CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.838155

Available online at: http://www.iajps.com Research Article

OPTIMIZATION OF FERMENTATION CONDITIONS FOR RED PIGMENT PRODUCTION FROM *ASPERGILLUS FLAVUS* UNDER SUBMERGED CULTIVATION AND ANALYSE ITS ANTIOXIDANT PROPERTIES

Gurupavithra. S, Rajalakshmi. A and Jayachitra. A*
Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai-625021.

Abstract:
Aspergillus flavus endophytic fungi was isolated from Ocimum sanctum, produced red pigment. A culture medium composition was established for the production of red pigment, optimum medium composition and environmental conditions were investigated in submerged flask cultures. The optimum carbon, nitrogen sources were determined to be 20g/L of [glucose, sucrose, dextrose] and 5g/L of [ammonium nitrate(NH_4NO_3), yeast, peptone]. The optimal temperatures [25^0C,30^0C 37^0C] and pH [pH-4.5,5,5.5,6.5] for pigment production in submerged fermentation. Under these conditions peak biomass concentration was 10.7gL^{-1} in NH_4NO_3 containing medium in a 15-days culture. The highest volumetric productivity of red pigment was obtained in a batch culture (30^0C, initial pH-6.5) with a defined medium of the following composition (gL^{-1}): dextrose(20) yeast(5). The biomass specific productivity of red pigment was analyzed in extracellular pigment. Extracellular pigment shows maximum absorbance at 750nm. Pigment was characterized with FT-IR shows strong peak at 3421.283nm of phenol group and HPLC resembles 62.978% at the retention time of 9.426. Their antioxidative activities were also estimated by an indication of DPPH, SO, NO and protein content also analyzed. The cytotoxicity of pigment analyzed using Hep-2 cell line and the IC50 value shows 50% inhibition at the concentration of 20µg/ml.

Keywords: ***A.flavus, mycopigment, submerged culture, antioxidant.***

Corresponding Author:
Dr.A.Jayachitra,
Assistant professor,
Department of plant Biotechnology,
School of Biotechnology, Madurai Kamaraj University,
Madurai-625021.
Email: jchitralab@gmail.com

QR code

Please cite this article in press as Gurupavithra. S et al, ***Optimization of Fermentation Conditions for Red Pigment Production from Aspergillus Flavus under Submerged Cultivation and Analyze its Antioxidant Properties*** *, Indo Am. J. P. Sci, 2017; 4(07).*

INTRODUCTION:

Plant endophytic fungi are colonizing inter or intra-cellularly living apparently symptomless fungi which spend the whole or part of their lifecycle inside the healthy tissues of the host plants. These lower eukaryotic fungi are important components of plant micro-ecosystems [1-3]. Endophytes have proven to be rich sources of novel natural compounds with a wide-spectrumof biological activities, biocatalysts in the biotransformation process of natural products and recently several bioactive substances have been isolated from these microorganisms [4,5]. Fungi are attractive organisms for production of useful proteins and biologically active secondary metabolites. Natural pigments have potential uses as colorants in food and cosmetics [6-8] are extracted from plants, animals and microorganisms. Under controlled conditions the pigments produced using microorganisms has advantages over other sources, as microorganisms can grow rapidly. This results in a high productivity of the pigments [9,10]. The alternative for harmful synthetic colorants used in food and pharmaceutical industries are natural pigments. The opened pathways for exploring pigments from microbes are less availability of accessible natural food colorants, serious environment and safety problems caused by synthetic pigments, and the increasing demand of consumers for natural products as compared with synthetic ones [11,12]. There is now strong evidence supporting secondary metabolites that allow organisms, particularly microorganisms, to carve out an ecological niche[13,14].The fungi suitable for spalting work, those that produce extracellular pigment capable of complete wood permeation,are known for having pigments that are UV stable [S C Robinson, D Tudor]. Potent pigment producing microorganisms are fungi [15]. Hamlyn (1995) reported the importance of pigments such as anthraquinone, anthraquinone carboxylic acids, pre-anthraquinones extracted from filamentous fungi. The important alternative to potentially harmful synthetic dyes Natural dyes and pigments [16]. Moreover, the total soluble phenolic content of the extract and fractions was determined, and the quantitative analysis of flavone C-glycosides was obtained by an HPLC analytical method [17]. The structurally similar pigments are produced by *Monascus* (monascorubrine and monascuscorubramine) [18]. Lovastatins or monacolins produced by *Penicillium, Monascus* and *Aspergillus[19]* inhibits cholesterol biosynthesis by binding to catalytic site of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis [20] and scavenged DPPH radicals[21-23]. Penicillenols secreted by *Penicillium sp* showed biological activity against HL-60 cell lines [24] . In general, compounds with antioxidant activity proved to possess anticancer, anticardio vascular, anti-inflammation and many other activities [25]. In this study, we have investigated the endophytic fungal strain *Aspergillus flavus* which was isolated from the leaves of the traditional medicinal plant *Ocimum sanctum.* The vast application of this plant is widely reported in Indian traditional medicine. In this study *Aspergillus sp.*, culture conditions were optimized for red pigment production, pigment was extracted and its biological characters are analysed.

MATERIALS AND METHODS:

Plant material

The *Ocimum sanctum* plant sample was collected from Madurai district and aseptic techniques were followed for further procedures.

Isolation and identification of endophytic fungi

The collected plant samples were surface sterilized and inoculated in potato dextrose agar (PDA) medium for fungal spores. Fungal identification methods were based on Scanning Electron Microscopy (SEM) morphology of fungal spores.

Optimization of Fermentation conditions

In order to optimize the fermentation conditions of *A.flavus* using potato dextrose broth (PDB) medium, different sources were used. The initial pH in the PDA medium. The optimum carbon sources were determined to be 20g/L of [glucose, sucrose, dextrose]and nitrogen sources were 5g/L of [ammonium nitrate(NH_4NO_3), yeast, peptone]. The optimal temperatures are [25^0C, 30^0C, 37^0C] and pH [pH-4.5,5,5.5,6.5]. During time factor study, the flasks were incubated at design temperature in a static phase. All experiments were performed in triplicates to ensure reproducibility.

Extraction of pigment

One-hundred ml fermentation broth was centrifuged at 3000 *g* to remove the mycelium, spore and other non-soluble particles. The red supernatant solution was vacuum concentrated on a rotary vacuum evaporator and extracted with water saturated n-butanol for several times till the lower layer was free of red colour. The organic upper layer was vacuum dried and then mixed with 10 ml chloroform. The dark orange-colour chloroform layer was discarded and the undissolved red residual was dried under nitrogen gas before being dissolved in 5 ml n-butanol **[9].** The n butanol solution was filtered through a 0.45 μm Millipore® filter and then directly injected into the HPLC machine for the determination of red pigment.

Characterization of extracted fungal pigment

UV-Visible Spectroscopy

The extracted pigment were analyzed by using UV-Visible Spectroscopy (Shimadzu UV 1700). The measurements were carried out at a resolution of 1nm.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopic analysis was carried out using a Jasco Fourier Transform Infrared Spectrometer 410. FTIR spectrophotometer was connected to a photo acoustic cell in the spectral range from 4000 to 400 cm-1.

Chromatographic system

HPLC (High Performance Liquid Chromatography) of the methanol extract

HPLC was performed on a Shimadzu model LC 10AD equipped with a detector SPD10A. The methanolic extract (20µL) was subjected to HPLC analysis under the following operating conditions. Column – C18 LiChroCART-merck (4.6 x 30cm), Stationary phase - Octa decyl silane, Mobile phase - Methanol: water (60:40, v/v), Flow rate - 1mL/min, Injection volume-500 ul, Detector – UV, Wave length - 254nm and Flow rate - 1mL/min, Detector – UV.The analysis was monitored at 220nm.

In order to study the effect of pH on elution of birch flavonoids. Individual glycosides were isolated by repeated injection of combined flavonoids glycoside fractions of the polyamide eluate of pigment extract on to the analytical column. The solvents were A (methanol) and B (water). The elution system was 0-30min, 10-30% of B in A. the flow- rate was 2ml/min and the injection volume 20µl. For most of the compounds, the separation was repeated by using methanol as solvent system.

Antioxidant assays

DPPH photometric assay

A 0.4mM concentration methanolic solution of DPPH was added with different concentrations of pigment (1mg/ml) and 0.48ml of methanol, and allowed to stand at room temperature for 30 minutes. Methanol served as the blank **[**32]. After 30 minutes, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows:

% DPPH radical scavenging = 100 x(Control OD-sample OD) / Control OD

Inhibition of *in vitro* superoxide generation

The assay contained with different concentrations of pigment samples (1mg/ml) with 0.2ml EDTA, 0.1 ml nitroblue tetrazolium, 0.05ml riboflavin and 2.64ml phosphate buffer. The control tubes were set up without sample, where DMSO was added. The initial optical densities of the solutions were recorded at 560nm and the tubes were illuminated uniformly with the fluorescent lamp for 30 minutes. A_{560} was measured again and the difference in O.D was taken as the quantum of superoxide production [30]. The percentage inhibitions by the samples were calculated by comparing with the O.D of the control tubes.

% of *in vitro* superoxide generation=1-OD Sample/OD Control x 100

Inhibition of *in vitro* nitric oxide generation

The assay containing 0.3ml of sodium nitroprusside, 2.68ml PBS and different concentrations of pigment samples (1mg/ml) was incubated at 25˚C for 15 minutes. Control tubes (100% generation) were prepared without the pigment extracts. After incubation, 0.5ml of the Griess reagent was added [29]. The absorbance of the chromophore formed, indicative of the quantum of NO generated, were read at 546 nm.

% *in vitro* nitric oxide generation = 100 x(Control OD- sample OD) / Control OD

Determination of hydroxyl radical scavenging

The hydroxyl radical scavenging activity of the pigment extracts was quantified by the method reported by [28].

The assay mixture (0.1ml) contained 0.1ml of deoxyribose, 0.1ml of $FeCl_3$, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 0.1ml of KH_2PO_4-KOH buffer and different concentrations of pigment samples (1mg/ml) in a final volume of 1.0ml. The reaction mixture was incubated for 1 hour at 37˚C. Then 1.0ml of TBA was added and heated in a boiling water bath for 20 minutes. The pink colour produced was measured at 535 nm in a spectrophotometer. Deoxyribose degradation was measured as TBARS and the per cent inhibition was calculated.

% hydroxyl radical scavenging = 100 x(Control OD- sample OD) / Control OD

MTT assay

Culturing of cells

The Hep-2 cells were seeded onto 13-mm glass coverslips in a 24-well plate at a density of 1 * 10 cells per well in 1 ml of complete medium for 24 h. After 24 h, medium was removed and the cells were washed with phosphate-buffered saline followed by viability staining.

***In-vitro* cell viability studies.**

The MTT assay is a simple, nonradioactive colorimetric assay to measure cell viability. Metabolically active cells are able to convert this dye into a water-insoluble dark purple formazan by reductive cleavage of the tetrazolium ring. Formazan crystals, then, can be dissolved in an organic solvent such as dimethyl sulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 545 nm, and the resultant value is related to the number of living cells. To determine cell cytotoxicity/viability, the cells were plated at a density of 1* 10 cells/well in a 96-well plate at 37˚C in 5% CO_2 incubator. After 24 h of culture, the medium in the wells was replaced with the fresh medium containing crude pigment in varying concentrations. After 24 h, 20 µl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4) was added to each well. After 4 h of incubation at 37˚C and 5% CO_2, the medium was removed and formazan crystals were solubilized with 200 µl of DMSO and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader at 545 nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing pigment was calculated by the following formula:

% of cell viability = 100 * (Sample absorbance / Control absorbance)

RESULTS AND DISCUSSION:

Isolation and identification [SEM]

The endophytic fungus was isolated from *Ocimum sanctum.L* medicinal plant. The green coloured spores surrounded with whitish matrix. Based on spore morphology of scanning electron microscope the isolated strain was identified as *Aspergillus flavus***[Figure-1&2]**.

Fig 1: *Aspergillus flavus*

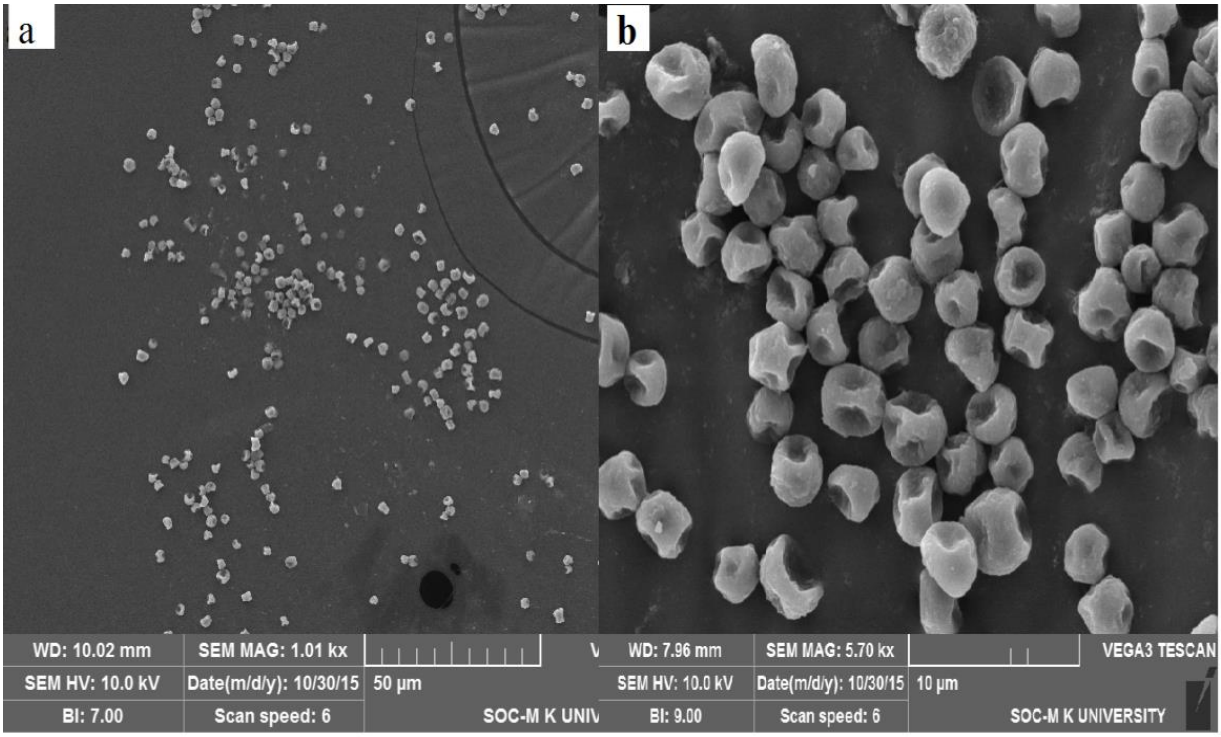


Fig 2: SEM image-*A.flavus* spore
Note:a-magnification X2000-50µm;b-magnification X6000-10µm

Optimization of culture medium

The PDA broth for submerged cultivation of endophytic fungi was optimized with different carbon and nitrogen source, pH and incubation period for pigment production. The carbon source containing medium shows no pigment production. But the medium containing yeast as a nitrogen source with dextrose shows pigment production at 30^0C with the pH of 6.5, the red pigment production was observed with 15 days culture. It has been reported that various nitrogen sources such as yeast [10], peptone supported greater pigment production in many kinds of pigment-producing fungi **[Table-1].**

Table-1: Optimization parameters for pigment production

Carbon source(20g/L)	Nitrogen source(5g/L)	pH	Temperature
glucose	NH_4NO_3,	4.5	25^0 C
sucrose	yeast	5.5	30^0 C
Dextrose	Peptone	6.5	37^0 C

Extraction of red pigment

The red coloured mycopigment was extracted from the optimized medium. The intracellular and extracellular pigments were isolated from the mycelia and fermentation broth using methanol extraction **[Figure-3]**. The pigments are brownish red in color as well as the extracellular pigments are thick in nature but the intracellular pigments are slimy in nature.

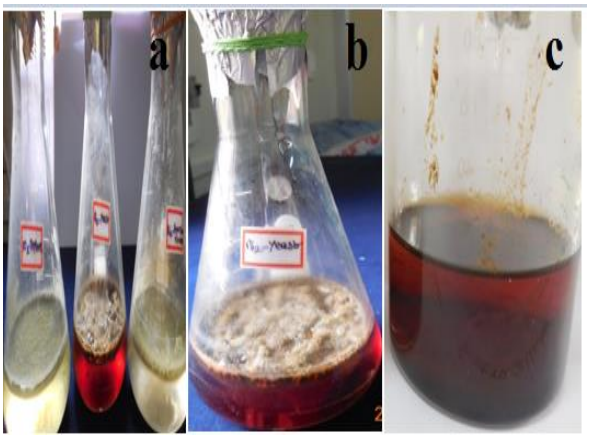


Fig 3: Pigment extraction
Note: a,b-Pigment production; c-Extracted pigment

UV

The extracellular pigment shows maximum absorbance of at 750nm **[Figure-4]**. The UV-visible spectra of pigment extracted from solid-state cultures showed λ max at 482-485nm indicated the production of a red pigment [26].

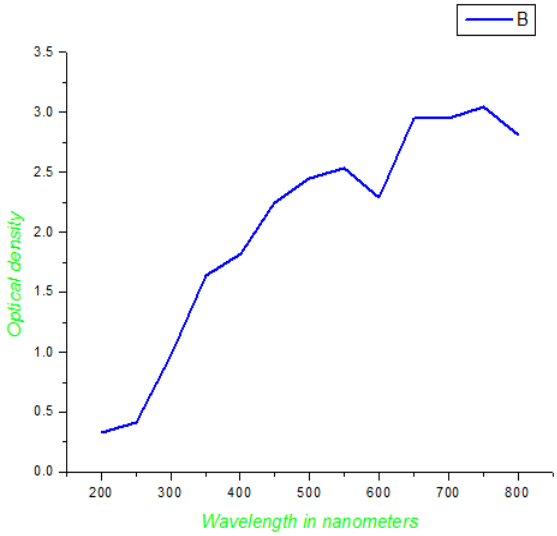


Fig 4: UV spectrum

Fourier Transforms Infra-Red analysis

The spectrum was recorded in the wavelength region between 350 cm^{-1} to 4000 cm^{-1}. The FT-IR spectrum showed single peak at 3421.283 cm^{-1} which indicates the presence of O-H stretching of alcohols and phenols **[Figure-5].** The peak observed at 1627.97 cm^{-1} represents the C=C stretch of olefins. [1]. Further, the peaks at 1388.83 cm^{-1}, 1315.50 cm^{-1}, 11120.68 cm^{-1} correspond to functional groups.

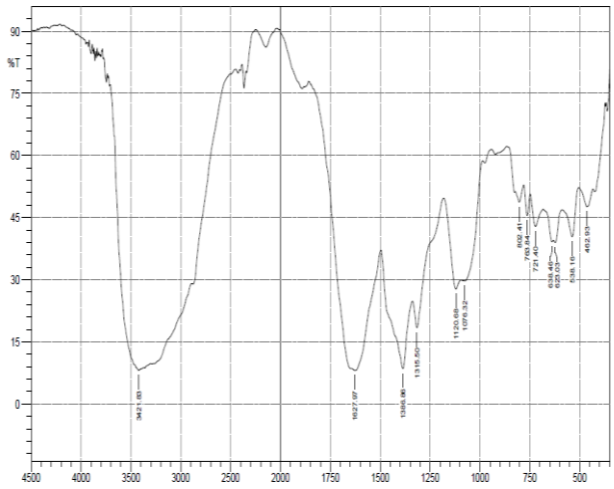
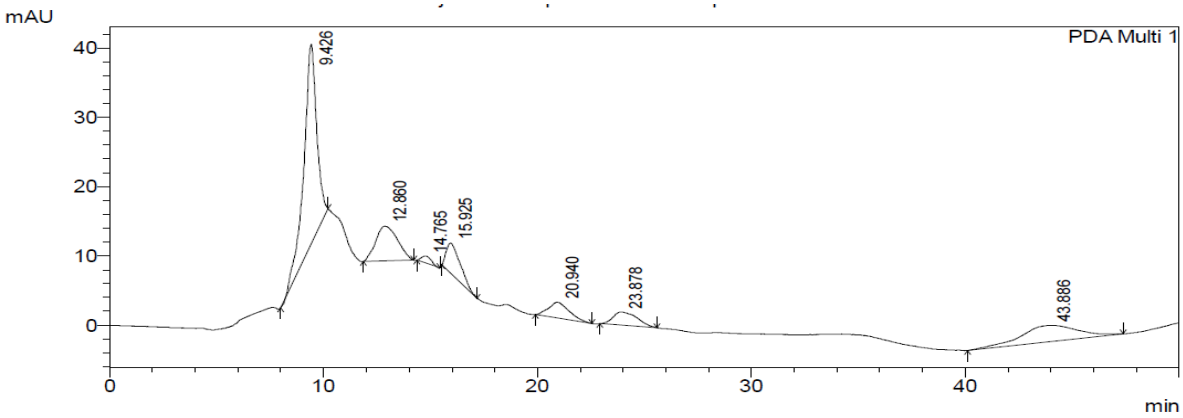


Fig 5: FT-IR spectrum

HPLC

The result of HPLC analysis of extracted pigment has been depicted in the chromatograph shows the presence of 7 peaks. The retention time of major peak was 9.426 min. all other peaks were tiny and appeared insignificant. The percent contributions of the area and height of the major peak were 47.411 and 62.978 respectively **[Figure-6**].

The antioxidant characteristics (DPPH, SO, NO, Hydrogen radical) of the extracted pigment shows good scavenging activity with increasing concentration shows increasing antioxidant activity **[Figure-7,8,9 & 10**].



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	9.426	1201891	28771	47.411	62.978
2	12.860	358375	5034	14.137	11.020
3	14.765	31479	1006	1.242	2.201
4	15.925	200625	4279	7.914	9.366
5	20.940	154367	2293	6.089	5.019
6	23.878	150294	1884	5.929	4.125
7	43.886	438022	2417	17.279	5.291
Total		2535053	45684	100.000	100.000

Fig 6: HPLC Chromatogram

Antioxidant study

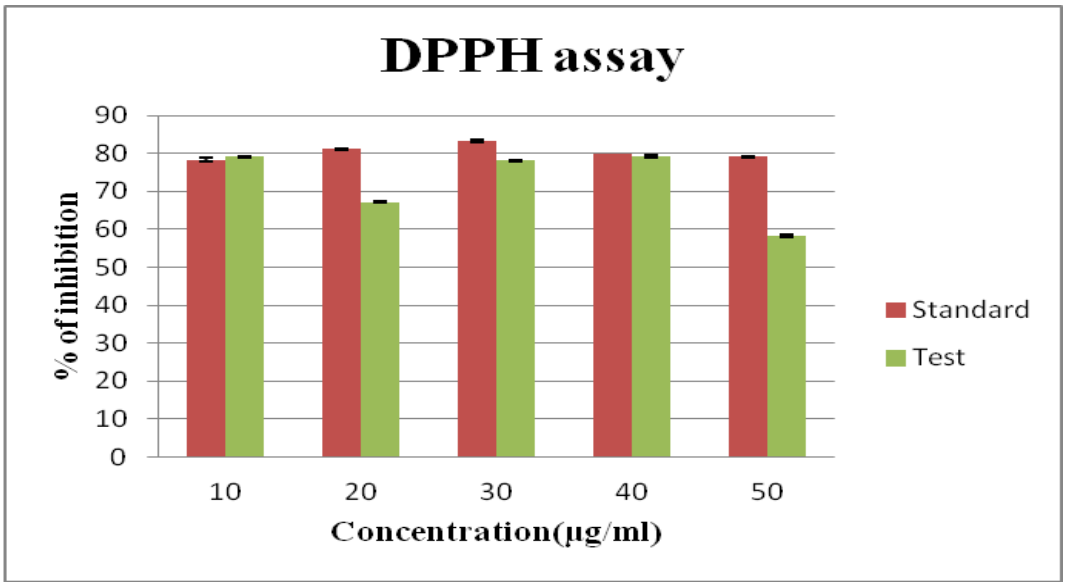


Fig 7: DPPH assay

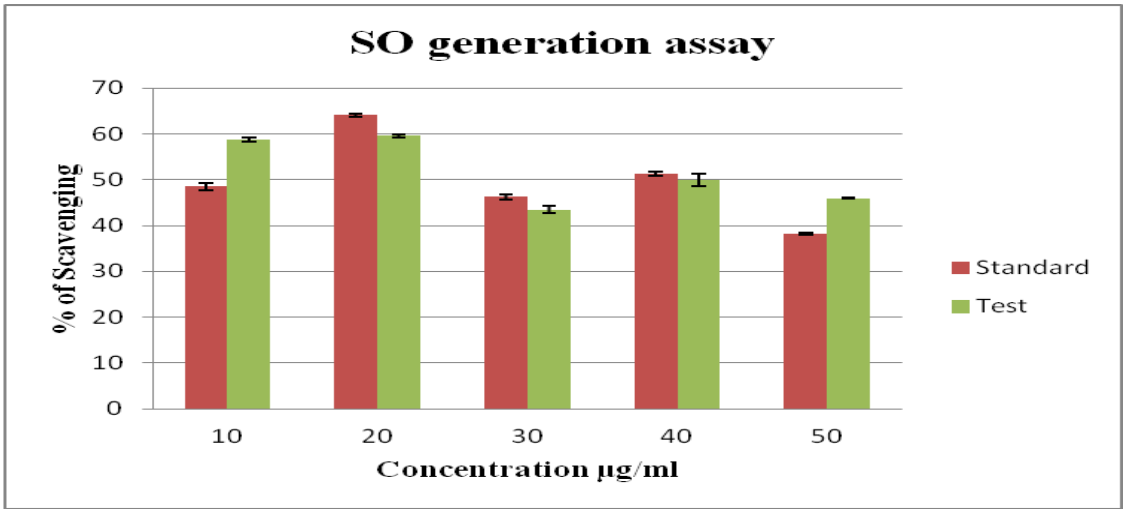


Fig 8: SO generation assay

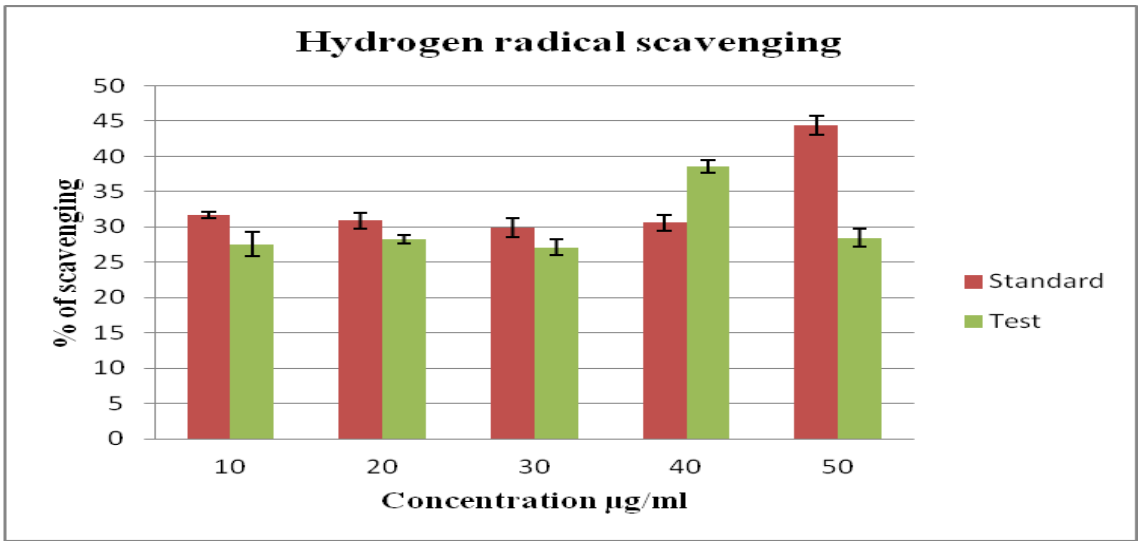


Fig 9: Hydrogen radical scavenging

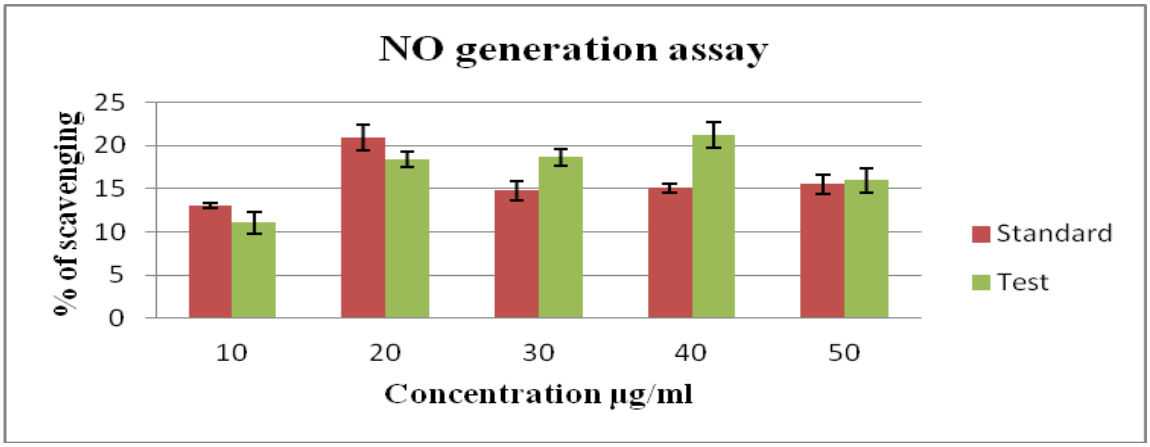


Fig 10: NO generation assay

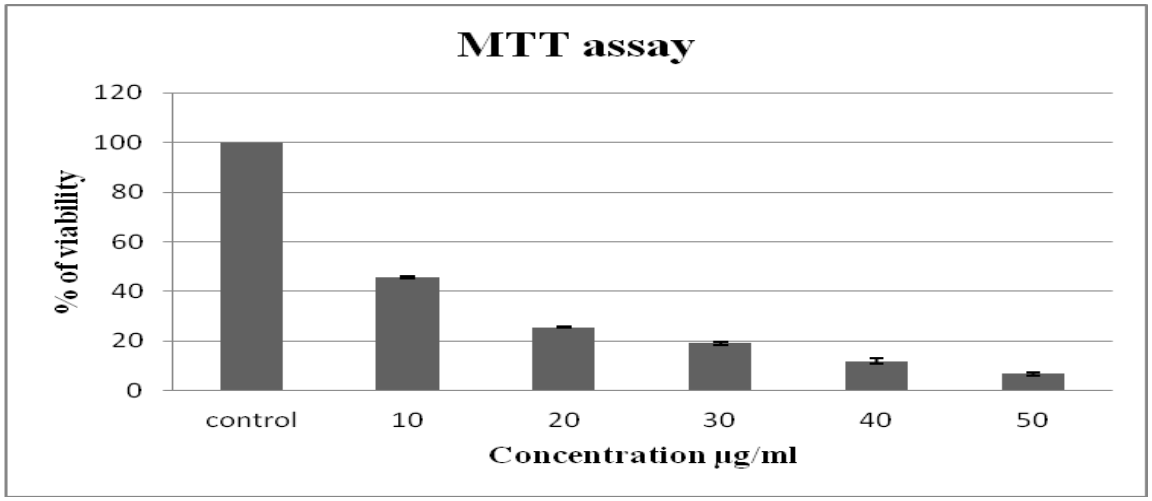


Fig 11: MTT cell viability assay

Cytotoxicity:

The cytotoxicity was analyzed using fungal pigment against Hep-2 cell lines shows effective results. The IC50 value shows 50% inhibition at the concentration of 20µg/ml **[Figure-11**].

DISCUSSION:

In production of the red pigment by submerged batch cultivation (30 ^{0}C) of *Aspergillus flavus*, the pigment production appeared to be growth associated. A carbon-nitrogen mole ratio of 9:1 maximized both the biomass growth and pigment development. An initial glucose concentration of 10 g L-1 promoted growth and pigment production. Yeast was the best nitrogen source. The optimal medium formulation for production of the red pigment contained the following components (g L-1): glucose (10), yeast (5). This medium afforded a peak red pigment productivity of 3.042 at 750nm AU L-1 h-1. The UV-visible spectra of pigment extracted from solid-state cultures showed λ max at 482-485 nm indicated the production of a red pigment [33]. Consequently, the optimal temperature for both mycelia growth and pigment production was found to be 30°C. The fungi usually require long periods for submerged culture, exposing them to contamination risk; which was similar to anthroquinone production by *P.oxalicum* [27].

This can be acceptable by the IR spectrum showing broad stretching at 3379 cm-1 for hydroxyl group of phenolics. In the IR spectrum stretching frequency were also observed at 1643 and 1414 cm-1 assignable to C=C and C-H of the aromatic ring respectively [31]. The extracts of *P. terrestre* tested by [26] exhibited cytotoxic effects (HL-60, MOLT-4, BEL-7402, and A-549 cell lines).

ACKNOWLEDGEMENT

This work was supported with Madurai Kamaraj University, Madurai-21. UGC-NON-NET fellowship for funding support and DST-PURSE for Instrumentation facility.

REFERENCES:

1.Tan RX, Zhou WX. Endophytes: a rich source of functional metabolites. Natural Product Reports.2001;18: 448-459.

2. Zhang HW, Song YC, Tan RX. Biology and chemistry of endophytes. Natural Product Reports.2006; 23: 753-771.

3.Rodriguez RJ, White JF, Arnold AE, Redman RS. Fungal endophytes: diversity and functional roles. New Phytologist.2009;182: 314-330.

4.Pimentel, Mariana Recco, et al, 2011 (2010). The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnology research international.

5.Nuclear, Paulwatt, et al. Butenolide and furandione from an endophytic Aspergillus terreus. Chemical and Pharmaceutical Bulletin. 2010; 58(9): 1221-1223.

6. Dufosse L, Fouillaud M, Caro Y, Mapari SAS, Sutthiwong N. Filamentous fungi are large-scale producers of pigments and colorants for the food industry. Curr Opin Biotechnol. 2014;26:56–61.

7. Furlow B. Cola colourant carcinogenicity claims. Lancet Oncol 13:e146. Kim C-H, Kim S-W, Hong S-

I, 1999. An integrated fermentation– separation process for the production of red pigment by Serratia sp. KH-95. Process Biochem. 2012;35:485–490.
8. Mapari SAS, Hansen ME, Meyer AS, Thrane U. Computerizedscreening for novel producers of Monascus-like food pigments inPenicillium species. J Agric Food Chem. 2008; 56:9981–9989.
9. Carvalho JCD, Oishi BO, Woiciechowski AL, Pandey A, Babitha S, Soccol CR. Effect of substrates on the production of Monascusbiopigments by solid-state fermentation and pigment extraction using different solvents. Indian J Biotechnol. 2007;6:194–199.
10. Cho YJ, Park JP, Hwang HJ, Kim SW, Choi JW, Yun JW, 2002. Production of red pigment by submerged culture of Paecilomyces sinclairii. Lett. Appl. Microbiol. 35(3): 195-202.
11. Mapari SAS, Nielsen KF, Larsen TO, Frisvad JC, Meyer AS, Thrane U. Exploring fungal biodiversity for the production of watersolublepigments as potential natural food colorants. CurrOpinBiotechnol. 2005;16:231–238.
12.Méndez A, Pérez C, Montañéz JC, Martínez G, Aguilar CN, 2011. Redpigment production by Penicilliumpurpurogenum GH2 is influencedby pH and temperature. J Zhejiang Univ-Ssci B. 2011;12:961–968.
13. Gloer J, 2007. Applications of fungal ecology in the search for new bioactive natural products. Environmental and Microbial Relationships, 2nd Edition. 257-283.
14. Keller NP, Turner G, Bennett J. Fungal secondary metabolism - from biochemistry to genomics. Nature Reviews in Microbiology.2005; 3: 937-947.
15. Babitha, S., Soccol, C. R., &Pandey, A, 2007. Effect of stress on growth, pigmentproduction and morphology of Monascus sp. in solid cultures. Journal of BasicnMicrobiology. 47:118–126.
16. Sivakumar, V., Lakshmi, A. J., Vijayeeswaree, J., &Swaminathan, G.Ultrasound assisted enhancement in natural dye extraction from beetroot forindustrial applications and natural dyeing of leather. UltrasonicsSonochemistry.2009;16: 782–789.
17. Aquino R., Morelli S., Lauro M. R., Abdo S., Saija A., and Tomaino A. Phenolic constituents and an- magtioxidant activity of an extract of Anthurium versi- color leaves. J. Nat. Prod. 2001;**64**: 1019D1023.
18. Ogihara, J. &Oishi, K. Effect of ammonium nitrate on the production of PP-V and monascorubrin homologues by Penicilliumsp. AZ. Journal of Bioscience and Bioengineering.2002;**93**:54-59.
19. Endo, A., Kuroda, M. Tsujita, Y. ML-236A, ML-236B, ML-236C, new inhibitors of cholestrogenisis produced by Penicilliumcitrinium. Journal of Antibiotics (Tokyo).1979;**29**:1346-1348.
20.Albert, A.W, 1990. Lovastatin and Simvastatin-inhibitors of HMG-CoA reductase and cholesterol biosynthesis. Cardiology. **77**:14-21.
21. Aniya, Y., Yokomakura, T., Yonamine, M., Shimada, K., Nagamine, T., Shimabukuro, M. &Gibo H, 1999. Screening of antioxidant action of various molds and protection of Monascusanka against experimentally induced liver injuries of rats. General of Pharmacology1999;, **32**:225-231.
22.Dhale, M.A., Divakar, S., Umesh-Kumar, S. &Vijayalakshmi, G. Isolation and characterization of dihydromonacolin-MV from Monascus purpureusfor antioxidant properties. Applied Microbiology and Biotechnology.2007;**73**:1197-1202.
23.Dhale, M.A., Divakar, S., Umesh-Kumar, S. &Vijayalakshmi, G. Characterization of dehydromonacolin-MV2 from Monascus purpureus mutant. Journal of Applied Microbiology.2007b; **103**:2168-2173.
24. Lin, Z.J., Lu, Z.Y., Zhu, T.J., Fang, Y.C., Gu, Q.Q. & Zhu, W.M. Penicillenols from Penicilliumsp. GQ-7, an endophytic fungus associated with Aegicerasorniculatum. Chemical and Pharmaceutical Bulletin (Tokyo). 2008;**56**:217-222.
25. Reis, M., Lobato, B., Lameira, J., Santos, A.S. and Alves, C.N. A theoretical study of phenolic compounds with antioxidant properties. European Journal of Medicinal Chemistry. 2007;42:440-446.
26.Chen, L., Fang, Y., Zhu, T., Gu, Q. & Zhu, W, 2008. Gentisyl alcohol derivatives from the marinederived fungus Penicillium terrestre. Journal of Natural Products. **71**: 66-70.
27.Sardaryan E, Zihlova H, Strnad R, Cermakova Z, 2004. Arpink Red – Meet a New Natural Red Food Colorant of MicrobialOrigin. In: Pigments in Food, More than Colours…, L.Dufossé (Ed.), Université de Bretagne Occidentale Publ.,Quimper, France pp. 207-208.
28.Elizabeth K, Rao MWA, Oxygen radical scavenging activity of Curcumin, Int. J. Pharmaceu., Vol. 58, p.237-240, 1990.

29.Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and (15N) nitrate in biological fluid. Anal Biochem, 1982; 126: 131-138.
30.Winterbourn CC, Hawkins RW, Brain M, Carell RW. The estimation of red cell superoxide dismutase activity. J Lab Clin Med, 1975; 85: 337-341.
31.Parekh J, Jadeja D, Chanda S (2005). Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. Turk. J. Biol. 29: 203-210.
32.Calvo, A. M., R. A. Wilson, J. W. Bok, and N. P. Keller. 2002. Relationship between secondary metabolism and fungal development. Microbiology and Molecular Biology Reviews 66 (3):447-+
33.Rasheva, T.V., Nedeva, T.S., Hallet, N. & Kujumdzieva, A. (2003). Chareterization of a non-pigment producing Monascus purpureus mutant strain. Antonie Van Leeuwenhoek, 83, 333-340.

Abbreviations
AU-Absorbance unit
PDB-Potato dextrose broth
Xmax-Maximum biomass concentration (g L^{-1})