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Tyrosinase from *Sepiella inermis* (Van Hasselt [Ferussac & d'Orbigny], 1835) and its Phenol Removal Activity

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ABSTRACT

Tyrosinase is a ubiquitous enzyme found in most pigmented animals. In the present study, the tyrosinase enzyme was isolated and purified from *Sepiella inermis* ink and its application in phenolic compounds removal from aqueous solution. The presence of the tyrosinase in the *S. inermis* ink was read at 280nm and the crude enzyme was purified by dialysis, ion exchange chromatography, and high-performance liquid chromatography (HPLC). The molecular weight of the purified enzyme was revealed as 30kDa through the SDS-PAGE analysis with 12% polyacrylamide gel. The optimum pH and temperature of the enzyme were found to be 6°C and 55°C respectively. The tyrosinase enzyme was immobilized with sodium alginate for the removal of phenolic contaminants in water. The accelerator with efficiency removed the phenols from the solution at intervals within a few hours. Hence, this study indicated that the isolated tyrosinase enzyme of *S.inermis* ink acts as an accelerator and could be used for the removal of hazardous phenol substances from wastewaters.

INTRODUCTION

Phenol and its derivatives are released as contaminants from different industrial activities. It is no less than two-thirds of phenols formed worldwide are engaged in the chemical synthesis of bisphenol A and phenolic resins (Palma et al. 2010). Apart from colouring and conferring smell to water these phenols and their derivatives are proven toxic substances for aquatic creatures even at mild concentrations (5-25mg/mL). Being toxic to aquatic organisms it is necessary to remove these kinds of pollutants from wastewaters. The available techniques such as recovery and destruction and among the later are being the biological treatments (Palma et al. 2010). These biological methods are considered to be competitive which can able to meet quality standards in a cost-effective manner (Zilli et al. 2010).

Tyrosinase is the enzymes proven polyphenol oxidizer ubiquitously distributed in several living beings that act as a catalyst using O_2 as an oxidant monophenols hydroxylation to o-diphenols and subsequent dehydrogenation of o-diphenols to o-quinones. (Halaouli et al. 2016). Huge innovation exists by means of underutilized waste and its by-products to build new healthy products for consumers (Fatimah & Rabeta 2018). Squid, cuttlefish, and octopus are major seawater catch other than fishes and prawns. These organisms are grouped under the phylum of invertebrate known as Mollusca and the family of Cephalopoda (Fatimah & Rabeta 2018).

In the connection cephalopod, ink is native stuff discharged by cephalopods from their ink sac when they try to get away from predators (Hossain et al. 2018). This ink is produced from the secretion of two glands and at the end process of maturation in a viscous colourless medium (Liu et al. 2011). This by-product can be a possible source of good quality bioactive compounds (Vate & Benjukul 2017). Apart from that squid is an excellent food source for zinc and manganese and high in copper, selenium, vitamin B12, and riboflavin. There is an ever-increasing demand for different enzymes in current industries. Besides, despite the outstanding advancement in chemistry, there is yet no efficient reagent for synthesizing some chemical substances.

One such best example is ortho-hydroxylation of phenolic compounds. This reaction happens in the majority organisms and ends up information for necessary organic chemistry like neurotransmitters of Bendopa family, coumestrol, polyphenolic acids (Haghbeen et al. 2004). Squid ink is used as an additive in food processing. It consists of melanin granules in a viscous colourless medium (Russo et al. 2003). Besides, this squid ink is also applicable for anti-tumour activity, antimicrobial property, phenol removal property, and skin

rejuvenation property. So this study aimed at the isolation of tyrosinase from squid ink and its phenol removal activity from aqueous for the benefit of the environment.

MATERIALS AND METHODS

Sample Collection and Processing

The cephalopod species *S. inermis* was collected from Mudasal Odai landing centre, Tamilnadu coast, India and brought to the laboratory. All the collected species were washed thoroughly twice with distilled water. The squids were dissected and ink glands were removed from the viscera.

Isolation of Cuttlefish Ink

The ink glands were placed in clean plastic containers before placing them into the freezer (-20°C). The ink duct, each weighing 800mg, was cut with sterile scissors, gently squeezed; milked ink was collected and stored at 4°C. The frozen ink was kept in the freeze dryer (-60°C), (Delvac, Chennai, India) for the lyophilization process. The freeze-dried ink kept in an airtight plastic container and then covered with aluminium foil to prevent light penetration (Fatimah & Rabeca 2018). The dried samples were stored in a freezer at -20°C before further analysis.

Extraction of Tyrosinase

The ink sample was lyophilized and obtained as a black powder. The pH was determined with a pH meter (Neifar et al. 2008). This powder was extracted with 40 volumes of 0.1 M tris-HCl buffer. Then it was centrifuged at 18,000 rpm for 30 min at 4°C to remove the melanin using a refrigerated centrifuge (Vate 2017). The pellet was removed and the supernatant was dialyzed against distilled water at 4°C for 48 h and then lyophilized to obtain an off grey powder (270 mg). This powder was re-dissolved in normal saline and used for further studies.

$$Yeild\ of\ tyrosinase\ (\%) = \frac{Weight\ of\ tyrosinase\ obtained}{Weight\ of\ ink\ obtained} \times\ 100 \quad \ldots(1)$$

Dialysis

Activation of the membrane (cellulose membrane) was performed as follows. 100 mL of distilled water was kept boiling for 30 min. Dialysis membrane (HiMedia) was placed in water for 10 min. Finally, the membrane was transferred to freshwater. The sample was dialyzed against distilled water for 42 hours at 4°C. The dialyzed sample was lyophilized to obtain a grey powder (Roy et al. 2014).

Ion Exchange Chromatography Purification of Tyrosinase

5 g of crude extract powder was dissolved in 20mL of 0.5M Tris-HCl buffer (pH 6.8), the solution was applied to a column (1.6 × 38 cm) of DEAE Cellulose (HiMedia) equilibrated with the same buffer, and then the column was eluted with a stepwise gradient of 0.05, 0.1, and 0.5 M in the same buffer at a flow rate of 1mL/min. Fractions of 5 mL were collected and analysed for tyrosinase activity (Naroaka 2000). Each fraction was examined under a UV spectrophotometer.

Effect of pH and Temperature on Enzyme Activity

The effect of temperature on tyrosinase activity was measured in standard assay conditions. Activity assay was performed at different pH and temperatures and the UV absorbance was measured at 475 nm (Neifar et al. 2012). The optimum temperature for enzyme activity was determined by incubating the standard reaction mixture at temperatures ranging from 35°C to 65°C. The activity of tyrosinase was evaluated at different pH values in the range between pH 3 and 10 under assay conditions and the amount of tyrosinase was determined (Zaidi et al. 2015). Buffers used were Tris-glycine (pH 3.0-10.0).

Molecular Weight Determination of Tyrosinase using SDS-PAGE

SDS-PAGE was done with 12% polyacrylamide gel using Tris-glycine buffer (pH 8.3). The crude and purified enzyme was loaded onto a denaturing polyacrylamide gel and compared with standard tyrosinase. The gel preparation method was carried out by referring to Laemlli (1970). The protein patterns of tyrosinase were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Tyrosinase samples (0.5 mg/2µL) were dissolved in 0.05M tris-HCl buffer (pH 7.2) and mixed with the sample buffer (0.5 M tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol) with 10% (v/v) β-mercaptoethanol (β-ME), using the sample to sample buffer ratio of 1:1(30:30µL) (v/v). Samples were loaded onto a polyacrylamide gel made of 12% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 20mA per gel, using a mini-protein II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with silver staining.

Preparative-RP-HPLC Purification of Tyrosinase

The culture free supernatant was analysed by high-performance liquid chromatography (Shimadzu, Japan) using C_{18}

column (Luna 5µ, Phenomenex 250mm × 4.6mm). The HPLC gradient program was starting at 8% solvent B and holding for 1 min, then ramping to 75% solvent B in 20 min, holding at 75% solvent B for 10 min, backing to 8% solvent B in 1 min and holding at 8% solvent B for 5min. Solvent A was 98:2 (v/v) water: methanol with 0.1% acetic acid, and solvent B is 10:90 (v/v) water: methanol with 0.1% acetic acid. The HPLC analysis was performed at a flow rate of 1.0mL/min (Roy et al. 2014).

Phenol Removal from Aqueous Environment by Using Immobilized Tyrosinase Enzyme

All phenol enzymatic removal experiments were carried out with constant stirring. The reaction mixture consists of 250mL aqueous phenol solution and crude extract with different tyrosinase concentrations (Kameda et al. 2006) with immobilization of tyrosinase in sodium alginate beads. The partially purified enzyme solution was mixed with a sodium alginate solution in a 2:1 ratio. The mixture was added dropwise into calcium chloride (0.2M) solution with shaking at 4°C. The beads were allowed to wash with distilled water followed by a phosphate buffer of pH 7 (Anwar et al. 2009). The immobilized beads of 20mL of enzymes were prepared and exposed to various concentration of phenol in water. The phenol concentration varied from 1mM to 5mM. The concentration of phenol that remained was estimated by Folin- Ciocalteu reagent after every half an hour. The phenol concentration was determined for each set of concentration to 4h (Roy et al. 2014).

RESULTS AND DISCUSSION

Yield Calculation

The yield calculation was estimated for 20g of ink duct which yields 2g of ink like grey powder. The yield of ink powder was found to be 10% (w/w). The yield of tyrosinase was estimated as 4.18% on the dry weighted basis (w/w). The yield calculation was determined for 2g of lyophilized cuttlefish ink powder extracted by tris-HCL and then tyrosinase was isolated from the ink and the yield was calculated as 80.25mg. In a study conducted by Roy et al. (2014) the net yield of the tyrosinase enzyme was determined as 50.69% from marine actinobacteria. The value of fat content in squid ink powder is slightly higher compared with raw squid which was about 1.0 to 2.0%, where the value considered lowest among all types of seafood (Okuzumi & Fujii 2000).

Anion-Exchange Chromatography Purification of Tyrosinase

The eluted fractions obtained by tris-HCL buffer with different concentrations of 0.05M, 1M, 1.5M through DEAE cellulose column and 0.05M was collected which showed high tyrosinase content. These results are illustrated in Fig. 1. The collected fraction was obtained as a grey colour powder. Purification is usually achieved by filtration, centrifugation, and precipitation and also by chromatographic techniques such as ion exchange, gel permeation, and affinity chromatography. According to Zaidi et al. (2015), the button mushroom of tyrosinase enzyme was purified under Sephadex G-100 column further with the ion exchange chromatography using DEAE-Cellulose column (20 × 1cm).

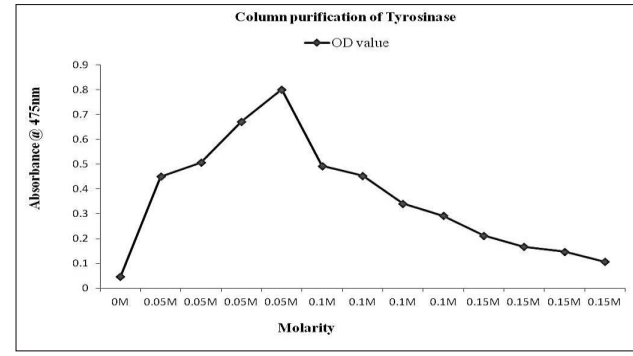

Fig. 1: Purification of tyrosinase using anion-exchange chromatography.

Effect of pH and Temperature on Tyrosinase

Tyrosinases with various physicochemical features have been reported from various organisms. These enzymes generally have a pH optimum in the neutral or slightly acidic range. The results revealed that pH 6.0 was the optimal pH for tyrosinase from *S. inermis* (Fig. 2) using Tris-HCl buffer. Our results also demonstrated that tyrosinase retained about 65 % of its activity after storing at pH 7.0 for 24 h. This means tyrosinase of *S.inermis* had higher pH stability over a wide range of pH values. The influence of pH on tyrosinase activity in crude melanin-free ink was studied in the range of pH 3 to 12 using citric acid/sodium phosphate buffer at 0.1M (Neifar et al. 2012).

The purified tyrosinase was active at a wide range of temperature from 30°C to 65°C with an optimal at 55° C and holding 35% of tyrosinase activity at 55°C, but it lost its activity at 60°C (Fig. 3). Our results were in agreement with the optimum temperature for tyrosinase activity obtained from *S. officinalis* and the enzyme was sustained at 55°C (Neifar et al. 2012). It has been reported that the stability toward the temperature of squid tyrosinase activity of the cephalopod mollusc *Illex argentinus* was stable up to 30°C and complete inactivation was observed at 70°C (Naraoka et al. 2003).

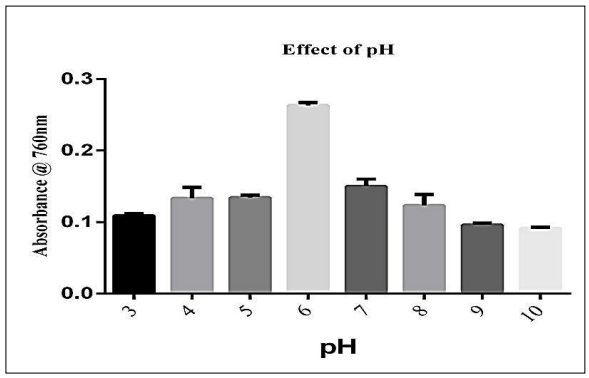

Fig. 2: Optimum pH of Tyrosinase from *S. inermis.*

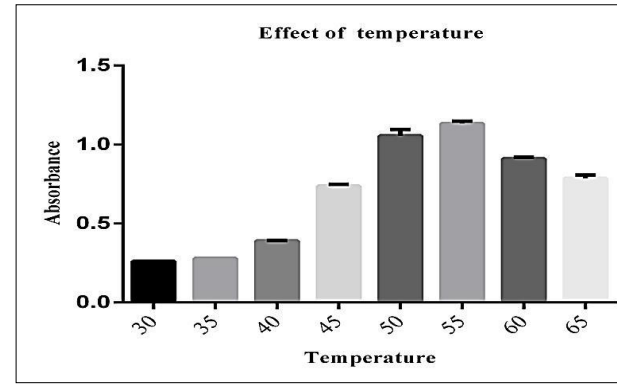

Fig. 3: Optimum temperature of Tyrosinase from *S. inermis.*

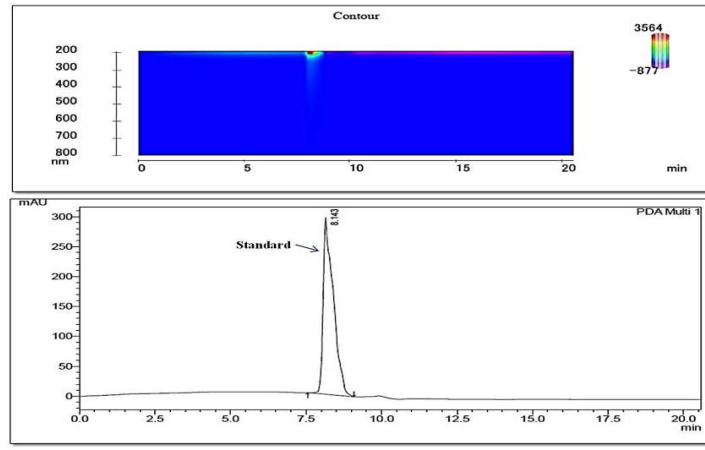

Fig. 4: HPLC chromatogram and contour view for standard Tyrosinase.

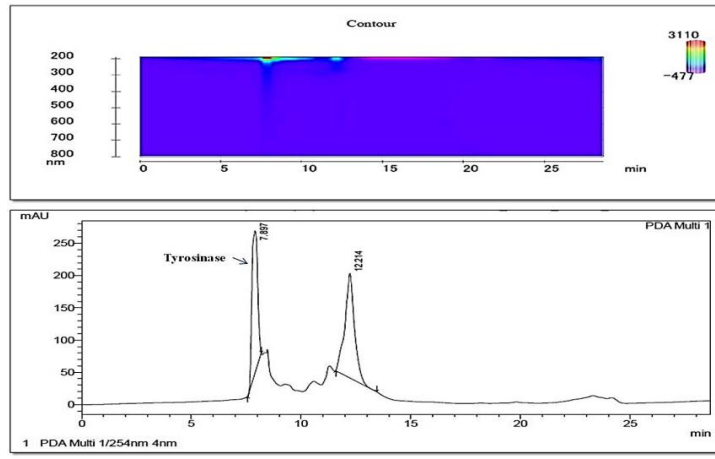

Fig. 5: HPLC chromatogram and contour view for purified Tyrosinase from *S.inermis*.

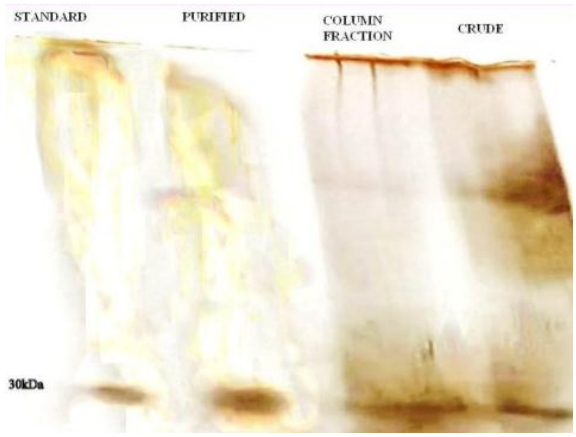

Fig. 6: SDS-PAGE for the purified and crude sample.

HPLC Analysis of Tyrosinase from *S.inermis*

The HPLC chromatogram of the partially purified enzyme showed a double peak at retention times of 7.897 and 12.214 mins. In which the major peak at R_t 7.897 corresponds to the tyrosinase were confirmed with the standard tyrosinase enzyme. The HPLC analysis of the partially purified tyrosinase enzyme with phosphate buffer as mobile phase revealed a single intense peak which confirmed the purity of the enzyme (Dolashki et al. 2009). Hence the present study revealed that the solvent was also used as a mobile phase in the HPLC purification of the enzyme. Fig.4 & 5 represents the standard, purified tyrosinase chromatogram, and contour view.

SDS PAGE of Tyrosinase

The purity of the enzyme and to determine the molecular weight, electrophoresis is performed using denaturing polyacrylamide gel electrophoresis. The molecular weight of the purified enzyme was determined as 30kDa, analysed by SDS PAGE. Fig. 6 shows the single band, which denotes the presences of tyrosinase when compared with standard tyrosinase from the mushroom. Likewise, Roy et al. (2014) revealed the same range of molecular weight enzyme through SDS-PAGE analysis by the presence of a single protein band that corresponds to approximately 30 kDa.

Phenol Removal Activity of Purified Tyrosinase

The amount of total phenol was determined with the Folin-Ciocalteau reagent. Phenolic compounds are a class of antioxidant agents that acts as free radical terminators. The immobilized enzyme provides improved resistance to alteration in conditions such as temperature or pH. In such a condition, enzymes remain held in position throughout the reaction which leads to easy separation from product, reusability and continuous operation. It is an efficient technique that is being used in industry for enzyme-catalysed reactions. In regards to enzymatic water treatment, immobilized tyrosinase showed better efficiency in terms of reusability, stability and longer viability (Bevilaqua et al. 2002, Molina et al. 2003, Kameda et al. 2006). The immobilization of tyrosinase improved the thermal stability and the gel-entrapped tyrosinase was almost entirely preserved from proteolysis maintaining more than 80% of its activity (Crecchio et al. 1995). The use of cheaper supports for the preparation of immobilized enzyme for such applications is always considered necessary. Hence, sodium alginate was used to immobilize the enzyme to remove phenol from the aqueous solution. Tyrosinase can oxidize a wide range of polyphenolic and phenolics components to their related nontoxic quinones and hence this enzyme has been utilized to remove hazardous and toxic phenolic contaminants from effluent and wastewater (Robb 1995). In the present study, the partially purified enzyme was immo-

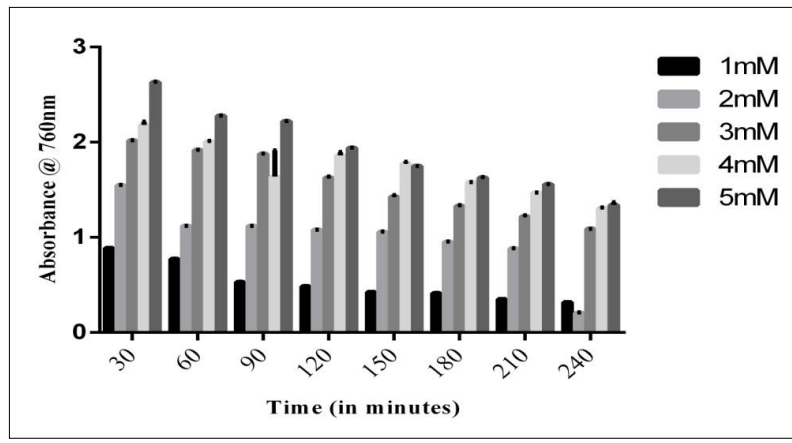

Fig. 7: Phenol removal activity under various concentrations of 1mM to 5mM.

bilized in sodium alginate to remove the phenol from the water. The efficiency came around 48% which showed a better result (Anwar et al. 2009), where the efficiency of the immobilized protease enzyme was found to be 45%. Thus the phenol removal activity was done through several concentrations of phenolic assays by using the immobilized beads. The immobilized enzyme was exposed to various concentration of phenol ranging from 1mM to 5mM for a period of 4 h which resulted in the reduction in the concentration of phenol with an increase in exposure time shown in Fig. 7. The reduction of phenolic components was a function of exposure time. There was a gradual decrease in the percentage of phenol removal from 1mM of phenol to 5mM of phenol. The result of phenol removal showed a slight similarity with the work of Shesterenko et al. (2012). Whereas Maurya & Singh (2010) estimated that the number of total phenolics in extracts was determined with the Folin- Ciocalteu reagent. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Thus total phenolic content can be determined.

CONCLUSIONS

The marine cephalopod *Sepialla inermis* was found to be the gifted producer of tyrosinase as well as the potent remover of phenol from aqueous solution. The enzyme was found to be stable even at high temperature, pH and it is also having high enzymatic activity and greater stability than the mushroom tyrosinase enzyme. Immobilized tyrosinase enzyme from *Sepialla inermis* can act as a promising technique for phenol removal from wastewater along with maintaining high stability of the enzyme. Hence, it can be concluded that the tyrosinase enzyme from thiscephalopod can be potentially used in industries to remove phenol from wastewater. Further studies on this particular enzyme can lead to producing a new skin lightening cream and could be used for hyperpigmentation as well in near future.

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