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Extraction, Purification and Characterization of Tyrosinase from Button Mushroom and its Environmental Application

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ABSTRACT

Tyrosinase (E.C.1.14.18.1) is a copper containing enzyme found mainly in different groups of bacteria, fungi and animals that uses phenolic compounds as substrate and produce compounds like melanin and many other polyphenolic products which are biologically important compounds within living cells. Edible mushrooms are considered as a clean, enriched and cheap source of this. This study aims to demonstrate theenvironmental application of tyrosinase from Agaricus bisporus (Button Mushroom) and to study its characteristics. The crude tyrosinase enzyme was obtained by homogenization of Button Mushroom. Optimization studies of crude enzyme was carried out byvarying pH and temperature. Purification of enzyme was obtained using ammoniumsulphate precipitation and membrane dialysis methods .Effect of Murraya koenigii leaves extract and orange peel extract on tyrosinase activity was investigated as per method of Chang et.al. Artificial sewage with phenol was treated with mushroom extract and reduction in amount of phenol was checked every day. Tyrosinase from the Agaricus bisporous was purified by Ammonium sulphate precipitation and membrane dialysis .About 0.75 fold purification was achieved to give 61.3% yield. Specific activity of this purified tyrosinase was found to be 1.46 uM/mg highest tyrosinase activity was found to be at pH 7 and temperature 37^{0}c .Murraya koenigii leaves shows increase in activity of tyrosinase. Orange peel extract showed inhibition on tyrosinase activity. TheKm and Vmax value of tyrosinase were determined from Line Weaver Burke Plot. Km calculated was 0.5mM/ml and Vma,41µMol/ml/min.Tyrosinase treatment of artificial sewage shows detoxification of phenolic compounds.Maximum reduction was observed on 5 th day.Tyrosinase shows successful detoxification of phenolic compounds in artificial sewage sample.So this can be promising method at field level for minimising pollution of water bodies.

Key words: *Tyrosinase, Kinetic analysis, Purification, Dialysis, Detoxification, Phenolic compounds*

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INTRODUCTION

Tyrosinase (E.C.1.14.18.1) is a copper containing enzyme found mainly in different groups of bacteria, fungi and animals that uses phenolic compounds as substrate and produce compounds like melanin and many other polyphenolic products which are biologically very important. Edible mushrooms are considered as a clean, enriched and cheap source of this enzyme. In fungi, melanins are involved in defence mechanisms against stress factors such as uv or gamma radiations, free radicals, dehydration and extreme temperatures. In addition, tyrosinases are associated with wound healing, with the immune response in plants. In humans, tyrosinase is involved in the pigmentation in melanocytes. Tyrosinase is a cuproenzyme responsible for formation of the pigments of skin, hair, and eye [1]. It carries out important reactions known as cresolase and catecholase. Tyrosinase is has been extracted from different sources such as fungi, fruits .But edible mushroom is a clean, enriched, and cheap source of this enzyme. The active site of mushroom tyrosinase is close to the surface of the protein skeleton which makes it accessible for substrates with different sizes [2].This enzyme has variety of applications food, agricultural and pharmaceutical industries .It can be used in solving some environmental problems like sewage treatment. Mammalian tyrosinase has a tetrameric structure. Mushroom Tyrosinase is also tetramer and i has structural simililarity with it It is used for variety of clinical purposes [3][4].So it is necessary to investigate simple tecnniques to purify this enzyme from musroom with economic procedures . Ortho hydroxylation of phenolic compounds occurs naturally in living organisms .This is responsible for formation of important biochemical such as neurotransmitter like L-Dopa family,tannins and polyphenolic acids. Tyrosinase catalyses two different reactions. The primary reaction is hydroxylation of monophenols resulting in o-diphenols, commonly known as monophenolase or cresolase. The second reaction is the oxidation of o-diphenols to o-quinones, often known as o-phenolase

or catecholase. The conversion of diphenols is a potentially catalytic ability and thus tyrosinase has attracted lot of attention with respect to industrial application.
This enzyme could also be used for oxidation of many type of phenolic compounds such as methyl phenols, naphtanols and chlorophenols. Phenolic compounds are mainly found in wastewater of many industries such as plastic and petroleum refineries, resins and coal conversion. These compounds are toxic to human health. There is lot of progress in synthetic chemistry .But there is no chemical reagent which can carry out such reactions .So the demand for enzyme tyrosinase which carry out this reaction is increasing in industry. Tyrosinase (EC.1.14. 18.1) is the only known enzyme for this purpose.So it has also therapeutic value.Phenolic compounds are mostly found in industrial waste water are the major pollutants of water bodies and they are even toxic in their small concentrations. Literature has proved the role of this enzyme to remove such compounds from industrial sewage. Therefore, some steps should be taken to treat wastewater. In environment technology, tyrosinase is used for detoxification of phenolic compound present in wastewater and soil contaminated with phenolic compounds [5]. So the present investigation was carried out to investigate the same application of Tyrosinase. This investigation also aims to extract ant purify Tyrosinase from Mushroom extract and characterize it .It is also used in cosmetic and food industries as important catalyticenzyme. This study during this study the laboratorybased experiment was carried out forremoval of phenolic compound. Tyrosinase play important role in rate limiting step of biosynthesis of melanogenisis.[6] Literature shows that citrus peel has antioxidant and antityrosinase activity [7] the active component present in citrus peel is noblietin act as tyrosinase inhibitor. It also suggest that the peel of citrus food could be anew source in the field of hyper pigmentation with potential as a skin whitening cosmetics[7].

MATERIALS AND METHODS

Sample Collection and Preparation

The Button Mushroom (*Agaricus bisporus*) sample were collected from Mahatma Phule Market, Pune. Authentication of sample was carried out by "BOTANICAL SURVEY OF INDIA", Pune Crude extract of Tyrosinase was prepared using method of kamalhdin et.al. The sliced mushrooms were homogenized by using waring blender. Enzyme extraction was prepared with 300mL of cold 100mM phosphate buffer (pH 5.8) for 200g of mushroom. The homogenate was centrifuged at 5000 rpm for 30min and supernatant was collected.The sediments were mixed with cold phosphate buffer and subjected to centrifugation onceagain to collect supernatant.The supernatant was used as a source of enzyme.

Purification of Crude Extract by Ammonium Sulphate Precipitation and Dialysis (By Method of Kamalhdin et.al, 2014)

Ammonium sulphate precipitation was done in an ice bath using the finely grounded ammonium sulfate. The powder was weighed and added slowly to the extract by constant stirring to ensure complete solubility, and the solution was centrifuged at 5000 rpm for 30min. Different precipitation steps were carried out for tyrosinase enzyme precipitation (45–80%) and precipitates were collected. The precipitate was dialyzed against 100mM potassium phosphate buffer (pH7.0) for 24hrs.The dialyzed fractionwas used for tyrosinase activity and protein content

Assay of Tyrosinase Activity as Per Method of Sung and Cho, 1992[9]

An aliquot containing tyrosinase (0.5) and 1 ml L-DOPA (4mg/ml) was incubated for 5min at 35^0C at time '0', was measured at 475 nm. After incubation for additional 5min, the mixture was shaken again and a second reading was determined and was measured for '3' minutes.The change in absorbance was proportional to enzyme concentration.One unit of enzyme corresponded to the amount which catalyzes the transformation of 1 μmol of substrate to product per min under the above conditions and produced 1.35 changes in absorbance. Specific activity was expressed as enzyme unit per milligram of protein.The protein content of the enzyme was determined by the method of Lowry.

Effect of pH on Enzyme Activity

The activity of tyrosinase was evaluated at different pHvalues in the range between pH 3 and 10 and the amount of dopachrome was determined. Buffers used were citrate phosphate (pH 3), potassium phosphate (pH 7), Tris-HCl (pH 8), and glycine-NaOH (pH 9).

Effect of Temperature on Enzyme Activity

Optimum temperature for enzyme activity was determined by incubating the standard reaction mixture at temperatures ranging from 35^0 to 65^0C.Kinetic Analysis.
The enzyme kinetics as measured by the Michaelis constant (Km) is defined as the substrate concentration at half the maximum velocity, the rate of enzymatic reactions, by relating reaction rate to the concentration of a substrate. The Michaelis constant (Km) value of the purified enzyme was estimated in a range of tyrosinase concentrations. The apparent Km value of purified tyrosinase. Was calculated from the Line Weaver-Burk plots relating 1/V to 1/[S].

Effect of *Murraya koenigii* (Curry leaves) Extract as Per Method of Sunanda and Varsha, 2018 [10]
Murraya koenigii leaves were collected from Mahatma Phule Market, Pune Leaves of murraya koenigii were dried in oven until the moisture was removed. 20 gm of leaves was kept in 80 ml solvent system (Ethyl acetate: Ethanol) (3:1) for 3 days at room temperature. Evaporation of solvent extract was carried out in water bath. Residue was dissolved in dimethyl-sulphoxide (1mg in 1000µl proportion) .At different concentration of Murrya koenigii extract 0.5 ml crude enzyme and 1 ml L-DOPAwas added and Incubation for 5 ml .Absorbance was taken at 475nm.

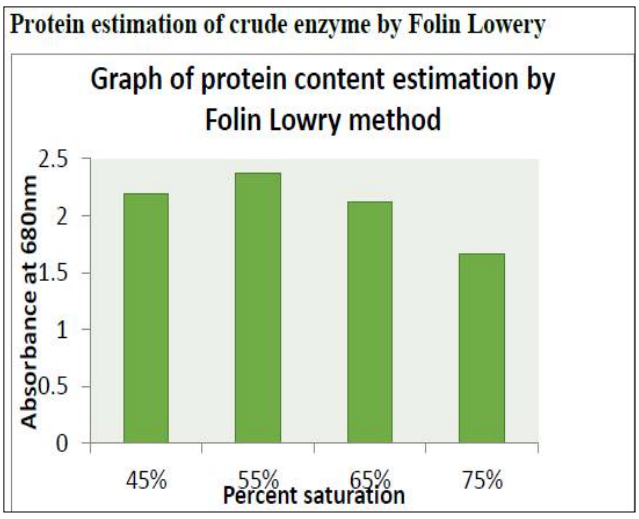
Anti-Tyrosinase Activity of Orange Peel Extract
Preparation of extract: Collection of Oranges from Mahatma phule Market, Pune. Orange peels were cut into small pieces and dried in hot air oven at 50°C for 48 hours dried orange peel was soaked in 95% ethanol and Incubated at 50°C for 72hrs then Filtered through the Whatman no. 1 The Filtrate was evaporated to yield crude extract and kept in refrigerator until used.
Antityrosinase activity of orange peel extract was determined using L-DOPA as substrate and orange peel extract as inhibitor[11] (One ku(1000 units/ml) mushroom tyrosinase was preincubated with orange peel extract (at various conc.) in 50mM phosphate buffer(pH-6.8),total volume of reaction mixture tube was 1.80 ml at 37^0C for 5 min. L-DOPA (25mM, 0.20 ml)was added to the reaction mixture and incubate 37°C for 10 min. Absorbance at 475nm. The % inhibition of tyrosinase activity was calculated by using equation of
% inhibition = A475Control - A475Sample × 100

TLC Detection of Flavonoids in Orange Peel Extract
Method: 100 ml volume of solvent system (Toulene:Chloroform:Acetone(40:25:45))was placed in glass tank and allowed to stand for 45min before use.The spot of orange peel extract given above 1cm on silica plate and kept in solvent system to run.TLC was observed in laminar air flow.Detection of flavonoids were done by calculating Rf value by comparing standard value.

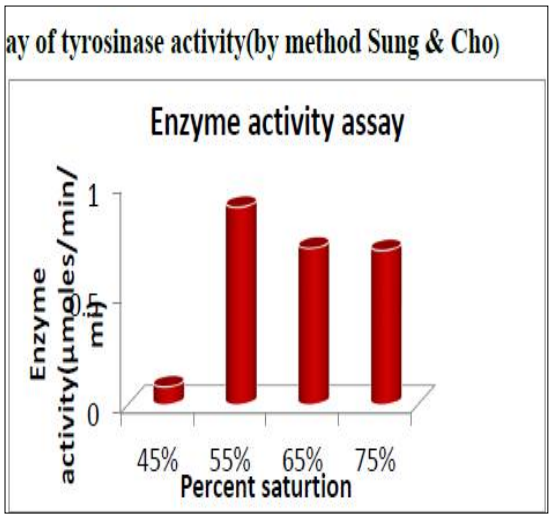
Laboratory based Experiment to study Application. Removal of Phenol Catalysed By Mushroom Extract from Synthetic Wastewater Containing Phenolic Compounds [12]

Preparation of synthetic waste water containing Phenolic compounds
1 ml phenol solution in 100 ml of Pond water sample which was collected from Sarasbag,Pune was prepared to use as a synthetic wastewater containing phenolic compounds.
2ml of mushroom extract was added to 15 ml of phenol solution and the reaction mixture was stirred. 1ml of mixture was withdrawn for every per day. Centrifuged to remove the precipitate formed and assayed for phenol. To different aliquots of phenol solution (0.1 to 1 ml),0.05 ml 6M Ammonium hydroxide was added and stirred. Afterstirring,0.05 ml Aminoantipyrine (2%) and 0.1 ml Aq. solutionof Potassium ferricynide (8%) were added. Incubate for 15 minutes for the development of colour The dye was extracted with 5 ml of chloroform. Absorbance of the extract was measured at 457nm The absorbance is proportional to the concentration of phenol.

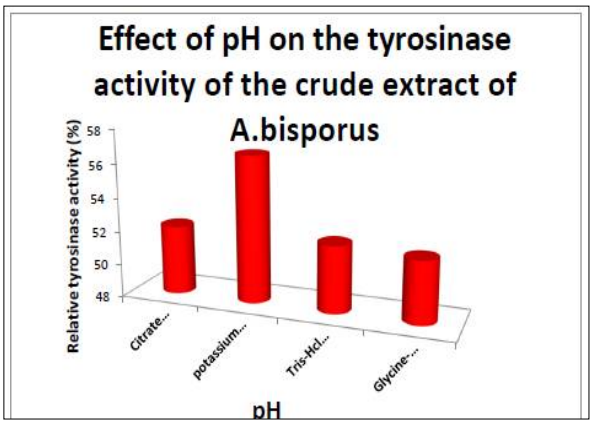
RESULT AND DISCUSSION:



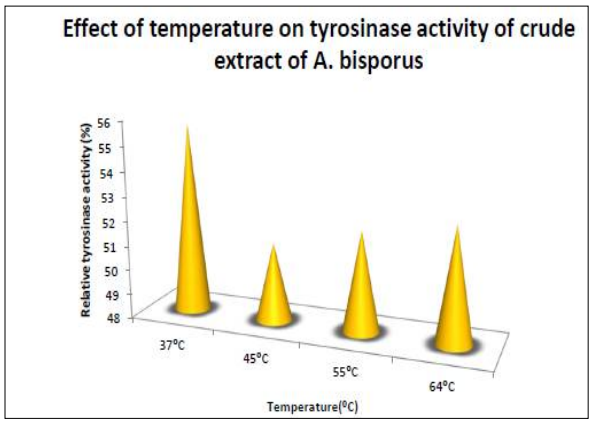
Graph:1: Protein estimation by Folin Lowery



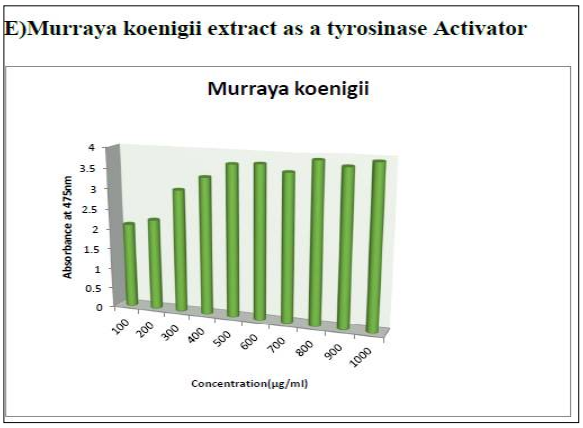
Graph2: Enzyme Activity Assay



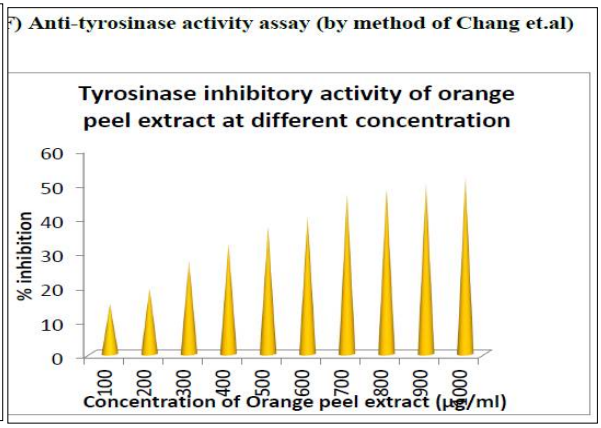
Graph3: Effect of pH on Tyrosinase activity



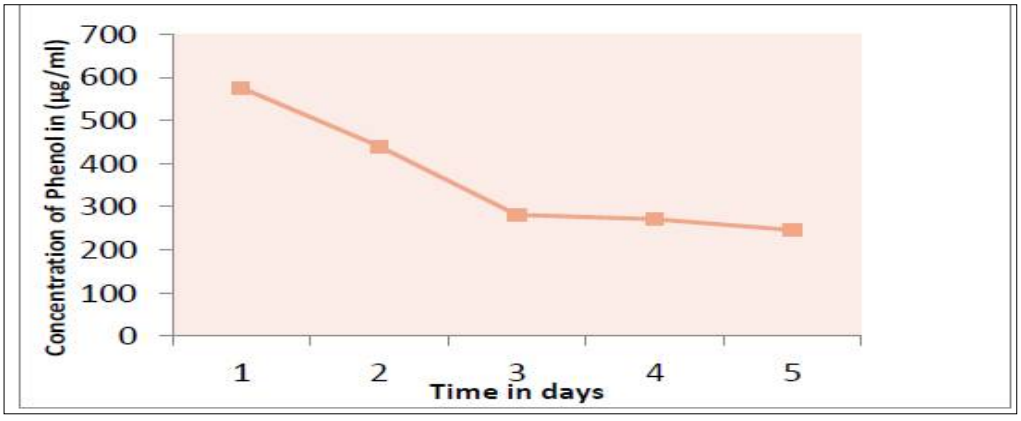
Graph 4: Effect of temperature on Enzyme activity



Graph 5: Effect of Curry Leaves Extract



Graph 6: Effect of Orange Peel Extract on Tyrosinase Activity



Graph7: Removal of phenol from artificial sewage using Tyrosinase

Maximum protein content and enzyme activity was observed at 55% saturation of ammonium Sulphate. This investigation revealed 37^0C**. as** optimum temperature for activity of Tyrosinase .Maximum catalytic activity of tyrosinase was found to be at pH 7.In similar experiments carried out by Ikehata, Keisuke & Nicell, Jim [13] same observations were detected Phenol is called as priority pollutant by environmental protection agency of US . There are strict discharge limits for phenol in surface waters .Less than 1ppb of limit of phenol has been recommended by EPA. Also toxicity levels of phenol are found to be 9-25 mg/liter for humans and aquatic life. Human exposure to phenols leads to number of problems like rashes and irritation of skin, eyes and mucus membranes. Chronic exposure leads to vertigo, weight reduction and irritation of gastrointestinal tract, liver, kidney and CNS. Animal studies have shown effect on growth and also abnormalities in offsprings [14]. Mushroom tyrosinase (polyphenol oxidase, EC 1.14.18.1) was investigated as an alternative to peroxidase enzymes for the catalytic removal of phenolic compounds from wastewaters. Phenol was successfully transformed by tyrosinase over wide ranges of

pH 5–8 and initial phenol concentration (0.5–10 mM, 47–940 mg/l). Several chlorinated phenols were also successfully transformed [13]

CONCLUSIONS

Extraction, Purification of mushroom tyrosinase was done successfully. Tyrosinase converts L-DOPA to red coloured oxidation product Dopachrome. Highest tyrosinase activity was found to be optimum at pH-7 and temperature 37^0C. *Murraya koenigii* leaves shows increase in activity of tyrosinase. Orange peelextract shows inhibition on tyrosniase activity. The Km & Vmax values of tyrosinase were determined from Line Weaver Burke Plot, where Km value is0.5mM/ml & Vmax is 42 µM/ml/min.Tyrosinase shows successful detoxification of phenolic compounds in artificial sewage sample. Tyrosinase from Muhroom was found to be the promising candidate for sewage detoxification.

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