Journal of Applied Biology & Biotechnology Vol. 8(02), pp. 22-27, March-April, 2020
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2020.80204

A simple and efficient method for removal of phenolic contaminants in wastewater using polyacrylamide entrapped mushroom tyrosinase

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ARTICLE INFO

Article history:
Received on: September 06, 2019
Accepted on: November 25, 2019
Available online: March 26, 2020

Key words:
Phenolics, mushroom tyrosinase, immobilization, polyacrylamide gel immobilization, wastewater.

ABSTRACT

Phenolic compounds are priority pollutants with high toxicity even at low concentrations. Ever-increasing burden of pollutants in major rivers and other water bodies along with stringent environmental legislation and focus on adaptation to eco-friendly treatment approaches have necessitated the need for the removal of these phenolics before being discharged to rivers and other freshwater bodies. Compared to physicochemical treatment, enzymatic treatment has proven to be the best way to treat various phenolic compounds under mild conditions with different enzymes such as peroxidases, laccases, and tyrosinases. In this study, we have designed a simple and efficient method for removal of phenols from effluent wastewater using an immobilized preparation of mushroom tyrosinase. The enzyme was isolated from *Agaricus bisporus* (button mushroom) and partially purified, and subsequently, various immobilization matrices were evaluated for their efficiency of immobilization, reproducibility, rate of degradation of phenolics, stability, and reusability. Experiments showed that the *in situ* polymerization of acrylamide monomer along with the enzyme gave the most effective entrapment with high reproducibility among the tested methods. Immobilized tyrosinase was much more stable than the free tyrosinase in storage and that the immobilized tyrosinase could even retain about most of its original activity after repeated use of 10 times in a batch system. This method could provide and an economical and stabilized immobilized-enzyme method for the removal of phenol in wastewater.

1. INTRODUCTION

Phenols and their derivatives are widely distributed environmental pollutants in many industrial effluents particularly from oil refineries, petrochemical plants, coal conversion plants, and phenolic resin industries [1]. In addition to coloring and conferring smell to the water, phenols have been reported to be toxic to aquatic organisms even at relatively low concentrations (5–25 mg/L), and their adverse effects on human health are well documented [2]. The effects of phenolic compounds on humans include the irritation of nose, throat, and eyes, digestive difficulties, nervous problems, headaches, and skin burns. Prolonged ingestion of phenol at concentration levels ranging 10–240 mg/L can cause mouth sore, diarrhea, and impaired vision [3]. Apart from this, some phenolic compounds are also known to accelerate tumor formation leading to carcinogenicity. Furthermore, the soil and surface waters of the contiguous areas are also contaminated by such compounds leading to increased risk of groundwater resources. Hence, screening, monitoring, and control of these pollutants are of utmost importance. As per Indian Standard Institution limits, the permissible limit of phenol/phenolic compounds in inland water resources should not cross 0.001 mg/L (permissible limit in the absence of alternate source is 0.002 mg/L) [4]. For these reasons, wastewaters exceeding the limit of 0.05 ppm of phenols are not permitted to be discharged into the main streams. In order to bring the phenol concentrations within the permissible limit of the set standards, it is necessary to treat refinery wastewater before discharging into the main stream.

Conventional processes for the removal of phenols from wastewater include extraction, adsorption, ion exchange, steam distillation, bacterial and chemical oxidation, and electrochemical techniques [5]. All these processes have drawbacks of high cost, incomplete removal of phenols, low efficiency, and problems of disposal of spent solution. In addition, they are also sometimes responsible for the formation of undesirable by-products [6]. Innovative biological/enzymatic methods offer an effective and

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competitive alternative to conventional treatments and have the potential to meet the increasingly stringent quality standards at relatively low costs [7].

Microbial enzymes have been reported to be quite effective in the assay and treatment of phenolic compounds. Earlier treatments were focused on the usage of peroxidases and laccases which can act upon a broad range of substrates indicating that these enzymes are not very substrate-specific. These enzymes also suffer from disadvantages that they are difficult to isolate and the treatment with either of these enzymes is expensive [8]. Polyphenol oxidases, usually called tyrosinases, are enzymes ubiquitously distributed in nature that catalyze, using O_2 as an oxidant, monophenols hydroxylation to o-diphenols and subsequent dehydrogenation of o-diphenols to o-quinones [9]. These are then non-enzymatically converted to more stable intermediates, which are subsequently transformed into insoluble, high molecular weight polyphenolics which can be easily removed by solid–liquid separation. Tyrosinase-catalyzed oxidation is an eco-friendly and sustainable method to treat phenol contaminated wastewaters; however, enzymes are often quickly inactivated, thus increasing the process cost. The use of tyrosinases from plant organisms, especially mushroom (*Agaricus bisporus*), would make the treatment less costly as compared to commercial enzymes. Wada et al. [10] have investigated the use of mushroom tyrosinase for removal of phenols [10]. Mushroom tyrosinase (1.14.18.1; monophenol dihydroxyphenyl alanine oxidoreductase) is a copper-containing tetrameric enzyme which can be used to oxidize phenols and aromatic amines to free radicals or quinones that are subjected to polymerization and partial precipitation from aqueous solutions. The use of this enzyme is of particular interest because it oxidizes phenols to oligomers similar to melanins and imparts a brown color to the reaction medium, making it possible to quantify in real time the degradation of phenolic compounds [11]. Tyrosinase-catalyzed oxidation may be an ecofriendly and sustainable method to treat phenol contaminated wastewaters. However, free enzyme, whether used for analysis or for treatment of phenolic wastes, suffers from operational instability. The drawback can be removed by immobilizing the enzyme on a suitable matrix. Even though immobilization of tyrosinases has been attempted on various matrices including nylon 6, 6 membranes and cinnamoylated derivative of d-sorbitol [12,13], still the area needs to be explored further to discover new methods of tyrosinase immobilization for effective phenolics treatment of wastewater.

This study was carried out to study the prospect of partially purified and immobilized mushroom tyrosinase as a cost-effective method for removing a broad spectrum of phenolic compounds present in industrial wastewaters. The kinetic parameters and operational stability of the immobilized enzyme were also investigated in this study.

2. MATERIALS AND METHODS

2.1. Material

Mushrooms (*Agaricus bisporus*) were procured from the local supermarket. L-Tyrosine and riboflavin were purchased from HiMedia Chemicals, Mumbai, India. Ammonium sulfate, calcium phosphate gel, p-cresol, catechol, Folin's reagent, glutaraldehyde, bovine serum albumin, N, N′ methylene bisacrylamide, acrylamide monomer, and sodium alginate were purchased from SRL India Pvt. Ltd. All other chemicals used in this investigation were of analytical grade. All the experiments were carried out in triplicates. The absorbance of samples was recorded in a Shimadzu UV-Vis spectrophotometer.

2.2. Extraction and Purification

Extraction and purification of tyrosinase were performed from fresh mushrooms as described by Duckworth and Coleman [14]. In brief, 2 Kg fresh mushrooms were homogenized in 2 L of homogenization buffer (15% ethanol containing 50 mM acetate at pH 5.6). This was followed by centrifugation and the pulp was resuspended in 0.1 M NaCl. This was again centrifuged to yield the first salt extract. The pulp was subsequently soaked overnight in 0.1 M NaCl to yield further salt extracts. The first five extracts were pooled together and further processed. These extracts were precipitated with ammonium sulfate in the fraction range of 0%–30%, 30%–60%, and 60%–90% by adding required amounts of the salt. The maximum activity fraction was treated with calcium phosphate gel twice to remove colored pigments. After each step of purification, the fractions were assayed for cresolase and catecholase enzyme activity using 1 mg/ml *p*-cresol and catechol, respectively, as substrates. The protein content was determined by the method of Lowry et al. [15].

2.3. Enzyme Assay

The cresolase and catecholase activities during the extraction and purification of the enzyme were determined using p-cresol and catechol as substrates as described by Pérez-Gilabert and García Caramona [16]. In brief, in a 3 ml reaction mixture containing 1 ml of 1 mg/ml substrate, 1 ml of 0.1 M phosphate buffer of pH 6.9, 0.1 ml of enzyme extract, and 0.9 ml of distilled water. The reaction was initiated by the addition of the substrate and the incubation was done at 25°C for 15 minutes. Suitable enzyme and substrate controls were also taken and the difference in absorbance at 380 nm between the test and controls was taken as a measure of enzyme activity. The protein content of all fractions was done by the method of Lowry et al. [15] using 1 mg/ml of Bovine serum albumin (BSA) as standard.

2.4. Enzyme Immobilization

Experiments were carried out to immobilize the enzyme using various methods including cross-linking with glutaraldehyde, entrapment within polyacrylamide (photopolymerization, photopolymerized gel activation and *in situ* polymerization), and entrapment within alginate. The efficiency of these different techniques for immobilization was assessed through the determination of enzyme activity for the immobilized enzyme with respect to the free enzyme and calculating the percentage of immobilization achieved by each method.

2.4.1. Immobilization by the covalent cross-linking method

Immobilization by the covalent cross-linking method was carried out as described by Shah et al. [17], in which 100 µl of the enzyme was mixed with 200 µl of 1 mg/ml of BSA in phosphate

buffer. This was followed by the addition of 200 µl of 2.5% glutaraldehyde. The resulting solution was mixed and allowed to dry at room temperature. The membrane thus formed was washed with distilled water followed by glycine buffer to remove excess glutaraldehyde and finally again washed with distilled water [17]. The membranes were stored at 4°C in phosphate buffer and enzyme activity was determined.

2.4.2. Physical entrapment within polyacrylamide gel

2.4.2.1. Photopolymerization

1.15 grams of N, N′ methylene bisacrylamide was dissolved in 40 ml of phosphate buffer (pH 6.4) and 6.06 g of acrylamide monomer was added to it (monomer: crosslinker: 1:4). This was poured into a 50 ml volumetric flask containing 5.5 mg riboflavin and potassium persulfate and the volume was made up to 50 ml. The gel thus formed was stored in dark. For carrying out enzyme immobilization, 1 ml of this gel was taken along with 100 ml of enzyme and photopolymerization was initiated by irradiating with 150 W lamp for one hour [18]. Excess riboflavin was removed by excessive washing with phosphate buffer and the amount of enzyme immobilized was determined.

2.4.2.2. Photopolymerized gel activation

Blank polyacrylamide gel was prepared as described in the previous section and was then activated using BSA and Glutaraldehyde. For this, 200 µl of 1 mg/ml BSA was pipetted out on the blank polyacrylamide gel and followed by the addition of 50 µl of enzyme and 50 µl of 2.5% glutaraldehyde. This was allowed to dry at room temperature and washed with distilled water, glycine buffer, and distilled water in that order. This gel was then used for the determination of the activity of the immobilized enzyme.

2.4.2.3. In situ polymerization of acrylamide monomer

1 ml of monomer and dimer was taken to which 100 µl of 4% Tetramethylethylenediamine (TEMED) (N, N′-methylene bis acrylamide, N,N,N′,N′-tetramethyl ethylene diamine) and 100 µl of 2% ammonium persulfate and 100 µl of enzyme stock (containing 88 enzyme units) were added. The gel thus formed was transparent and was washed with phosphate buffer. This gel was then used for the determination of enzyme activity.

2.4.3. Entrapment within alginate beads

Calcium alginate gel was prepared for the concentration of 4% by mixing calcium chloride and sodium alginate as described by Mahajan et al. [19]. Calcium chloride was taken in a beaker and sodium alginate containing 88 enzyme units was dropped into the beaker using a micropipette. As the spherical drops of alginate and enzyme came in contact with the calcium chloride, calcium alginate spherical beads were formed inside which the enzyme was entrapped. The same procedure was repeated with barium and strontium chloride, to test which method is most suitable for enzyme entrapment. Beads were also prepared with a mixture of all the three chlorides in the ratio of 4:3:3 (calcium chloride: barium chloride: strontium chloride).

2.5. Operational Stability of the Immobilized Enzyme

The *in situ* polymerized enzyme was then tested for its operational stability and shelf life by storing the membrane in 0.1 M phosphate buffer, pH 6.5, and carrying out the enzyme assay periodically for a period of one month. After each assay, the membrane was washed thrice to remove traces of any residual substrate and was further stored in phosphate buffer.

2.6. Effect on Reaction Rate Upon Enzyme Immobilization

Since the binding of the enzyme to the support matrix might lead to structural alterations or inactivation of the binding site, it is necessary to redetermine the enzyme-catalyzed reaction rates. Enzyme activity of the polyacrylamide-entrapped enzyme was further studied by plotting absorbance against time for the immobilized enzyme and this graph was compared with that of free enzyme.

3. RESULT AND DISCUSSION

3.1. Enzyme Extraction and Purification

The crude extract was partially purified by salt precipitation followed by ammonium sulfate fractionation and a final treatment with calcium phosphate gel. At each step of enzyme purification, cresolase and catecholase activities along with the total protein content were determined. The results are shown in Figure 1.

The purification of mushroom tyrosinase is moderately more difficult as the mushrooms contain a significant amount of various phenolic compounds which are readily oxidized and polymerized to macromolecules of melanins during the homogenizing process. However, in our process, we could obtain significant activity in the first five salt extracts after homogenization and hence no further extraction with the salt was done. These fractions were pooled and precipitated with ammonium sulfate in different fractionation range. Maximum activity was found in the 30%–60% fraction followed by 60%–90% fraction. Little or no activity was found in the 0%–30% fraction. This is supported by the earlier literature where 70% of saturation with ammonium sulfate was done to fractionate the enzyme [20]. The most active fractions (30%–90%) were pooled and treated with calcium phosphate gel thrice and a decrease in enzyme activity was observed in the supernatant due to the absorption of the enzyme on the calcium phosphate gel. The maximum activity fraction (30%–90%) was selected for enzyme immobilization.

3.2. Enzyme Immobilization

The activity of the immobilized enzyme was compared with the free enzyme to determine the % immobilization achieved by each method. The results are shown in Table 1.

Immobilization by covalent cross-linking with glutaraldehyde showed very poor protein binding or immobilization, this method was rejected for further study. Physical entrapment within polyacrylamide gel resulted in effective tyrosinase immobilization ranging from 5% to 40% depending upon the method used for activation of the polymerization process. The photopolymerization method gave up to 20% immobilization but suffered from a critical

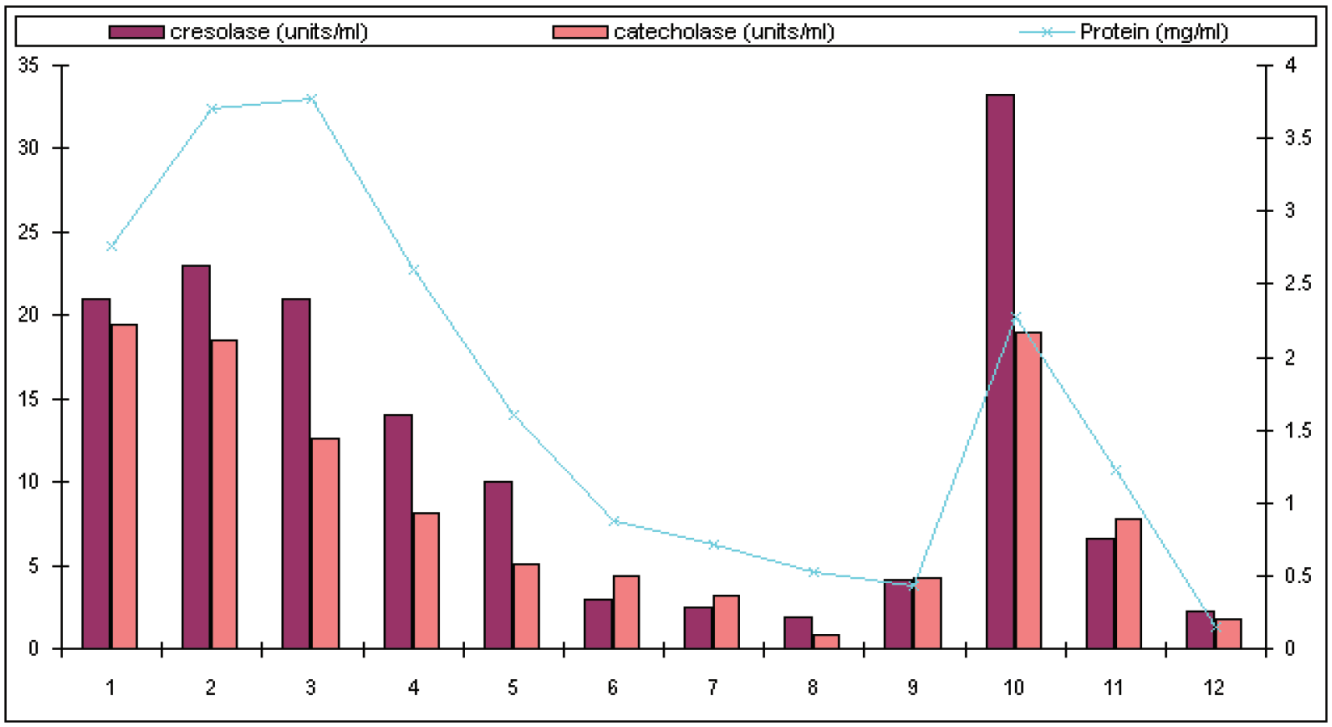

Figure 1: Activity and protein content at various stages of tyrosinase purification (procedure followed as per Duckworth and Coleman [14]. **1**: crude extract; **2**: ethanol fraction; **3**: first salt extract; **4**: second salt extract; **5**: third salt extract; **6**: fourth salt extract; **7**: fifth salt extract; **8**: sixth salt extract; **9**: 0%–30% ammonium sulfate; **10**: 30%–60% ammonium sulfate; **11**: 60%–90% ammonium sulfate; **12**: calcium phosphate gel.

Table 1: Efficiency of the immobilizates prepared by various techniques.

Method	Percentage Immobilization (%)
Cross-linking with glutaraldehyde	Negligible
Photopolymerization of polyacrylamide gel	Up to 20%
Activated polyacrylamide gel	5%–6%
In situ polymerized polyacrylamide gel	35%–40%
Entrapment in alginate	2%–20%

drawback that it had low reproducibility. The photopolymerized gel activation method of immobilization showed retention of only 5%–6% of the enzyme activity. Hence, this method was also rejected for further study. *In situ* polymerization of acrylamide monomer gave 35%–40% of effective entrapment. It was also found to be reproducible upon repeated immobilization. Since this method gave maximum protein binding and was found to be reproducible, it was selected for further study. Different degrees of immobilizations were observed on gels of calcium alginate, barium alginate, and strontium alginate. Thecalcium alginate beads gave maximum retention of enzyme activity but were highly unstable and disintegrated very fast. Other gels gave more stability but the amount of enzyme retained was relatively low. The stability of the beads was inversely related to the enzyme activity as more stable beads had lower pore size making the diffusion of substrate and product more difficult, and hence low enzyme activity was detected.

3.2.1. Effect of enzyme immobilization on reaction rate

Comparing the absorbance *versus* time plot of free enzyme and the polyacrylamide entrapped enzyme showed a similar pattern as shown in Figure 2, using phenol as a substrate. Both the graphs show an initial lag of 10 minutes, after which the reaction rate was linear, inferring that immobilization does not have any significant effect on the response time.

3.2.2. Operational stability of the in situ polymerized enzyme

The *in situ* polymerized enzyme was found to be stable for up to 8–10 usages after which it showed a fall in activity by up to 60%, facilitating its reusability for about 10 times. The results are shown in Figure 3.

Our results have indicated improved tyrosinase stability upon immobilization with additional benefits of reuse or application in continuous processes. A recent study to evaluate to what extent enzyme immobilization is economically justified with tyrosine immobilized on a cellulose-based carrier [Diethylaminoethyl (DEAE)-Granocel] by covalent attachment via the hydroxyl groups of the carrier has demonstrated that immobilized tyrosinase does not suffer from adsorption of reactants or diffusion limitations. In addition, the native enzyme is recommended for single usage, whereas for multiple usages immobilized tyrosinase is more efficient [21].

Furthermore, our results are in agreement with various other recent methods for the development of immobilized tyrosinase-based processes for phenol removal. In a recent study on polyacrylonitrile beads immobilized tyrosinase, it was seen that the removal percentage of phenol by immobilized tyrosinase was nearly equal to the free enzyme and could achieve up to 90% removal of toxic phenols in a 12-hour period. At higher phenolics concentration, the efficiency of removal was found to be higher with immobilized tyrosinase. It was suggested that the process of

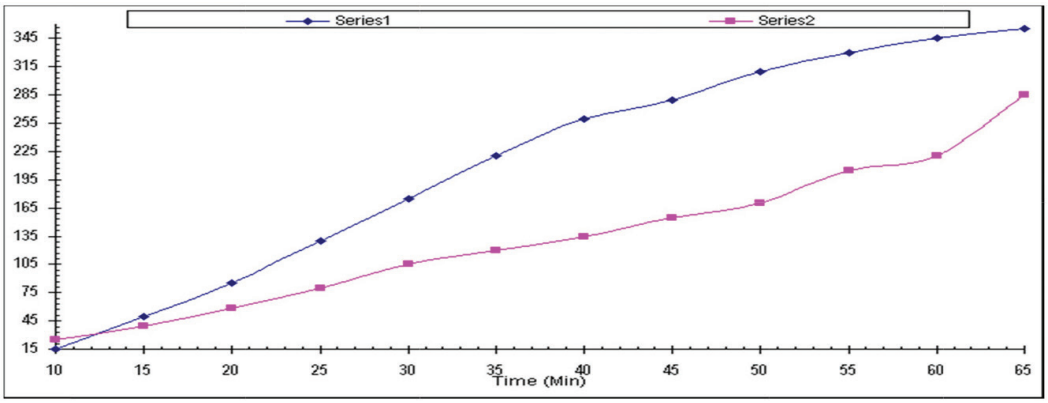

Figure 2: Progress of the enzymatic action with free and immobilized forms. **Series1**: Free enzyme; **Series2**: Immobilized enzyme.

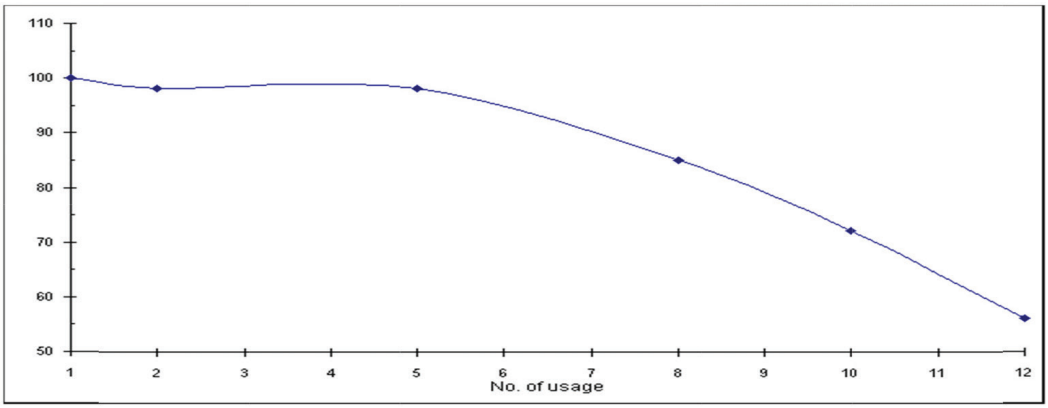

Figure 3: Operational stability of tyrosinase immobilized through *in situ* polymerization in terms of (%) relative activity.

immobilization exerts some sort of protective effect on Tyr against the inactivation/inhibition effects which has been accentuated by polyacrylonitrile [22].

4. CONCLUSION

In this study, effective immobilization strategies for partially purified mushroom tyrosinases were evaluated for cost-effective removal of phenolics from wastewater. *In situ* polymerization of acrylamide monomer with tyrosinases was found to be the most effective in terms of percentage immobilization and reproducibility. Monitoring the progress of the reaction with free and *in situ* polymerized immobilized enzyme showed a similar pattern, showing that immobilization does not affect the substrate-binding site of the enzyme. The *in situ* polymerized, polyacrylamide entrapped enzyme showed operational stability of up to 10 usages without any significant decline in enzyme activity. Mushroom tyrosinases would seem to be natural candidates for the establishment of commercial processes for cost-effective removal of phenolics present in wastewater.

ACKNOWLEDGEMENTS

The authors acknowledge Dr. A.K. Chauhan, Founder President, Amity University, for his continuous motivation and encouragement and Dr. Chanderdeep Tandon, Director, Amity Institute of Biotechnology, Amity University for providing the infrastructure to carry out the investigations.

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How to cite this article:
Avasthi AS, Ghosal S. A simple and efficient method for removal of phenolic contaminants in wastewater using polyacrylamide entrapped mushroom tyrosinase. J Appl Biol Biotech 2020;8(02):22–27. DOI: 10.7324/JABB.2020.80204.