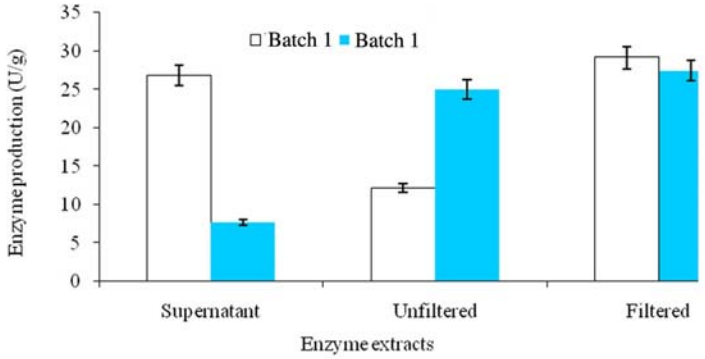

Fig. 1. Dilution effect on laccase production of *A. nidulans* IMPP-0785.

The rate of laccase production from *A. nidulans* IMPP-0785 in SSF was studied (Fig. 2). The enzyme stability was recorded to a maximal of 25.95 U/g with supernatant and 30.24 U/g for crude enzyme. Afterwards, enzyme stability was declined gradually up to 23.98 U/g with filtered crude enzyme. Hence, 20 min time of incubation was optimized for enzyme production.

The effect of incubation period (5, 10, 15, 20, 25, 30, 35 and 40 min) on laccase production was studied in sodium acetate buffer and 1 µm of PDC solution (at 620 nm), highest enzyme activity (20, 40, 60, 80, 100 and 120 min) (Fig. 3). Best results in terms of enzyme activity achieved (29.24 U/g) when incubated for 25 min. Enzyme production declined gradually up to 10.02 U/g at an incubation of 40 min. Hence, 25 min time of incubation was optimized as holding time for maximal enzyme activity.

The effect of incubation temperature (Fig. 4) at different temperature (15, 20, 25, 30, 35, 40, 45, 50, 55 and 60ºC) was applied to observe enzyme activity. A maximal enzyme activity (34.28 U/g) was found when incubated temperature was 35ºC and minimal (6.66 U/g) was achieved when temperature was raised up to 55ºC.

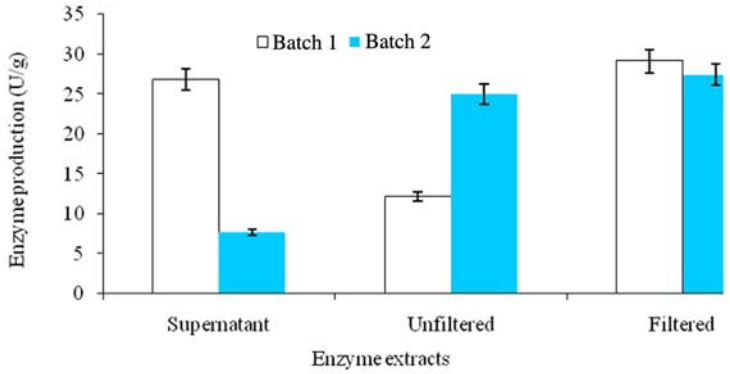

Fig. 2. Effect of different level of diluents on the production of an extracellular laccase from *A. nidulans* IMPP-0785 under SSF.

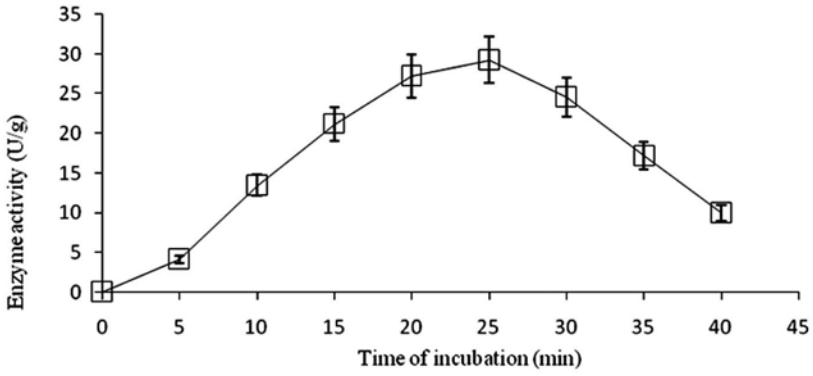

Fig. 3. Effect of different incubation periods on the activity of immobilized laccase from *A. nidulans* IMPP-0785 under SSF.

Effect to different dilutions of enzyme (2.5, 5, 7.5, 10, 12.5 and 15%) was a gradual increase in enzyme activity. Dilution effect increased from 2.5 to 10% and then abruptly decrease from 12.5 to 15% dilution factors and minimal activity (24.25 U/g) noticed at 15% (Fig. 5).

Maximal decolorizing percentage (82.55) was achieved with immobilized laccase of methylene blue and eosin gelblich dye was least decolorized (32.63%). While malachite green dye decolorized 71.2% with laccase enzyme (Fig. 6).

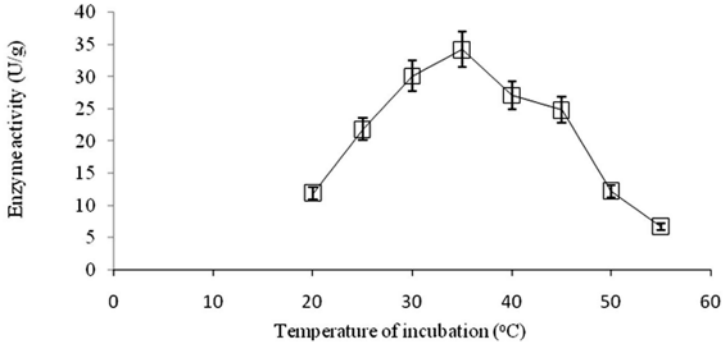

Fig. 4. Effect of temperature on activities and stability of immobilized laccase produced from *A. nidulans* IMPP-0785 under SSF.

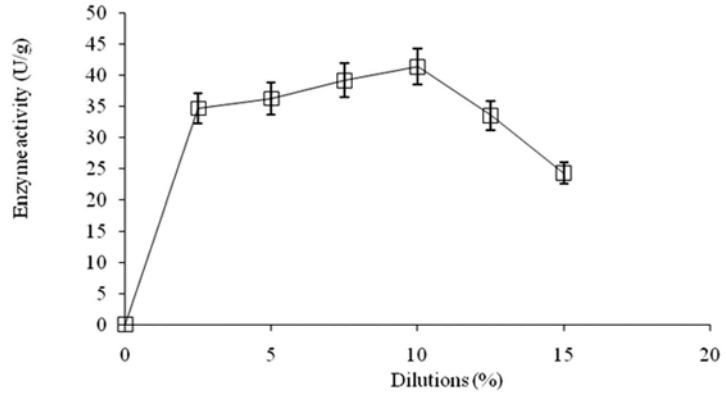

Fig. 5. Effect of different enzyme dilutions on activity of immobilized laccase.

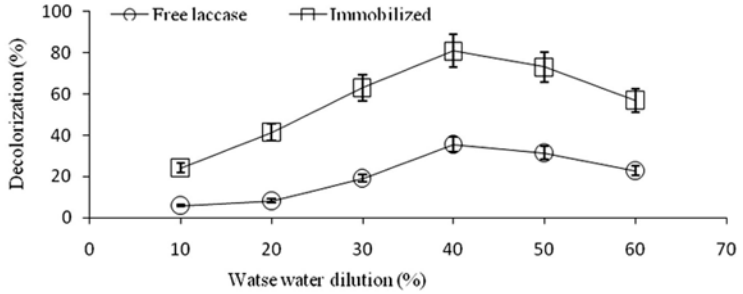

Fig. 6. Effect of free laccase and immobilized laccase on decolorizing activity of different dyes.

Enzyme production, immobilization, time of incubation, different dilutions, thermo stability and decolorizing activity with different dyes and industrial waste water were investigated, respectively. A large number of agricultural wastes are used as substrate for example cotton stalk, molasses waste water, wheat and barley, rice, bran, grape seeds and grape stalks which resulted highest enzyme production (Souza *et al.* 2002, Couto *et al.* 2002, Lorenzo *et al.* 2002 and Chawachart *et al.* 2004). Barley bran was used for maximum enzyme production as it contained

21% lignin with 75% moisture content (Pant and Adholeya *et al*. 2006), production of laccase was found on day 7 as reported by (Xin and Geng *et al*. 2010). The species of fungus by used Viswanath *et al.* (2008), Produced more enzyme as compared to the enzymes studied by Pant and Adholeya *et al*. (2006).

When waste water dilutions (10, 20, 30, 40, 50 and 60%) Nishat mills industry LTD were treated with free and immobilized laccase (Fig. 7), the maximal decolorizing per cent was seen with 40% dilution (68.64%) and minimal results were found with 10 time dilution factor (29.42%). Haroon textile industry LTD waste water dilutions (10, 20, 30, 40, 50 and 60%) treated with free and immobilized laccase (Fig. 8a), vital textile industry waste water dilutions (10, 20, 30, 40, 50 and 60%) treated with free and immobilized laccase (Fig. 8b) With increase in dilution effect activity gradually increase up to 50 % dilution and then at 60 % decolorizing ability was decreased.

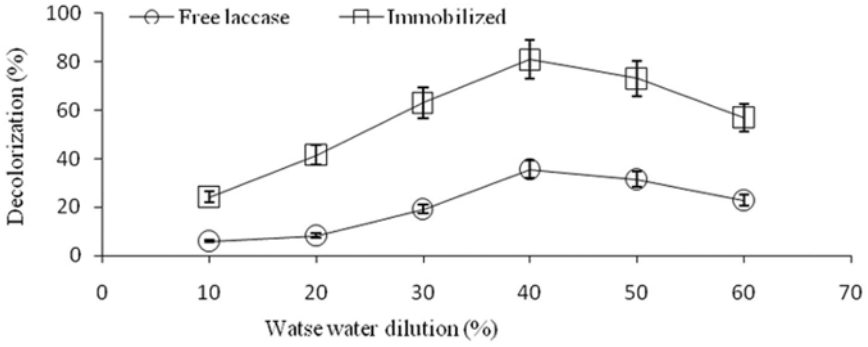

Fig. 7. Effect of free laccase and immobilized laccase on decolorizing activity of Nishat mills industrial waste water concentration.

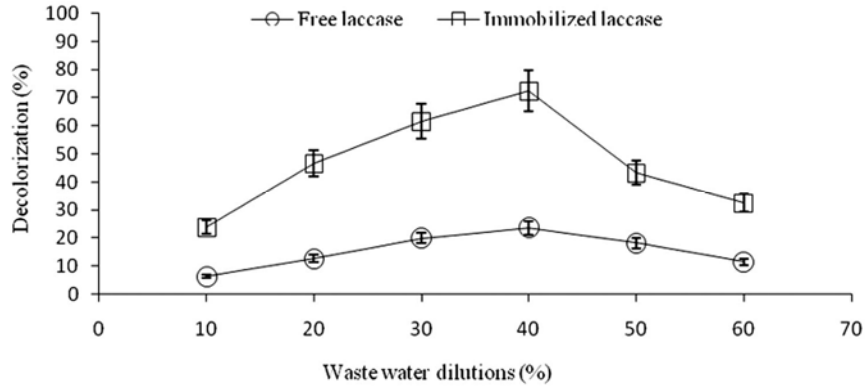

Fig. 8(a). Effect of free laccase and immobilized laccase on decolorizing activity of Haroon textile industrial waste water concentrations.

The immobilization procedure was accomplished by adding alginate to a gelatin solution containing the enzyme and the subsequent dropwise addition of the mixture into stirred $CaCl_2$ solution. The most frequently used stabilization method is immobilization of enzyme and reuse of the catalyst and assistance of reaction control (Cao *et al.* 2003 and Mateo *et al.* 2006). Gelatin-alginate was prepared by Mogharabi *et al.* (2012). He entrapped laccase in gelatin-alginate mixed

gel by adding 0.1 g sodium alginate to 10 ml of solution with gelatin and laccase range of 5 - 50 mg. Galhaup *et al.* (2002) obtained a maximum laccase activity of 740,000 U/l by *T. pubescens* cultured in a 20-l STR with a stirring speed of 100 rpm and with 2 mM Cu^{+2} after immobilization of enzyme. Font *et al.* (2003) obtained a maximum laccase activity of 16,000 U/l by free pellets of *T. versicolor* in a 0.5 - l pulsed-bed reactor.

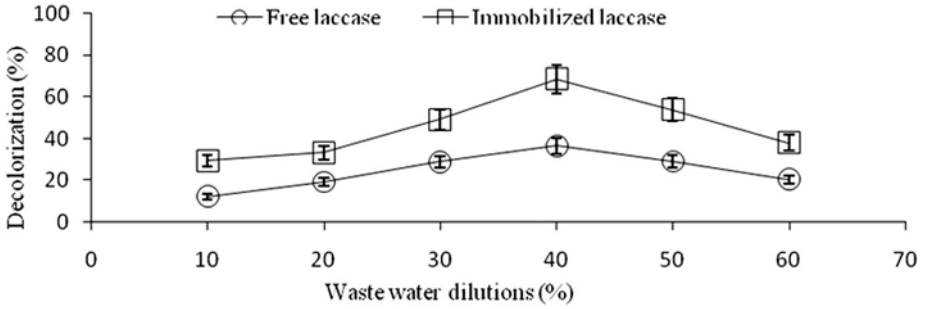

Fig. 8(b). Dye decolorizing effect of immobilized laccase produced from *A. nidulans* IMPP-0785 under SSF on Vital textile industrial waste water.

Wang *et al*. (2010) studied decolorization ability of wool dyes by laccase. He used two commercial wool dyes (azo dye diamond black PV and anthraquinone dye weak acid blue AS) and incubated reaction mixture at 20-70°C. The maximal decolorization rates of tested wool dyes were both observed at 40°C. The decolorization percentage for weak acid blue AS was more than 88% after incubation at 40°C for 2 hrs at pH 4.0, whereas, it was lower for diamond black PV with only a decolorization percentage of 74.7% at 40°C and pH 4.5.

Similar results were obtained by Lu *et al.* (2007), who stabilized enzyme at 25°C for 1 hr in the pH range from 2.0 to 5.0, an increase in temperature of the fermented mass due to respiration. *A. nidulans* IMPP-0785 was evaluated at 570 nm absorption.

Harron and Vital textile industries waste water decolorize maximally at (72.44 and 68.64%) with immobilized enzyme and (23.66 and 36.61%) with free laccase at 40% dilutions, respectively. Dilutions of the entrapped laccase was also studied and the results showed that 10% concentration gave optimal results (41.36 U/g). Related results were found by Asgher *et al.* (2012) for different textile industries (Sitara textile, Nishat textile, K&N textile and Crescent textile units of Faisalabad) effluents.

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