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Effect of fermentation medium, pH and temperature variations on antibacterial soil fungal metabolite production

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Infectious diseases caused by pathogenic microorganisms have been the leading cause of morbidity and mortality in human history. Despite critical need for new antibiotics to treat drug-resistant infections and other infectious diseases, very few new antibiotics are being developed. Natural products offer a vast source of chemical diversity and yield unusual and unexpected lead structures. Since microorganisms grow in unique and extreme habitats, they may have the capability to produce unique and unusual metabolites. Because only a tiny fraction of soil microbes from soil are readily cultured, soil might be the greatest untapped resource for novel chemistry. In the present investigation, the soil mold *Aspergillus terreus* was isolated and screened for antibiotic production and effect of medium, pH and temperature variations for the maximum production of antibacterial metabolite were optimized for maximum antimicrobial metabolite production. The maximum production of antibacterial metabolite was observed in Potato dextrose broth under pH 6.0 and incubation temperature of 25^0C. The minimum inhibitory concentration of the fungal metabolite against the test pathogens ranged between 1 to 4 µg/ml. It may be suggested from the present study 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.

Key words: *Aspergillus terreus*, antibacterial metabolite, optimization, fermentation

Introduction

Infectious diseases caused by pathogenic microorganisms have been the leading cause of morbidity and mortality in human history. The discovery of the antibacterial compound from the mold *Penicillium notatum* by Alexander Fleming led to the development of antibiotics, which are still the main weapons for combating the deadly bacterial infections at the present time. However, over

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60 years of application of antibiotics leads to the development of antibiotic resistance of many bacterial pathogens (Emmert *et al*., 2004). Consequently, microbial infections have again become the most common and deadly causes of human diseases.

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 (Luzhetskyy *et al*. 2007). 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 (Luzhetskyy *et al*. 2007).
Natural products have a long history of success as biologically active leads for therapeutic agents. Natural products offer a vast source of chemical diversity and yield unusual and unexpected lead structures. In recent years, pharmaceutical natural products research has tended to favour compounds derived from bacteria and fungi because of their diversity of species and, ease of culture and because if active compounds are discovered, generally it is possible to culture and manipulate microorganisms to enhance the production of the wanted compounds (Luzhetskyy *et al*., 2007). Microorganisms are a virtually unlimited source of novel chemical structures with many potential therapeutic applications (Behal, 2000). 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 (Gallo *et al*., 2004). One gram of soil may harbour up to 10 billion microorganisms of possible thousands of different species (Rosello-Mora and Amann, 2001). As less than 1% of the microorganisms observed under microscope, soil ecosystems are to large extent, uncharted (Torsvik and Ovreas, 2002). Because only a tiny fraction of soil microbes from soil are readily cultured, soil might be the greatest untapped resource for novel chemistry (Handelsman *et al*., 1998). Although several hundreds of compounds with antibiotic activity have been isolated from

microorganisms over the years, but only a few of them are clinically useful (Thomashow *et al*., 2008). The reason is that only compounds with selective toxicity can be used clinically - they must be highly effective against a microorganism but have minimal toxicity to humans. The discovery and development of effective antimicrobial drugs with novel mechanisms of action have become an urgent task for infectious disease research programs (Thomashow *et al*. 2008). Keeping in view the above justifications, the objectives of the present research were to isolate and screen the soil fungi for antibiotic production and to observe the effect of medium, pH and temperature for antibacterial metabolite production from soil fungi and to purify and determine the minimum inhibitory concentration of the fungal metabolite against the test pathogens.

Materials and methods

Isolation and identification of soil antibiotic producing fungi

The soil samples were collected from various other 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 fungi from the soil samples (Cappuccino and Sherman, 1996; Aneja, 2003). All the fungal cultures were identified up to generic/species level following volumes/monographs/manuals (Gilman, 1967; Ellis, 1971; Barron, 1972; Domsch *et al*., 1980; Sutton, 1980; Alexopoulos *et al*. 1996; Kirk *et al*. 2001).

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 Nos. 1943, 890 and 497) *Staphylococcus aureus* (MTCC No.3160) and *Lactobacillus casei* (MTCC No. 1423); Gram-negative bacteria, *Pseudomonas aeruginosa* (MTCC No. 2295) and *Escherichia coli* (MTCC No. 43) and two yeasts namely *Candida albicans* (MTCC Nos. 3017, 227 and 183) and *Saccharomyces cerevisiae* (MTCC No. 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 (Andrews, 2001).

Evaluation of antimicrobial activity of fungal isolates by using overlay culture plate method

The antimicrobial activity of purified fungal isolates was tested by using overlay culture plate method (Casida, 1997; Corrado and Rodrigues, 2004). Of the 200 soil samples screened, 127 isolates of fungi were obtained following serial dilution agar plate method. Out of 127 fungal isolates, the 10 fungal isolates: 7, 12, 19, 28, 107 (*Aspergillus* spp.), 50 (*Cephalosporium* sp.), 70, 85, 98 (*Penicillium* spp.) and 103 (*Cladosporiun* sp.) were effective against test pathogens.

On the basis of maximum diameter of zone of growth inhibition against test pathogens obtained from screening of fungal isolates, the fungal isolate no. 107 was the most promising and was selected for detailed study. Identification of the fungal isolate no. 107 was done on the basis of colony characteristics (colony growth, colour and production of exudate) and sporulating structures (conidiogenous cells, vesicle, conidial head and conidia) by following various manuals) (Gilman, 1967; Ellis, 1971; Barron, 1972; Domsch *et al*., 1980; Sutton, 1980; Alexopoulos *et al*. 1996; Kirk *et al*. 2001).

Effect of fermentation medium for antimicrobial metabolite production

The production of antimicrobial metabolite by the selected *Aspergillus terreus* was studied in five fermentation broths (Gupte and Kulkarni, 2002) namely Potato dextrose broth, Malt extract broth, Malt extract peptone broth, Yeast extract broth and Czepak dox broth. Two hundred ml of each broth was taken in 500 ml Erlenmeyer flasks. These flasks were autoclaved at 121°C, for 15 minutes. Each flask was inoculated with six mm disk of the fungus inoculum grown on PDA. The inoculated flasks were incubated at 25°C for 8 days under stationary condition. The broth was filtered through sterilized Whatman filter paper 1 and the culture filtrates were then tested for antimicrobial activity against test pathogens using agar well diffusion assay.

Effect of pH and Temperature variations on antimicrobial fungal metabolite production

Effect of pH and temperature variations for the maximum production of antimicrobial metabolite from *A. terreus*, was done by using the modified methods outlined by several workers (Stanbury et al., 1997; Cazar *et al*., 2004; El-Tayeb *et al*., 2004; Rizk *et al*., 2007).

Effect of pH

The optimization of pH of the fermentation broth for antimicrobial metabolite production was done by carrying out the fermentation at six different pH values viz. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (Furtado *et al*., 2005). For each pH value, 200 ml of PDB (adjusted to desired pH by using either 1N NaOH or 0.1 N HCI) was taken in 500 ml Erlenmeyer's flasks. These flasks were autoc1aved at 121°C for 15 minutes. Three replicates were used for each pH values. One, disk of six mm diameter, cut from four days old colony of *A. terreus*, grown on PDA was added as an inoculum in 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 tested for antimicrobial activity against the test pathogens by using agar well diffusion assay.

Effect of incubation temperature

The optimization temperature for antimicrobial metabolite production in *A. terreus* was carried out at five different incubation temperatures viz. 20, 25, 30, 35, and 40°C (Cazar *et al*., 2004). 200 ml of PDB was taken in 500 ml Erlenmeyer's flasks and autoc1aved at 121°C for 15 minutes. Three replicates were used for each chosen temperature. Each flask was inoculated with inoculum of the fungus with one, six mm disk, cut from four days old colony. The inoculated flasks were incubated at the five incubation temperatures for 8 days under stationary condition. The filtration was done through sterilized Whatman filter paper no. 1 and various filtrates were tested for antimicrobial activity against test pathogens by using agar well diffusion assay.

Extraction and Partial Purification of antimicrobial metabolite with various solvents

Five different organic solvents namely acetone (100%) (Ranbaxy), chloroform (100%) (Ranbaxy), ethyl acetate (100%) (Ranbaxy), dichloromethane (100%) (Ranbaxy) and dimethylsulphoxide (100%) (Ranbaxy) were tested for extraction of antimicrobial metabolite from *A. terreus* (Saxena et al., 2007). Fermentation broth and solvent (1:1) were taken in separating funnel and shaken vigorously for 5 minutes and kept without any disturbance for another 15 minutes to separate the solvent from aqueous phase. Antimicrobial activity of each extract extracted with different solvents was carried out by using agar well diffusion assay. The maximum extraction of

antimicrobial metabolite was observed with chloroform which exhibited the best antimicrobial activity against all the test pathogens. The chloroform extract was dried by the repeated addition of Na_2S0_4 and concentrated by distillation which resulted in golden oily metabolite.

Thin Layer Chromatography (TLC)

The golden coloured oily metabolite obtained after chloroform extraction was subjected to thin layer chromatography to detect the various components present in the crude antimicrobial metabolite of *A. terreus* (Thomashow *et al*., 2008). After TLC, the golden oily metabolite was further subjected to hexane: petroleum ether (95:5) solvent system to remove remaining impurities. The procedure was repeated until the pure white powdered metabolite was obtained.
Determination of minimum inhibitory concentration (MIC) of powdered metabolite

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). MIC of the powdered antimicrobial metabolite against all the test pathogens was determined by a macrodilution broth assay. In this method, the test concentrations of white powdered antimicrobial metabolite were made from 64 to 0.125 µg/ml in ten sterile tubes labelled1-10. 100µl sterile Mueller Hinton broth (HiMedia) was poured in each sterile tube and then 200µl metabolite was added in tube 1. Two fold serial dilutions were carried out from tube 1 to the tube 10 and excess broth (100µl) was discarded from last tube no. l0. To each tube, 100µl of inoculum, adjusted to 0.5 McFarland standard was added. Positive control (containing inoculum but no metabolite) and negative control (containing metabolite but no inoculum) were made for the comparison. All the tubes were incubated for 24h at 37°C.

Results

In the present endeavour, 200 soil samples were collected to isolate antibiotic producing fungi by using the serial dilution agar plate method. 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.

During the isolation of antibiotic producing microorganisms from 200 soil samples, a total 127 fungal isolates were obtained. The fungal isolates identified mainly belonged to species of *Aspergillus* (20), *Penicillium* (25), *Alternaria*

(20), *Fusarium* (23), *Mucor* (20), *Rhizopus* (10), *Cladosporium* (02), *Cephalosporium* (02), *Gliocladium* (03), and *Colletotrichum* (02).

The fungal isolates were identified on the basis of colony characteristics (colony growth, colour and production of exudate) and sporulating structures (conidiogenous cells, vesicle, conidial head and conidia) by consulting manuals as referred above.

The fungal isolates were screened against test pathogens by agar overlay culture method. Out of 127 fungal isolates, 10 fungal isolates: 7, 12, 19, 28, 107 (*Aspergillus* sp.), 50 (*Cephalosporium* sp.), 70, 85, 98 (*Penicillium* sp.) and 103 (*Cladosporiun* sp.) were effective against test pathogens.

The 10 promising fungal isolates exhibiting antibacterial as well as antifungal activity were further evaluated for production of antimicrobial metabolite by inoculating them in the Potato dextrose broth (PDB) and incubating them at 25^0C for 8 days. After the eighth day, the liquid metabolite produced from these isolates were evaluated against test pathogens by agar well diffusion assay. These 10 promising fungal isolates which were antifungal as well as antibacterial were mainly species of *Aspergillus* (5), *Penicillium* (3), *Cladosporium* (1) and *Colletotrichum* (1). The fungal isolates were categorized highly effective, moderately effective and ineffective on the basis of diameter of zone of growth inhibition showed against test organisms (Table 1 and Fig.1). The moderate antimicrobial activity was shown by the following fungal isolates: 19, 50 and 70. The isolates 7, 12, 28, and 85 were ineffective as most of the test organisms were resistant to the antimicrobial metabolite secreted by these fungal isolates. Isolates 98, 103 and 107 were highly effective against all the organisms as they showed susceptibility towards the antimicrobial metabolites.

On the basis of the results obtained in the form of diameter of zone of growth inhibition of test pathogens, isolate 107 was found to be most effective of all the fungal and bacterial isolates and seems to be wide spectrum in its mode of action as it inhibited the growth of all the test pathogens. The fungal isolate 107 was subjected to identification using parameters such as colony morphology, colour of the colony and the sporulating structures by referring to various manuals and monographs mentioned earlier. The isolate was identified as *Aspergillus terreus*, which is a deuteromycetous mold, belonging to the class *Hyphomycetes*, order *Moniliales* and family *Moniliaceae* and which shows optimum growth on Potato dextrose agar at 25^0C.

The present study revealed that out of the five fermentation broths tested namely Potato dextrose broth (PDB), Malt extract broth (MEB), Malt extract peptone broth (MEPB), Yeast extract broth (YEB) and Czepak dox broth (CDB), PDB was found to be the best for the production of antimicrobial

metabolite in *A. terreus* as it showed maximum diameter of zone of growth inhibition 30 to 37 mm against all the three strains of *S. mutans*, 17mm diameter zone of inhibition against *S. aureus, L. casei, E. coli* and *P. aeruginosa*, 28 to 30mm diameter zone of inhibition against three strains of *C. albicans* and *S. cerevisiae* (Table 2 and Fig.2). The other four fermentation broths could not be selected as there was negligible activity shown towards the test pathogens when the culture filtrate was used for evaluating antimicrobial activity.

In the present study, *A. terreus* grew at all pH values tested viz. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (Table 3 and Fig. 3). The antimicrobial activity assayed by culture filtrate against test pathogens was found maximum at pH 6.0 as the diameter of zone of growth inhibition was ranging from 22 to 28 mm. The diameter of zone of inhibition was 25 to 28mm against three strains of *S. mutans*, 24mm against *S. auerus*, 25mm against *L. casei*, 26mm against *E. coli*, 25mm against *P. aeruginosa*, 22 to 23mm against three strains of *C. albicans* and 24mm against *S. cerevisiae*. The diameter of zone of growth inhibition was found to reduce at pH 5.0 and 5.5 (19 to 24 mm), pH 7.0 (19 to 24 mm) and pH value 7.5 (18 to 21mm) and reduced inhibition zones (10 to15mm) at pH 8.0.
The present study also revealed that the production of the antimicrobial metabolite in *A. terreus* has been found in the temperature range of 20 to 35^0C (Table 4 and Fig. 4). The metabolite when filtered and assayed against test pathogens showed maximum inhibitory activity at the incubation temperature of 25^0C (22 to 28 mm). The diameter of zone of inhibition was 25 to 28mm against three strains of *S. mutans*, 26mm against *S. aureus*, 25mm against *L. casei*, 24mm against *E. coli* 25mm against *P. aeruginosa* 22 to 23mm against three strains of *C. albicans* and 24mm against *S. cerevisiae.* The antimicrobial activity was decreased at 20^0C (10 to13 mm), at 30^0C (20 to 23mm), at 35^0C (20 to 23mm) and completely inhibited at 40^0C. Antimicrobial metabolite production increased with the increase in temperature from 20 to 25^0C. However, as temperature was increased from 25 to 35^0C, there was a decline in antimicrobial metabolite production. Thus, 25^0C is the most suitable temperature for the antimicrobial metabolite production.

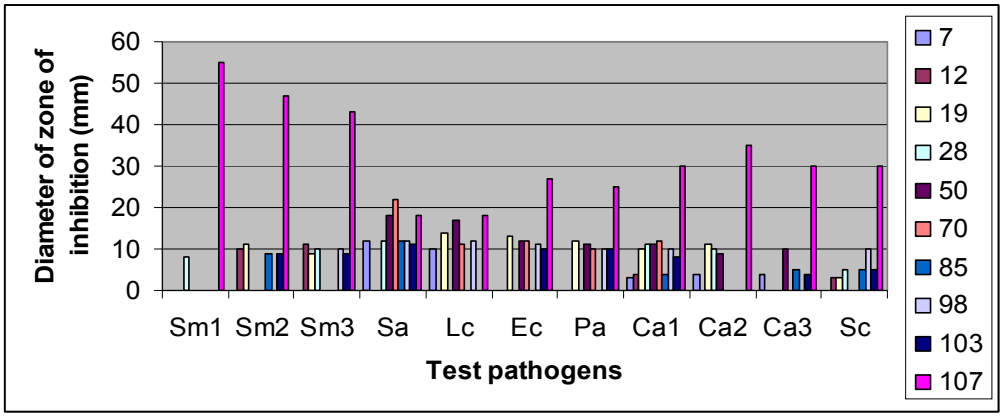
Of the five organic solvents namely acetone, chloroform, ethylacetate, dichloromethane and dimethylsulphoxide (DMSO) (Table 5 and Fig.5) used for the extraction of antimicrobial metabolite from *A. terreus*, the maximum extraction resulted with chloroform in all the test microorganisms (22 to 26mm) when the metabolite was filtered and assayed. The diameter of zone of inhibition was 25 to 26 mm against three strains of *S. mutans*, 26mm against *S. aureus*, 25mm against *L. casei*, 24mm against *E. coli,* 25mm against *P. aeruginosa,* 22 to 23mm against three strains of *C. albicans* and 24mm against

S. cerevisiae. The antimicrobial activity of the metabolite was reduced in ethyl acetate (11 to 23mm), dichloromethane (8 to 15mm) and dimethylsulphoxide (10 to 12mm) against all the test pathogens. In acetone, the metabolite lost its complete antimicrobial activity.

The chloroform-extracted fraction concentrated by distillation resulted to 700 mg purified golden coloured, oily residue after extraction of 20 liters of fermentation broth. This residue on subjecting to TLC showed single band with relative flow (R_f) of 0.86 respectively.

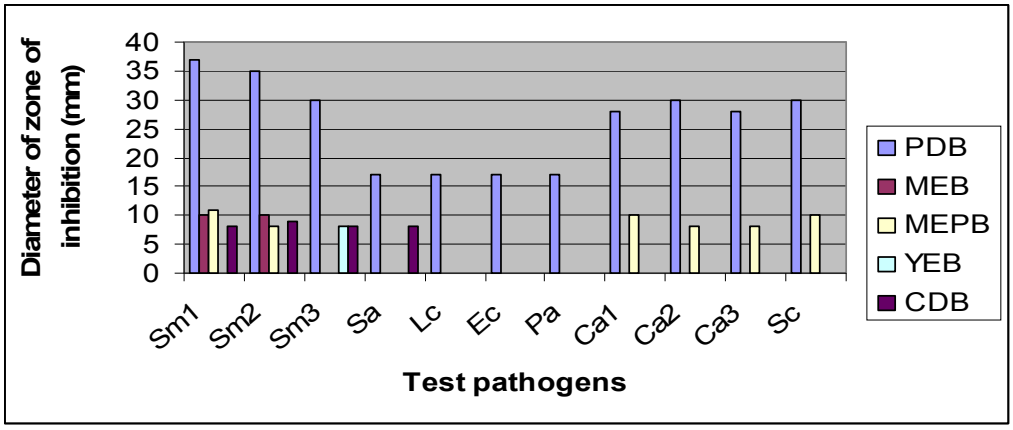
After TLC, the golden oily metabolite was further subjected to hexane: petroleum ether (95:5) solvent system to remove remaining impurities. The procedure was repeated until the pure white powdered metabolite was obtained. The antimicrobial activity tested of the amorphous white powder from the major band, dissolved in DMSO at the concentration of lmg/ml, showed inhibitory activity against all the test pathogens that ranged between 22 and 26 mm in diameter (Table 6). The diameter of zone of inhibition was 25 to 26 mm against three strains of *S. mutans*, 26mm against *S. aureus*, 25mm against *L. casei*, 24mm against *E. coli,* 25mm against *P. aeruginosa,* 22 to 23mm against three strains of *C. albicans* and 24mm against *S. cerevisiae*.

The *in vitro* minimum inhibitory concentration (MIC) of the purified and powdered antimicrobial metabolite was determined by a macrodilution broth assay. On testing against the pathogens, it was revealed that MIC values differed in different test pathogens (Table 7). MIC was found to be 1µg/ml in three strains of *S. mutans*, *S. aureus*, *E. coli* and *P. aeruginosa*; 2 µg/ml in *L. casei* and 4 µg/ml in three strains of *C. albicans* and *S. cerevisiae*. Ciprofloxacin and Ketoconazole were used as a positive control at the concentration of 5 and 10µg/ml. All the bacterial pathogens were found to be resistant to Ciprofloxacin and in case of yeasts, only one strain of *C. albicans* was found to be sensitive to the antibiotic Ketoconazole. Thus, it is inferred that the white powdered metabolite of *A. terreus* is highly effective against Gram--positive and Gram-negative bacteria and yeasts, thus broad-spectrum in action and MIC ranging from 1 to 4 µg/ml.



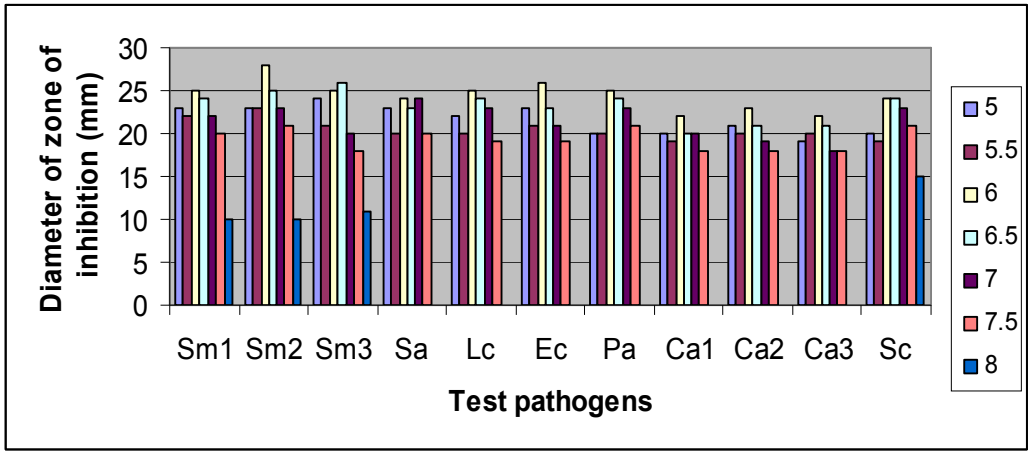
Sm1- *Streptococcus mutans* (MTCC No. 1943), Sm2- *S. mutans* (MTCC No. 890), Sm3- *S. mutans* (MTCC No. 497), Sa- *Staphylococcus aureus* (MTCC No. 3160), Lc- *Lactobacillus casei* (MTCC No. 1423), Ec-*Escherichia coli* (MTCC No. 43), Pa-*Pseudomonas aeruginosa* (MTCC No. 2295), Ca1- *Candida albicans*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

Fig.1. Antibacterial and antifungal activity of ten selected soil molds against test pathogens.



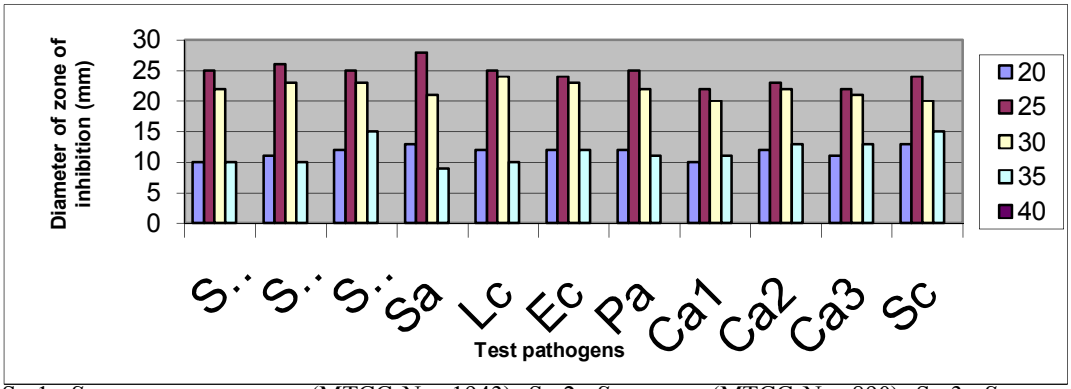
Sm1- *Streptococcus mutans* (MTCC No. 1943), Sm2- *S. mutans* (MTCC No. 890), Sm3- *S. mutans* (MTCC No. 497), Sa- *Staphylococcus aureus* (MTCC No. 3160), Lc- *Lactobacillus casei* (MTCC No. 1423), Ec-*Escherichia coli* (MTCC No. 43), Pa-*Pseudomonas aeruginosa* (MTCC No. 2295), Ca1- *Candida albicans*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

Fig. 2. Effect of various fermentation broths on the production of antimicrobial metabolite from *Aspergillus terreus*, by using agar well diffusion assay as measured by diameter of zone of growth inhibition of test pathogens.



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)

Fig.3. Effect of pH on the production of antimicrobial metabolite in *A. terreus* tested by agar well-diffusion assay against test pathogens



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)

Fig. 4. Effect of incubation temperature on the production of antimicrobial metabolite in *A. terreus* tested by agar well-diffusion assay against test pathogens.

Table 1. Antibacterial and antifungal activity of ten selected soil molds against test pathogens.

Isolate No.	Zone of growth inhibition (mm)[a]										
	Sm1	**Sm2**	**Sm3**	**Sa**	**Lc**	**Ec**	**Pa**	**Ca1**	**Ca2**	**Ca3**	**Sc**
7	NA	NA	NA	12±0.57	10±0.37	NA	NA	3±0.57	4±0.81	4±0.57	NA
12	NA	10±0.57	11±0.37	NA	NA	NA	NA	4±0.81	NA	NA	3±0.57
19	NA	11±0.57	9±0.57	NA	14±0.57	13±0.57	12±0.37	10±0.81	11±0.57	NA	3±0.57
28	8±0.57	NA	10±0.57	12±0.57	NA	NA	NA	11±0.57	10±0.57	NA	5±0.37
50	NA	NA	NA	18±0.81	17±0.57	12±0.81	11±0.57	11±0.57	9±0.57	10±0.81	NA
70	NA	15±0.81	15±0.81	22±0.81	11±0.81	12±0.57	10±0.57	12±0.37	NA	NA	NA
85	NA	9±0.57	NA	12±0.57	NA	NA	NA	4±0.57	NA	5±0.57	5±0.57
98	NA	16±0.57	10±0.57	12±0.57	12±0.57	11±0.57	10±0.81	10±0.81	15±0.57	15±0.57	10±0.37
103	15±0.57	9±0.57	9±0.57	11±0.57	NA	10±0.57	10±0.81	8±0.57	15±0.37	4±0.57	5±0.57
107	55±0.57	47±0.81	43±0.37	18±0.37	18±0.57	27±0.81	25±0.57	30±0.57	35±0.57	30±0.37	30±0.57

NA- No Activity; [a] Mean of diameter of zones of inhibition in three replicates; ± Standard deviation

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)

Table 2. Effect of various fermentation broths on the production of antimicrobial metabolite from *Aspergillus terreus*, by using agar well diffusion assay as measured by diameter of zone of growth inhibition of test pathogens.

Medium	Zone of growth inhibition (mm)[a]										
	Sm1	**Sm2**	**Sm3**	**Sa**	**Lc**	**Ec**	**Pa**	**Ca1**	**Ca2**	**Ca3**	**Sc**
PDB	37± 0.5	35± 0.8	30± 0.5	17± 0.8	17± 0.5	17±0.3	17±0.5	28±0.8	30±0.5	28±0.5	30± 0.5
MEB	10± 0.3	10± 0.5	NA	NA	NA	NA	NA	NA	NA	NA	NA
MEPB	11± 0.8	8± 0.5	NA	NA	NA	NA	NA	10±0.5	8±0.5	8±0.3	10±0.5
YEB	NA	NA	8± 0.5	NA	NA	NA	NA	NA	NA	NA	NA
CDB	8± 0.5	9± 0.3	8± 0.5	8± 0.5	NA	NA	NA	NA	NA	NA	NA

NA -No Activity; [a] Mean of three replicates; ± Standard deviation
PDB- Potato dextrose broth, MEB- Malt extract broth, MEPB- Malt extract peptone broth, YEB- Yeast extract broth
CDB- Czepak Dox broth
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)

Table 3. Effect of pH on the production of antimicrobial metabolite in *A. terreus* tested by agar well-diffusion assay against test pathogens.

pH of PDB	Zone of growth inhibition (mm)[a]										
	Sm1	**Sm2**	**Sm3**	**Sa**	**Lc**	**Ec**	**Pa**	**Ca1**	**Ca2**	**Ca3**	**Sc**
5.0	23±0.57	23±0.81	24±0.57	23±0.57	22±0.57	23±0.57	20±0.37	20±0.57	21±0.81	19±0.57	20±0.37
5.5	22±0.81	23±0.57	21±0.57	20±0.81	20±0.81	21±0.81	20±0.57	19±0.37	20±0.57	20±0.57	19±0.37
6.0	25±0.37	28±0.37	25±0.81	24±0.57	25±0.57	26±0.37	25±0.81	22±0.81	23±0.57	22±0.81	24±0.57
6.5	24±0.57	25±0.57	26±0.37	23±0.37	24±0.57	23±0.81	24±0.57	20±0.57	21±0.81	21±0.57	24±0.81
7.0	22±0.57	23±0.81	20±0.57	24±0.57	23±0.81	21±0.57	23±0.57	20±0.81	19±0.57	18±0.57	23±0.57
7.5	20±0.57	21±0.57	18±0.81	20±0.81	19±0.57	19±0.57	21±0.81	18±0.57	18±0.57	18±0.57	21±0.57
8.0	10±0.37	10±0.81	11±0.57	NA	NA	NA	NA	NA	NA	NA	15±0.81

NA -No Activity; [a] Mean of diameter of zones of inhibition in three replicates; ± Standard deviation

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)

Table 4. Effect of incubation temperature on the production of antimicrobial metabolite in *A. terreus* tested by agar well-diffusion assay against test pathogens.

Incubation temperature	Zone of growth inhibition (mm)[a]										
	Sm1	Sm2	Sm3	Sa	Lc	Ec	Pa	Ca1	Ca2	Ca3	Sc
20	10±0.57	11±0.57	12±0.81	13±0.57	12±0.37	12±0.57	12±0.81	10±0.81	12±0.37	11±0.57	13±0.81
25	25±0.81	28±0.57	25±0.57	26±0.37	25±0.57	24±0.37	25±0.37	22±0.37	23±0.37	22±0.37	24±0.57
30	22 ±0.57	23±0.81	23±0.37	21±0.37	24±0.37	23±0.57	22±0.37	20±0.37	22±0.37	21± 0.57	20±0.57
35	10±0.57	10±0.81	15±0.37	9±0.57	10±0.57	12±0.37	11±0.57	11±0.57	13±0.37	13±0.57	15±0.37
40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA- No Activity; [a] Mean of diameter of zones of inhibition in three replicates; ± Standard deviation
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)

Table 5. Comparison of antimicrobial activity of the metabolite in *A. terreus* extracted with various solvents tested following agar well-diffusion assay against test pathogens.

Organic solvents	Zone of growth inhibition (mm)[a]										
	Sm1	**Sm2**	**Sm3**	**Sa**	**Lc**	**Ec**	**Pa**	**Ca1**	**Ca2**	**Ca3**	**Sc**
Chloroform	25±0.57	26±0.37	25±0.37	26±0.37	25±0.37	24±0.37	25±0.37	22±0.57	23±0.57	22±0.57	24±0.57
Ethyl acetate	12±0.81	13±0.57	13±0.81	13±0.81	12±0.81	12±0.81	11±0.57	22±0.81	23±0.81	22±0.81	18±0.37
Dichloro methane	11±0.37	12±0.57	12±0.81	12±0.57	11±0.57	11±0.57	11±0.57	8±0.57	9±0.57	10±0.57	15±0.81
Di-methyl sulphoxide	10±0.57	11±0.57	12±0.37	11±0.57	10±0.57	11±0.57	12±0.81	9±0.57	10±0.57	11±0.37	12±0.57
Acetone	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA- No Activity; [a] Mean of diameter of zones of inhibition in three replicates; ± Standard deviation
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)

Table 6. Antimicrobial activity of component of metabolite separated through TLC by following agar well-diffusion assay against test pathogens.

Component	Zone of growth inhibition (mm)[a]										
	Sm1	**Sm2**	**Sm3**	**Sa**	**Lc**	**Ec**	**Pa**	**Ca1**	**Ca2**	**Ca3**	**Sc**
Single major band	25±0.81	26±0.57	25±0.57	26±0.81	25±0.57	24±0.57	25±0.81	22±0.37	23±0.57	22±0.81	24±0.57

[a] Mean of diameter of zones of inhibition in three replicates; ± Standard deviation

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)

Table 7. Evaluation of Minimum Inhibitory Concentration (MIC) of white powdered metabolite against test pathogens.

Organism	Concentration of metabolite (µg/ml)										
	0.125	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**12.0**	**32.0**	**64.0**	**MIC**
Sm1	+	+	+	-	-	-	-	-	-	-	1
Sm2	+	+	+	-	-	-	-	-	-	-	1
Sm3	+	+	+	-	-	-	-	-	-	-	1
Sa	+	+	+	-	-	-	-	-	-	-	1
Lc	+	+	+	+	-	-	-	-	-	-	2
Ec	+	+	+	-	-	-	-	-	-	-	1
Pa	+	+	+	-	-	-	-	-	-	-	1
Ca1	+	+	+	+	+	-	-	-	-	-	4
Ca2	+	+	+	+	+	-	-	-	-	-	4
Ca3	+	+	+	+	+	-	-	-	-	-	4
Sc	+	+	+	+	+	-	-	-	-	-	4

No growth; + Growth

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 represents the traditional source of new drug candidates (Luzhetskyy *et al*., 2007).

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 (Hara Kishore *et al*., 2007). 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. Keeping in view this fact, searches are to be made for the isolation of novel compounds from the soil microorganisms to fight against pathogenic microorganisms involved in dental caries and periodontal disease. Isolating microorganisms from the environment is the first step in screening for natural products such as secondary metabolites and enzymes (Hunter-Cevera *et al*., 1999).

Aspergillus species isolated from various soils have been found to produce antibacterial, antifungal and antitumour metabolites. Species of *Aspergillus* are known to produce mycotoxin, organic acids and antibiotics (Domsch *et al*., 1980; Roy *et al*., 1999; Hasegawa *et al*., 2007). *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 (Vinci *et al*., 1991; Waters *et al*., 1994).

The choice of a good fermentation medium is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation (El-Tayeb *et al*., 2004). The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production (Stanbury *et al*., 1997). Various aspects of microbial media such as

carbon and nitrogen sources, minimal salts, trace elements, vitamins and pH have been reviewed (El-Tayeb *et al*., 2004; Rizk *et al*., 2007).

Potato dextrose broth has been used for the production of tropolone, an antimalarial antibiotic from the insect pathogenic fungus *Cordyceps* sp. (Seephonkai *et al*., 2001). Similar medium has been used for the production of cladospolide D antifungal antibiotic from *Cladosporium* sp. (Zhang *et al*., 2001) antifungal antibiotic production by *Streptomyces chattanoogensis* against *C. albicans* (Gupte and Kulkarni, 2002); diketopiperazine novel antifungal antibiotic from marine fungus (Byun *et al*., 2003); asperaldin, a new aldose reductase inhibitor from *Aspergillus niger* (Rao *et al*., 2003); and, secondary metabolite production in *Penicillium sclerotiorum* with antibiotic activities (Lucas *et al*., 2007). The results in the present study are in accordance with findings of various workers cited above who also find the best production of fungal metabolites in PDB.

The pH level of the growth medium has a marked effect on secondary metabolite production with synthesis falling rapidly either side of an optimal level. The hydrogen or hydroxyl ion concentration may have a direct effect on cell, or it may act indirectly by varying the degree of dissociation of substances in the medium. Therefore, the change of pH is also important for the enzyme activity of microorganisms, for the intermediate products, their dissociation and solubility (Rizk *et al*., 2007). Thus, pH 6.0 is the best for the production of antimicrobial metabolite by *A. terreus*. Similar result had been reported earlier by Nishihara *et al.* (2001) during the production of FR198248, a new anti influenza agent at pH value between 6.3 to 6.4 from *A. terreus*.

Physical factors such as incubation temperature, can exert different effects on the growth and production phases of secondary metabolism (Rizk *et al*., 2007). Our results substantiate the findings of Suzuki *et al.* (1997) for the production of insecticidal compounds from *Aspergillus* sp. at 25^0C. The incubation temperature of 27°C was used by Fang *et al.* [41] for the production of aspochalasins antimalarial antibiotic from *Aspergillus* sp.

The purification of bioactive metabolites from fermented broth of microorganisms largely depends upon the physico-chemical properties of metabolite. Thus, our results substantiate with those of Saxena *et al*. (2007) who also extracted the antimicrobial metabolite obtained from *A. terreus* by chloroform which showed potent anticandidal activity.

Our results in the present study substantiate with the findings of Furtado *et al*. (2005) who also recorded antimicrobial activity of metabolite extracted from *A. fumigatus* within R_f values of 0.33 to 0.91. In a study carried out by Fang *et al*. (1997), CJ-17665 antibacterial antibiotic from *Aspergillus ochraceus* showed MIC of 12.5, 12.5 and 25 μg/ml against multi drug resistant

organisms such as *S. aureus, S. pyogenes* and *E. faecalis,* respectively. Suzuki *et al*. (1997) reported that NF00659 A1, A2, A3, Bl and B2 novel metabolite produced by *Aspergillus* sp. NF00659 showed antimicrobial activity at concentration of 1mg/ml against Gram-positive and Gram-negative bacteria and fungi.

Thus, the results of antimicrobial susceptibility tests indicated that white powdered metabolite purified from *A. terreus,* when compared with commercial antibiotics, has been found to be better than the commercial antibiotics evaluated in the present study. From the present study, it may be concluded that the white powdered antimicrobial metabolite obtained from *A. terreus* may be used for the treatment of dental caries and periodontal disease causing microorganisms. 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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