



Production and purification of the Tyrosinase enzyme from Soil bacteria

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Abstract:

Tyrosinases are a group of bifunctional enzymes that are involved in the hydroxylation of mono phenols to o-diphenols. These enzymes have their potential applications in several domains like Biosensors, biocatalysis in several pathways, and production of L-DOPA from tyrosine. Detection and removal of phenolic compounds from wastewater also involves the use of tyrosinases. In view of the extensive roles played by these enzymes the current work aimed to isolate and identify the bacteria capable of producing this enzyme in the invitro study followed by the enzyme extraction and purification. The work dealt with the maximum production of the enzyme in two different media by bacterial isolates so as to detect the best media for the maximum yield. Once the production was done the crude enzyme has been extracted and is subjected to purification based on different aspects and methodologies. Various purification protocols like Ammonium sulphate precipitation, Dialysis, Ion exchange chromatography, gel filtration etc have been employed for the maximum purification. The enzyme assay and concentration estimations were also performed and Medium A and C7-*Pseudomonas geniculata* resulted in the maximum yield and activity of the enzyme.

Keywords: Tyrosinases, L-DOPA, Optimization, Guaze Agar, Production Media, Salt Precipitation, Gel Filtration, Ion Exchange Chromatography.

Introduction:

Tyrosinases are the enzymes that are majorly involved in the formation of the naturally occurring pigment Melanin. Tyrosinases being involved in the melanin formation have inherent properties like absorption of UV radiation, metals, sound and also have anti-oxidant and semi-conductor properties ^[1] thus are used in the production of complex biopolymers. Cultures of streptomycetes have been

used in the commercial scale production of Tyrosinases ^[2]. The best production yield reported till date for tyrosinase was about 1g/l, which was produced by cultivating the *Trichoderma reesei* a filamentous fungus for 6days^[3]. Immobilized tyrosinase enzymes are used in the production of L DOPA ^[4]. Several organisms have been used in the production of these enzymes at the commercial scale like Mushrooms, Bacteria, and Fungus etc. a study was undertaken on the production of tyrosinases from *Verrucomicrobium spinosum*. These organisms were later expressed in was expressed in *Escherichia coli* and the enzyme production was studied in both batch and fed-batch cultivations ^[5]. One of the studies was carried out on the biochemical characterizations of human tyrosinase and to provide an unlimited source of the enzyme for further study, an expression plasmid, pHis-Tyrosinase, which contains the entire coding sequence except the signal sequence of a human tyrosinase, was constructed and expressed in *Escherichia coli* ^[6]. Some strains of *Streptomyces* isolated from soil were also used in the invitro production of the tyrosinase enzyme extracellularly The enzyme was purified using eries of column chromatographies on Duolite A-2 and CM-cellulose and gel filtration on Sephadex G-100 ^[7]

In view of the extensive applicability and vital role of the tyrosinase enzymes the current research was focused onto the production of maximum yield from the bacteria followed by the purification and enzyme estimations at various stages of purification.

Materials and Methods:

Sample collection:

In order to obtain the bacterial species capable of producing tyrosinases, soil sample from slaughter house was collected and purified further by subcultures and labeled as C4, C7 and C10.

Grams staining:

Isolated three cultures were analyzed for Gram nature to determine the shape and stain taken ^[9].

Gause agar pigmentation test:

A specialized media called Gause agar media has been used to detect the pigment formation capability of the isolates.

Oxidase test:

Oxidase test was performed to detect the ability of the isolates to produce the enzyme oxidase. This test is one of the biochemical characterization tests as per Bergey's manual ^[10,11].

Melanin formation test:

This test was performed to identify the melanin formation ^[12] ability of the selected bacteria. A test served as a qualitative test for the production of tyrosinases by the bacterial isolates. Coloration results from the dopachrome formation read in spectrophotometer at 480nm.

Production of the Enzyme in vitro:

A specific production medium ^[13] has been used for the production of the enzyme in vitro conditions. These production medias are available commercially and study was conducted to determine the culture capability to show enhance ability to produce enzyme when grown in both media. The medium named as production medium A and B and the composition as given:

Production medium A: - starch 20g/L, KNO₃ 1g/L , K₂HPO₄ 0.5g/L , MgSO₄.7H₂O 0.1g/L ,FeSO₄.7H₂O 0.1g/L, Tyrosine 10g/L , pH 7 .

Production medium B: - Casein hydrolysate 10g/L, K₂HPO₄ 0.5g/L, MgSO₄ 0.25g/L, L- tyrosine 1.0 g/L , pH 7 The cultures were incubated at 28°C and 200 rpm for 72 hrs.

Crude enzyme extraction and Assay of tyrosinase activity in the medium A and B:

After given period of incubation the broths were centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected containing crude enzyme. Continuous spectrophotometric rate determination ^[14] has been employed for the assay of the enzyme produced. The major principle in this assay was the

determination of tyrosinase activity upon the substrate tyrosine in a suitably buffered solution.

Purification of the enzyme from the crude extract:

Crude enzyme extract further purified by salt precipitation, Dialysis, Gel filtration, Ion exchange chromatographies etc have been employed in series so as to obtain the enzyme in its purest form. The pure enzyme thus produced can be used for the further analysis.

a) **Salt precipitation** ^[15] using Ammonium sulphate:

The crude extract was used for the precipitation with ammonium sulphate till the saturation point under ice cold conditions. The procedure will not only help the pure protein to get precipitated out but also enables the extracted protein to retain its function.

b) **Dialysis** ^[16]: The precipitate obtained in the previous step dialyzed against normal water so as to filter out the particulate impurities by dialysis.

c) **Ion Exchange chromatography** ^[17]: This step would enable the removal of the charged impurities. Based on the principle of charge based filtration the purification is achieved in this step DEAE Cellulose membrane has been used.

d) **Gel Filtration Chromatography** ^[18]: This step recovered the final and complete purification of the protein. Sephadex G75 membrane has been used for the gel filtration and bed formation. Elutes were collected and the purification was performed.

Protein Estimation by Lawry's method:

Once the protein was obtained in its pure form the next step performed was the estimation of the protein concentration. Concentration was evaluated by Lawry's method ^[19] for protein. The enzyme produced is measured spectrophotometrically and the concentration was estimated on the standard graph.

Results and Discussion:

Gram's nature and biochemical characterization

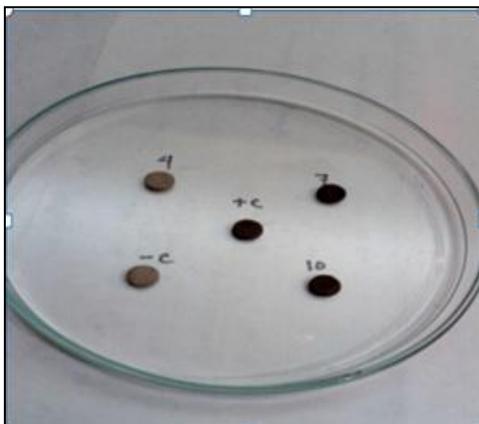
Obtained three cultures have been analyzed for Gram nature and biochemical test performed as in Table1 and Fig 1:

Table 1: Results for the Gram's Nature, Motility and Biochemical tests:

Tests	C4	C7	C10
Gram's	+ve cocco bacilli	-ve bacilli	-ve bacilli
Catalase	+ve	+ve	+ve
Motility	-ve	+ve	+ve
Gelatin Hydrolysis	+ve	+ve	+ve
Oxidase	-ve	+ve	+ve

These cultures were further identified as C4 as *Rhodococcus ruber*, C7 as *Pseudomonas geniculata* and C10 as *Pseudomonas geniculata* by 16S rRNA (Data not shown).

Fig 1: The figure showing the results for the oxidative test:



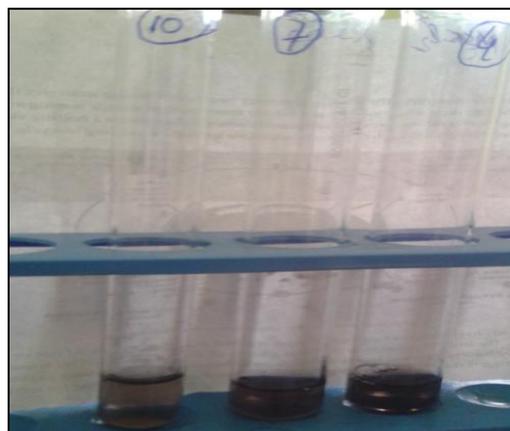
The above Fig. 1 shows the results of Oxidative test for the three selected strains. The plate also includes a positive control and a negative control. Test culture C 4 is found to be Oxidase –ve whereas the other C7 and C10 are positive.

Gause agar pigmentation test:

The pigmentation test showed positive results for the selected bacterial cultures suggested the three isolates capability of production of the enzyme tyrosinase.

Melanin Formation test:

Fig 2: Tubes showing the results of Melanin formation test



As shown by the Fig. 2 three cultures were tested for their ability to produce melanin. The results indicated that all the three were positive for the melanin formation test. The formation of the dopachrome was detected at 480nm. The optical densities obtained for C4, C7 and C10 were 1.92, 1.78, and 1.37 respectively.

Production of the enzyme in two media A and B:

The inoculation of the three strains in two different production media was performed and incubated for the production of the enzyme invitro. The two media differed in their composition. The best media can be finalized based upon the results of Enzyme Assay.

Enzyme Assay:

In order to analyze the activity of the enzyme produced, assay has been performed. The enzyme assay is based on the Continuous Spectrophotometric Rate Determination. The results showed that the enzyme produced in the media A was more active than the enzyme produced in media B. Thus the Media A enzyme has been used for further analysis. (Table 2)

Enzyme Purification:

The crude enzyme produced was purified using Salt Precipitation, Dialysis, and Ion exchange chromatography and Gel filtration.

The pure enzyme thus produced is used for any other analysis.

Table 2: The table showing the Enzyme assay values, Total enzyme concentration and Protein fold for the produced protein at each step of purification along with the respective values for crude enzyme:

SR. No.	Sample	OD at 280nm/min	Units/ml (Enzyme activity)	Total protein in mg	Specific activity Units/mg	Protein fold
1.	Blank	0.419	--			
2.	Production media A (C-4), crude extract	1.141	7220	0.86	8400	1
3.	Production media A (C-7), crude extract	1.304	8850	0.92	9619.5	1
4.	Production media A (C-10), crude extract	1.287	8680	0.77	11272	1
5.	Production media B (C-4), crude extract	0.69	2710	0.67	4044.7	-
6.	Production media B (C-7), crude extract	0.78	3610	0.73	4945.2	-
7.	Production media B (C-10), crude extract	0.76	3410	0.83	4108.4	-
8.	Ammonium sulphate precipitation A-C-4	1.313	8940	0.56	15964	1.9
9.	Ammonium sulphate precipitation A-C-7	1.315	8960	0.49	18285	1.9
10.	Ammonium sulphate precipitation A-C-10	1.305	8860	0.38	23315.7	2.0
11.	Dialysis fractionation A-C-4	1.301	8820	0.31	28451.6	3.2
12.	Dialysis fractionation A-C-7	1.293	8740	0.34	25705.8	2.6
13.	Dialysis fractionation A-C-10	1.314	8950	0.34	26323.5	2.3
14.	DEAE cellulose, IEX-chromatography A-C-4(elute-6)	0.86	4410	0.13	33923	4.0
15.	DEAE cellulose ,IEX-chromatography A-C-7(elute-4)	0.95	5310	0.12	44250	4.6
16.	DEAE cellulose ,IEX-chromatography A-C-10(elute-10)	0.78	3610	0.10	36100	3.1
17.	Sephadex G-75 – C4(18- fraction)	0.87	4710	0.12	39250	4.6
18.	Sephadex G-75 – C7(21- fraction)	0.98	5610	0.11	51000	4.9
19.	Sephadex G-75 – C10(13- fraction)	0.83	4110	0.10	41100	3.3

From the above table 2 it can be inferred that the maximum enzyme activity was shown by the tube Ammonium sulphate precipitation A-C-7 (*Pseudomonas geniculata*). The media A and the *Pseudomonas geniculata* was the best combination for the maximum enzyme activity. The specific activity in Units per mg was highest for Sephadex G-75 – C7 (*Pseudomonas geniculata* C7). The results conclude that the *Pseudomonas geniculata* (C7) out of the three selected cultures was having high potential of enzyme production and has good enzyme activity. Thus the culture can be applied at the commercial scale for enzyme production.

Conclusion:

The current study involved the use of bacteria isolated from the soil near slaughter house in the production of tyrosinase enzymes. From the master plate three cultures were selected for the study and labeled as C4, C7 and C10 and identified as *Rhodococcus ruber* strain C4, *C7-Pseudomonas geniculata* strain C7 and C10 as *Pseudomonas geniculata* strain C10. The cultures were also shown positive test for the tyrosinase enzyme production like melanin formation and pigmentation on gauze media. The cultures were further used for the production of enzyme in two different medias namely A and B that differ in their composition. The enzyme assay study proved that the Media A can be the best source for maximum enzyme activity when compared to media B. In which the media A and the *Pseudomonas geniculata* strain C7 was the best combination for the maximum enzyme activity. The purification of the enzyme was performed using various methodologies and the enzyme produced was evaluated by Lawry's method at each step of purification. The work concludes that the *Pseudomonas geniculata* with the media A is the best combination for the maximum production of the enzyme. The work can further be extended for the optimization of the culture conditions for the maximum production of the enzyme.

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