Research Article

EFFECT OF DIFFERENT CARBON AND NITROGEN SOURCES ON *ASPERGILLUS TERREUS* ANTIMICROBIAL METABOLITE PRODUCTION

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ABSTRACT

In the present investigation, the soil mold *Aspergillus terreus* was isolated and screened for antibiotic production and effect of variation of carbon and nitrogen sources was observed for maximum antimicrobial metabolite production. The antimicrobial substance production in *A. terreus* was greatly influenced by addition of dextrose reaching the highest antimicrobial activity (followed by fructose, xylose and negligible antimicrobial activity was observed in case of mannose and maltose. In case of nitrogen sources, the maximum production of the antimicrobial metabolite in *A. terreus* was observed in the presence of sodium nitrate The antimicrobial activity reduced to about half in the presence of ammonium sulphate, and no production of metabolite in the presence of ammonium chloride. There was a negligible antimicrobial activity when yeast extract and peptone were used as nitrogen sources. It may be suggested from the present study that dextrose may be used as carbon source and sodium nitrate as nitrogen source for maximum production of antimicrobial metabolite from *Aspergillus terreus*.

Keywords: *Aspergillus terreus*, antimicrobial metabolite, fermentation.

INTRODUCTION

Despite critical need for new antibiotics to treat drug-resistant infections and other infectious diseases, very few new antibiotics are being developed. There is a huge variation in the time for emergence of resistance, which varies among organisms and antibiotics. At this point, a new antibiotic is required, which is active against resistant bacteria. In response to microbial resistance, the pharmaceutical industry has produced a remarkable range of antibiotics[1]. Not only is there a problem in finding new antibiotics to fight old diseases (because resistant strains of bacteria have emerged), there is a parallel problem to find new antibiotics to fight new diseases. Keeping in view these facts, there is an urgent need to discover new antibiotics to treat patients infected with multidrug-resistant infections[1].

Microorganisms are a virtually unlimited source of novel chemical structures with many potential therapeutic applications[2]. Complex products derived from plants and animals may prove more difficult due to the rarity of the species and difficulty in cultivation or collecting raw materials. Microbiological diversity is enormous and has only partially been investigated. Since microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites. Generally, the reason why they produce such metabolites is not known, but it is believed that many of these metabolites may act as chemical defense as an adaptation of fungi competing for substrates[3].

Aspergillus species isolated from various soils have been found to produce antibacterial, antifungal and antitumour metabolites. Species of the mold *Aspergillus* are known to produce mycotoxins, organic acids and antibiotics[4-6]. *A. terreus* is an especially prolific producer of secondary metabolites. A few of the compounds that are produced by *A. terreus* are aspulvinone, asterric acid, asterriquinone, butyrolactone I, citrinin, emodin, geodin, itaconate, lovastatin, questrin, sulochrin, and terrecyclic acid[7,8]. Keeping in view the above justifications, the objective of the present research was to isolate and screen the soil fungi for antibiotic production and to observe the effect of variations in carbon and nitrogen sources for maximum metabolite production from *A. terreus*.

MATERIALS AND METHODS

Isolation and identification of soil antibiotic producing fungi

The soil samples were collected from various sites rich in organic matter e.g. areas receiving industrial wastes, mushroom farm, crop fields, rotten wood soil, leaf litter, farmhouse backyards, household wastes and vegetables refuses. After collection, the sample bags were labeled with date and site of collection. The soil samples were processed immediately for determining moisture content and pH. The serial dilution agar plate method was used for the isolation of *A. terreus* from the soil samples [9, 10]. The fungus was identified following volumes/ monographs/ manuals [11, 12].

Procurement of microbial cultures

The selected test pathogens were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, which included Gram positive bacteria, *Streptococcus mutans*

(MTCC 1943, 890 and 497) *Staphylococcus aureus* (MTCC 3160) and *Lactobacillus casei* (MTCC 1423); Gram-negative bacteria, *Pseudomonas aeruginosa* (MTCC 2295) and *Escherichia coli* (MTCC 43) and two yeasts namely *Candida albicans* (MTCC 3017, 227 and 183) and *Saccharomyces cerevisiae* (MTCC 170). The slants of Brain heart infusion agar were used for maintaining *S. mutans* strains, Rogosa agar for *L. casei*, Nutrient agar for *P. aeruginosa, S. aureus* and *E. coli* and Malt extract agar for *C. albicans* strains and *S. cerevisiae*. All the slants were kept at 4^0C in the refrigerator for future studies. The inoculum of different test pathogens was adjusted according to above prepared 0.5 McFarland standard. The McFarland tube was stored at 4-5^0C and was prepared afresh after every 3 to 4 months [13].

Evaluation of antimicrobial activity of *A. terreus* by using overlay culture plate method

The antimicrobial activity of purified *A. terreus* isolate was tested by using overlay culture plate method. The fungal isolates tested for antimicrobial activity were inoculated into the center of the presolidified Czepak Dox Agar plates and incubated at 25° C for 3 to 4 days. The plates were overlaid with a thin film of molten malt extract agar medium (45°C) containing 24 hrs old cultures of test pathogens. All the overlaid plates were incubated at 35°C for 24 hrs to allow the growth of test pathogens. Antimicrobial activity of fungal isolates against test pathogens was indicated by zone of growth inhibition around the fungal colonies showing positive test for its antimicrobial activity [14, 15].

Effect of carbon sources

The optimization of carbon source used in fermentation broth (Czepak Dox Broth) during the antimicrobial metabolite production in *A. terreus* was carried out by employing various carbon sources such as dextrose, xylose, fructose, maltose and mannose[16, 17]. For each sugar, 200 ml of the fermentation broth was substituted with dextrose, maltose, mannose, xylose and fructose in 500 ml Erlenmeyer's flasks and autoclaved at 121°C for 15 minutes. Three replicates were used for each sugar. Unmodified fermentation broth was used as negative control and ciprofloxacin and ketoconazole as positive control. One, six mm disk, cut from four days old fungal colony of isolate grown on Czepak Dox agar was added to each flask. The inoculated flasks were incubated at 25°C for 8 days under stationary condition. The filtration was done through sterilized Whatman filter paper no. 1 and various filtrates were assayed for antimicrobial activity against test pathogens using agar well diffusion assay.

Effect of nitrogen sources

For evaluating the most suitable nitrogen source for the antimicrobial metabolite production, the fermentation broth (Czepak Dox Broth) was supplemented with various organic (peptone and yeast extract) and inorganic (ammonium sulphate [$(NH_4)_2SO_4$]; sodium nitrate [$NaNO_3$] and ammonium chloride, [NH_4Cl]) nitrogen sources [16, 17]. 200 ml of CDB was taken in each 500 ml Erlenmeyer's flasks and was supplemented with different nitrogen sources (1 %) and autoclaved at 121°C for 15 minutes. Three replicates were used for each nitrogen source. Unmodified CDB was used as negative control and ciprofloxacin and ketoconazole as positive control. Each flask was inoculated with one, six mm disk, cut form four days old colony of fungal isolate, grown on Czepak Dox Agar. Inoculated flasks were incubated at 25°C for 8 days under stationary condition. The filtration was done through sterilized Whatman filter paper no. 1 and various filtrates were used for testing the antimicrobial activity against test pathogens using agar well diffusion assay.

RESULTS

In the present endeavour, the soil samples were collected from various sites rich in organic matter like areas receiving food and rice industry waste, mushroom farm, crop fields, rotten wood soil, leaf litter, farmhouse backyards, household wastes and vegetables refuses. The soil fungal isolate was identified as *Aspergillus terreus* using parameters such as colony morphology, colour of the colony and the sporulating structures, it is a deuteromycetous mold, belongs to the class *Hyphomycetes*, order *Moniliales* and family *Moniliaceae*.

There was a high degree of variation in the level of antimicrobial activity in the present study when the different carbon sources were tested in the fermentation medium (Table 1). The antimicrobial substance production in *A. terreus* was greatly influenced by addition of dextrose reaching the highest antimicrobial activity (22 to 26mm), followed by fructose (12 to 16mm), xylose (11 to 12mm) (inhibiting only few test organisms), and in case of mannose and maltose, there was a negligible antimicrobial activity.

In the present study, sodium nitrate, ammonium sulphate, ammonium chloride, yeast extract and peptone were used as nitrogen sources (Table 2). The maximum production of the antimicrobial metabolite in *A. terreus* was observed in the presence of sodium nitrate as nitrogen source (22 to 28mm) when the metabolite was filtered and assayed against test pathogens. The diameter of zone of inhibition was 25 to 28mm against three strains of *S. mutans*, 25mm against *S. aureus*, 24mm against *L. casei*, 24mm against *E. coli,* 23mm against *P. aeruginosa*, 20 to 23mm against three strains of *C. albicans* and 20mm against *S. cerevisiae.* The antimicrobial activity reduced to about half in the presence of ammonium sulphate (9 to 12mm), and no production of metabolite in the presence of ammonium chloride. There was a negligible antimicrobial activity when yeast extract and peptone were used as nitrogen sources.

Table 1: Effect of various carbon sources on the production of antimicrobial metabolite in *Aspergillus terreus* tested by agar well-diffusion assay against test pathogens.

Carbon source	Zone of growth inhibition (mm)[a]										
	Sm1	Sm2	Sm3	Sa	Lc	Ec	Pa	Ca1	Ca2	Ca3	Sc
Dextrose	25±0.57	26±0.81	25±0.37	26±0.57	25±0.57	24±0.37	25±0.57	22±0.37	23±0.57	22±0.81	24±0.57
Maltose	NA	NA	NA	17±0.37	NA	NA	NA	NA	NA	NA	17
Mannose	13±0.57	NA	NA	12±0.37	NA	NA	NA	NA	NA	NA	NA
Xylose	13±0.37	12±0.57	12±0.37	NA	NA	NA	NA	NA	NA	NA	NA
Fructose	12±0.57	13±0.57	NA	NA	13±0.57	17±0.57	16±0.81	NA	NA	NA	NA
Ciprofloxacin (5µg)	NA	NA	NA	NA	NA	NA	NA	ND	ND	ND	ND
Ketoconazole (10µg)	ND	ND	ND	ND	ND	ND	ND	10±0.37	NA	NA	NA

Table 2: Effect of various nitrogen sources on the production of antimicrobial metabolite in *Aspergillus terreus* tested by agar well-diffusion assay against test pathogens.

Nitrogen source	Zone of growth inhibition (mm)[a]										
	Sm1	Sm2	Sm3	Sa	Lc	Ec	Pa	Ca1	Ca2	Ca3	Sc
Ammonium sulphate	9±0.57	10±0.81	9±0.57	12±0.57	10±0.81	9±0.57	10±0.57	10±0.81	11±0.57	10±0.81	9±0.57
Sodium nitrate	28±0.81	25±0.37	25±0.57	25±0.57	24±0.81	24±0.57	23±0.57	20±0.81	22±0.57	23±0.37	20±0.57
Peptone	NA	NA	NA	10±0.37	NA	NA	NA	NA	NA	NA	10±0.57
Yeast Extract	NA	NA	NA	10±0.37	NA	11±0.57	10±0.37	NA	NA	NA	NA
Ammonium chloride	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Czepak Dox Broth	25±0.57	26±0.57	25±0.37	26±0.57	25±0.57	24±0.57	25±0.81	22±0.57	23±0.57	22±0.37	24±0.37
Ciprofloxacin (5µg)	NA	NA	NA	NA	NA	NA	NA	ND	ND	ND	ND
Ketoconazole (10µg)	ND	ND	ND	ND	ND	ND	ND	10±0.37	NA	NA	NA

NA- No Activity; ND- Activity Not Determined[a] Mean of diameter of zones of inhibition in three replicates; ± Standard deviation

Abbreviations:

Sm1- *Streptococcus mutans* (MTCC No. 1943), Sm2- *S. mutans* (MTCC No. 890), Sm3- *S. mutans* (MTCC No. 497), Sa- *Staphylococcus aureus* (MTCC No. 3160), Lc- *Lactobacillus casei* (MTCC No. 1423), Ec-*Escherichia coli* (MTCC No. 43), Pa-*Pseudomonas aeruginosa* (MTCC No. 2295), Ca1- *Candida albicans* (MTCC No. 3017), Ca2-*C. albicans* (MTCC No. 227), Ca3- *C. albicans* (MTCC No. 183), Sc-*Saccharomyces cerevisiae* (MTCC No. 170)

DISCUSSION

For the past five decades, the need for new antibiotics has been met largely by semisynthetic tailoring of natural product scaffolds discovered in the middle of the 20th century. More recently, however, advances in technology have sparked a resurgence in the discovery of natural product antibiotics from microbial sources. In particular, efforts have refocused on finding new antibiotics from old sources (for example, streptomycetes) and new sources (for example, other actinomycetes, cyanobacteria, uncultured bacteria and fungi). This has resulted in several newly discovered antibiotics with unique scaffolds and/or novel mechanisms of action, with the potential to form a basis for new antibiotic classes addressing bacterial targets that are currently underexploited. Natural products represent the traditional source of new drug candidates [1]

Soil sustains an immerse diversity of microbes, which to a large extent, remains unexplored. Bacteria including actinomycetes and fungi are most preferably used as screening sources from various habitats. Fungi are well known as prolific producers of biologically active natural products. Most of the naturally occurring antibiotics have been isolated from soil microorganisms. These substances play a significant role in their establishment on (rhizoplane) and around (rhizosphere) the roots of plants [18].

Variations in the fermentation environment often result in an alteration in antibiotic production. The alteration involves changes both in yields and in the composition of the substances. The choice of carbon sources greatly influenced secondary metabolism and therefore antibiotic production [19, 20]. Different carbon sources, like dextrose[21], lactose[22], sucrose[23], fructose[24], starch[25] and glycerol[26] have been reported to be suitable for production of secondary metabolites in different microorganisms. Sometimes quickly metabolized substrate such as glucose may achieve maximum cell growth rates, but is known to inhibit the production of many secondary metabolites. This "catabolite repression" is thought to be due to intermediates generated from the rapid catabolism of

glucose interfering with enzymes in the secondary metabolism process[20].

The nature of the nitrogen source used has a notable effect on the production of the antimicrobial metabolite in *A. terreus.* High nitrogen levels have been noted to repress idiophase production of antibiotics[27]. Control of ammonia concentration during the mid-cycle was found to be important in the optimization of idiophase secondary metabolite production[28], though this may reflect the role of nitrogen in growth promotion. The use of certain amino acids as a nitrogen source can inhibit good synthesis of secondary metabolites[19]. Various nitrogen sources such as soybean flour[29], fish meal[30], peptone[31], potassium nitrate[32], sodium nitrate[33], asparagine[34], arginin[26], valine and isoleucine and L-histidine[35] have been reported by various workers, while studying the suitability of nitrogen sources for the production of metabolites from microorganisms.

Thus, the results of antimicrobial susceptibility tests indicated that antimicrobial metabolite obtained from *A. terreus* may be produced optimally in the presence of dextrose sugar and sodium nitrate as a nitrogen source. It may be suggested that further research is needed for determining the chemical structure of metabolite which is responsible for bioefficacy and to determine the cytotoxicity before it is used for commercialization purposes.

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