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# Isolation and Characterization of Tyrosinase (A Carbon Trapping Enzyme) Producing Microorganisms, in the Agricultural Soil of Western Uttar Pradesh and the Study of Enzymatic Activity of Tyrosinase Produced

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## Abstract

**Tyrosinase is an enzyme which shows a carbon trapping activity in soil. Carbon, being one of the main component of the greenhouse gases needs to be reduced in the earth's atmosphere. Hence, tyrosinase producing microorganismin the soil plays a vital role in reducing carbon content and hence the global warming. Taking in view the importance of tyrosinase producing microorganism in the soil, present study is conducted to isolate and characterize the microflora which is the potent producer of tyrosinase in the soil of western Uttar Pradesh. The work dealt with the growth of soil dwelling microorganism on suitable growth medium. Once they were characterized as enzyme producer, the enzyme was produced and crude enzyme was then extracted. Finally, the activity and kinetic studies of the enzyme were performed from a very potent bacterial population of *micromonospora* found among them.**

***Keywords: Tyrosinase; Micromonospora; Carbon trapping; Soil microflora west U.P.; Enzyme kinetics; Dopachrome***

## Introduction

Greenhouse gases in atmosphere plays a major role in trapping heat of the earth's atmosphere. These greenhouse gases include carbon dioxide, chlorofluorocarbons (CFC's), methane, tetrafluoromethane, etc. The main component of the entire above stated greenhouse gases is Carbon. Thus, lesser carbon in the atmosphere means lesser greenhouse gases and hence lesser is the global warming. Nowadays, several efforts are being made to reduce the emission of carbon and its derivatives from automobiles, factories & other manmade machines, in the earth's atmosphere. But the effects seen are not really big.

But, what if our soil can help us in this? Our soil contains a varying range of flora including Tyrosinase producing bacterias and fungus. Tyrosinase (EC 1.14.18.1) is a copper containing mono oxygenase enzyme which is widely distributed in nature. This enzyme has a very unique ability to trap and absorb the atmospheric carbon [7]. But, adding tyrosinase artificially to the

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soil is a cost effective process. Moreover, the enzyme added artificially to the soil will have only a specific lifetime in the soil and cannot be regenerated by itself.

However, Tyrosinase producing bacterias and fungi which can live in soil [1,5,6,9,12], can help us in a long way towards increasing the tyrosinase content in the soil and hence improving soil's efficiency of carbon uptake thereby reducing global tempreture[4]. The present work reports the different tyrosinase producing microorganism found in the soil of western Uttar Pradesh (India) along with the activity & kinetic studies of the tyrosine enzyme extracted from one of the potent bacteria found among them, of Genus *micromonospora*.

## Material and Methods

### Culture medium

### Enrichment media

Separate enrichment media were used for increasing the relative concentration/ population of bacteria and fungi inherent in the soil samples. Enrichment media used were bacterial enrichment broth (which consisted of 2.0g potato dextrose broth powder) & fungal enrichment broth (1.0g Tryptone, 0.2g Glucose & 0.5g Sodium chloride).

### Media preparation

### Culture Medias used were

NAM (Nutrient Agar Medium MM012500G), by Himedia, MSM (Mineral Salt Medium), MMB (Mineral Medium Broth), Streptomyces isolation media i.e. Casein broth & Nutrient broth, *Aspergillus* isolation media, Starch Casein Agar media & TES (Trace element solution).

### Collection of soil sample

Various soil samples were collected from agricultural land in Meerut (Uttar Pradesh), dug about 2-3 inches deep and weighed accordingly (one gm soil for 100ml enrichment media, and accordingly for other volumes. The conglomeration was incubated at 30°C (for increasing fungal micro flora) and 37°C (for increasing bacterial micro flora)

### Serial dilution of soil sample

The original enrichment suspension served as zero dilution. Further dilutions were prepared in factors of 10, up to 107. 100µl zero dilution suspension was mixed with 900 µl autoclaved distilled water in an 1.5ml eppendorf tube. 100µl of this suspension (101) when mixed with the 900µl autoclaved distilled water served as 102 dilutions and so on until 107 dilutions is achieved.

### Spread plate method

The spread plate method includes the spreading of 100 µl of sample from each dilution (100 to 107) on the solidified media petriplate with the help of sterilized glass spreader. The Petri plates were incubated at 28±20C (for fungal culture) and 28±20C (for bacterial culture). Observations were recorded daily.

### Examination of microorganism

Slide was prepared by heat fix method and was stained using gram staining method and was observed under microscope for identification. Some of the selected strain’s genus were identified using Bergey’s Manual of systematic Bacteriology[2].

### Enzyme production

The inoculums were taken from the culture plates and were inoculated in the respective medium broth with the appropriate amount of enhancer (L-Tyrosine) and inducer (copper sulphate) and were incubated at 25±50C on shaking condition. Observations were recorded on alternate day.

### Qualitative enzyme assay

Around 1.5ml broths were taken in 2ml eppendorf tube from each inoculated flask and were subjected to centrifugation (5000rpm for 10 minutes) and clear supernatant was collected. The supernatant was taken in varying concentration mixed with 500µl of L-DOPA and 1.5ml phosphate buffer (pH-6.5) and was subjected to spectrophotometer (›’= 475 nm) for analysis. A graph was plotted accordingly for the analysis of the results.

## Results and Discussion

Numerous colonies developed on the selective media plates after suitable incubation period. They were examined by eye for distinct morphological features of the appearance of brown black pigmentation or melanin formation [10]. A particular colony of *Micromonospora* exhibiting wrinkled and reddish orange morphology as shown in Figure 1 was taken from *Actinomycetes* isolation agar (AIA) media plates and preserved on NAM plates. This organism was further tested for qualitative tyrosinase enzyme production activity[3], along with Gram staining, morphological and microscopic (Figure 2) characterization.

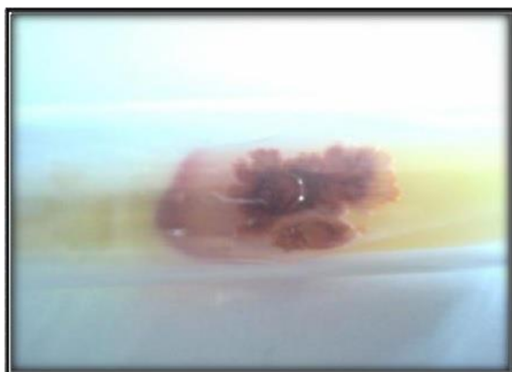

**Figure 1:** Slant of *Micromonospora*

**Figure 2:** Microscopic view

The pH of the phosphate buffer (prepared from 0.1M KH2PO4 and 0.1M K2HPO4 buffers) was adjusted to 6.50. L-DOPA solution was prepared in this buffer to improve its shelf life and stability along with preventing its unwanted oxidation to dopachrome[8]. The crude enzyme extract was prepared by centrifuging the culture broth of the organism at 5000rpm for 10 min. Required quantity of the supernatant was mixed homogeneously in the phosphate buffer to provide a suitable microenvironment to the secreted enzyme and hence prevent its untimely degradation.

Special care was done in adding the L-DOPA solution to the phosphate added crude enzyme extract. Recording of the absorbance values was started immediately after the addition of the L-DOPA solution, so as to infer the initial velocity of the catalysis of the enzymatic extract, which is highest in the beginning and reduces as the time proceeds.

**TABLE 1: Quantities of phosphate buffer and L-DOPA taken in blank and test sample**

| 0 min reading (λ= 475nm) | 0.1 M phosphate buffer (µl) | 0.02M L-DOPA (µl) |
|---|---|---|
| 0.0 (Blank) | 2250 | 750 |
| 1500 (Test) | 750 | 750 |

The qualitative tyrosinase enzyme secretion data analyzed spectrophotometrically is tabulated below:

**TABLE 2 : Spectrometric Absorbance value of reaction of crude enzyme with L-DOPA as a substrate at 475nm wavelength**

| Time (min) | Absorbance (λ= 475nm) |
|---|---|
| 1 | 0.1772 |
| 2 | 0.2120 |
| 3 | 0.2154 |
| 4 | 0.2215 |
| 5 | 0.2224 |
| 6 | 0.2225 |
| 7 | 0.2245 |
| 8 | 0.2278 |
| 9 | 0.2284 |
| 10 | 0.2283 |
| 11 | 0.2300 |
| 12 | 0.2308 |
| 13 | 0.2287 |
| 14 | 0.2300 |
| 15 | 0.2308 |

## Graphical analysis

In the above graph, the X-axis corresponds to the time interval (in minutes), while the Y-axis corresponds to the absorbance of the dopachrome compound formed as a result of the catalytic activity of the crude enzyme extract. There is a linear correlation found between the absorbance of the dopachrome formed and the initial velocity of the enzyme catalyzed reaction [11].

Another assumption that is followed is that at zero time, the amount of dopachrome formed is zero moles and hence the absorbance at that time is zero. However, when the L-DOPA solution is added to the enzyme extract dissolved in the buffer, the biocatalytic activity of the enzyme is started immediately at a very high rate (this depends on the affinity of the enzyme towards its substrate). Initially, the number of the enzyme molecules is very high, due to which the initial velocity of the reaction is very great. This high value of the initial velocity reduces sharply, and by the time the absorbance value is taken, the initial velocity value is considerably reduced. It is this reduced value which corresponds to the first absorbance reading.

The most suitable time interval for measuring the initial velocity readings is taken to be 1 minute, both because of its precision and ease of calculation.

The overall shape of the graph is a hyperbola. This corresponds to the sigmoidal kinetics of the crude enzyme extract towards its dopachrome substrate. With time, the amount of the substrate converted to product increases, resulting in the increase in the absorbance of the test sample, hence the slope of the graph is positive.

However, as the number of free substrate molecules available for conversion to product diminishes with time, the amount of the fresh dopachrome formed also decreases, resulting in the stabilization of the graph (ie. the plateau like region). The slope of the graph at this moment approaches zero (ie. parallel to the X-axis.) In terms of the double derivative analogy, this graph demonstrates that the rate of change of absorbance with respect to time is decreasing, thus the double derivative is negative throughout. This negative value of the double derivative is a direct consequence of the sigmoidal kinetics of the crude enzyme extract towards its dopachrome substrate.

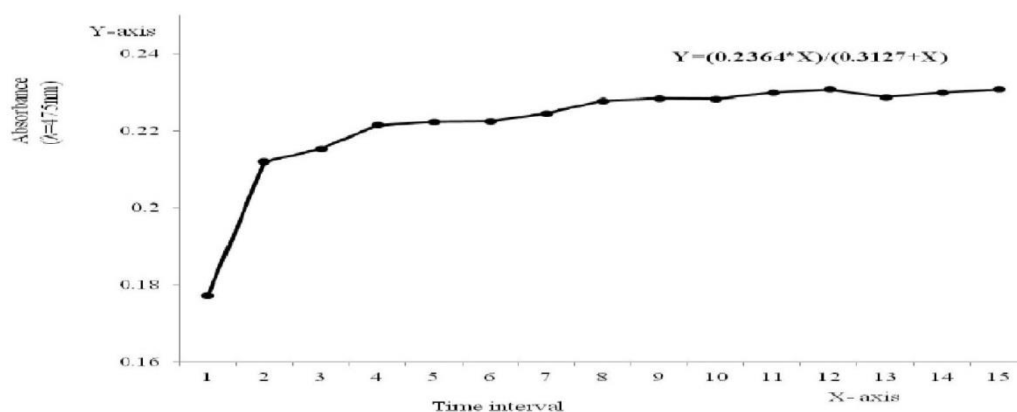


**Figure 3:** Graph depicting the rate of change of initial velocity (and hence the enzymatic activity) of crude enzyme extract processed from micromonospora culture with time.

## Calculations

### Estimation of initial velocity

First value of absorbance = 0.0000 (at time t=0 min) Second value of absorbance = 0.1772 (at time t=1 min).

Since the molar extinction coefficient ([) of dopachrome is 3600 L mol-1cm-1, we can infer the relative concentrations of the dopachrome formed in the test sample solution according to the BeerLambert Law:

Absorbance (A) = [ * c * l Where

[ = molar extinction coefficient of the product formed (L mol-1cm-1)

c = concentration of the product formed (Molar)

l = path length of the monochromatic light rays which pass through the test sample (cm)

Since there is a linear correlation between the Absorbance values and the concentration, a change in the absorbance values will result in a corresponding linear change in the concentration values.

Hence we may safely conclude that:

ΔA = [ * Δc * l

Where the Ä denotes the change in the value of a quantity (ie. the initial value subtracted from the final value). Substituting values, we get

ΔA = A2 – A1

= 0.1772-0.0000

= 0.1772

Here, the path length of the quartz cuvette was 1.0 cm. Substituting, we get

Δc = 4.922 * 10-5 M Or 0.04922 mM Or 49.222 μM

Thus, 49.222 μM of dopachrome is produced in the reaction mixture in 1 second. So, the amount formed in 1 minute would be 2953.33 μM dopachrome. This value corresponds to the initial reaction velocity of the crude tyrosinase enzyme extract. Thus, the initial reaction velocity of the crude tyrosinase enzyme extract is 2953.33μM dopachrome per minute.

Estimation of the maximum amount of dopachrome product formed. The maximum value of the absorbance was recorded at time t = 12 seconds, which was 0.2308. Applying BeerLambert law again, we can estimate the maximum concentration of dopachrome formed in the test sample, which is 6.4 * 10-5M or 0.0641 mM, or 64.11μM. Thus, the maximum amount of dopachrome which is formed due to the catalytic activity of the crude enzyme extract is 64.11μM.

### Estimation of maximum reaction velocity (Vmax)

Consequently, the (theoretical) maximum reaction velocity that can be reached is estimated by assuming that the reaction proceeds at the same rate as the initial reaction velocity.

The maximum reaction velocity represents the theoretical case when the maximum absorbance (and hence the concentration) values are attained during the first second of the reaction time.

Proceeding along the same lines, the maximum amount of dopachrome formed which can theoretically be formed in 1 second is 64.11μM. So, the same amount formed in 1 minute will be 3846.67μM.

Thus the maximum reaction velocity of the crude enzyme extract can be estimated to be 3846.67μM dopachrome product formed per minute.

Concluding, the initial reaction velocity of the crude tyrosinase enzyme extract = 2953.33μM dopachrome per minute, the maximum amount of dopachrome which is formed due to the catalytic activity of the crude enzyme extract is 64.11μM, the maximum reaction velocity of the crude enzyme extract can be estimated to be 3846.67μM dopachrome product formed per minute.

## Conclusion

The tyrosinase producing microbial flora that was identified in the variety of soil samples taken from Meerut, west U.P., (India) includes Bacillus, Aspergillus, Pseudomonas, Micromonospora. A very potent tyrosinase producer from soil found to be Micromonospora, whose enzyme kinetics was studied in detail. It was found that there is a linear correlation between the

absorbance of the dopachrome formed and the initial velocity of the enzyme catalyzed reaction. The overall shape of the graph is a hyperbola. This corresponds to the sigmoidal kinetics of the crude enzyme extract towards its dopachrome substrate. This work can further be extended for the determination of specific amount of the nutrient which we can add in the soil so as to increase the amount and activity of the tyrosinase enzyme produced by the above said variety of microflora, which can further increase the efficiency of carbon trapping and hence will contribute more towards reducing the global warming.

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