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Use of Tyrosinase Enzyme from *Bacillus thuringiensis* for the Decontamination of Water Polluted with Phenols

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Abstract: The present study aimed to evaluate the enzymatic treatment using tyrosinase (E.C. 1.14.18.1) from *Bacillus thuringiensis* in phenol removal from wastewater. In this study, the tyrosinase was isolated and purified from *Bacillus thuringiensis*. The purification process included ammonium sulfate precipitation, DEAE-Sepharose and phenyl Sepharose. The final specific activity was 130 U mg^{-1} protein and purification fold of 31. The enzyme had maximal activity at 35°C. Sixty nine percent of its activity was recovered at 40°C. Polyethyleneglycol (PEG), Tween 20, SDS and Triton X-100 were tested for their ability to prevent the inactivation of tyrosinase during removal of 2,4-dichlorophenol (2,4-DCP) from water. 2,4-DCP was disappeared greatly in tyrosinase-treated water samples amended with the various tested compounds particularly PEG over that observed in nonamended samples. The enhancement of phenol removal by PEG was dependent on its molecular weight. In case of 4-chlorophenol, increasing the PEG concentration to 0.6 g L^{-1} caused a substantial increase in the pollutant removal. The transformation of 4-chlorophenol by tyrosinase was inferior as compared with that of 2,4-DCP, which may indicate that the reaction products from 4-chlorophenol were stronger inhibitors of tyrosinase activity than those originating from 2,4-DCP. The commercially available carbon completely removed the colored products from the treated water without reducing the removal efficiency of tyrosinase. Chitosan eliminated the colored products of the reaction but the decolorization was accompanied by a reduction in 2,4-DCP removals. On the basis of the present results it is evident that using the different additives to polluted water may improve considerably the quality of wastewater treated with the bacterial tyrosinase.

Key words: *Bacillus thuringiensis*, tyrosinase, pollution, phenol, water

INTRODUCTION

Phenolic compounds are present in the wastewater of a number of industries such as coal conversion, resin and plastics, petroleum refineries, textiles, dyes, iron and steel and pulp and papa (Brasquet *et al*., 1999). Phenols are toxic pollutants in industrial wastes imposing several risks to human health and some are suspected carcinogens. In addition, phenols cause coloration of the receiving waters; it is therefore essential to decontaminate such compounds (Singh and Singh, 2002).

Commonly used conventional treatments (biological, chemical oxidation and adsorption) often fail to generate final effluents with the required discharge quality at affordable costs. Alternative technologies are sought for the treatment of phenolic wastewaters to overcome conventional biological treatment drawbacks such as the narrow range of contaminant concentrations that can be treated, delays associated with the biological acclimatization and generation of high sludge volumes.

Several researchers have studied the use of enzymes in wastewater treatment (Karam and Nicell, 1997). The application of oxidoreductive enzymes such as tyrosinase, peroxidase and laccase in removal of phenol and its derivatives has become very important and effective method (Ikehata and Nicell, 2000; Krastanov, 2000). Tyrosinase (EC 1.14.18.1) offers the advantage over other enzyme systems that have been used for phenol removal in that molecular oxygen rather than hydrogen peroxide is the oxidant, theoretically reducing the potential cost of applying the technology (Wu *et al*., 2001; Bevilaqua *et al*., 2002).

Tyrosinase catalyses two different reactions. The first reaction is the hydroxylation of monophenols leading to *o*-diphenols, often known as monophenolase (Ikehata and Nicell, 2000). The second reaction is the oxidation of *o*-diphenols to *o*-quinones, often referred to as *o*-diphenolase. In both of these oxidation reactions, oxygen is used as an oxidant and *o*-quinone as product can inactivate the tyrosinase. Quinones are usually

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formed rapidly and undergo non-enzymic conversion to form more stable intermediates. These intermediates subsequently undergo slow oligomerization reactions that ultimately yield high molecular weight, insoluble polyphenolics.

Since precipitate has been observed in phenol solutions treated with tyrosinase, one must remove the colored product remaining in the solution. Chitosan and other natural or synthetic cationic polymers were investigated to accomplish the removal of color from solutions (Wada *et al*., 1995).

The present study addressed the efficiency of enzymatic treatment using tyrosinase from *Bacillus thuringiensis* (either for pretreatment or polishing with chitosan as a coagulant) in phenol removal together to conventional aerobic biological treatment. *Bacillus thuringiensis* is a bacterium known for producing protein crystals with insecticidal properties (Rampersad and Ammons, 2005).

MATERIALS AND METHODS

Microorganism and culture maintenance: The investigated organism was isolated from sample collected from El-Mahala El-Kubra for Dyes Company, Gharbia Governorate, Egypt and according to Bergey (1989) and Sensitive Microbiology System (SMS) it was identified as *Bacillus thuringiensis*. The medium used was mineral salts medium (MSM) supplemented with phenol, buffered at optimal pH 7.0. The medium contained $(NH_4)_2SO_4$, 1.0 g; $MgCl_2$. $6H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.02 g; NaCl, 0.1 g; $CaCl_2$, 0.01 g; K_2HPO_4, 2.75 g; KH_2PO_4, 2.25 g and distilled water 1000 mL. The medium was inoculated with bacteria and incubated at 30°C for 3 days on an incubator shaker at 180 rpm.

Enzyme preparation: The culture media were centrifuged at 3500 rpm for 10 min and the clear supernatant was collected which represents the cell free extract.

Enzyme purification: The method of tyrosinase purification was initially of Munjal and Sawhney (2002). The cell free extract was resuspended in 15 mL of 50 mM Tris-HCl buffer (pH 7.4) and precipitated with ammonium sulfate at 40-60% saturation for 1 h with gentle stirring. After fractionation with ammonium sulphate the precipitated proteins were recovered by centrifugation at 8000 g for 30 min. and were dialyzed against 10 mM Tris-buffer (pH 7.4) supplemented with 0.15 M NaCl.

Approximately 10 mL of clear crude extract was loaded onto a DEAE-cellulose fast flow anion-exchange column (17.5 by 1.6 cm, 1 mL min^{-1}) previously equilibrated in 100 mM Tris-HCl buffer (pH 6.5). Tyrosinase was eluted in 50 mM Tris-HCl-buffer (pH 6.5) with a 0.1 to 0.5 M NaCl gradient. Fractions (I.5 mL) were collected and tested for tyrosinase activity. Activity fractions were pooled, dialyzed and equilibrated with 50 mM Tris-HCl buffer (pH 6.5).

Later tyrosinase was separated by hydrophobic interaction chromatography with Phenyl-Sepharose where the active fractions were loaded onto a Phenyl-Sepharose column (11.5 by 1.6 cm, 1 mL min^{-1}). Previously equilibrated in 50 mM Tris-HCl buffer (pH 6.5). Active fractions were pooled, dialyzed and concentrated and used for assay.

Enzyme assay: The method is that adopted by Ikehata and Nicell (2000). The total volume of the reaction medium was 3 mL containing: 2 mL of L-tyrosine (0.5 mM) in potassium phosphate buffer (50 M) pH 6.5 and 1 mL of tyrosinase at temperature of 25°C. One activity unit was defined as the amount of enzyme that increased absorbance 0.001 (λ = 280 nm) per minute.

Electrophoretic study (SDS-PAGE): Electrophoresis was performed by the method of Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue. The protein standards were transferrin (80 kDa), bovine serum albumin (68 kDa), fumarase (49 kDa), pyruvate kinase (58 kDa), lactate dehydrogenase (35 kDa) and chymotrypsinogen (25 kDa).

Protein determination: Protein was estimated according to Lowry *et al*. (1951) after precipitation with equal volumes of 20% TCA and using bovine serum albumin as standard.

Polishing of phenolic solutions: Phenol removal catalyzed by tyrosinase was initially tested in experiments using buffer solutions (potassium phosphate 50 mM, pH 6.5) containing phenol at the tested concentrations. Reactions were performed at room temperature in 200 mL aerated and magnetically stirred reactors. The working volume was 50 mL and samples of 3 mL were taken for analysis. Adding 0.1 mL of H_3PO_4 8.5% (w/v) stopped reactions. Phenol concentration was analyzed at the beginning and after appointed periods. Chitosan solution was prepared by dissolving it in acetic acid 0.5% v/v.

All values are the means of three measurements±SE.

RESULTS AND DISCUSSION

In a preliminary experiment using different bacteria for testing their productivity of tyrosinase we found that

Table 1: Purification of tyrosinase from *Bacillus thuringiensis*

Step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
[Crude extract]	45.00	127.6	2.8	1.0	100.0
[$(NH_4)_2SO_4$ (40-60%)]	23.30	98.0	4.2	1.5	76.8
[DEAE- Cellulose]	0.80	65.9	82.4	29.4	51.6
[Phenyl Sepharose]	0.38	48.8	130.0	31.0	38.2

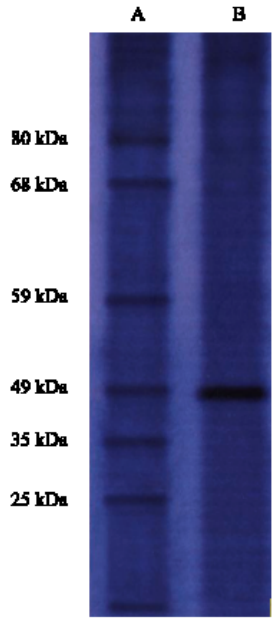

Fig. 1: Gel electrophoresis of cell free extract from *Bacillus thuringiensis* (A) and pure tyrosinase (B)

Bacillus thuringiensis expressed the higher productivity of the enzyme compared to the other tested ones. Therefore, we have chosen *Bacillus thuringiensis* for this present study. Table 1 shows a summary of the purification procedure that was used for tyrosinase from *Bacillus thuringiensis*. The purification schedule included ammonium sulfate precipitation, DEAE-cellulose and phenyl Sepharose. The final specific activity was 130 U mg^{-1} protein and purification fold of 31. The purified enzyme appeared homogeneous as judged by disc gel electrophoresis (Fig. 1). The molecular weight appears to be 49 kDa for the enzyme. Tyrosinase was also purified to homogeneity from other microorganisms such as *Thermomicrobium roseum* (Kong *et al*., 2000).

On studying the relationship between the temperature of the incubation medium and tyrosinase activity from *Bacillus thuringiensis* (Fig. 2) it was found that tyrosinase has maximal activity at 35°C and retained 69% of its activity at 40°C. This optimum temperature was also reported for tyrosinase from *Agaricus bisporus* (Khan *et al*., 2005).

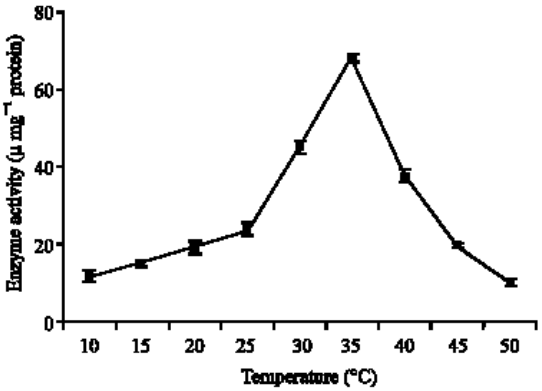

Fig. 2: Effect of different temperatures on tyrosinase activity

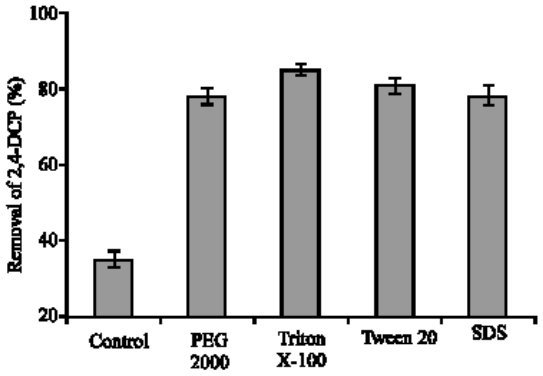

Fig. 3: Effect of polyethylene glycol and some surfactants on the removal of 2,4- DCP by tyrosinase

The results in Fig. 3 presents the effect of Polyethylene glycol (PEG) and different surfactants (Tween 20, SDS, Triton X-100) on the removal of 2,4-DCP from water treated for 3 h with tyrosinase and H_2O_2. It is observed that in the absence of the additives the removal of 2,4-DCP was small and increased dramatically when 0.1 g L^{-1} of PEG or surfactants was added. The enhancement was probably due to reduced adsorption of the reaction products on tyrosinase molecules (Wu *et al*., 1993).

PEG and the different tested surfactants vary in their chemical structures and molecular weights. They are commonly used as detergents, solubilizing agents, or lubricants. Other chemicals, such as borate, gelatin and polyvinyl alcohol, also can suppress enzyme inhibition by reaction products (Nakamoto and Machida, 1992). Therefore, it is not clear which chemical or physical factors may be critical for the protective effect of the additives.

Using peroxidase as another oxidoreductive enzyme, other workers (Nakamoto and Machida, 1992; Wu *et al*.,

1993) showed that PEG could suppress the adsorption of reaction products on enzyme molecules and thus reduce the inhibition. In the study of Wu *et al.* (1993), the removal of phenol was almost doubled in the presence of PEG, as compared with the removal achieved in the absence of PEG. A similar enhancement of the removal potential by PEG was observed in present investigation using bacterial tyrosinase for the treatment of water polluted with 2,4-DCP (2-10 mM).

In the present investigation, the effect of molecular weight of PEG on the tyrosinase treatment (Fig. 4) showed the pattern of increasing 2,4-DCP removals with increasing molecular weight. 2,4-DCP removals was increased (Fig. 4) with increasing molecular weight of PEG from 33% for molecular weight = 200 (as compared with a 27% removal in the control sample) to 74% for molecular weight = 400. The removal increased to 82% for molecular weight = 1000 and to 99% for molecular weight = 2000. These results are similar to those reported by Nakamoto and Machida (1992) for peroxidase as another candidate of oxidoreductases used for removal of phenol. The only difference was that in the study of Nakamoto and Machida (1992), PEG of molecular weight = 400 was ineffective in suppressing the inhibition of the enzyme whereas when applied with tyrosinase in the present work was reasonably effective in improving the 2,4-DCP removal. The difference was probably due to differences in the reaction conditions: using different pollutants, different pollutants concentrations, different form of the enzyme used, different buffers and different pH values of the reaction mixture (Nakamoto and Machida, 1992).

The effect of 0.1 g L^{-1} PEG (molecular weight = 400) on removal potential of tyrosinase for 2,4-DCP at different concentrations (2-10 mM) in the aqueous medium was investigated (Fig. 5). The results indicate that the concentration of 2,4-DCP has no effect on the removal of 2,4-DCP in the presence of 0.1 g L^{-1} PEG. In contrast, in the control samples containing no PEG, the removal decreased dramatically with increasing 2,4-DCP concentrations. Polymerized phenols have a number of hydroxyl groups in their structure. Nakamoto and Machida (1992) suggested that these highly hydrophilic polymers might react with the enzyme and form hydrogen bonds. This results in inactivation of the enzymes. Additives such as PEG can also interact with polymerized phenols and prevent the inactivation of enzymes. The suppression effect of PEG depended on PEG molecular weight. It is possible that PEG protection of the enzymes may be related to the water binding properties of PEG.

On studying the effect of different concentrations of PEG (0.2-1.2 g L^{-1}) on the removal of phenol, 2,4-DCP and

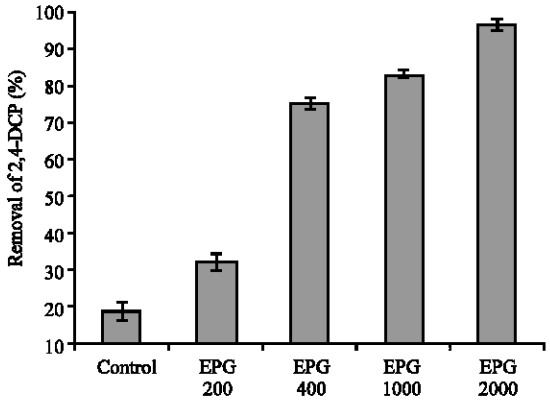

Fig. 4: Effect of molecular weight of PEG on the removal of 2,4- DCP by tyrosinase

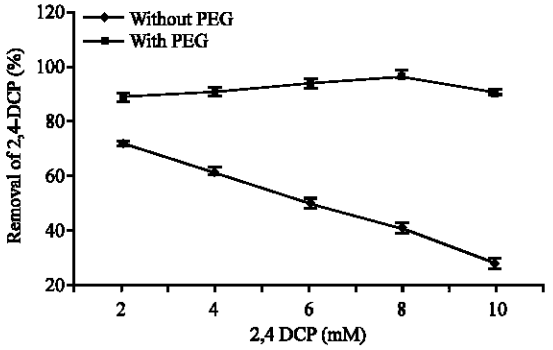

Fig. 5: Effect PEG 2000 on the removal of various concentrations (2-10 mM) of 2,4- DCP by tyrosinase

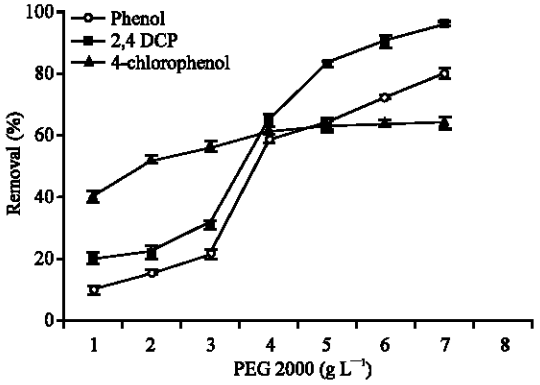

Fig. 6: Effect of different concentrations of PEG on removal of phenol, 2,4- DCP and 4-chlorophenol

4-chlorophenol (Fig. 6), it was found that after addition of 0.6 g L^{-1} PEG there was no marked increase in the removal of 4-chlorophenol. In contrast, the removal of phenol and

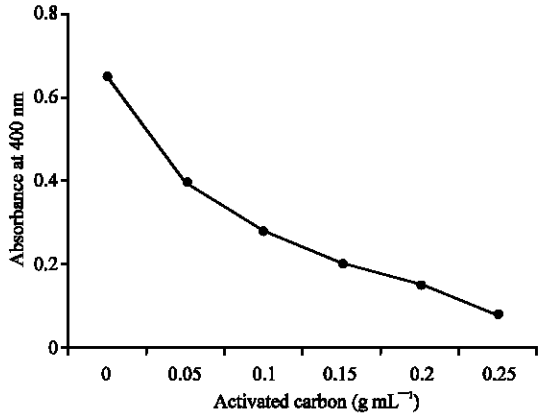

Fig. 7: Effect of activated carbon on color removal after tyrosinase treatment

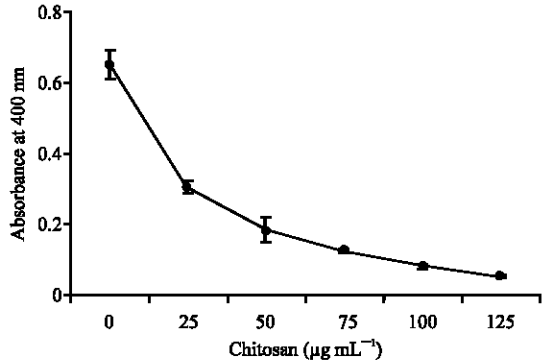

Fig. 8: Effect of chitosan on color removal of tyrosinase treatment

2,4-DCP was remarkable after 0.6 g L^{-1} PEG. These observations confirm that all phenols are not equally good substrates for tyrosinase and some may cause rapid inactivation of tyrosinase than others. Thus, the quantity of tyrosinase required to achieve a given degree of transformation will vary substantially between substrates. In support, Wu *et al.* (1993) found that increasing the concentration of PEG resulted in increased removal of phenol and 2,4-DCP during incubation with peroxidase. Also, Dec and Bollag (1994) reported that the transformation of 4-chlorophenol by peroxidase was inferior as compared with that of 2,4-DCP. It is possible that the reaction products from 4-chlorophenol were stronger inhibitors of enzyme activity than those originating from 2,4-DCP. If this is the case, the transformation of 4-chlorophenol could be influenced to a greater degree by increasing concentration of PEG than the transformation of 2,4-DCP.

On studying the effect of chitosan as a natural polymer of glucosamine on removing the colored products from aqueous solution of tyrosinase, it was found that chitosan was efficiently removed colored reaction products from the aqueous solution of tyrosinase from *B. thuringiensis* (Fig. 7). In support, the colored products of mushroom tyrosinase were removed by chitosan and the rate of phenols was observed to be accelerated in the presence of chitosan (Wada *et al.*, 2004). Also, Sun and Payne (1996) successfully used chitosan to encapsulate tyrosinase and removed colored reaction products originating from the oxidation of phenol. The presence of chitosan with tyrosinase showed encouraging effects as a color adsorbent (Wada *et al.*, 1995; Ganjidoust *et al.*, 1996; Sun and Payne, 1996).

On studying the effect of activated carbon on color removal by bacterial tyrosinase, it was observed that light absorbance at 400 nm, which was 0.45 in the absence of chitosan dropped to only 0.34 when chitosan was added. Thus, activated carbon proved to be an efficient approach for color removal from reaction mixture involving phenols and tyrosinase from *Bacillus thuringiensis* (Fig. 8). A maximum color removal (reduction of the absorbance from 0.45 to below 0.15) was achieved within a relatively short time (3 h) after carbon addition. These results are in agreement with those reported by (Tonegawa *et al.*, 2003). Activated carbons are common adsorbents widely used in a variety of industries. Therefore, due to the relatively low amount required for achieving a satisfactory color removal, activated carbons could be considered cost-effective decolorization agents.

CONCLUSION

In conclusion, tyrosinase and the tested additives in the present investigation proved to be a promising combination for the removal of phenols from aqueous solutions and for improving the quality of treated water at the same time. However, the choice of additives and the development of application methods for specific situations (e.g., pollutant combinations, environmental conditions, the amounts of pollution etc.) require further studies.

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