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Production and Characterization of Fusaric Acid from *Fusarium Species* Isolated from Different Soil Samples

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Abstract
Fusaric acid (FA) is one of the most important secondary metabolites produced by *Fusarium oxysporum*. It is toxic to humans, many plants, and microorganisms and it enhances the toxicity of fumonisin and trichothecene. *Fusarium oxysporum* strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes and degrade lignin and complex carbohydrates with soil debris. Rapid growth rate of fungi and increased fungal biomass was obtained in Potato dextrose agar medium where the mixed populations of microbes exist from the natural soil sample. Discrete colonies of various fungal species were obtained *Fusarium oxysporum* was identified microscopically by slide preparation. This fungus was individually isolated in PDA slants and stored in refrigerated conditions. The colony diameter, the conidia germinating rate and the conidia yield were increased. In this research we performed qualitative and quantitative analysis of Fusaric acid production in Potato sucrose broth (PSB) with TLC method finally the samples were detected by FT-IR test.

Keywords
Fusaric acid, Fumonisin, TLC, FT-IR

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INTRODUCTION
The predominant role of these fungi in native soils may be as harmless or even beneficial plant endophytes or soil saprophytes, many strains within the *Fusarium oxysporum* complex are pathogenic to plant, especially in agricultural settings. Pathogenic fungi produce various biologically highly active substances (organic acids, specific polysaccharides) into the growth *milieu*, many of which are toxic to both plants and microorganisms [1]. Specific in this sense is Fusaric acid (FA), an important nonspecific mycotoxin. The effects of root exudates and decaying residues on methanogenic microbial communities on rice or maize roots and in the rhizosphere [2], bacterial community composition, microbial biomass, stimulation of ginseng root exudates on growth of phytophthora Cactorum and Pythium irregular I, rhizobacterial populations [3] and fungal species abundantly rich in the desert [4] have been studied. Fusaric acid (FA), also known as 5-butylpicolinic acid, is a non-host specific phytotoxin of *Fusarium species* and suspected of being involved in pathogenecity [5].

FA has been shown to stimulate the rapid development of some disease symptoms, such as interveinal necrosis and foliar desiccation [6]. Fusaric acid is a mycotoxin with low to moderate toxicity. Which is of concern since it might be synergistic with other cooccurring mycotoxins. Fusaric acid is widespread on corn and corn-based food and feeds and is frequently found in grain, where *Fusarium spp.* are also isolated. To determine the effects of concentration of Fusaric acid on the growth of several strains of the biocontrol bacterial endophytes *Bacillus mojavensis* and other species within the *Bacillus subtilis* group, as well as the genetic relationships within this small group of Gram-positive bacteria, and their antagonisms to *Fusarium verticillioides*.

Fusarium oxysporum is toxic to humans, many plants, and microorganisms and it enhances the toxicity of fumonisin and trichothecene. A simple and rapid method for Fusaric acid (FA) screening in *Fusarium* isolates was developed. In this study, several strains of *Fusarium oxysporum* were tested for their ability to produce FA by using a suitable race of *Bacillus subtilis* as the bioassay. A modified method using small agar blocks with the fungus producing FA was applied in the screening test. *Fusarium oxysporum* strains are ubiquitous that have the ability to exist as saprophytes and degrade lignin and complex carbohydrates associated with soil debris. *Fusarium* toxins are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize. They include a range of mycotoxins, such as: Fusaric acid; the fumonisins, beauvercin and enniatins, and fusarins. Rapid growth rate of fungi and increased fungal biomass was obtained in Potato dextrose agar medium where the mixed populations of microbes exist from the natural soil sample, Discrete colonies of various fungal species were obtained. Higher plants synthesize various secondary metabolites including tannic acid and its derivatives, such as ellagic or gallic acid [7]. The genus *Fusarium* is one of most of the economically important genera of fungi and includes many pathogenic species that cause a wide range of plant disease [8]. It also includes endophytic and saprophytic species found in association with plants in agricultural and natural ecosystem [9,10].

MATERIALS AND METHODS

Sample Collection

Using sterile polythene bags, soil samples were collected from area in Vellore.

Isolation of Fungi from Soil Sample

Solid Potato Dextrose Agar (PDA) medium has been used for the surface growth of fungi. The soil sample was diluted by serial dilution technique, this serially diluted up to 7 dilutions and sample was utilized for further dilution. Spreading was done using a bent glass rod. 1ml of diluted soil suspension was placed in the center of the plate using a sterile pipette and the spreaded it. This method was utilized for rapid growth rate of fungi and increased fungal biomass.

Morphological Analysis

The mixed fungal colonies initiated after3-4 days of incubation at 30°C, which contain varied fungal species.[11] The plates were incubated further for 10 days for colony development and maturation. From these mixed colonies, single fungal colony could be identified microscopically based on its morphological (size, shape, color) differences etc.

Isolation of *Fusarium Sp* From Fungal Consortium

After microscopic identification fungal biomass was touched by inoculating loop and streaked on PDA plate. This plate was incubated for 10-15 days at 30°C and obtained as pure culture and stored for further studies.

Assessment of Conidial Germination

To determine the effect of gallic acid on conidial germination, FON was grown in 2% water agar. A 5-mm agar plug taken from a 7-day old PDA culture and incubated at 28°C for 7 days. The broth was filtered to collect conidia. [12] Conidial suspension was filtered to collect conidia. Conidial suspension was diluted water. Some 0.5ml of the diluted suspension was spreaded on plates and incubated at 28°C for 3 days. The number of colonies was counted daily.

Fungal Culture Filtrate

To obtain fungal culture filtrate, flask containing 100ml of Potato Sucrose Broth were inoculated with 6mm diameter mycelia disc of inoculums and incubated at 26°C. Filtrate was obtained by filtration through 4 layers of cheese cloth, twice through whatman No.1 paper and centrifugation at 3000rpm for 30 min to sediment spores and mycelia and stored at 5°C in sterile bottles.

Extraction of Fusaric Acid (Fa)

FA from culture filtrate was extracted briefly PH of the filtrate adjusted to 3.9-4.0 with 2N HCL and FA was extracted thrice with ethyl acetate. Organic extracts were pooled and evaporated at room temperature samples were dissolved in 1000µl of 80% methanol and stored at 20°C until for the use.

Qualitative and Quantitative Analysis of Fusaric Acid

Samples were applied on TLC plate (TLC silica gel) together with FA standard. The plates were developed in n-butanol, acetic acid, ethyl acetate, water (3:2:2:2, v/v) solvent, dried at 80°C and FA was detected under UV light (λ=254). The UV spectrophotometric assay for FA was performed using UV grade methanol with λ max 260 nm.[13]

Time Course for Fusaric Acid Production

100ml of Potato Sucrose Broth medium in 250ml flask, the fungal culture was inoculated in PSB medium. And incubated at 26°C. Culture filtrate was harvested at 4,6,8,9,10,11. And 13 days by filtration through Whatman No.1 filter paper and used for FA quantification by spectrophotometric method.

Effect of PH

100ml of Potato Sucrose Broth medium in ten 250ml flasks, adjusted to different pH of 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 7.5, 8.0, and 9.0 using 1M NaOH or 1M HCL. Each pH medium is sterilized at 121°C for 15 min. The fungal culture was inoculated into all the flasks containing medium. Then the flasks were incubated at 26°C for 19 days in static condition. Clear filtrate was used for FA quantification by spectrophotometric method.

Maintenance Of Pure Culture

Test Organism

Test organisms were collected from college, a antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Bacillus species, Salmonella typhi, Shigella flexinarii.* And antifungal activity against *Aspergillus niger, Aspergillus flavus, Mucor species, Penicillium species* these cultures was used in Fusaric acid production of *Fusarium species*

Antibacterial Activity Test

Antibacterial activity of the Fusaric acid from methanol extracts was evaluated by agar well diffusion method. For the determination of antibacterial activity, the 24 hours fresh bacterial cultures were taken for the determination of antimicrobial for all bacteria.

The organisms were swabbed on Muller Hinton Agar. The wells were cutted by sterile well border. The well contains 20µl of Fusaric acid extract were delivered to respected well and the plates were incubated at 37°C for 24 hours. After incubation DIZ (Diameter Inhibition Zone) were measured by using Zone measuring scale.

Antifungal Activity

Antifungal activity of the Fusaric acid methanol extract of the *Fusarium species* was evaluated by agar well diffusion method. For the determination of antifungal activity. The 48 hours fresh cultures were taken for the determination of antifungal activity for all fungi.

The organisms were swabbed on Sabouraud Dextrose Agar. The wells were cutted by sterile well border. The well contains 20µl concentration of Fusaric acid methanol extract were delivered to respected well and the plates were incubated at room temperature for 48 hours. After incubation each plate was examined and measured the diameter of the zone of inhibition.

FT-IR Spectral Analysis

FTIR spectra were performed and recorded with a Fourier-transform infrared spectrophotometer.

RESULTS AND DISCUSSION

Microscopic observation shows the colonies are pale coloured, Felty, cottony or wooly or spares and wet-looking. The colony form was circular, irregular to filamentous, with flat or raised elevation. The margin was undulate or filiform. The colour of the organism is orange. In the microscopic observation shows hyaline septate hyphae, conidiogenous cells are awl-shaped phialides. Phialides may have one (mono phialides) or more than one (poly phialides) openings. Two types of conidia are seen. Macroconidia are multicelled, with two to five septa. They are fusiform or sickle shaped and often has a distinct notched basal cell. The Macroconidia that are produced in aerial mycelium are less typical than those produced in sporodochia. Microconidia are oval, globose, ellipsodical and clavate or kidney shaped.

The Conidial germination produces number of conidia. In TLC analysis a dark intense band was observed, and Rf value were tabulated. And also tested effect of incubation time and effect of pH by using spectrophotometric method. And finally, antimicrobial activity test and FTIR test is performed.

Fusaric acid is a major toxin secreted by all *Fusarium species.* And it is the key factor with many applications

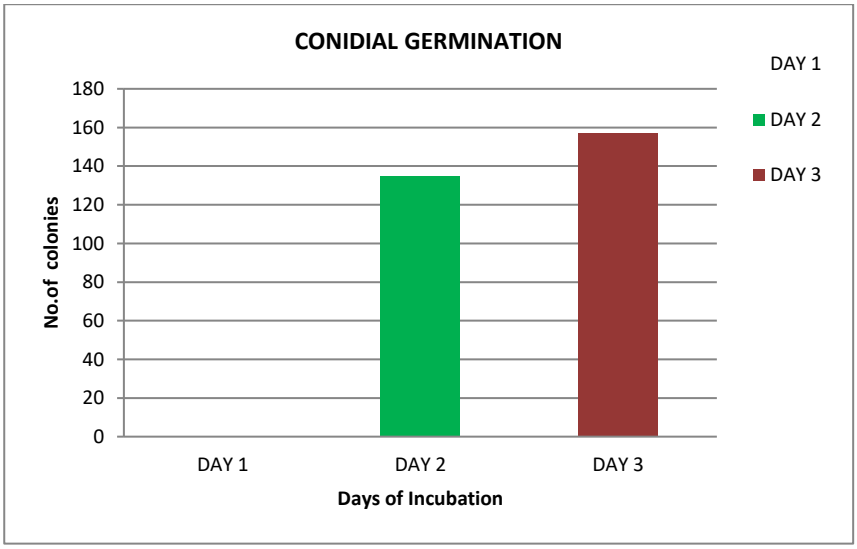
lie it use as chelating agent, antihypertensive agent etc.

Fusaric acid (FA) is a mycotoxin produced by the *Fusarium species*, among which the highest yielding was reported to be *Fusarium oxysporum* [14]. It is moderately toxic to animals. It has antibiotic, insecticidal and pharmaceutical activity. *Fusarium species* are found worldwide in soil as both pathogenic and non-pathogenic strains. Large concentrations of Fusaric acid reduce growth of root and root tubers. The biosynthesis of Fusaric acid involves condensation reaction of polyacetate and aspartic acid units.

Toxic metabolites on Potato Dextrose Broth reached a peak after 25 days of static incubation at 25°C under illumination. In the present investigation, similar were achieved with fungal biomass production but the toxic metabolites. i.e growth level reached a peak at 10 days and declined subsequently. The biomass production was intentionally studied to establish a reaction between the growth of fungus and toxin production but in this case, it found to be negatively related with each other. It was observed that various growth conditions influenced the production of toxins by *Fusarium oxysporum*. A change in pH before the media sterilization led to an increase in growth level, peaking at pH, suggesting that a higher initial pH of the culture medium may stimulate the production of toxic metabolites.[15]

MACROSCOPIC IDENTIFICATION

Fusarium species



TLC CHROMATOGRAPHY

FIGURE: 1

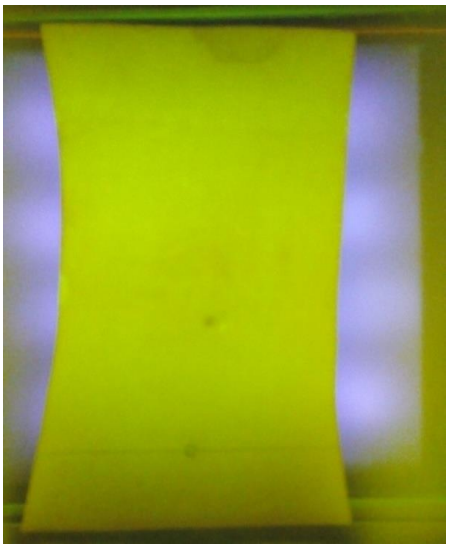


FIGURE:2

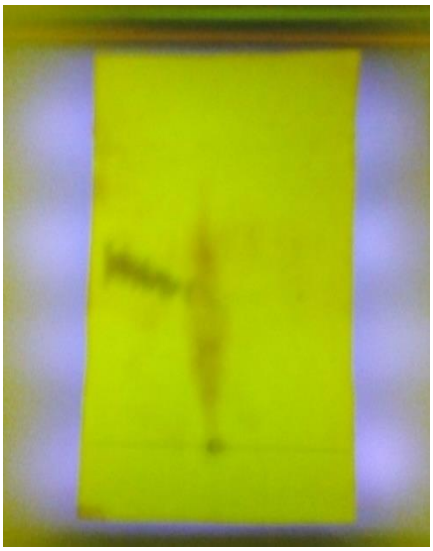


FIGURE:3

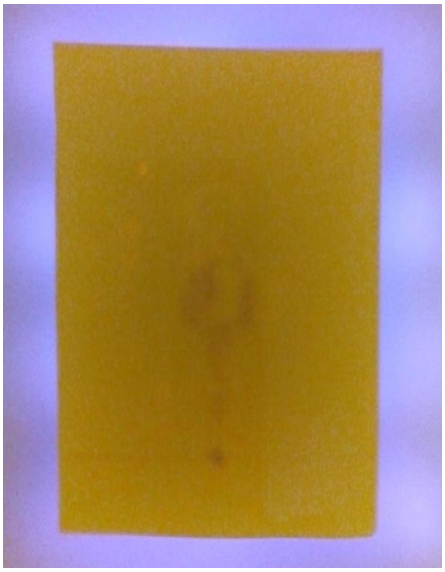
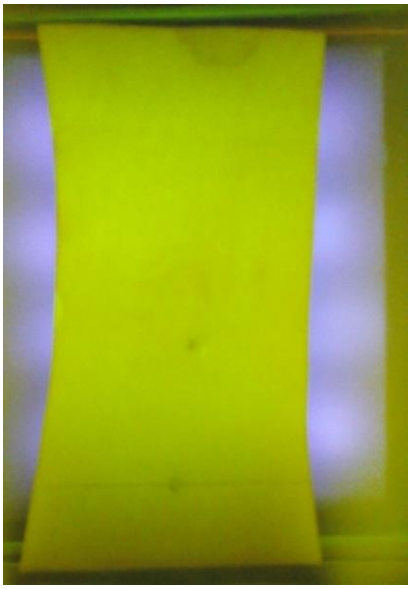
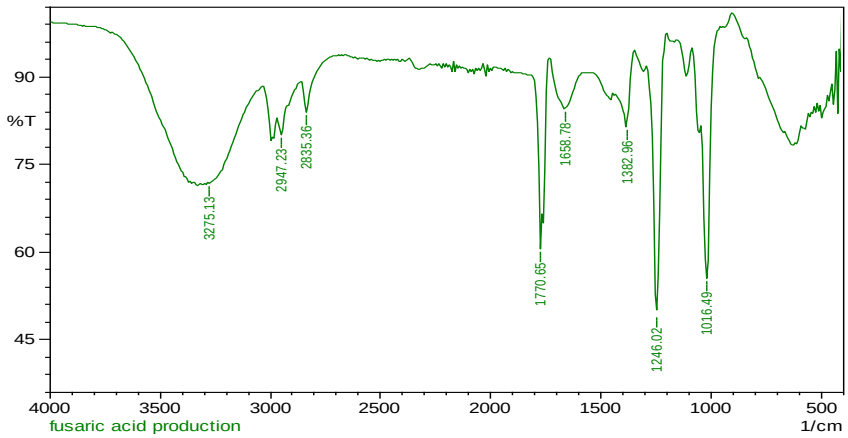


FIGURE:4



FT-IR ANALYSIS



FUSARIC ACID PRODUCTION

WAVE NUMBER	RANGE	BOND	FUNCTIONAL GROUP
3275	3100-3010	=CH stretch	Alkenes
2947	2950-2800	C-H stretch	Alkanes
1770	-1725	C=O stretch	Aldehydes
1246	-1250&-1120	C-O stretch	Ethers
1016	1050	S=O stretch	Sulfoxide

CONCLUSION

Fusaric acid is produced by using *Fusarium species.* And it has many applications it includes; FA strongly inhibits root and leaf cell function physiologically responsible for *Fusarium* wilt of watermelon. Fusaric acid induces endogenous ethylene production in tomato cutting. FA is used to examine the cell death in saffron roots. FA increases the mitochondrial and plasma membrane permeability. FA is used for selecting resistance in barely plants. FA is known as an inhibitor of metal containing oxidative enzymes mycotoxins and antibiotics. It is used as a marker compound for *Fusarium* contamination of grains. Fusaric acid is used as chelating agent, anti-hypersensitive agent etc.

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