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Production of griseofulvin from marine fungi *Penicillium fellutanum*

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Abstract
The marine world offers an extremely rich resource for important compounds of structurally novel and biologically active metabolites. Among them Griseofulvin is one of the representative antifungal agent which is metabolic product of many species of *Penicillium.* The Present study is carried out in production of griseofulvin from marine fungus *Penicillium fellutanum* using three different media (semisolid medium, potato dextrose agar medium and minimal medium) for maximum production of Griseofulvin. TLC and FT-IR techniques were carried out for determination of Griseofulvin from crude extracts. Among these three media were tested, semisolid media exhibited more biomass and Griseofulvin production when compared with others.

Keywords: Griseofulvin, antifungal antibiotics, *Penicillium fellutanum*

1. Introduction
Toxicological, pharmacological and ecologically important Secondary metabolites like alkaloids, phenolics, steroids and terpenoids have been characterized from mangroves. The continuing success of microbiologists in the search among microbial metabolites for use as antibiotics in combating human, animal and plant diseases has stimulated the belief that micro-organisms constitute an inexhaustible reservoir of interesting compounds [2]. The fungal species are known for the production of enzymes and secondary metabolites, which have not been exploited completely. Fungal secondary metabolites possess unusual chemical linkages, such as β —lactam rings, cyclic peptides made of normal and modified amino acids, unsaturated bonds of polyacetylenes and polyenes, and large macrolide rings. The best known fungal secondary metabolites that are subjected to commercial production are the β-lactum antibiotics. Among them Griseofulvin is one of the representative antifungal antibiotics and has been widely used as an antifungal drug, particularly against dermatophytes. It is a metabolic product of many species of *Penicillium* and affects the growth characteristics of various fungi.
This drug kills young and actively metabolizing cells, but it inhibits the growth of older and dormant cells. Different strains of *P. griseofulvum* produce either patulin a hepatotoxic and carcinogenic metabolite or griseofulvin, an antibiotic compound. Griseofulvin is a systemic antifungal antibiotic with low toxicity. Griseofulvin, an antifungal antibiotic produced by the fungus *P. griseofulvum*, has been widely studied with respect to fermentation conditions [8, 12, 14], downstream processing and purification [9, 10, 14], applications, and pharmaceutical formulations [5]. Griseofulvin is commercially produced by submerged fermentation (SmF). The mycelial morphology of fungi is well-suited to invasive growth on solid media [1]. It has been used for production of enzymes, antibiotics, silage, and primary metabolites such as citric acid, gallic acid, fermented foods, and alcohol [4, 13]. Studying marine fungal metabolites is relevant both for the understanding of their ecological role. As a result, antifungal metabolites biosynthesis could be induced in response to other fungal components, thus proving a selective advantage for their development in natural marine environment.

2. Materials and Methods
2.1. Sample collection
Sediment samples were collected from Vellar estuary at morning, it was aseptically transferred to clean polythene bags for mycological analysis. All samples are transferred to

the laboratory for analysis in a portable ice box. Mycological analysis was performed with in 3-4 hours immediately after collection.

2.2. Isolation and Identification of Fungal Strain
P. fellutanum was isolated from mangrove sediment sample and identified based on the growth, morphological and Microscopic characteristics

2.3. Seed culture medium
The seed culture medium was inoculated with 1ml of spore suspension (in sterile distilled water) containing 18 x10 ^{6} spores from a 9 days slant. The culture was incubated for 4 days on a rotary shaker at 160 rpm per min and at 30 °C.

2.4. Culture conditions
One milliliter of spore suspension (18 x10 ^{6}) was inoculated into a 500 ml Erlenmeyer flasks containing 300 ml minimal broth, semisolid medium, potato dextrose broth and incubated in orbital shaker at 200 rpm for 14 days at 28 °C.

2.5. Extraction of Griseofulvin from minimal broth
Triplicate flasks were harvested at intervals and analyzed for biomass, p^{H} and griseofulvin production. Mycelia were recovered by filtration, freeze dried, and weighed. Extraction with broth was carried out using distilled methanol (3×15 ml). Methanol solutions were pooled and evaporated and checked for the griseofulvin production.

2.6. Extraction of Griseofulvin from semi solid medium
Cultures was stopped by warming on a hot plate (60 °C) to allow the semi-solid agar to melt and the extraction procedure was done with warm methyl acetate. After shaking, suspension was left at room temperature until the re-solidification of the agar phase. The liquid organic phase was then removed, filtered under vacuum by aseptic condition. The obtained dry component was mixed into the three solvent such as ethanol, methanol, chloroform in the component solvent ratio of 25:1 (mg/ml). This mixture was used to check antimicrobial activity of semisolid extracted compound.

2.7. Extraction of Griseofulvin from Potato dextrose broth
Potato Dextrose broth was filtered to separate mycelia. After filtration, the culture filtrate was extracted twice with equal volumes of ethyl acetate and the organic phase was concentrated to dryness.

2.8. Analytical methods
2.8.1 Determination of Dry Cell Weight
The three culture broth was filtered through pre weighed Whatmann No.1 filter papers. The cells were then dried at 80 °C to a constant weight prior to measuring dry weight.

2.8.2 Qualitative Determination of Griseofulvin by TLC
Griseofulvin was qualitatively determined using pre-coated Thin Layer Chromatographic (TLC) plates with silica gel GF-254. The methanolic extract samples are spotted on the chromatograms and developed in the solvent system of chloroform – n-hexane (1:1 v/v). The TLC plates were removed from the solvent before they reach the end of the slide. The reaching point of the solvent marked and allowed to air dry for few minutes. Spots were visualized under vapors of iodine crystals. Then, the Rf value was calculated for identification of which fungal metabolites are present in this sample.

Rf = Distance traveled by components / Distance traveled by solvent

2.8.3 FT-IR spectroscopy
FTIR used to investigate the vibrations of molecules and polar bonds between the different atoms. Structures of Cy-A, such as, glucosidic bonds and functional groups can be analyzed using FTIR spectroscopy. After running TLC, samples were scrapped off from the plates, pooled and eluted with distilled water and dried to get the compounds of interest in a solid form. 2 mg of samples were mixed with potassium permanganate and pressed into pellets of 13 mm size and infrared spectrum was recorded using Perkin - Elmer IR spectrophotometer (Model IR-577).

3. Results
3.1. Production of Griseofulvin
The production of griseofulvin by *P. fellutanum* was tested with three different media viz., Potato Dextrose agar, Minimal medium and Semi solid media. These three media were used to check biomass production ability of the *P. fellutanum* (table1) and methyl acetone extraction was used to check Griseofulvin production. Among these media were tested semi solid media exhibited more biomass (2.36g/ 100ml) and ethanolic extract of griseofulvin (86 mg/ 100 ml) production when compared other two different media.

Potato dextrose agar also produced satisfactory amount of biomass (1.88 g/100ml) but not in a griseofulvin (32 mg/100 ml) production. In case of minimal medium exhibited least biomass (1.52 g/100ml) but high rate of griseofulvin production (56mg/100ml).

Table: Effects of three different media on the production of biomass and griseofulvin

Different medium	Biomass (g/100ml)	Griseofulvin(mg/100ml)
Potato Dextrose agar medium	1.88	32
Semi-solid medium	2.36	86
Minimal medium	1.52	56

3.2 Chromatographic analysis
Griseofulvin was approximately determined in crude extracts from *P. fellutanum* by TLC. This compound appeared as a dark brown spot at Rf 0.65 on our TLC system.

3.3 FTIR- spectroscopy
The IR spectra of *P. fellutanum* crude extract were shown in (graph) This crude extracts exhibited broadly stretched intense peak at around 3284 cm^{-1}characteristrics of hydroxyl (O-H) groups and peak around 1442 cm^{-1} characteristics of methane stretching frequency (CH). The relatively strong adsorption peak at around 3400cm^{-1} indicated the characteristics of amine (NH) stretching vibration and the weak one at around 3400 cm^{-1} also indicated the characteristics of IR adsorption of griseofulvin.

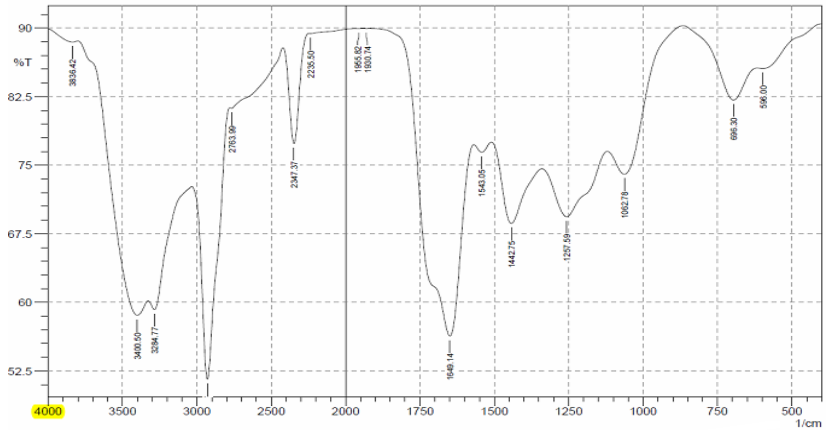

Fig 1: FT-IR identification of griseofulvin compound from extract of *Penicillium fellutanum*

4. Discussion

Griseofulvin, an orally non-toxic antifungal antimitotic drug derived from several species of *Penicillium* has been used for many years for the treatment of Tinea capitis (ringworm) and other dermatophytic infections [3]. Its mechanisms have been thought to involve the selective inhibition of fungal cell mitosis in association with its accumulation in the keratin layers of the epidermis. This work aimed to study, marine fungi as a new source of bioactive compounds. Griseofulvin is not the first antifungal metabolite isolated from a marine fungus [11, 16] indicating that marine adapted strains could synthesize those bioactive compounds in marine culture conditions.

In the present study, 3 different viz., PDA, semi-solid and minimal media were tested for the production of both biomass and griseofulvin. Among these media were tested semi solid media exhibited more biomass (2.36g/ 100ml) and griseofulvin (86 mg/ 100 ml) production when compared other two different media. Potato dextrose agar also produced satisfactory amount of biomass (1.88 g/100ml) but not in griseofulvin (32 mg/100 ml) production. In case of minimal medium exhibited least biomass (1.52 g/100ml) and highest griseofulvin production (56mg/100ml). This was more or less similar with the results of [18]. The reason for high biomass production from semi solid medium is could be the fact that glucose together with fructose is the main carbohydrate in the semi-solid media. Our results are lined with those of [15] and [18]. Since the microorganisms can use sugars other than glucose in the biosynthesis of griseofulvin. The only source of carbon in the PDA medium is the glucose, therefore it decreasing the production of griseofulvin.

State of antibiotic molecules was determined using FT-IR. (Graph). This crude extracts exhibited broadly stretched intense peak at around 3284 cm^{-1}characteristrics of hydroxyl (O-H) groups and peak around 1442 cm^{-1} characteristics of methane stretching frequency (CH). The relatively strong adsorption peak at around 3400 cm^{-1} indicated the characteristics of amine (NH) stretching vibration and the weak one at around 3400 cm^{-1} also indicated the characteristics of IR adsorption of griseofulvin. These peaks are similar with the results of [17].

5. Conclusion

Our study brings here new evidences of potential development of filamentous fungi in mangrove environment. The original warm semi solid extraction protocol was used in this study seems to be a powerful technique for fungal compounds extraction from semi solid culture medium. It is rapid and effective and it allows extraction of excreted metabolites as well as mycelial compounds. No specific site of action of griseofulvin is demonstrated by the findings presented here. Special interest is drawn, however, to the alterations in nucleic acid and protein metabolism, because of the observation (6,7) that large amounts of griseofulvin are taken up by sensitive organisms and are present as complexes with the cellular nucleic acid and protein.

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