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Production of *Streptomyces* Melanozymes through SsF & its Antioxidant Attributes

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Abstract: *Streptomyces* tyrosinases are the most thoroughly characterized enzymes of bacterial origin. Being a widest genera among actinomycetes these soil-dwelling mycelial forms with sporulating aerial and substrate type, produce large extracellular enzymes & other important secondary metabolites. Tyrosinase a melanogenic enzyme found to be quite encouraging in bioprospecting attributes in comparison to commercialized mushroom tyrosinase. Solid state bioprocess being more favourable for mycelial organisms than bacteria due to their bulk biomass has achieved an unprecedented improvement in SsF employing agro-industrial residues, leading to the production of value-added 'low volume-high cost' products. In the preset study, myriad tyrosine containing substrates like wheat bran, rice bran, pigeon pea waste, bengal gram husk, black gram husk were employed with available strain of *Streptomyces rubrocyanodiastaticus* UGC13 (NCBI,GENBANK ID 51$$381) giving tyrosinase yield by bengal gram husk (72.5U/ml) being a better substrate followed by corn husk(14.72 U/ml), wheat bran (12.5 U/ml) for the production. The enzyme showed maximum percent scavenging activity employing DPPH & ABTS at100µl (92.53%) and 100µl (99.426%) respectively showing a linear relationship between the concentration of sample antioxidant & percent scavenging activity.

Keywords: SsF, DPPH, ABTS, percent scavenging activity, tyrosinase

1 INTRODUCTION:

Tyrosinase or Tyrosine hydroxylase (EC (1.14.18.1) an oxidoreductase enzyme that is the rate-limiting enzyme for controlling the production of melanin. It is mainly involved in two distinct reactions of melanin synthesis; firstly, the hydroxylation of a monophenol and secondly, the conversion of an o-diphenol to the corresponding o-quinone. o-Quinone undergoes several reactions to eventually form melanin. Being a copper-containing enzyme present in plant and animal tissues it catalyzes the production of melanin and other pigments from tyrosine by oxidation, as in the blackening of a peeled or sliced potato exposed to air. It is found inside melanosomes which are synthesised in the skin melanocytes.The two copper atoms within the active site of tyrosinase enzymes interact with dioxygen to form a highly reactive chemical intermediate that then oxidizes the substrate. The activity of tyrosinase is similar to catechol oxidase, a related class of copperoxidase. Tyrosinases and catechol oxidases are collectively termed polyphenol oxidases Tyrosinase activity is very important. If uncontrolled during melanoma, it results in increased melanin synthesis. Decreasing tyrosinase activity has been targeted for the betterment or prevention of conditions related to the hyperpigmentation of the skin, such as melasma and age spots.(Jeanette schmaler,2008)

Actinomycetes are the organisms with characteristics common to both bacteria and fungi but yet possessing distinctive features to delimit them into a distinct category. Their presence is unanimous. In the strict taxonomic sense, *actinomycetes* are clubbed with bacteria the same class of *Streptomyces* and confined to the order Actinomycetales.They are prokaryotic ,unicellular bacteria, mycelial in njature, non-septate (coenocytic) slender resembling true bacteria. On culture media unlike slimy distinct colonies of true bacteria which grow quickly, *actinomycetes* colonies grow slowly, show powdery consistency and stick firmly to agar surface. (Labeda)They produce hyphae and conidia / sporangia like fungi. Certain actinomycetes whose hyphae undergo segmentation resemble bacteria, both morphologically and physiologically. *Streptomyces*

is the largest genus of *Actinobacteria* and the type genus of the family *Streptomycetaceae.* Over 500 species of *Streptomyces* bacteria have been described.As the other *Actinobacteria, streptomycetes* are Gram-positive, and have genomes with high GC content. Found predominantly in soil and decaying vegetation, most Streptomycetes produce spores, and are noted for their distinct "earthy" odor that results from production of a volatile metabolite, geosmin tyrosine and glutaminase (Lerch et Ettlinger,1972).

Solid State Fermentation(SsF) is a process where microorganism uses a undissolved materials for growth and metabolism.Usually simpler with low energy requirements. Here growth is taken place at absence of free water. It has a higher volumetric productivity.SsF is an economically viable fermentation process specially for filamentous organism and widely used for secondary metabolite production from actinomycetes (Robinson et at, 2001).The streptomyces tyrosinase is a more novel enzyme compared to the mushroom tyrosinase . Presently there is an increasing interest in using tyrosinase in industrial application : environmental technology for the detoxification of Phenols – contaning waste waters ,contaminated soil.(Claus and Filip,1990)

The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. Antioxidants (AOX) are considered a promising therapeutic agents as they may be playing neuroprotective (preventing apoptosis) and neurodegenerative roles (Moosmann and Behl, 2002). Biological systems have developed mechanisms to control ROS levels, including enzymatic (superoxide dismutase, glutathione peroxidase, catalase) and nonenzymatic antioxidant agents (®-tocopherol, ascorbic acid, α-carotene, and glutathione, among others (Johnson and Nasr-Esfahani, 1994). Although, both ABTS and DPPH methods are commonly being used by several researchers, no literature is available on the comparative account. However, according to a report (Payet *et al.,* 2005) brown sugar aqueous solutions exhibited weak free radical scavenging activity in the DPPH assay and higher antioxidant activity in the ABTS assay at relatively high concentration.Report of Neethu et al.(2015) was found to record DPPH scavenging assay (88.20%) by streptomyces tyrosinase .

2 METHODOLOGY:

2.1. Screening of substrates employed in Solid State Bioprocess (SsF) for the production of tyrosinase:

Solid state fermentation was carried out in a 250 ml Erlenmeyer flask containing 25 g substrates like Black gram husk, Corn husk, Wheat bran as sole carbon source and Tyrosine Gelatin broth(TGB) (Gelatin -5g,Beef extract-3g,Tyrosine-5g,Agar-20g,Distilled water-1000ml) as moistening agent. The above mentioned substrates were procured from APMC yard, Tumkur and cleared the debris, other impurities by sieving and dried in hot air oven at 50^0C for overnight. The substrates were sieved to approx 2 cm particle size employing grinder.

2.1.1. Preparation of the substrates:

Solid state fermentation was carried out in a 250 ml Erlenmeyer flask containing 25 g of cleaned dried and sieved substrates as per the standard procedure. The final moisture content of the medium was adjusted to approximately 65% employing TGB broth (pH 8.0-8.5) substituting tyrosine (Lerch and Ettlinger,1972). All substrates were autoclaved and sterilized at 121°C, 15 lb for 15 minutes. The contents were tapped in palm for even distribution of moisture. After sterilization, the cooled flasks were inoculated with previously prepared 1ml spore suspension of five days old standard isolate *Streptomyces rubrocyanodiastaticus* UGC13 (already isolated and screened and confirmed for tyrosinase activity earlier at Dept of Biotechnology, SIT, Tumkur under the guidance of Dr. Mousumi Das, Asst Prof and submitted to NCBI with submission number GENBANK ID 51$$381) . The scraped spore suspension from the Tween 80 slant with spore count equal to $1x10^8$ spores/ml was inoculated in the autoclaved substrate containing flasks & kept for incubation followed by tapping over palm, at 35^0 C for a five days in a humid chamber incubator at 45^0 to get even dissipaiation of heat all over the flasks containing substrates and inoculum. At every 24 hr of incubation period the enzyme assay was carried out with the spent substrates.(

2.1.(2) Enzyme Extraction & Assay :

10ml of sterile distilled water is added to the 5g spent substrate and stirred for 30 minutes in a rotary shaker (KEMI) at 100 rpm. The mixture then centrifuged at 10,000 rpm for 5 minutes (Pandey *et al*., 2005).The supernatant was collected and used as crude extract for tyrosinase assay by following the procedure of Lerch and Ettlinger, (1972) as explained earlier.Tyrosine gelatin broth (pH 8.0-8.5) (100 ml) with 1 ml spore suspension (0.01% tween 80 scraped in 4-5 days old slant) and incubated at stipulated temp. that is 35^0C for 6 days. Every 24 hrs old spent broth (5 ml) undrgone centrifugation at 10.000rpm for 5 min (cooling centrifuge) 4mM L-Dopa for every Assay . Only colourless substrate (L-Dopa) is used for the assay. It gets black when exposed to light

and it cannot be used, it can stay colourless only for 3 to 4 days once prepared. Preparation of 4mM L-Dopa : (0.04mg of L-Dopa in 50ml of Sodium Phosphate buffer-SPB)

Enzyme Assay Mixture contains 0.5ml supernatant + 0.5ml L-Dopa + 0.5ml SPB-sodium phosphate buffer (pH 6.8 or 7.2). If one cannot read with total volume of 1.5ml, one can increase the values to its double (i.e., Supernatant 1ml, L-Dopa 1ml but not buffer, buffer is constant). The mixture was incubated at 35^{0}C for 10 min. Absorbance was read at 475nm and always initial absorbance will be zero. How much one read in the spectrophotometer that will be the final value for the sample.

The activity of the enzyme was calculated by using the formula as under.

$$\text{Activity} = \frac{\text{change in absorbance X volume of assay (1.5ml)}}{\epsilon \text{ X incubation time(30min)X sample volume (0.5ml)}}$$

Molecular coefficients (ϵ) = $3700\ M^{-1}cm^{-1}$.

Antioxidant activity:

In vitro antioxidant activity of purified tyrosinase was studied against ABTS (2, 2'-azino-bis 3-ethylbezothiazoline-6-sulfonic) radicals and ascorbic used as standard for fair comparison with tyrosinase from

ABTS Assay

Preparation of ABTS solution:

0.3840g of ABTS was dissovled in 100ml of distilled water to obtain 7mM concentration and to this 100ml (2.45mM) of potassium persulphate was added .The reaction mixture was left to stand at room temperature overnite in dark before use.

Working solution : 6ml of stock in 55ml of 50% of methanol.

Assay: The different volume of enzyme (10,50,100 µl) and make with 150 µl milliQ water were taken alone reacts with 2850 µl of ABTS(0.7mM). Then kept it for 10 min in incubation. Absorbance was measured at 734nm.

$$\% \text{ Inhibition} = \left(\frac{\text{Mean OD of individual conc. of test sample}}{\text{Mean OD of control group}} \right) \text{X } 100$$

Since in the present study with 11 different concentrations for single test sample based on that there will be plotting of a graph of X-axis indicating the concentration and Y-axis indication % inhibition

Preparation of DPPH reagent:

Stock solution: - 12mg DPPH of dissolved in 50ml of methanol stored at 20'C.

Working solution:-4.5ml of methanol to 1ml stock solution to obtain absorbance value ± 1.1.Firstly it was found 1:3 ratio of stock methanol solution of DPPH and methanol to get absorbance but it was found 1.3137 absorbance.To obtain the 1.1 absorbance at 517nm it was diluted to 1:4.5 to get 1.1 absorbance 10:45 ml of DPPH stock and methanol working solution was used.Then 55 ml of working solution for DPPH assay was prepared

Preparation of Standard Ascorbic acid:

0.014 gm of ascorbic acid in 100 ml of distilled water (0.8 mM)

Preparation of enzyme sample

5 ml of crude spent broth was carefully withdrawn at every 24 h and centrifuged at 10,000 rpm for 5 min. The supernatant thus obtained was used as enzyme sample for tyrosinase assay.

Assay: The different volume of enzyme (10,50,100 µl) were taken alone reacts with DPPH solution and final volume were made up to 3 ml.The mixture was shaken vigorously and allowed stand in dark at room temperature for 30 minutes and decrease in absorbance of resulting solution was then spectrophotometrically measured at 517 nm. Control sample without enzyme containing the same volume of solvent was also analyzed and results were expressed as radical scavenging capacity was calculated using the following equation

Formula:- % SCA=(A control – A sample /A control) ×100

3 RESULTS & DISCUSSION:

Solid State Fermentation of tyrosinase

Most of the biotechnology industries relied on submerged liquid fermentation (SLF)process,where the microorganisms are grown in liquid media, some of the biotransformation reactions can be carried out in a different fermentation process that is known as solid-state fermentation (SsF). SsF proves to be a better option than the SLF (Manpreet *et al.*, 2005). Commercially, tyrosinase has been produced by submerged fermentation technique. Many researchers have reported a quite encouraging production of tyrosinase

by different strains of bacteria and fungi such as in reports of *Streptomyces antibioticus* (12.60 U/ml, Prakasham reddy setty *et al*.,2013), *Aspergillus niger* LMA (20.14U/ml, Ahamed and vemette,2008), *Tricoderma viride* ATCC (28.00 FPU , Montiel-Gonzalez et al.,2002).

Plate-1: Regional agro wastes screened for the synthesis of tyrosinase under solid state bioprocess

In the present study, solid state bioprocess was carried out employing various agro waste substrates like Black gram husk, Corn husk and Wheat bran using tyrosine-gelatin broth medium as moistening solution, and incubated at different period of fermentations.. Plate -1 represents the agrowastes employed in the present study. Plate 2 indicates the inoculated flasks with different agrowastes employed for SsF employing the standard strain.

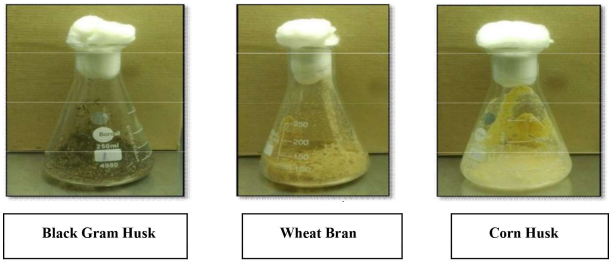

Plate-2: Inoculated flasks employing SsF by *S. rubrocyanodiastaticus* UGC 13

Figure -1 represents the graphical view of production of tyrosinase employing different agrowastes by the standard strain. Bengal gram husk (72.5U/ml) was found to be better substrate followed by corn husk(14.72 U/ml) for the production of tyrosinase by standard strain UGC13 on 2nd day of fermentation. On the other hand wheat bran (12.5 U/ml) was served as better substrates followed by corn husk (14.72 U/ml) for tyrosinase production by UGC13 on 4th day of fermentation. Agro-industrial residues are processed using SSF because it has lower energy requirement, produce lesser waste water and are environment-friendly. In recent years, tyrosinase is also being produced under solid state fermentation (Pandey, 2003), using natural (e.g., brans, husks, oil cakes etc.) and inert solid materials (e.g., polystyrene beads). Although, the activity is reported to be much better under SmF by bacteria than SsF. Fungal tyrosinase production by SsF is much better than SmF. Tyrosinase production in SsF by *Auricularia auricula* (17.22 U/ml, Zou et al., 2012), *Lensinus edodus(*19.67 U/ml*, A. Perani et al.,*1994 *)*.Till date Streptomyces mediated SsF data to produce tyrosinase are not so plenty and still there are lots of avenue of research in this line.Black gram is showing the highest activity because its tyrosine content in 49% where as in corn husk and wheat bran the tyrosine content is 21% . Thus, black gram shows more activity compared to other agrowastes

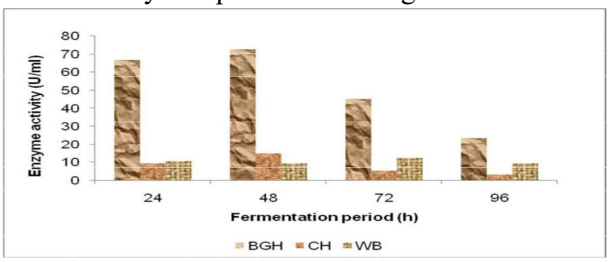

Figure 1 : Production of tyrosinase employing different agro wastes under solid state bioprocess by *S.rubrocyanodiastaticus* UGC 13

Antioxidant Attributes :

Antioxidants are used to reverse the harmful effects of the free radicals by scavenging the free radicals and detoxifying the physiological system. Assays based on the use of 1,1-diphenyl-2-picryl-hydrazyl(DPPH) and 2,22 -azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals are among the most commonly used spectrophotometric methods for determination of the antioxidant capacity of foods, beverages, plant extracts and pure compounds due to the simple, rapid, sensitive and reproducible procedures involved (Celik *et al.,* 2003; Gulcin, 2009).The enzyme showed in this an ABTS scavenging activity with increasing concentration of the tyrosinase.The DPPH and ABTS shows highest activity at 100 µl (92.53%) ,100 µl(99.426%) respectively .

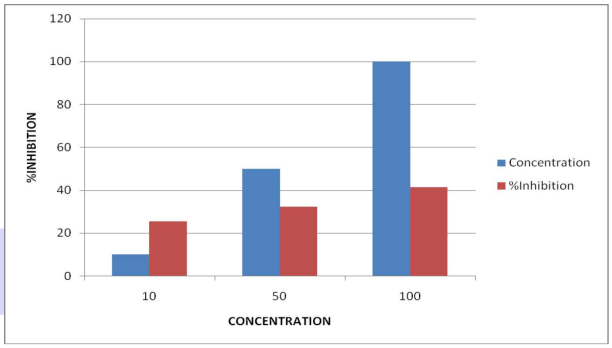

Figure 2 : Antioxidant activity of Ascorbic acid by ABTS method

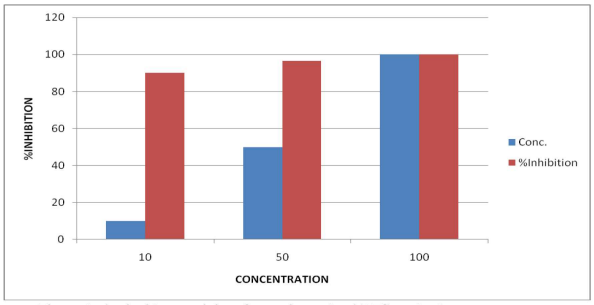

Figure 3: Antioxidant activity of tyrosinase by ABTS method

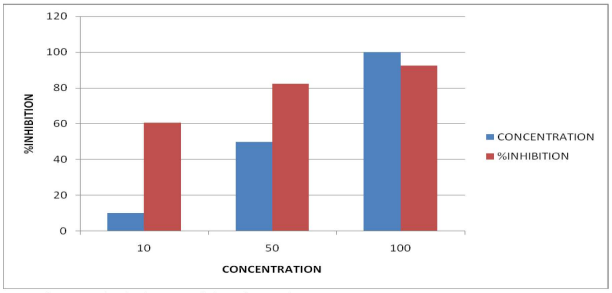

Figure 4: Antioxidant activity of tyrosinase by DPPH method

CONCLUSION :

Enzymes are among the most important products for human needs. Tyrosinase a therapeutically and industrially important enzyme is ubiquitously present in various organisms including microbes. Commercially, the demand for extracelluar microbial tyrosinase is more and its use is also safe as therapeutic agent, especially in treatment of Leukaemia. Actinomycetes are a unique group of microorganism having diverse metabolic potential with a rich receptoire of secondary metabolites or bioactive molecules specially enzymes. In spite of, a wider exploitation of bacteria and fungi for the production of tyrosinase, reports on efficient production of this enzyme from actinomycetes is still not so satisfactory and there is plenty room of research.

In the present study, an attempt was made to study the production of tyrosinase from a standard strain of Streptomyces by developing a suitable bioprocess that is SsF with an activity of tyrosinase 72.5 U/ml employing Black gram husk at 48 hrs of incubation. Pigmented organisms especially melanoid pigmentation bearing Streptomyces species found to be more potential for providing antioxidant attributes due to its maximum extent of trapping free radicals which oxidise reactive oxygen species (Xu *et al*.,2005) as also found in the present study (99.426 % and 92.53% by employing ABTS and DPPH free radicals).

REFERENCE:

Abdallah, A.N., Amer, K.S. and Habeeb, K.M., 2012. Screening of L-tyrosinase produced by actinomycetes isolated from different soils in Egypt. *International Journal of Chem. Tech. Research*., **4(4):**1451-1460.

Agate, A.D. and Bhat, J.V., 1963. A method for the preferential isolation of actinomycetes from soils. Antonie van Leeuwenhoek. *J. Microbiol. Serol*.,**29:** 297-304.

Aidoo, K.E., Hendry, R. and Wood, B.J.B., 1982. Solid-substrate fermentations. *Advances in Applied Microbiology,***28:**201-238.

Andrew, E., CookPaul, Meyers, R.,2003. Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *International Journal of Systematic and Evolutionary Microbiology*, **53:**1907–1915.

Aneja, K.R., 1996. *Practical Microbiology*. p. 435, 202, 416, 417.

J.Xu, S. Chen and Q. Hu, Antioxidant activity of brown pigment and extracts from black sesame seed (Sesamumindicum L.),*Food Chemistry* ,91(2005),7-83

Labeda, P. David,. Actinomycete taxonomy: generic characterization. *J. Ind. Microbiol*., **28(2):** 1986 ,115-121.

Lechevalier, H.A. and Lechevalier, M.P., 1967. Biology of actinomycetes. *Annu. Rev. Microbiol.,***21:** 71-100.

Lerch, Konrad, and Leopold Ettlinger. "Purification and characterization of a tyrosinase from Streptomyces glaucescens." *The FEBS Journal* 31.3 (1972): 427-437.

Montiel-González, Alba Mónica, et al. "Invertase production on solid-state fermentation by Aspergillus niger strains improved by parasexual recombination." *Applied biochemistry and biotechnology* 102.1-6 (2002): 63-70.

Neethu,K.,Satish Kumar ,K.R. and Bhaskar Rao,K.V. 2015.Antioxidant and haemolytic activity of tyrosinase producing marine actinobacteria from salterns.*DerPharmarcia letter*,7.1:172-178

Omenn, Gilbert S., et al. "Overview of the HUPO Plasma Proteome Project: Results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly available database." *Proteomics* 5.13 (2005): 3226-3245.

Robinson T, D.Singh and P.Nigam, Solid-state fermentation:A Promising microbial technology for secondary metabolites production ,*Appl Microbial Biotechnol*,55(2001),284-289.

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