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Analysis of antiproliferative activity of Laccase from Fusarium Species

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ABSTRACT

The production of laccase enzyme from four Ascomycetes species was studied in detail by in silico and in vitro analyses. Initial screening of crude enzyme showed complete oxidation of ABTS and Guaiacol after 7 days of incubation. Alternaria brasicola showed maximum production of enzyme (800U/l) at 30^0 C in 4.5 pH followed by Fusarium oxysporium with 600 U/l at 45^0 C in 5 pH after 15 days of incubation. Enzymes were subjected to analyse anticancer activity. Laccase enzyme isolated from Fusarium oxysporium shows 8% cytotoxicity in 10µg concentration and 64% effect in 200µg concentration in short term in vitro cytotoxicity test.

Keywords: Laccase, Ascomycetes, Guaiacol, ABTS, Fusarium, Antiproliferative.

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INTRODUCTION

Cancer is a class of diseases in which the body cells become abnormal and divide indiscriminately Cancer may also be initiated by carcinogens, tobacco smoke, radiation, chemicals or infectious agents, especially some viruses. Cancers cause annually more than 13% of all human deaths. More than 70% of all cancer deaths occurred in low and middle income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (WHO estimate).

The HEp-2 cell line was established by A. E. Moore, L. Sabachewsky, and H. W. Toolan from tumours that had been produced in irradiated-cortisonized weanling rats after injection with epidermoid carcinoma tissue from the larynx of a 56-year-old male [1]. Hardy cell line, HEp-2 resists temperature, nutritional, and environmental changes without loss of viability it has been used for experimental studies of tumor production in rats, hamsters, mice, embryonated eggs and terminal cancer patient volunteers.HEp-2 cell line has a high proliferation rate and a 23 hours cell
Cycle [2]

The use of fungal enzymes in the diverse fields of biotechnological based industries has been increased in recent years. The search for efficient and green oxidation technologies has increased the interest in the use of enzymes to replace the conventional non biological methods. Among the different existing oxidant enzymes the fungal laccases have been are of great interest since they have low substrate specificity; do not require the addition or synthesis of a low molecular weight cofactor ; more stable and utilize the enzyme in an immobilized state.

Laccases are belonging to the group of oxidases. Laccases (Benzenediol; oxygen oxidoreductase EC 1.10.3.2) also called as a blue copper oxidases or blue copper proteins. They are sometimes refers to as polyphenol oxidases (PPOs). They are extra cellular enzymes. The proteins of laccases are characterized by containing 4 catalytic copper atoms, present in their catalytic sites. Laccases catalyze the oxidation of a variety of phenolic compounds diamines and aromatic amines pigment formation, lignin degradation and detoxification[3].The production of laccase is affected by many typical fermentation factors .More over many aromatic compounds have been widely used to stimulate production of laccase. Laccase activity in fungal culture can be increased by the addition of different aromatic compounds [4].

The use of laccase in pharmaceutical industry is in progress and is growing very fast. Besides their use in industrial applications for biodegradation, laccases are used for organic synthesis of several novel compounds that exhibit beneficial antibiotic properties[5] enhancing anti oxidant capability[6] antiproliferative activity[7] reducing the effect of poison ivy dermatitis, oxidizing iodide to iodine a disinfectant widely used in medical field [8]

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In view of the importance of laccases, the present study aimed to isolate a laccase from four fungal isolates has been taken to analyse their ability to produce laccase enzyme and to check its effect on cancer cell lines.

MATERIALS AND METHODS

Organism and Culture condition

Soil samples were collected at Anamalai Hills Village Coimbatore District Tamil nadu India, a region with moderate rain fall and a temperature of 30^0 - 19^0 C .Using serial dilution technique, fungal cultures were isolated and tested for its production of laccase in B&K agar medium with ABTS.The Production of green colour in and around the fungal colony was considered as a positive reaction resulting from ABTS oxidation. The Production of green colour in and around the fungal colony was considered as a positive reaction resulting from ABTS oxidation. Positive cultures were identified by both morphologically and using sequencing of the DNA and preserved it in slats for further use.

Enzyme Preparation

3 mL mycelial or conidial suspension got by mixing with 10 mL sterile distilled water was inoculated to 50 mL altered Basal medium and were left to grow in a shaking incubator at 150 r.p.m at 30^oC for 12 days. After 4 days of growth period, 0.01 M of 2, 5 Xylidine (Sigma Co.) was added to the culture flask to induce the laccase synthesis. After the incubation period, the contents of the each flask were filtered through Whatmann filter paper No.1 and the filtrate was centrifuged at 10,000 rpm for 10 min at 4^oC. The supernatant thus obtained was treated as the enzyme extract for the study.

Pharmacological properties of the enzyme

Purified enzymes was tested for its anticancer activity in cancer cell line- Hep 2.Cell lines were obtained from Pune and maintained in Dulbeco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units /ml of penicillin, and 100 µg/ml of streptomycin at 37^0 C in a humidified atmosphere with 5% CO_2 and the medium was changed every 2 days. After confluence, the cells were subcultred following trypsinization.

Experimental Design: Cell lines were divided into four Groups. Group I serves as control, Group II Group III and Group IV with varying concentration of the enzyme.

Determination of cell viability by tryphan blue dye exclusion Technique

The monolayer cell culture was trypsinizing and the cell count was adjusted to $1.0X\ 10^5$ cells/ml, each well of the 96 well microtitre plate, 0.1 ml of the cell suspension approximately 10,000 cells was added. When partial monolayer was formed the supernatant was flicked off,

washed the monolayer once and 100 ml of the different drug concentration was added to the cells in microtitre plates. The plates were then incubated at 37°c for 24 hrs in 5% CO_2 atmosphere and microscopic examination was carried out. The medium from all the wells were removed. The trypsin EDTA was added to all the wells (0.1 ml/well) and incubated at 37^0 c for 3 to 5 minutes. Then 1 ml growth medium was added to all the wells. The cells were dispersed and cell suspension was pooled from the wells to eppendorf tubes. The equal amount of tryphan blue dye was added and the cell count was taken using haemocytometer .The percentage of viability was calculated [9].

MTT Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0X10^5$ /cells/ml using medium containing 10% serum. To each well of 96 well microtitre plates 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and enzyme was added in different concentration and incubated for 24 hours and 48 hours to see the cell viability in dose and time dependent manner. To each well add 10 µl of MTT dissolved in PBS at different concentration of 5 mg/ml and incubated for a period of 5 hours at 37^0C .100 µl of DMSO was added and mixed thoroughly to dissolve dark blue crystals. After few min the absorbance was read at 570nm.[10]

Assay of LPO GSH and LDH

To a set of tubes, 1.0 ml of the buffered substrate and 0.1 ml of the media were added and the tubes were incubated at 37 ^{0} C for 15 minutes. After adding 0.2 ml of NAD^+ solution, the incubation was continued for another 15 minutes. The reaction was then arrested by adding 1.0 ml of DNPH reagent and the tubes were incubated for further 15 minutes at 37° C.0.1 ml of media was added to blank tubes after arresting the reaction with DNPH.7.0 ml of 0.4N Sodium hydroxide solution was added and the colour developed was measured at 420 nm in shimadzu spectrometer, suitable aliquots of the standard were also analysed by the spectrophotometer. The enzyme activity was expressed as IU/L. [11]

0.5 ml of cell suspension was mixed with 0.16 ml of 10% SDS and 3 ml of 0.4 %TBA in 10% acetic acid pH 5.The mixture was adjusted to 4.0 ml with distilled water and then heated to 90^0 C for 60 minutes. After cooling the contents to room temperature, 4 ml of butanol was added and the mixture was shaken vigorously. After centrifugation at 1500 rpm for 10 minutes, the organic layer was taken and its absorbance was read at 532nm. [12]

0.5 ml of the cell suspension was precipitated with 55 TCA.The contents were mixed well for complete precipitation of protein and centrifuged. To an aliquot of clear supernatant, were added 2.0 ml of DNEB reagent and 0.2 M- phosphate buffer to a final volume of 4.0 ml. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series

of standards treated in a similar manner were also run to determine glutathione content. Amount of GSH was expressed as n moles of GSH/10 ^{6} Cells. [13]

Extraction of Nucleic acid.

Known amount of the cell suspension was added to 5.0 ml of 5% TCA and kept in Ice for 30 min to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% TCA. Then it was treated with 95% ethanol to remove lipids. The final precipitate was dissolved in 5.0 ml of TCA and kept in a water bath maintained at 90 ^{0} C for 15 min with occasional shaking to facilitate the quantitated separation of nucleic acids from proteins. The supernatant after centrifugation was used for the estimation of DNA.

Estimation of DNA

A known volume of the nucleic acid extract was made up to 3.0 ml with 1N perichloric acid. This was mixed with 2.0 ml of diphenyl amine reagent. Reagents blank and standard were also carried out concurrently. This was kept in boiling water bath for 10 min and the blue colour developed was read at 640 nm in a shimadzu spectrophotometer.[14]

Estimation of RNA.

Aliquots of nucleic acid extracts were made up to 2.0 ml with 5 % TCA. To this 3.0 ml of orcinol ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 min than cooled. The colour developed was measured at 640 nm in a shimadzu spectrophotometer.[15]

Estimation of Protein.

Aliquots of suitably diluted cells were made up with water to 1 ml and 4.5 ml of alkaline copper reagent was added to all the tubes including blanks. Blank containing 1.0 ml water and standard containing aliquots of BSA were also treated similarly. The contents were left to stand for 10 minutes at room temperature. Then 0.5 ml of diluted folin's phenol reagent was added. The blue colour developed was read at 640 nm after 20 min.[16]

RESULTS AND DISCUSSION

Out of 30 fungi isolated from forestry soil samples 4 fungi showed positive reaction when grown in the presence of Guaiacol and ABTS. The edge of the fungal colonies showed the green to blue colour .Cultures were sequenced for its identification using ITSF and ITSR primers and were found that all the four sequences were belonging to Ascomycetes family. Culture C1 as found to be *Aspergillus niger*, C2 Fusarium *oxysporium,* C4 *Alternaria arborescens* and C6 *Penicillium marnefei.* .

As reported by Hu *et al* 2011 [18] so far *A. cylindracea* laccase has highly potent antiproliferative activity against hepatoma Hep G2 cells and breast cancer MCF-7 cells, with an [IC.sub.50] less than 10 [micro]M. activity. Previously no laccases with antiproliferative activity against tumor cells have been reported. Many works in laccase enzymes have been focused on its dye Decolourization property and in textile works. Detailed study on its pharmacological property in cancer cell line is limited.

Enzymes from four cultures isolates were subjected to check their Anticarcinogenic property in Hep2 cell line. Among the four isolates. *Fusarium oxysporium* (c2) shows maximum cytotoxic activity in short term compared to other species.(Result not shown) Hence this culture filtrate was further analysed to its cytostatic effect by estimating the Percentage of inhibition (Graph 1) ,levels of DNA, RNA and Protein. Number of cells in laccase treated groups decreased in dose dependent manner when observed in the light microscopic examination and percentage of inhibition studies. This is further confirmed by the results obtained from the contents of DNA, RNA and protein (Graph 2). Cytotoxic effect of laccase on Hep2 cells was assayed by LPO, MTT assay; LDH assay and GSH assay The Cytotoxic index as observed by the MTT assay (Graph 7) confirmed the Cytotoxic effect of laccase in dose dependent manner in Hep2 cells. LDH release (Graph 5) was increased in dose dependant manner which also confirms the cytotoxic effect. The LPO levels (Graph 4) and GSH (Graph 6) in enzyme treated cells were significantly reduced when compared to control cells.

CONCLUSION

From a four number of fungal strains isolated from different regions of hill area, the fungal isolate Fusarium oxysporium was chosen because it exhibited a large and fast oxidation of ABTS on agar plates and liquid cultivation of the fungus in low nitrogen medium with half strength of sea water, initial pH of 5 showed that, only laccase of the three major ligninolytic enzymes was detected in culture supernatant. Showed a final specific activity of higher laccase activity at 600 IU/l, and it's recommended that the isolate *Fusarium oxysporium* was the most promising one for the laccase enzyme production.

In the present Study we found that *F.oxysporium* markedly reduced the cell viability of Hep2 cancer cells in dose dependant manner. The suppression of cell proliferation is may be due to induction of apoptosis. These results are in consistent with the finding of Oh *et al* 2007 [17] and Hu *et al* 2011.[18]. *C.sphecocephala* 'an s polysaccharide peptide complex (PPC) shows cytotoxicity n HepG2 and SK-N-SH cancer cells. *A. cylindracea* laccase, like lectins and antifungal proteins which are defense or antipathogenic proteins, exhibit antiproliferative activity toward tumor cells and inhibitory activity toward HIV-1 reverse transcriptase.[18]

We still not identified the most active component responsible for the Cytotoxic and cytostatic effects against the cancer cell line, the qualitative chemical analysis indicates that the fungal laccase has anticancer activity.

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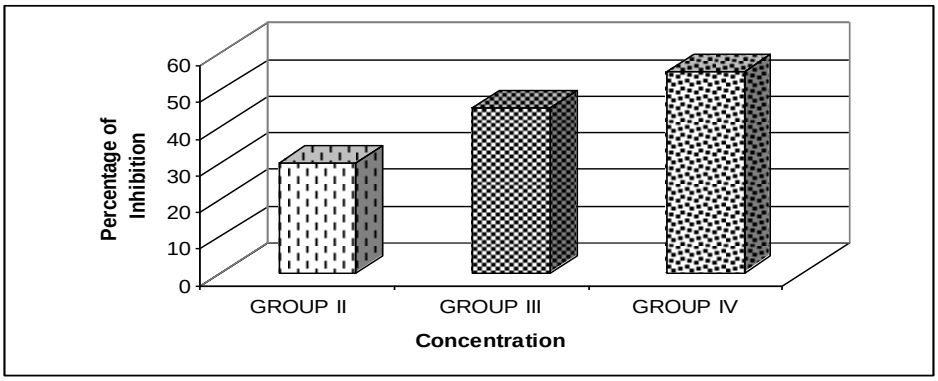
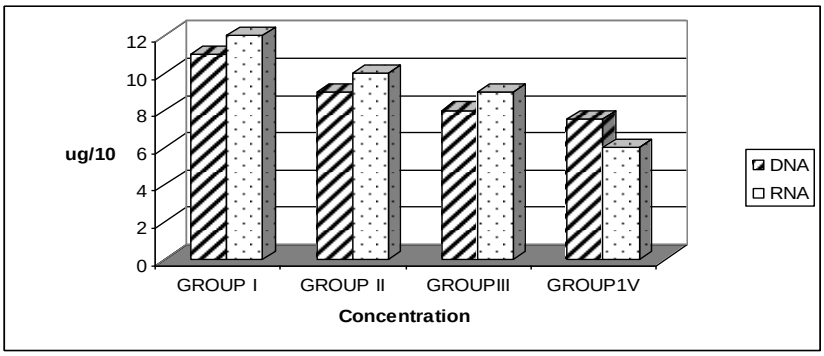
In conclusion, this study the anticancer activity of laccase enzyme which was isolated from *F.oxysporium* species was studied only against one cancer cell line with qualitative chemical analysis. Further work is extended for the possible activity of this enzyme against other cancer cell lines and to design a 3D structure for the isolates. Find out the actual compound responsible for this anticancer property and to design a drug using bioinformatics software.

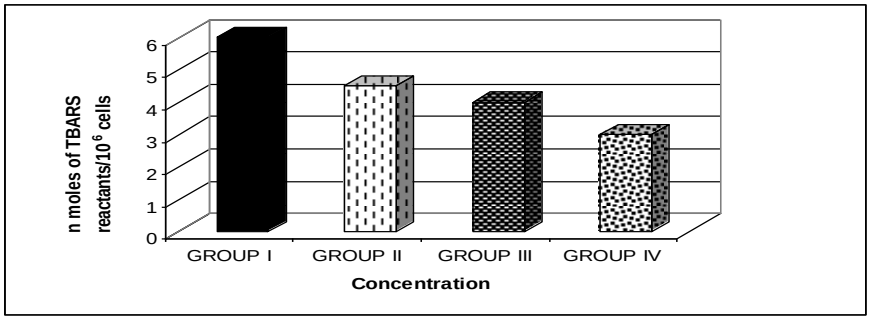
Graph 1: Effect of Laccase on Percentage of inhibition of cell proliferation when compared to control



Graph 2: Effect of Laccase on DNA RNA levels in Hep 2



Graph 3: Effect of Laccase on Protein levels in Hep 2



Graph 4: Effect of Laccase on LPO level in Hep 2 cell line.

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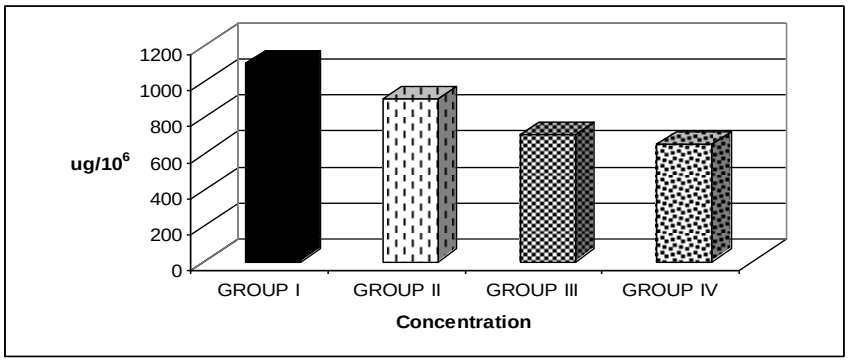
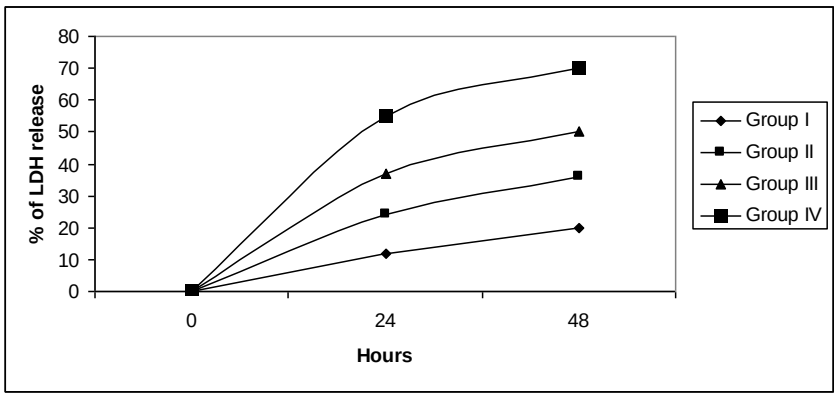
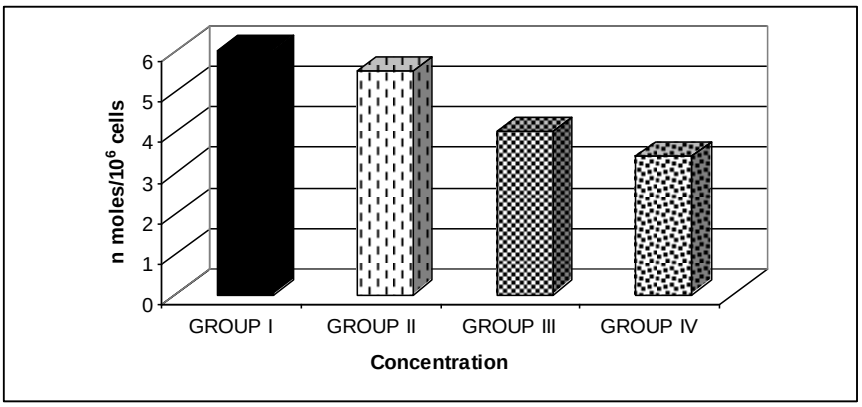


Graph 5: LDH Assay

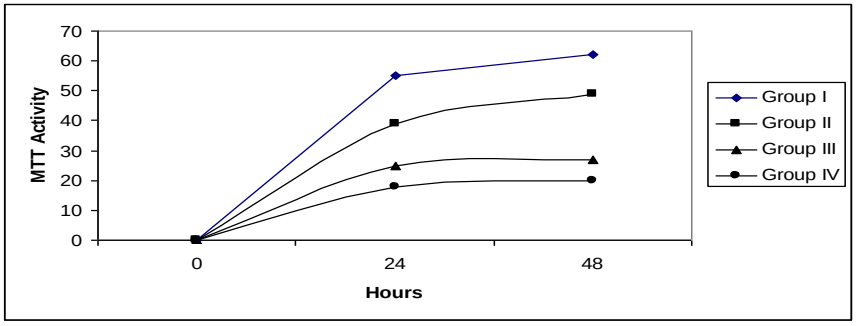


Graph 6: GSH Assay



ISSN: 0975-8585

Graph 7: MTT Activity



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