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Studies on production of griseofulvin

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Abstract Griseofulvin is an antifungal antibiotic and is used for the treatment of mycotic diseases of human, veterinary and plant systems. It is synthesised by many species of *Penicillium*. The exploitation of fermentation process plays a vital role in the industrial applications. This leads to techno-economic feasibility of the processes. The present review mainly discusses on the production of griseofulvin in soil, in plants and by various micro-organisms. This communication also summarises the various factors which influence the griseofulvin production and strain improvement by mutation for enhanced production.

1
Introduction

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. *Penicillium janczewski* was found to produce a substance capable of shrinking and stunting of fungal hyphae. It was named curling factor, which was later found to be griseofulvin [1]. During the next decade this antibiotic was widely employed for treating a variety of fungal diseases. This drug is fungistatic in vitro for various species of dermatophytes, for example *Microsporum*, *Epidermophyton* and *Trichophyton*. The drug has no effect on other *fungi* including *yeast*, *actinomyces* and *Nocardia*. The drug kills young and actively metabolizing cells, but it inhibits the growth of older and dormant cells. During the course for potential therapeutic compound for management of Scottish miners, it was observed that griseofulvin cured experimentally produced mycotic disease of guinea pigs [2]. The drug was then subjected to clinical trial and made available for general use.

Initially the structure of griseofulvin was reported to be 7-chloro-4,6 dimethoxy-3-cumarone-2-spiro-1′,2′-methoxy-6′-methyl-2′-cyclohexen-4′-one. The name grisan is given to the ring system, 7-chloro 2′,4,6 trimethoxy-6′ methyl 2′-grisan 3–4 dione [3]. The structures proposed by various researchers are inconsistent with the ultraviolet and infrared spectra [3, 4]. Later the structure of griseofulvin was determined by Grove et al., [5]. The present communication reviews the production of griseofulvin, factors which influence the griseofulvin synthesis, and strain improvement by mutation for enhanced griseofulvin production.

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2
Factors similarity with griseofulvin

The substance produced in liquid cultures of *Penicillium janczewskii* causes distortion of *Botrytis alli* and other fungi is found to be identical to griseofulvin [6, 7]. It has been reported that griseofulvin is a metabolic product of many species of *Penicillium* and curling factor is chemically identical to griseofulvin. It is shown to affect the growth charecteristics of many fungi. This antibiotic concentrations of 0.0001–0.001 kg/m^3 produce severe stunting, excessive branching, abnormal swelling and twisting of hyphae. The effects produced by griseofulvin are transient. It was observed that its action on *Botryis alli* is by stunting the germ tubes. This action has been demonstrated in other filamentous fungi having chitinous cell walls. Fungi such as *yeasts* and *Oomycetes* which lack chitinous cell walls are not affected. Transplants obtained from griseofulvin containing media produce normal growth [8]. Similarly it was found that esters of indole acetic acid and related compounds have similar effects on fungal hyphae but at higher concentration. Griseofulvin has no effect on seed germination and root extention of higher plants similar to that of indoleacetic acid and coumarin [1].

3
Production of griseofulvin

3.1
Production of griseofulvin in soil

Studies were carried out on the factors affecting the production of griseofulvin by *Penicillium nigricans* in an acidic sandy podzol and in a garden soil. The essential prerequisites for production of griseofulvin in both the types of soil are sterilization and enrichment with organic matter. Griseofulvin could be detected in unsupplemented autoclaved soil, or in normal soil. Garden soil was a better medium for the growth of *Penicillium nigricans*. Griseofulvin yield was decreased in soils reinfected by other soil organisms. In some cases *Penicillium nigricans* was itself antagonistic to other fungi irrespective of their ability to produce antibiotics or of fast growing habit [9]. A higher

level of nutrient was required for griseofulvin production than was the case with gliotoxin production from *Trichoderma viride*. When griseofulvin is added to fresh garden soil, it disappears rapidly after initial lag. This suggests a biological degradation of griseofulvin in soil [10].

3.2
Production of griseofulvin in plants

Griseofulvin and chloramphenicol were extracted from the tissues of broad bean (*Vicia faba*) plants grown in a solution containing the antibiotics using chloroform or ethyl acetate as solvent. Partial separation of griseofulvin and chloramphenicol from other constituents was effected by counter current distribution in CCl_4:CH_3OH:H_2O (62:35:3). Pure griseofulvin and chloramphenicol were obtained by recovery of the solute in appropriate tubes, followed by chromatography on activated Al_2O_3. A new technique was adopted for estimating griseofulvin in plant tisssue extracts based on this counter current procedure and examination of the fractions spectrophotometrically at 271 nm [11].

3.2.1
Study of uptake of griseofulvin in plants

Griseofulvin produced by *Penicillium nigricans* was translocated to the leaves through the roots of lettuce and oats in 7–14 days. The estimated concentration of griseofulvin was 0.01–0.1 kg/m^3. Roots were stunted even at the minimum concentration tested. Griseofulvin could be used as an partial systemic protectant against fungus attack [12]. Similarly, the uptake of griseofulvin in higher plants has been reported by Stokes [13]. Plants were grown in a nutrient medium containing antibiotic. Translocation was detected by bioassay of guttation drops. This was induced by transfer of plants to a humid atmosphere. It was toxic to wheat plants at a concentration of 0.005 kg/m^3 and higher concentrations were also found to be toxic. The concentration of griseofulvin in the guttation drops was directly proportional to its concentration in the nutrient medium. Griseofulvin is also accumulated in the leaves. Uptake and translocation were increased by atmospheric conditions. Conditions which favour transpiration increase uptake of griseofulvin. It was inhibited by inclusion of respiratory enzymes in the nutrient medium [13]. The translocation of griseofulvin in broad bean and tomato from water was determined by bioassay. The amount of antibiotic absorbed by broad bean was proportional to the volume of water transpired for any concentration of the solution which was treated. The decay was exponentially related to the time of exposure in tissues. This was same with tomato plants. The amount of antibiotic accumulated by both the plants was a linear function of the concentration of the treating solution. A new technique was developed for griseofulvin recovery from plants and for estimation of griseofulvin by spectrophotometric method [14].

3.3
Chemical synthesis of griseofulvin

Chemical synthesis of griseofulvin is economically not feasible, because a number of intermediate steps are involved for the final product formation. Although griseofulvin is probably prepared commercially by fermentation processes, chemical synthesis of the drug have been reported by Lednicer and Mitscher, [15]. The key step in the synthetic sequence involves an oxidative phenol coupling reaction patterned after biosynthesis of the natural product. Preparation of the moiety that is to become the aromatic ring starts by methylation of phloroglucinol with methanolic hydrogen chloride to give dimethyl ether. Treatment of that intermediate with sulfuryl chloride introduces the chlorine atom needed in the final product. Synthesis of the remaining half of the molecule starts with the formation of the monomethyl ether from orcinol. Latter method follows by a number of intermediate steps for the final product preparation. Second synthesis depend upon a double-Michael reaction to establish the spiran junction [15]. Chlorophenol is treated with chloroacetylchloride to give coumaranone. This is treated with methoxyethyl ketone in the presence of K-tert-butoxide catalyst. The ensuing double-Michael reaction gave the enantiomeric diastereoisomers of griseofulvin[15].

3.4
Microbial production of griseofulvin

3.4.1
Lab scale production

Griseofulvin was first isolated from *Penicillium griseofulvum* Dierckx [3]. It was observed that all strains of *Penicillium janczweskii* have the capacity to produce griseofulvin but all the strains of *Penicillium griseofulvum* do not have this feature. The optimum chloride concentration for optimal griseofulvin production was found to be 0.005–0.01% KCl [7]. The medium related to the Czapek-Dox, but with additional trace elements was used for the griseofulvin fermentation [16]. Bayan et al., [17] have reported the sporulation of *Penicillium griseofulvum*, *Penicillium nigricans* and *Penicillium patulum*. Permanent stocks were prepared by drying the spores grown on corn and storing the spores in sand. Both spore suspensions and vegetative cells have been reported as suitable inoculum for laboratory scale fermentation. The reported yield of griseofulvin in shake culture was 0.2 kg/m^3 by *Penicillium griseofulvum*. Strains of *Penicillium griseofulvum, Penicillium nigricans* and *Penicillium patulum* were compared for their griseofulvin synthesising ability in submerged shake culture. *Penicillium griseofulvum* was found to be the best. Similarlly, the medium constituents and additives for optimum production of griseofulvin were determined. Fe^{2+} shows a specific effect on the formation of mycelianamide, a second compound, which is considered as interfering substance. By proper selection of a strain and keeping $FeSO_4$ content in the media at 0.1% the formation of mycelianamide was eliminated [17].

The Glaxo group described the sporulation medium which provided the inoculum for their shake culture studies [18]. When the nitrogen level was increased (corn-steep liquor) to 4–5 kg/m^3, antibiotic yields improved to 11 kg/m^3 after 260 h [19]. In an another report, it was claimed that the corn-steep liquor requirement could be partially replaced by choline. The corn-steep liquor solids could be reduced to 1 kg/m^3 (nitrogen) if 1 kg/m^3 choline

was added. The resulting griseofulvin yield was superior to that produced in conventional medium. Other methyl donating compounds were also claimed to be of similar benefit [20].

The production of griseofulvin was compared using different carbon sources by *Penicillium griseofulvum*. In lactose-corn steep liquor medium, only 0.27 kg/m^3 of griseofulvin was produced as compared to 0.67 kg/m^3 in glucose medium for 35 days of fermentation. Intermittent addition of glucose during the fermentation further raised the maximum level of griseofulvin to 0.81 kg/m^3. In a sucrose medium the griseofulvin level could not reach more than 0.55 kg/m^3. In the absence of $CaCO_3$, the pH dropped to 4.4 using glucose in the nutrient medium, but with sucrose as medium constituent it dropped to 4.9. Again the pH was increased to 8.0 with corn steep liquor-lactose. The yield of griseofulvin was maximum (0.81 kg/m^3) when pH was maintained between 5.5–6.0 by the addition of $CaCO_3$ to the glucose medium. It was concluded that more griseofulvin was produced by *Penicillium griseofulvum* in the presence of glucose than other sugars [21]. Kimura et al., [22] have studied the production of griseofulvin by a soil isolate of *Penicillium* species using malt extract medium at 24 °C for 23 days. Griseofulvin and dechlorogriseofulvin were extracted using ethyl acetate as solvent. After evaporation of the solvent under vacuum, the extract is subjected to silica gel column chromatography and eluted stepwise with n-hexane-acetone [22]. Griseofulvin was isolated along with dechlorogriseofulvin from fermentation broth of *Penicillium griseofulvum* and some of these *Penicillium janczewskii*. Griseofulvin and dechlorogriseofulvin were isolated from the crude solid extraction by chromatography on alumina or by fractional crystallisation from CH_3OH or C_6H_6 [23]. Birch et al., [24] have studied the bioconversion of acetic acid into griseofulvin by *Penicillium griseofulvum*. In this study, acetic acid (carboxy C^{14}) was incorporated to an extent of at least 10% into *Penicillium griseofulvum* and griseofulvin was degraded. The activities of the various degradation products agreed within the limits of the experimental error having values calculated on the basis of activity of griseofulvin [24]. Incubation of *Penicillium urticae* with griseophenone B produced 9.9% of a dichloro analog and 2.6% of a 2′- propoxy analog of griseofulvin. The other reaction products were compared with those produced after incubation of *Penicillium urticae* with natural precursors of griseofulvin like 4-dimethyl dehydrogriseofulvin and dihydrogriseofulvin [25].

Nona et al., [26] have studied the effect of deuterium oxide on the culturing of *Penicillium janczewskii*. Fully deuterated griseofulvin was isolated in pure form from *Penicillium janczewskii* grown in a completely deuterated medium. Direct fermentation and a replacement culture technique were used for the production of griseofulvin. The isolated antibiotic was purified and the extent of incorporation of deuterium during biosynthesis at the various proton sites in the molecule was determined [26].

A new mechanism for griseofulvin production has been reported by Klimov and Efimova [27]. Experiments with the application of radioactive compounds showed that both acetate and malonate participated in the formation of griseofulvin by *Penicillium griseofulvum*. It was concluded that methanol and glycine stimulated the formation of griseofulvin [27]. However, the production of griseofulvin was improved by careful control of the growth conditions. The medium containing (in kg/m^3) glucose syrup 60, organic N (as corn steep liquor) 1.5, KH_2PO_4 4.0, KCl 1.0, $CaCO_3$ 8.0 was inoculated with cultures of *Penicillium urticae* and incubated for 13 days. At the end of fermentation, the production of griseofulvin was found to be 14 kg/m^3 [28]. To intensify the biosynthesis of griseofulvin and to increase the yield of griseofulvin, the unit rate of the growth of biomass was maintained in the range 0.004–0.007 h^{-1}. The carbon source was added at variable rates on consecutive days at 0.9, 0.4, 0.6, 0.75, 1.0, 1.25 and 1.5% by weight of the culture medium respectively [29].

The origin of the methoxy groups in griseofulvin has been described by Hockenhull and Faulds [30]. The methoxy groups of griseofulvin was labeled with C^{14}. The degradation of griseofulvin gave (β 7-chloro 2-hydroxy 4–6-dimethoxy cumaranon-2yl) butyric acid containing C^{14} labeled methyl group and CH_3 $(C_2H_5)_3$ N containing 3 labeled methyl groups per mole. Therefore, choline acts as a methyl donar in the biosynthesis of griseofulvin by *Penicillium patulum* [30].

Incorporation of doubly-labelled NaOAc (^{13}C-^{18}O) into the griseofulvin by *Penicillium griseofulvum* ATCC 1188 showed that all of its oxygen is derived from acetate, thereby confirming the earlier reports [27] on biosynthesis. The location of labelling was determined by new technique employing a spin echo pulse sequence in ^{13}C-NMR to detect ^{18}O-induced isotope shifts [31].

Terekhova et al., [32] have investigated the production of antibiotics identical to griseofulvin and ethamycin by *Streptomyces albolongus* 4297 II, isolated from actinomycetes complex 4297. Cell walls of *Streptomyces albolongus* 4297 II contained L-diaminopimelic acid, and aerial mycelium contained long, straight and wavy chains of spores. The strain antagonised growth of *Staphylococcus auereus* strains 209P and 209P//UV-2, *Bacillus subtilis* ATCC 6633, *Bacillus mycoides* R567, but it did not antagonise Gram-negative bacteria. The species were distinct from *Streptomyces griseus, Streptomyces conganiensis* and *Streptomyces griseoviridis*. Griseofulvin was a wide spectrum agent containing cystine and traces of serine and glycine, along with 2 hydroxyl groups. Mass spectrometric and x-ray analysis showed it to be griseofulvin [32]. Jimenez et al., [33] have compared the production of patulin and griseofulvin by 12 strains of *Penicillium griseofulvum* Dierckx. In this study, 11 strains were isolated from pistachio nuts and the other from the spanish type culture collection. In yeast extract medium for 6 of the 11 isolated strains did produce patulin and griseofulvin, whereas not a single strain could produce patulin in Wickerham medium. Griseofulvin production is higher in Wickerham medium and significant in the yeast extract sucrose medium. These antibiotics were separated and determined by HPLC after extraction of broth with $CHCl_3$ [33]. Two different groups of the strains have been tested which cannot be distinguished by morphological and cultural characteristics. The production of patulin and griseofulvin were found to be below the detection level on sucrose-yeast

extract and Wickerham media, whereas another groups produced in variable proportions. These isolates were differentiated by enzyme assays, viz., leucine arylamidase, phosphoamidase and β-D-glucosidase. This type of studies will help classification of the micro-organisms based on their morphological criteria [34]. Hammady [35] has studied the production of griseofulvin and patulin from an unidentified species of *Penicillium*. *Penicillium* was grown for 14 days in a sterile medium at 23 °C and the mycelium was separated from the culture broth. The solution was concentrated under vacuum and extracted three times with ethylacetate. The compound was purified with benzene and chloroform. It was identified as patulin. Mycelium was dried, pulverized and then extracted continuously in a soxhlet apparatus with petroleum ether and then with diethylether. The two solutions were concentrated leaving a residue. The residue was crystallized with absolute alcohol. The compound produced was found to be griseofulvin [35]. Similarly, Torres et al., [36] have reported the production of patulin and griseofulvin by *Penicillium griseofulvum* Dierckx using three different media. Griseofulvin was not produced with yeast extract medium. The apple medium and minimal medium were the best for griseofulvin production and patulin production respectively [36].

The production of griseofulvin was studied in a solid state fermentation using 33–70 strains of *Penicillium* and mixtures of corn mash and wheat bran as medium. The yield of griseofulvin significantly decreased when wheat bran was replaced with rice bran. However, the yield markedly increased when 8% vegetable oil or 0.1–1.0% (v/v) surfactants including detergents (Tween-80) were added to the fermentation medium. The addition of water further improved the yield of griseofulvin [37]. n-Tetracosanoic (lignoceric) acid was isolated as the byproduct in griseofulvin fermentation by *Penicillium nigricans*. Experiments were carried out to determine the rate of formation of the fatty acid, which could show marginal antibacterial activity [38].

3.4.2
Large scale production

Rhodes et al. [39] have reported the large scale production of griseofulvin under submerged aerobic conditions. 600 cm^3 medium containing, whey powder (to give 3.5% lactose and 0.05 N), 0.4% potassium hydrogen phosphate, 0.05% potassium chloride and 0.38% corn steep liquor solids was autoclaved for seed culture development to improve the griseofulvin production. It was then inoculated with a suspension of spores from Czapek-Dox agar culture of *Penicillium patulum*. It was then incubated on a shaker at 25 °C for 7 days to give a suspension containing 18×10^6 spores/cm^3. A medium (0.150 m^3) containing whey powder (to give 3.5% lactose and 0.11 N), 0.4% potassium hydrogen phosphate, 0.15% potassium chloride and 0.38% corn steep liquor solids (to give 0.04% N) in a stirred fermentor was sterilized for 20 minutes at 121 °C and inoculated with 150 cm^3 of the above seed culture. Air flow was maintained at 85 vvm for the first 24 h. It was increased to 226.5 vvm for the development of growth and foaming decreased. The temperature was 25 °C and stirring was at 350 rpm. After 28 h a satisfactory vegetative inoculum was obtained for fermentation. A medium (0.450 m^3) containing corn steep liquor solids (to give 0.21 N), 7% lactose, 0.8% limestone, 0.1% potassium chloride and 0.4% potassium hydrogen phosphate was sterilized at 121 °C for 20 minutes and inoculated with 10% (v/v) of the above vegetative inoculum. The air flow was maintained at 283.2 vvm for the first 8 h and then the air flow rate was maintained at 566.4 vvm. Temperature was 25 °C and stirring was at 350 rev/min. Mineral oil was used as antifoam agent. After 162 h the batch was filtered at pH 8.0. The filtrate was discarded and the mycelium was extracted several times with butyl acetate. It was dissolved in acetone and filtered. It was then precipitated with water to obtain 0.150 kg pure griseofulvin [39]. Similarly, large scale production of griseofulvin was carried out using thirty tons of culture medium containing corn seed 5–15, corn extract 5–8, lactose 10–14, glucose 7–15, $CaCO_3$ 10–14, KCl 1–2, KH_2PO_4 6–10, $MgSO_4$ 0.05–0.1, urea 1–2 and hydrogenated sunflower oil 2–10 kg/m^3. The medium was inoculated with 5–10% (v/v) precultivated inoculum of *Penicillium* and aerobically fermented for 300–350 h at pH 5.8–7.0 and at 26–30 °C The maximum griseofulvin production achieved 1.2 kg/m^3 without formation of intermediates [40]. Partial replacement of corn-steep liquor by ammonium sulphate has some advantage. This modification gave a yield of 14 kg/m^3 in large scale production of griseofulvin[41].

Wen [42] has discussed the development of antibiotics in China in this review which mainly focused on the development of various antibiotics including griseofulvin and their production in large scale [42]. A significant improvement in the fermentation technology of griseofulvin was developed by introduction of fed-batch process. Yield of 6 kg/m^3 was obtained after 200 h of cultivation. Application of fed-batch technique permitted an increase use in the medium nutrient concentration [43].

3.4.3
Factors influencing the griseofulvin production

(a) Effect of nitrogen

Soloveva et al., [44] have investigated the effect of nitrogen on the griseofulvin production by *Penicillium nigricans*. The medium contained (% w/v) glucose 4.0, KCl 0.1, KH_2PO_4 0.4, $CaCO_3$ 0.4% and different amount of $(NH_4)_2SO_4$ corresponding to 0.02–0.5 g% of N. If the N concentration was less than 0.04 g% or more than 0.4 g%, production of griseofulvin was inhibited by N. The retarding effect of the high N levels was explained by specific inhibition by NH_4^+ of oxaloacetate formation. The reduction of N concentration in medium did not effect the secondary metabolism of the cells [44]. Similarly, *Penicillium nigricans* (20% inoculation) was cultivated in 100 cm^3 medium at 200–220 rpm for 13 days. Griseofulvin was determined spectrophotometrically in a methylene chloride mycelium extract. Gluten was substituted for corn steep extract for the griseofulvin production in the nutrient medium. By using gluten as a nitrogen source in the medium the production of griseofulvin was found to be 0.312 and 0.426 kg/m^3 [45]. Growth of *Penicillium nigri-*

cans in a glucose mineral medium was enhanced by the addition of glucose and $NaNO_3$. Maximum yield of mycelia was observed upon addition of glucose at the time of inoculation or fractional addition of glucose-$NaNO_3$ mixture. The addition of $NaNO_3$ alone at the time of inoculation or on the fifth day of incubation inhibited subsequent development of the mycelia and maintained stable levels of ATP and ADP, whereas the addition of $NaNO_3$ on the seventh day stimulated growth and griseofulvin production. The combined addition of glucose and $NaNO_3$ eliminated the inhibitory effect, increased the ATP/ADP ratio and stimulated griseofulvin synthesis [46].

(b) Effect of inoculum

The effect of inoculum level on the production of penicillin and griseofulvin was studied by using *Penicillium chrysogenum* and *Penicillium patulum* respectively. It was reported that the biochemical factors such as the levels of the enzyme activity and efficiency were at least as important as morphology in determining the yield of the antibiotic [47].

(c) Effect of temperature

The effect of temperature on changes in ATP and ADP levels and on the growth of *Penicillium nigricans* 117 strains in the presence of different carbon sources for griseofulvin production has been investigated by Rogal and Malkov [48]. The growth of *Penicillium nigricans* 117 in mineral medium containing glucose, acetate or succinate as the C-source was found to stimulate at 28–28.6 °C. The low producing strain of griseofulvin was also stimulated by the high temperature when the medium contained acetate or glucose. It was inhibited when succinate was present in the medium. The ATP/ADP ratio paralleled the growth rate at all times, which indicates that temperature affected the energy metabolism of the microorganisms [48]. Similarly, the maximum ATP and minimum ADP concentrations were found on glucose medium and minimum ATP and maximum ADP concentrations occurred on acetate medium. The ATP/ADP ratio markedly increased at the time of maximum griseofulvin synthesis [49].

(d) Effect of pH

There is no specific reports available on the effect of pH on griseofulvin production. The yield of griseofulvin was maximum (0.81 kg/m^3) when pH was maintained between 5.5 and 6.0 by the addition of $CaCO_3$ to the glucose medium [18].

(e) Effect of addition of culture filtrate

Culture filtrate of *Candida, Rhodotorula* and *Trichosporon* inhibited the griseofulvin formation by *Penicillium nigricans*. However, the filtrate stimulates the formation of some antibiotics by specific organisms [50].

4
Bioconversion of griseofulvin

Bioconversion of griseofulvin by various fungi gave different demethylated products of griseofulvin. Incubation of griseofulvin with *Microsporum canis, Botrytis allii* and *Cerospora melonis* gave 4-demethyl griseofulvin, 2′-demethyl griseofulvin (griseofulvic acid) and 6-demethyl griseofulvin repectively [51].

5
High yielding strains of *Penicillium* for griseofulvin production

The strains of *Penicillium patulum* are induced by exposure to S^{35}. The parent culture is grown and sporulated in a synthetic medium containing sulphur mainly in the form of S^{35}. The percentage of viable mutant spores obtained is more. This technique was found to give improved yield of griseofulvin than any other mutation technique. The best strain isolated produced 2.964 kg/m^3 of griseofulvin after 13 days of incubation in shake culture containing a corn steep liquor-lactose medium in contrast to 1.640 kg/m^3 produced by the parent culture. This method promises new types of strains in large scale fermentation industry for the production of griseofulvin [52]. The biochemical characteristics of mutants of *Penicillium patulum* D-756 for higher production of griseofulvin were studied by Songgang and Yunshen [53]. Mutant showed high Cl^-tolerance. The production of griseofulvin increased with increasing Cl^- concentration and reached a maximum at 3% of Cl^- in the production medium. The mutant contained high amylase sources and the production of griseofulvin was markedly reduced when soybean or peanut powder was used as N-source. However, the production of griseofulvin was improved by the addition of defoaming agent. The optimum pH for griseofulvin production was found to be 4.8–5.2 [53].

Mutant strain was devoloped by irradiation of *Penicillium nigricans* 548 with radioactive S. The medium contained corn steep liquor as N source, glucose, lactose, and inorganic salts was used for this investigation. After 200 h of aerated fermentation, the maximum production of griseofulvin was found to be 0.17 kg/m^3 [54]. Similarly the mutagenic action on the morphology and physiology of *Penicillium nigricans* strain 548 was studied using S^{32}. It was inferred that the low lethal effect of S^{32} has got strong mutagenic action. Sulphur was used in the form of sodium sulphate ($Na_2S^{35}O_4$) at 0.001 kg/m^3 in Bonner and Czapek agar in which the fungus was grown for a period of 10–60 days at a temperature of 24 °C. The spores were then washed and grown on Czapek agar with 0.2 cm^3 commercial extract. The survival rate of spores were declined by 6-fold for culture medium having S and by100-fold for medium having isotope of S at 40^{th} day. Morphological variations was shown by 8.5–15.2% of colonies from treated spores. The variants were divided into five types. Type two which has an appearance of grey-white is more abundant on Czapek corn and has more range (40–160%) in antibiotic yield on Bonner's medium. With stable sulphur low producers were developed. Czapek medium was better to use in studies, both as control and with the isotope. Bonner isotope agar produced antibiotic with greater variablity (80–160%) and with Czapek medium the variablity was 60–100%. The high producing strains did not retain their superiority [55].

Kommmunarskaya and Zhukova [56] have studied the lethal and mutagenic effects of N-nitrosomethylurea on

Penicillium nigricans for griseofulvin production. The treatment of *Penicillium nigricans* conidia with N-nitrosomethylurea at pH 5.6, 6.4, or 8.4 for 2–7 h, decreased the survival rate and increased the frequency of mutation. The highest number of productive variants was obtained after treatment with the mutagen in 0.2% solutions at pH 5.6 and 6.4. Mutants requiring proline, arginine, threonine, adenine, uracil, riboflavin, biotin or ammonia N were obtained. Variants requiring adenine or uracil showed the lowest production of griseofulvin [56]. Similarly some morphological mutations in *Penicillium nigricans* was induced by exposing to UV radiation after pretreatment with 2% (v/v) of N-nitroso-N-methylbiuret. The variability of the fungi was increased with respect to griseofulvin production by 40–140%. Pretreatment of conidia with N-nitroso-N-methylbiuret decreased the lethal effect of UV light by 15–25 times and the variability of morphology decreased by 1.5–2.5 times. The possible use of N-nitroso-N-methylbiuret in the selection of *Penicillium* has increased the ability of antibiotic production [57].

Mutant of *Penicillium nigricans* was developed by exposing UV-radiation after pretreatment with N-nitroso-N-methylurea for enhanced griseofulvin production. The productivity of mutant was 1.33 times more than that of the parent strain [58].

Kiuchi et al., [59] have reported the transformation of *Penicillium urticae* with plasmids containing the hygromycin-B resistance. A protoplast transformation system was developed with efficiency of 6×10^3–50×10^3 stable transformants/cm^3 DNA for enhanced griseofulvin production. The transformation system used plasmid pDH25MC and its derivatives containing fragments of the *Penicillium urticae* genome. Tandem repeated integration and random integration of vector DNA were observed. Although *Penicillium urticae* was able to grow in the presence of 0.2 kg/m^3 hygromycin-B, transformation were resistant to more than 5.0 kg/m^3 hygromycin-B [59].

6
Determination of griseofulvin

Dasu et al., [60] have discussed the various analytical techniques, viz., spectrophotometry, spectroflurometry, paper chromatography, thin layer chromatography, gas chromatography, high performance liquid chromatography and microbiological assay for the determination of griseofulvin in pure form, in different chemical mixtures, in biological fluids and in fermentation samples [60].

7
Conclusions

The production of griseofulvin in soil, in plants and microbial production in lab scale and in large scale have been reviewed in this paper. It was also highlighted on the development of strains by mutation for higher production. Fed-batch and continuous processes are to be developed for enhanced griseofulvin production. Protoplast technology and r-DNA technology require to be developed for enhanced griseofulvin production to meet the required demand and the use of cellulosic material as alternative substrate.

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