JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

GROWTH AND MASS SPECTROMETRY PROFILE OF *ALTERNARIA ALTERNATA* PIGMENT GROWN IN MAIZE GRAIN EXTRACT

*Sagarika Devi**[1], Hemanth AK. Kumar*[2], Geetha Ramachandran*[2], Chandrasekarenthiran Subramanian*[1], Perumal Karuppan*[1]

Address(es): Sagarika Devi
[1]Shri AMM Murugappa Chettiar Research Centre, Taramani, Chennai-600113, India, Tel: +9144- 22430937, +919677225681.
[2]Department of Biochemistry & Clinical pharmacology, National Institute for Research in Tuberculosis, No.1, Sathiyamoorthy Road, Chetput, Chennai – 600031, India. Tel: +9144- 28369657, +919444076305.

*Corresponding author: sagarikadevi@mcrc.murugappa.org

doi: 10.15414/jmbfs.2014.4.2.179-184

ARTICLE INFO

Received 18. 6. 2014
Revised 12. 9. 2014
Accepted 22. 9. 2014
Published 1. 10. 2014

Regular article

OPEN ACCESS

ABSTRACT

Alternaria species are common saprophytes found in a variety of habitats as ubiquitous agents of decay. *Alternaria* spp. produces about sixty different secondary metabolites. In the present investigation, growth and production of pigment from *Alternaria alternata* was studied in maize grain extract at pH 4-9. The reddish brown pigment was extracted, estimated and partially purified by fractionation. Through mass spectrometry, major constituents of pigment from *Alternaria alternata* such as Tenuazoic acid (m/z 198), Stemphyperylenol (m/z 253), Alterperylenol (m/z 351), Alternariol (m/z 259.200), Altenuene (m/z 292), Alternarienoic acid (m/z 279.35) and Alternariol 5 methyl ether (m/z 273.20) were identified. The bio-prospecting of these secondary metabolites in industrial applications is also discussed.

Keywords: *Alternaria alternata*, fungal pigment, mass spectrometry, secondary metabolites

INTRODUCTION

Micro-organisms are potential biocatalysts in the biotransformation process of natural products and are sources of several novel bioactive substances **(Pimentel *et al.*, 2011)**. Similar to plants, there is a long history of the utilisation of fungi by mankind as remedies and in everyday life. Nearly 3000 years ago the Mayans used fungi to treat intestinal ailments **(Strobel *et al.*, 2004).** Without deeper knowledge about the mode of action, the transformation by fungi, they have been used for food production since Neolithic times. It has been well known that apart from a variety of plants and animals, microorganisms also produce pigments **(Lauro, 1991; Masahiro *et al.*, 1994; Kim *et al.*, 1995; 1998a, b).** The pigments produced by many fungi have been used as a natural food colourant for fish, bean curd and wine. The natural pigments produced by *Monascus spp.* have been looked upon with favour as safe natural colour and a replacement for synthetic pigments.In 1974, the American Miriam C. Rice published the first book concerning dyeing with fungi and since then, the custom has spread all over the world **(Aittomaki *et al.*, 2000).** An orange pigment from the fungi *Ganoderma applanatum*, *Coriolus versicolor* and *Amanita muscaria* was extracted from the basidiocarp and applied on silk and cotton fabrics **(Perumal *et al.*, 2004).** In this context, it is of great relevance that microbes are producers of large number of bioactive compounds **(Zhang *et al.*, 2006)** that have evidence of diverse application in agriculture, medicine and other industries **(Strobel, 2002)**. Therefore the understanding and detailed study on the production of these fungal pigments should thus be encouraged to provide ample solutions to the increasing global concerns of a better and safe environment. *Alternaria* spp. are widely distributed in soil and on aerial plant surfaces **(Ozcelik and Ozcelik, 1990)**. In addition to causing damage to vegetables, fruits and grains, *Alternaria* sp. produces several secondary metabolites. Metabolites of *Alternaria alternata* (Fries) Keissler (*A. tenuis* Auct.) were first isolated and characterized from 4 to 5 week cultures grown on Czapek-Dox liquid medium **(Raistrick *et al.*, 1953)**. The growth and production of secondary metabolites from micro organisms is predominantly dependent on the composition of growth medium and culture condition **(Bills, 1995)**. This makes the study of various types of growth medium and condition imperative to investigate and generate a broad range of secondary metabolite production in microbes **(Larsen *et al.*, 2005)**. *Alternaria sp*. grows over a wide range of pH, temperature and simple sugars. However the combined influence of these factors on biomass and pigment production has not been widely studied. In this study, we utilized cost effective maize grain extract (MGE) as culture medium to estimate the mycelial growth and production of pigment from *A.alternata* under the influence of pH 4-9. Also we documented the secondary metabolite constituents in the reddish brown pigment of *A.alternata* through mass spectrometry (LCMS).

MATERIAL AND METHODS

General

For MS analysis, SHIMADZU LCMS 2010 consisting of gradient Ultra-Fast Liquid Chromatography (UFLC 20AD) pumps, SIL-HTc auto sampler, photo diode array detector SPD-M20A, coupled to a 2010EV mass spectrometer with ESI probe was used. LC Column dimensions: ODS C18 column (Phenomenex, 50 mm x 5µm x 2.1 mm ID). Column chromatography was carried out in a glass column (32 cm × 1.5 cm) packed with silica gel (240-400 mesh). All UV-Visible spectra data of collected fractions were recorded using Varian Cary 50 spectrophotometer at scan mode of 200 - 800 nm.

Alternaria culture conditions

A. alternata, MTCC 2724 strain was obtained from Microbial Type Culture collection and Gene Bank (MTCC), IMTECH, Chandigarh, India. The culture was revived on sterile Potato Dextrose Agar (HIMEDIA, India) medium, incubated at 28°C and the 21st day mycelial culture served as inoculum for biomass and pigment production studies. Erlenmeyer flasks containing 50 mL maize grain broth (40g maize flour dissolved per litre deionized water) was taken individually as substrate and the pH value was adjusted to 4, 5, 6, 7, 8 and 9 using 1M HCl or 1M NaOH. The flasks containing medium were autoclaved at 121°C for 15 min. The flasks were inoculated with 6 mm mycelial culture discs of the 21 day old *A. alternata* and incubated at 28°C ± 2°C as stationary cultures. At every three days interval the flasks containing *A. alternata* were withdrawn and the mycelial mat was collected by filtration using filter paper. The harvested mycelial mat was air dried at 40 °C for 24hrs. The obtained dry mycelia were extracted and estimated for pigment content following the method as described by Gadd (1982). Standard Melanin pigment obtained from Sigma Aldrich served as standard for quantification of pigment. All experiments were maintained in triplicates and the data was analyzed statistically.

Pigment estimation and purification

Dry mycelium (2g) was pre weighed, soaked in 10mL of Methanol and well ground using a mortar and pestle. The mixture was allowed to stand overnight and then taken for extraction in soxhlet apparatus with 110 mL methanol. The extraction was continued for 10 hrs with 20 intermittent cycles lasting 30 min each to obtain a deep reddish brown pigment **(Chandrasekarenthiran, 2010 and Sumathi, 2008).** The whole vacuum dried crude pigment extract was introduced in column chromatography setup with solvent system of n-hexane (100%), Chloroform: Methanol (C: M) at 9:1, 8:2, 7:3, 5:5, 4:6, 3:7, 2:8, 1:9 followed by Methanol (100%) and Water (100%). The flow rate was maintained at 1mL/min, and 2mL of each fraction was collected in separate vials. The eluted fractions were determined at their respective absorbance maxima (λ max) in UV-Visible Spectrophotometer. Three coloured fractions namely 4, 13 and 16 were then submitted for LC-MS analysis.

LC-MS Analysis

The mobile phase A consisted of 0.1% Formic acid (v/v) in water (H2O), and the mobile phase B consisted of Methanol (MeOH) and 0.1% Formic acid (v/v). The following linear gradient used was; Solvent B - 0.01 min-10%; Solvent B - 15 min -100%; Solvent B - 25 min-100%. The samples were dissolved with 100% MeOH and 5 µl sample was injected into the UFLC system and data were acquired and processed in Shimadzu LabSolution V3. The processed mass spectra were matched with compounds reported in previous literatures and searched through Pubchem compound database and NIST similarity search.

NMR Mixture analysis of eluted fractions

Mixture analysis of the eluted fractions was carried out using proton NMR (500 MHz Bruker Avance III NMR) to confirm the presence of mass spectrometric based identified compounds viz., Altenuene, Alternarienoic acid, AlternariolMonoethyl Ether, Alternariol, Alterperylenol, Stemphyperylenol, Tenuazoic Acid. The eluted fractions 4, 13 and 16 were redissolved in deuterated chloroform and analysed for ppm shifts. The recorded ppm shifts were matched with existing literature of fungal pigments and the components were identified using TopSpin software.

RESULTS AND DISCUSSION

We observed the maximum mycelial growth (7.59 ± 0.02 g/L) at pH 5 on 9th day of culture grown in Maize grain broth without supplementation of any other additional carbon or nitrogen sources. However, maximum pigment production of 14.3 ± 0.86 mg/g dry mycelium was recorded in MGE at pH 6 on 9th day. Acidic pH supported better growth while at pH 7 and with increase in alkalinity there was a decline in the fresh and dry mycelial growth.Low pH (4- 4.5) is reported ideal for the growth of *A. alternata*, while the minimum growth was observed in pH 9, irrespective of isolates **(Ramjegathesh and Ebenezar, 2012).**The pigment production also declined after 15 days of mycelial growth. No mycelial growth and pigment was recorded at pH 9.The dry mycelial growth and production of pigment from *A. alternata* grown on MGE at various pH is furnished in Table 1. Natural raw materials and by-products of industry (sugar cane molasses, corn steep liquor, cheese whey) have wide use as culture media in fermentation processes because of their low cost since the medium components can represent from 38 to 73% of the total production cost. Several studies have also been reported about the influence of culture media on metabolite production of *Alternaria* **(Misaghi *et al.*, 1977)**. The interacting effects of time, temperature, pH and simple sugars on biomass and mycotoxin production is reported in three *Alternaria* sp. where 25°C at pH 8 is reported suitable for biomass production with enhanced toxin production at 15 °C **(Ozcelik and Ozcelik, 1990)**. *Alternaria citri* and *A. tenuis* grew over a temperature range of 15 to 35°C, and were able to grow at a pH range of 2.7 to 8.0, the optimum being at 5.4 **(Hasija, 1970)**. Maximum pigment production at 28°C at a standard time period of three weeks was also studied **(Sharma *et al.*, 2012)**. The maximum production of pigments was obtained when cultures were grown on potato dextrose agar (PDA) with 20% dextrose, an initial pH of 4.5 at 25–30°C under continuous darkness or diurnal light, and without wrapping the culture plates **(Shabana *et al.*, 2001)**.

The crude pigment extract (0.310 g) was purified using silica column chromatography. A total of 28 fractions were fractionated. The fraction no. 1& 2 eluted with n-hexane and fraction 3 eluted with Chloroform- Methanol at 9:1 were observed to be colourless at the retention time of 9.5 min. At the same solvent gradient, a red fraction (4th fraction) eluted using Chloroform and Methanol partition of 9:1 was observed to have maximum absorption at the wavelength of 205, 207, 364 and 371nm at the retention time of 13.5. At 23.50 minutes, a brown fraction (8th fraction) eluted using Chloroform and Methanol partition of 8:2 was observed to have maximum absorption at 203 and 208 nm. Similarly a violet fraction (Fraction 13 at retention time of 53.0 min) and a brown fraction (Fraction 16 at retention time of 74.00) were collected using partition of Chloroform and Methanol at 7:3 and 5:5 ratios having maximum absorption at 209, 217, 220 nm and 203nm respectively. The UV-Visible spectra of fragments eluted from each solvent have been summarized in Figure 1.

Table 1 Influence of pH on mycelial growth (g/litre medium) and pigment (mg/gram biomass) of *Alternaria alternata* in Maize Grain Extract medium (MGE)

	Days of Culture	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Dry Mycelial growth (g/L)	3	0.19 ± 0.01	1.24 ±0.04	0.54 ± 0.02	0.36 ± 0.01	0.34 ± 0.01	0 ± 0
	6	1.32 ± 0.02	3.78 ± 0.01	3.30 ± 0.03	1.70 ± 0.04	2.06 ± 0.03	0 ± 0
	9	1.62 ± 0.01	7.59 ± 0.02	2.61 ± 0.04	2.04± 0.02	2.14 ± 0.02	0 ± 0
	12	1.86 ± 0.06	3.29 ± 0.01	2.20 ± 0.01	2.01 ± 0.01	2.66 ± 0.04	0 ± 0
	15	1.13 ± 0.01	2.46 ± 0.02	1.82 ± 0.00	1.56 ± 0.02	1.98 ± 0.02	0 ± 0
	18	0.65 ± 0.01	2.29 ± 0.04	1.31 ± 0.01	1.33 ± 0.00	1.17 ± 0.02	0 ± 0
	21	0.45 ± 0.01	1.10 ± 0.03	0.77 ± 0.04	0.41 ± 0.00	0.82 ± 0.02	0 ± 0
Crude pigment(mg/g)	3	1.26 ± 0.06	5.26 ± 0.06	0.60 ± 0	0.80 ± 0	2.53 ± 0.06	0 ± 0
	6	1.66 ± 0.06	6.46 ± 0.06	1.66 ± 0.06	4.20 ± 0.23	4.60 ± 0	0 ± 0
	9	12.40 ± 0.11	7.33 ± 0.13	14.33 ± 0.86	4.46 ± 0.06	4.60 ± 0	0 ± 0
	12	11.33 ± 0.06	10.86 ± 0.06	13.00 ± 0	12.46 ± 0.06	3.2 ± 0	0 ± 0
	15	9.80 ± 0	6.00 ± 0	10.80 ± 0	3.30 ± 0.06	1.80 ± 0	0 ± 0
	18	5.46 ± 0.06	2.26 ± 0.06	4.66 ± 0.06	2.26 ± 0.06	0.40 ± 0	0 ± 0
	21	0.60 ± 0	2.40 ± 0	4.80 ± 0	1.73 ± 0.06	0.20 ± 0	0 ± 0

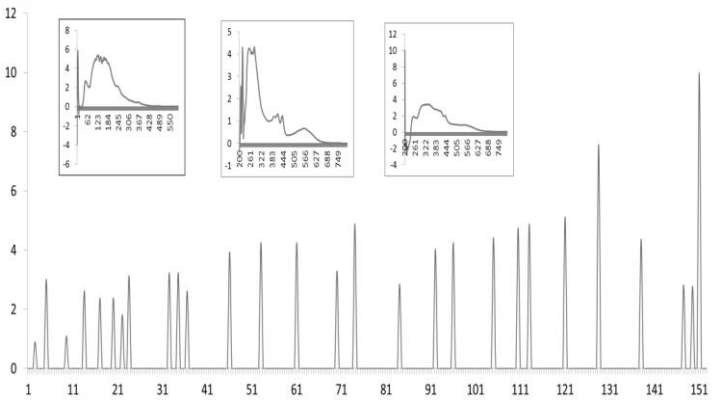


Figure1 Column chromatogram of *Alternaria alternata* crude extract run using solvent gradient of Hexane, Chloroform: Methanol (9:1, 8:2, 7:3, 5:5, 4:6, 3:7, 2:8, 1), Methanol, Water. Insets (a), (b) and (c) depicts UV-Visible scan of fractions 4, 13 and 16.

The LC chromatograms of the crude reddish brown pigment and eluted fractions i.e. 4, 13 & 16 showed the presence of different metabolites of *A. alternata*. The compounds identified in fraction 4, 13 and 16 through previous literatures match have been summarized in Figure 2. The mass spectra of identified metabolites have been furnished in supplementary figures S1-S7.

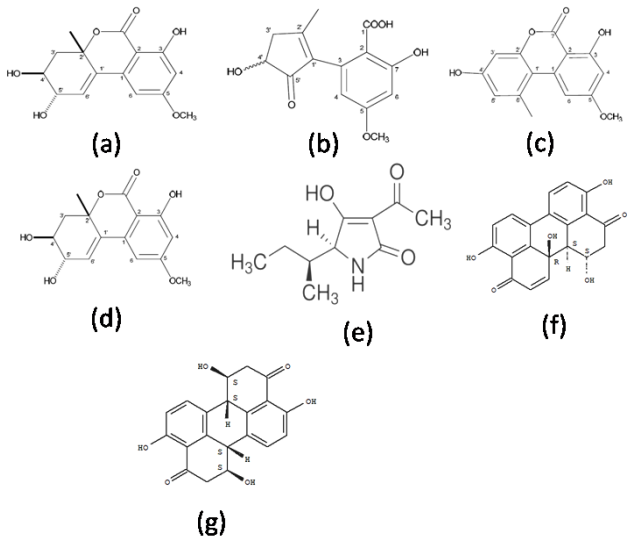
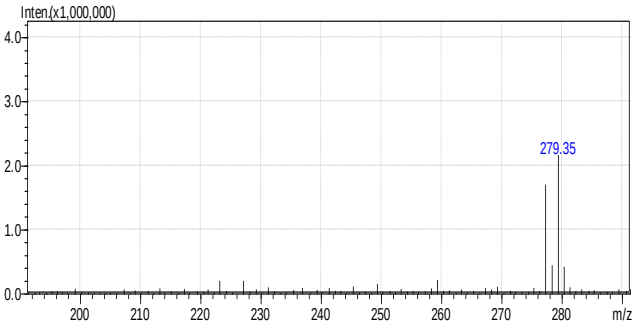
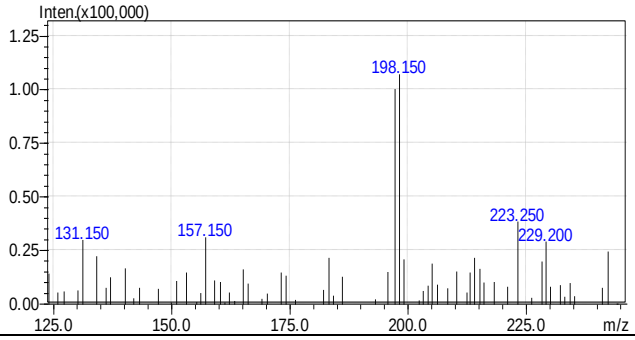
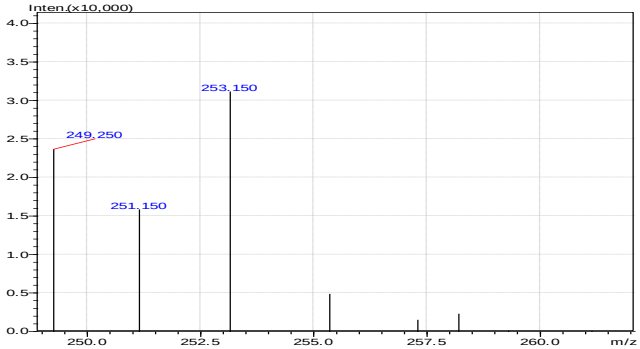
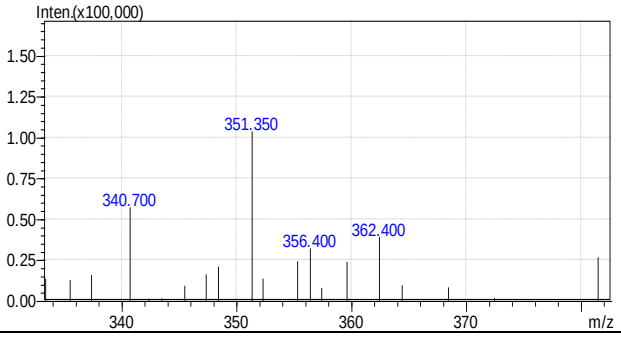
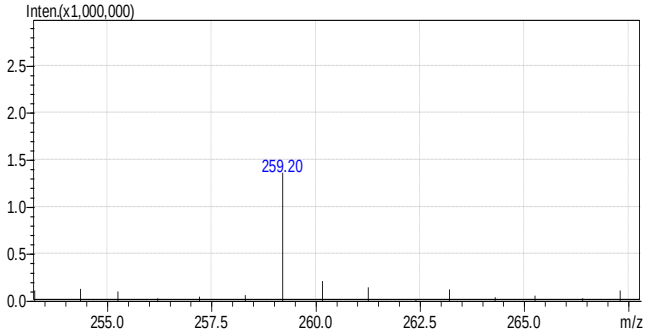
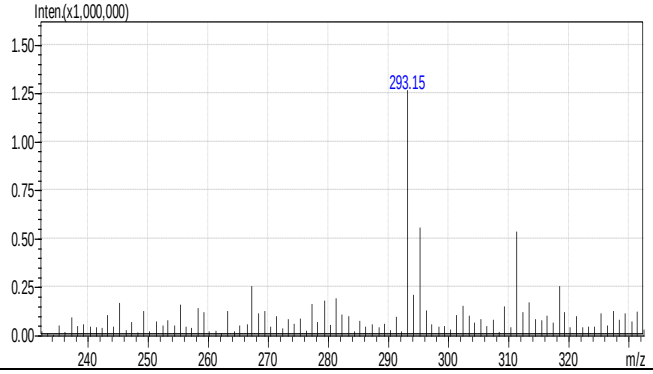
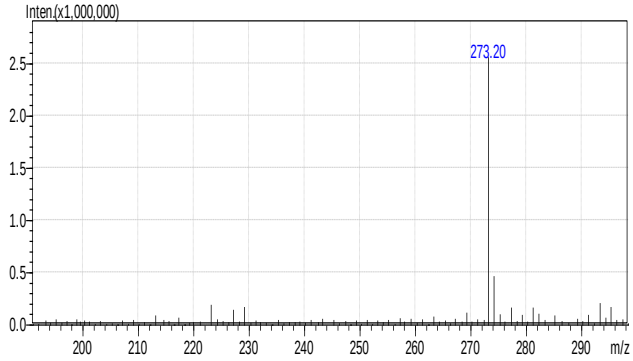


Figure 2 Identified compounds in Fraction 4, 13 and 16 [17] [19] (a) Alternariol, (b) Altenuene, (c) Alternarienoic acid, (d) Alternariol 5 methyl ether (e) Tenuazoic acid (f) Alterperylenol (g) Stemphyperylenol.

In the crude methanol extract a total of 25 components were observed out of which 5 compounds were identified through previous literatures. The rest of the 17 mass spectra were searched through similarity search in the NIST Library, of which three were identified. Alternarienonic acid, was isolated with m/z 279 at the retention time of 12.171 with characteristic UV absorbance maxima at 231 and 291 nm (supplementary figure S1). At a retention time of 12.518 min, Peak No 9, Tenuazoic acid was identified with m/z 198 and absorbance maxima at 229 and 288 nm (supplementary figure S2). At m/z 253 and retention time 14.932 min, Peak No13 was identified as Stemphyperylenol with absorbance maxima at 231, 263, 300 (supplementary figure S3). Peak No 25 was identified as Alterperylenol with RT: 21.443 min, absorbance maxima 256 nm and m/z 351 (supplementary figure S4). Other unknown masses were searched with NIST database, in which peak No 3 was identified as Glufosinate ($C_{15}H_{12}NO_4P$) with m/z181, peak No 8 was identified as 8-Linoleolyl-benzoylhypaconine ($C_{49}H_{73}NO_{10}$ with m/z 835 and peak No 21 was identified as 18-(6-Hydroxy-purin-9-yl)-benzo-18-crown-6 ($C_{21}H_{26}N_4O_7$) with m/z ratio of 446. The red fraction eluted with C: M: 9:1, showed 20 peaks out of which 4 were identified. Alternariol (Peak No 7) was identified at m/z [M+] 259.200 at RT 10.170 and absorbance maxima 255 nm (supplementary figure S5). Peak no 8 was identified as Altenuene at RT 10.170, m/z [M+] 292 and absorbance maxima 245, 312 nm (supplementary figure S5). Alternarienoic acid (Peak No 12) was identified at RT 14.054 and m/z [M+] 279.35 revealing absorbance maxima 250, 277 nm. At RT 12.009 and m/z [M+] 273.20, Alternariol 5 methyl ether (Peak No 9) was identified with absorbance maxima 338 nm (supplementary figure S7).

Table 2 LC/MS chromatograms of compounds identified in the reddish brown pigment of *A.alternata*

Fig No	Compound Name	Mass- charge ratio (m/z)	LC/MS chromatogram
S1	Alternarienoic acid	279.35	
S2	Tenuazoic acid	198	
S3	Stemphyperylenol	253	
S4	Alterperylenol	351	

S5	Alternariol	259.20	
S6	Altenuene	292	
S7	Alternariol 5 methyl ether	273.20	

The violet fraction eluted with C: M: 7:3, 11 peaks were observed out of which 1 was identified. Altenuene (Peak No2) was identified at m/z [M+] 293.30 at RT 1.186 and absorbance maxima 250 nm. From NIST Library search, Apigenin-7-O –glocoside, tms- was identified at RT 6.7000 having m/z 864.At RT 8.8000, m/z 698, 2,11-Dibromo-9,18-bis-(2-chloro-phenyl)-5,7,14,16-tetrahydro-5,7,8,14,16,17-hexaaza-dibenzo[a,H]cyclotetradecene-6,15-dione was identified. Thiabendazole (RT 0.8667, m/z 201), (2)-(1-Acetylazacyclooctacosane)(1-acetylazacyclohexacosane)-catenane (RT 6.2333, m/z 842) and L-Tryptophan, N-(N-acetyl-L-tyrosyl)-,butyl ester (RT 6.3500, m/z 465) was also identified based on similarity search using NIST Library.

The brown fraction showed 33 peaks of which 1 peak was identified as Alternariol 5 methyl ether at RT 11.497, m/z: 274 showing absorbance 274 nm. The other peaks were searched for similarity in NIST Library. From the Library search, Isopthalic acid, butyl 2 –methylcyclohexyl ester (RT 12.1833, m/z 318), 1,6-Dihydroxy-8-methoxy-3-methylanthraquinone, O, O'-bis (trimethylsilyl)-(RT 18.4000, m/z 428) and l- Alanine, N-(cyclopentylcarbonyl)-, dodecyl ester (RT 17.7167, m/z 353) were identified.

Mixture analysis of eluted fractions were carried out using proton NMR (500 MHz Bruker Avance III NMR) to confirm the presence of mass spectrometric based identified compounds viz., Altenuene, Alternarienoic acid, AlternariolMonoethyl Ether, Alternariol, Alterperylenol, Stemphyperylenol, Tenuazoic Acid. The proton ppm shifts of standard simulated spectra of each molecules were compared and matched with experimental spectra of each eluted fraction. The characteristic proton shifts of each experimental spectrum falling into particular shift range that represent specific structure also verified. First the experimental spectra of fraction 4, 13 and 16 were processed for binning and deconvulation using topspin software. Further the ppm shifts were adjusted in accordence with the internal standard (tetramethylsilane 0 ppm). Further the peaks were identified using peak identification algorithm in Topspin and peak lists were generated for each fractions. These peak shifts were matched with the simulated as well as experimental spectra (source: previous literature) of seven compounds which identified through LCMS. the following matches were recorded. 2(H-C-COOR ester), 2.36(H-C-COOH acid), 3.73(RCOO-C-H ester), 3.81(RCOO-C-H ester), 3.83(RCOO-C-H ester). Apart from that, the experimental spectra shows the presence of aromatic rings at the region of 5-6.8 ppm (Ar-H) which confirms the presence of aromatic esters and aromatic acids). The 13th fraction registered matching ppm values of 2.36 (Benzylic Ar-C-H), 1.62 (Tertiary R3-C-H). Likewise, 16th fraction registered matching shifts of 3.83 (RCOO-C-H ester), 3.81 (RCOO-C-H ester), 2.37 (Benzylic Ar-C-H), 1.62 (Tertiary R3-C-H). These results confirm the presence of the identified compounds which show peculiar structure as depicted above.

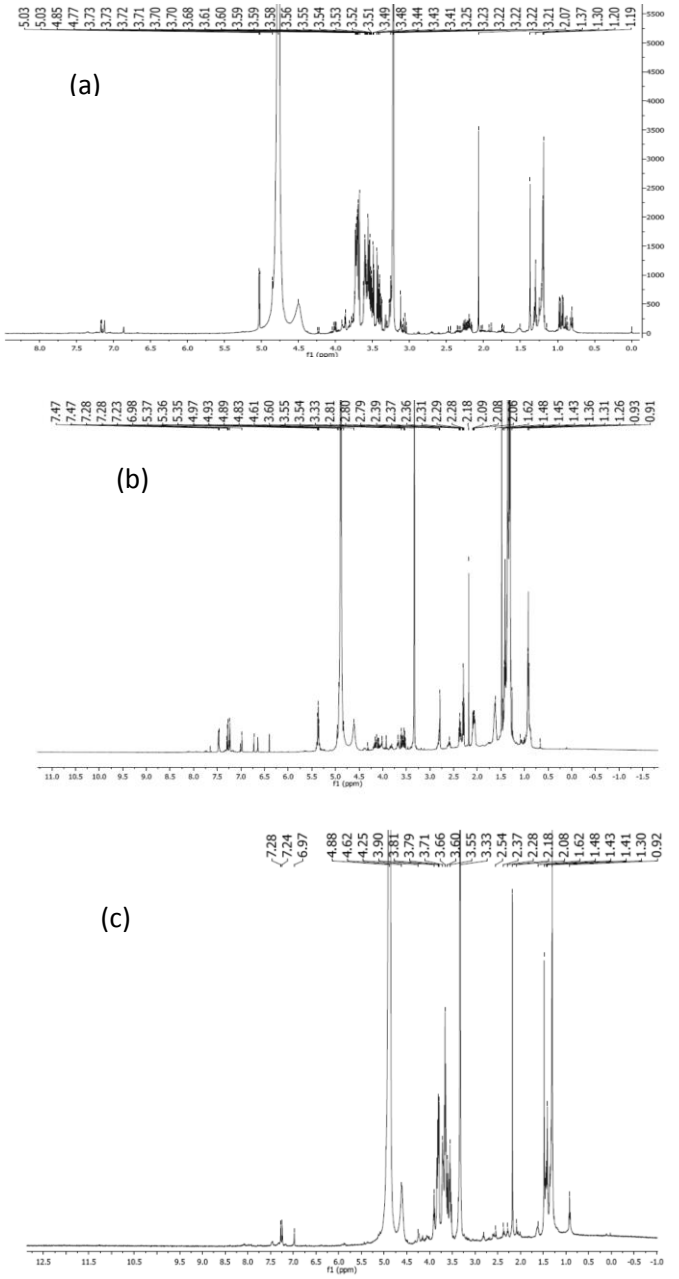


Figure 3 Proton NMR mixture analysis of eluted Fractions 4, 13 and 16 (a) 4th Fraction (b) 13th Fraction (c) 16th fraction.

The predominant classes of fungal secondary metabolites include polyketides, non-ribosomal peptides, terpenes and alkaloids **(Keller *et al.*, 2005)**. *Alternaria* sp. produce a wide assortment of toxic and non toxic secondary metabolites **(Stinson, 1985)**. *A. alternata* produces about 60 different metabolites **(Saha *et al.*, 2012)**. Isolation of secondary metabolites like Alteranariol, Altenuene, Alternarienoic acid and Alternariol 5 methyl ether have been reported from ethanolic extracts of *Alternaria* cultures grown in Rice medium **(Kjer, 2009)** whereas production of Alternariol is also reported in liquid cultures of *Alternaria* grown in liquid Wickerham medium in a study **(Hassan, 2007)**. The utilization of Sugarcane bagasse (SB), Corn Bran (CB), Wheat Bran (WB), Fenugreek Straw (FS) and solid medium Cooked Rice (CR) without any supplemental addition by *Alternaria alternata* and reported SB as productive media for the production of secondary metabolites like Alternariol, Alternariol methyl ether and Tenuzonic acid has also been studied **(Ashour *et al.*, 2011)**. Secondary metabolites from microbes can be utilized for industrial applications and leather dyeing **(Velmurugan *et al.*, 2010)**.The application of fungal pigments in dyeing of cotton, silk and wool have been reported in several studies **(Santis *et al.*, 2005; Nagia and El-Mohamedy, 2007; Perumal *et al.*, 2009)**. *Alternaria* metabolites exhibit a variety of biological activities such as phytotoxic, cytotoxic, and antimicrobial properties, which have drawn the attention of many chemists, pharmacologists, and plant pathologists in research programs as well as in application studies **(Brase *et al.*, 2009; Tsuge *et al.*, 2013)**. Brown pigments from *A. alternata* expressed good to very good fastness properties to washing, perspiration and light when dyed on wool **(Atalla *et al.*, 2011)**. Olive colour pigments from *A. alternata* dyed on wool shows 4-5 on grey scale rating meaning less staining and excellent wash fastness. The dyes were also tested for toxicity to human skin and have been found to be safe **(Sharma *et al.*, 2012)**. Microbial preparation of herbicide or bio-herbicide can control the growth of weeds. The first evidence that confirmed the herbicidal potential of tenuazonic acid against control of *Lantana Camara* produced by submerged fermentation of *A. alternata* **(Sanodiya *et al.*, 2010)**.

CONCLUSION

The potent applications of metabolites from *Alternaria* as antitumor agents, herbicides and antimicrobials as well as other promising bioactivities have led to considerable interest within the pharmaceutical community. It is evident from this study that the culture conditions can be modified to obtain maximum pigment production in *A. alternata*. In the present investigation secondary metabolites like Alternarienonic acid, Tenuazoic acid, Stemphyperylenol and Alterperylenol have been observed in the methanol extract of crude pigment whereas compounds like Alternariol, Altenuene, Alternarienoic acid, Alternariol 5 methyl ether were predominantly identified in the fractionations from column chromatography of the crude pigment. The *Alternaria* sp. could be the rich sources of biologically active compounds that are indispensable for medicinal and agricultural applications as they produce over sixty different metabolites. However there is very limited information on the utilization of pigments from *A .alternata*. Also, the interrelationship of factors such as nutrition, pH, and temperature, light exposure in bio regulation and production of secondary metabolites in the micro fungus *A.alternata* is not much understood. Hence further comprehensive study factors influencing growth, bio-synthesis, isolation and characterization of more secondary metabolites from *A. alternata* is inevitable and imperative to obtain and elucidate a wide range of secondary metabolites for for promotion of diversified application of these fungal dyes in agriculture, medicine and other industries.

Acknowledgments: We thank Dr. Soumya Swaminathan, Director, National Institute for Research in Tuberculosis, Chetput, Chennai-31 for providing LCMS infrastructure. We thank Dr. E. Murugan, Department of Physical Chemistry, School of Chemical Sciences, University of Madras, Guindy, Chennai-25 for his valuable inputs to conduct the Chromatographic studies. We are grateful to the kind support extended by Dr. V.Sivaram for his scholastic guidance in conducting the mass spectrometry experiments and interpretion of LCMS, FTIR and NMR spectra. We acknowledge Dr. A. Kiran Kumar, and Miss. V. Sudha for technical assistance throughout this work.

REFERENCES

AITTOMAKI, R., COLLIANDER, H., KOTIRANTA, H. 2000. Varia Luonnosta, 3rd Ed. *Heilsinki: Tammi*, p. 104

ASHOUR, M., YEHIA, H.M., PROKSCH, P. 2011. Utilization of Agro-industrial by-products for production of bioactive natural products from endophytic fungi. *Journal of Natural Products*, 4, 108-114.

ATALLA, M.M., EL-KHRISY, E.A.M., YOUSSEF, Y.A., MOHAMED, A. A. 2011. Production of textile reddish brown dyes by fungi. *Malaysian Journal of Microbiology*, 7, 33-40

BILLS, G. F. 1995. Analyses of micro fungal diversity from a user's perspective. *Can. J. Bot*, 73(1), 33-41. http://dx.doi.org/10.1139/b95-222

BRASE, S., ENCINAS, A., KECK, J., NISING, C. F. 2009. Chemistry and biology of mycotoxins and related fungal metabolites. *Chem. Rev*., 109, 3903–3990. http://dx.doi.org/10.1021/cr050001f

CHANDRASEKARENTHIRAN, S. 2010. "Production, characterization of pigment from selected basidiomycetes - *Pycnoporus sanguineus* L. Ex. Fries and *Coriolus versicolor* (L.) Quelet. and application of pigment in textile dyeing" *Academic Dissertation*, *University of Madras, Chennai. India*

HASIJA, S.K. 1970. Physiological studies of *Alternaria citri* and *A.tenuis. Mycologia*, 62, 289-295. http://dx.doi.org/10.2307/3757587

HASSAN, E. H. A. A. 2007. Novel Natural Products from Endophytic Fungi of Egyptian Medicinal Plants - Chemical and Biological Characterization. *Academic Dissertation, Heinrich Heine University Dusseldorf. Germany.*

KELLER, N. P., TURNER, G., BENNETT, J. W. 2005. Fungal Secondary Metabolism from Biochemistry to Genomics. *Microbiology*, 3, 937-947. http://dx.doi.org/10.1038/nrmicro1286

KIM J. K., PARK, S. M., LEE, S. J. 1995. Novel antimutagenic pigment produced by *Bacillus licheniformis* SSA3. *J. Microbiol. Biotechnol*., 5, 48-50

KIM, C. H., KIM, S. W., HONG S. I. 1998a. Production of red pigment by *Serratia* sp. KH-95 and its cultural properties. *Kor. J. Biotechnol. Bioeng*., 13, 431-437

KIM, C. H., KIM, S. H., HONG, S. I. 1998b. Isolation and characteristics of prodigiosin like red pigment produced by *Serratia* sp. KH-95. *Kor. J. Appl. Microbiol. Biotechnol*., 26, 283-289.

KJER, J. 2009. New Natural Products from Endophytic Fungi from Mangrove Plants – Structure Elucidation and Biological Screening. *Academic Dissertation, Heinrich Heine University Dusseldorf. Germany.*

LARSEN, T.O., SMEDSGAARD, J., NIELSEN, K. F., HANSEN, M. E, FRISVAD, J. C. 2005. Phenotypic taxonomy and metabolite profiling in microbial drug discovery. *Nat. Prod. R*, 22(6), 672-695. doi: http://dx.doi.org/10.1039/b404943h

LAURO, G. J. 1991. A primer on natural colours. *Cereal Foods World*, 36, 949-953

MASAHIRO, K. O., MINE, K., TAYA, M., TONE, S., ICHI, T. 1994. Production and release of anthraquinone pigments by hairy roots of madder (*Rubia tinctorum* L.) under improved culture conditions. *J. Ferment. Bioeng*., 77, 103-106. http://dx.doi.org/10.1016/0922-338x(94)90219-4

MISAGHI, I. J., GROGAN, R. G., DUNIWAY, J. M., KIMBLE, K. A. 1977. Influence of environment and culture media on spore morphology of *Alternaria alternata*. *Phytopathology*, 68, 29-34. http://dx.doi.org/10.1094/phyto-68-29

NAGIA, F. A., EL-MOHAMEDY, R. S. R. 2007. Dyeing of wool with natural anthraquinone dyes from Fusarium oxysporum. *Dyes and Pigments*, 75, 550–555. http://dx.doi.org/10.1016/j.dyepig.2006.07.002

OZCELIK, S., OZCELIK, N. 1990. Interacting effects of time, temperature, pH and simple sugars on biomass and toxic metabolite production by three *Alternaria* sp. *Mycopathologia*, 109,171-175. http://dx.doi.org/10.1007/bf00436806

PERUMAL, K., STANLEY ABRAHAM, L.,VATSALA, T. M. 2004. Isolation, extraction and dyeing performance of fungal pigment from *Ganoderma applanatum*, *Coriolus versicolor* and *Amanita muscaria*. *J. Mycol. Pl. Pathol*. 34, 690-691

PERUMAL, K., STALIN, V., CHANDRASEKARENTHIRAN, S., SUMATHI, E., SARAVANAKUMAR, A. 2009. Extraction and characterization of pigment from *Sclerotinia* sp. and its use in dyeing cotton. *Text. Res. J*, 79, 1178 - 1187. http://dx.doi.org/10.1177/0040517508087680

PIMENTEL, M. R., MOLINA, G., DIONISIO, A. P., JUNIOR, M. R. M., PASTORE, G. M. 2011. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. *Biotechnology Research International*, 1, 11. http://dx.doi.org/10.4061/2011/576286

RAISTRICK, H., STICKINGS, C. E., THOMAS, R. 1953. Studies in the biochemistry of micro organisms. 90. Alternariol and alternariol monomethyl ether, metabolic products of *Alternaria tenuis*. *Biochem. J*, 55, 421-433

RAMJEGATHESH, R. A., EBENEZAR, E. G. 2012. Morphological and physiological characters of *Alternaria alternata* causing leaf blight disease of onion. *Intl. J. Plant Pathol*.., 3, 34-44. http://dx.doi.org/10.3923/ijpp.2012.34.44

SAHA, D., FETZNER, R., BURKHARDT, B., PODLECH, J., METZLER, M., DANG, H. A, LAWRENCE, C., FISCHER, R. 2012. Identification of a Polyketide Synthase Required for Alternariol (AOH) and Alternariol-9- Methyl Ether (AME) Formation in *Alternaria alternata. PLoS ONE*, 7, 7: e40564. http://dx.doi.org/10.1371/journal.pone.0040564

SANODIYA, B. S., THAKUR, G. S., BAGHEL, R. K., PANDEY, A. K., PRASAD, G. K. S., BISEN, P. S. 2010. Isolation and Characterization of Tenuazonic Acid produced by *Alternaria Alternata*, A Potential Bioherbicidal Agent for Control of *Lantana Camara*. *Journal of Plant Protection Research*, 50, 2. http://dx.doi.org/10.2478/v10045-010-0023-3

SANTIS, D. D., MORESI, M., GALLO, A.M., PETRUCCIOLI, M. 2005. Assessment of the dyeing properties of pigments from *Monascus purpureus*. *J. Chem. Technol. Biotechnol*, 80, 1072 – 1079. http://dx.doi.org/10.1002/jctb.1285

SHABANA, M., ELWAKIL, M. A., CHARUDATTAN, R. 2001. Effect of Nutrition and Physical Factors on Mycelial Growth and Production of Pigments and Nonchromatic UV-absorbing Compounds of *Alternaria eichhorniae*. *Journal of Phytopathology*, 149, 21–27. http://dx.doi.org/10.1046/j.1439-0434.2001.00564.x

SHARMA, D., GUPTA, C., AGGARWAL, S., NAGPAL, N. 2012. Pigment extraction from fungus for textile dyeing. *Indian Journal of Fiber and Textile Research*, 37, 68-73.

STINSON, E. E. 1985. Mycotoxins-Their biosynthesis in *Alternaria*. *Journal of Food Protection*, 48.

STROBEL, G. A. 2002. Microbial gifts from rain forests. *Can J. Plant Pathol*, 24 (1), 14-20. http://dx.doi.org/10.1080/07060660109506965

SUMATHI, E. 2008. "Cultivation, extraction of pigment from basidiomata of *Ganoderma lucidum* (Fr.) P. Karst and its dyeing performance with selected textile yarns" *Academic Dissertation, University of Madras, Chennai. India*

TSUGE, T., HARIMOTO, Y., AKIMITSU, K., OHTANI, K., KODAMA, M., AKAGI, Y., EGUSA, M., YAMAMOTO, M., OTANI, H. 2013. Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. *FEMS Microbiol. Rev*., 37, 44–66. http://dx.doi.org/10.1111/j.1574-6976.2012.00350.x

VELMURUGAN, P., KAMALA-KANNAN, S., BALACHANDAR, V., LAKSHMANAPERUMALSAMY, P., CHAE JC, JONG-CHAN., OH, BT. 2010. Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. *Carbohydrate Polymers*, 79, 262–268. http://dx.doi.org/10.1016/j.carbpol.2009.07.058

ZHANG, H. W., SONG, Y. C., TAN, R. X. 2006. Biology and chemistry of endophytes. Nat. *Prod. R*, 23(5), 753-77. http://dx.doi.org/10.1039/b609472b