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OPTIMIZATION OF CULTURAL CONDITIONS AFFECTING GROWTH AND IMPROVED BIOACTIVE METABOLITE PRODUCTION BY A SUBSURFACE *ASPERGILLUS* STRAIN TSF 146

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ABSTRACT : The aim of this study was to optimize cultural conditions for optimum growth and bioactive metabolite production by *Aspergillus* strain TSF 146, isolated from the subsurface soils of Brahmaputra plains, Assam, India. Agar disc diffusion assay was used to examine the antagonistic activity of the strain. The effect of different culture media, temperature, pH, incubation period, shaking, inoculum size and various carbon and nitrogen sources on the mycelial growth and bioactive metabolite production in a fixed volume of culture broth were studied. *Aspergillus* strain TSF 146 grew well and produced optimum bioactive metabolites in Potato dextrose broth medium at 25 °C on the 14th day of the incubation. The optimal mycelial growth, however, was obtained at pH 5.5. Sucrose (2.0 g/l) and asparagine (1.0 g/l) were the best carbon and nitrogen sources respectively for optimum growth and production of active metabolites by the isolate. Inoculum size of 2x10^{10} spores/ml with periodic shaking at 150 rpm optimized production of bioactive molecules. The results of the present investigation indicated that cultural conditions like carbon, nitrogen and mineral sources as well as physical factors such as temperature, pH, incubation period, inoculum size and shaking greatly affected the growth and production of bioactive metabolites by *Aspergillus* strain TSF 146.

Keywords Agar disc diffusion assay, antagonistic activity, *Aspergillus* strain TSF 146, bioactive metabolite, inoculum size, North Brahmaputra plains

INTRODUCTION

Fungal antagonism has been reported for a wide variety of pathogenic organisms (Park et al., 2002; Rajakumar et al., 2005; Ramezani, 2008). Many new and interesting bioactive metabolites such as antibiotics, antiviral, anticancer and antioxidant compounds having pharmaceutical, industrial and agricultural importance are isolated and characterized from soil fungi (Stobel and Daisy, 2003). Soil Fungi are also the major sources of other industrially important compounds like enzyme inhibitors, antihelminthics, antitumor agents, insecticides, vitamins, immunosuppressant and immunomodulators (Makut and Owolewa, 2011). The antimicrobial properties of secondary metabolites derived from various groups of fungi are widely reported (Sekiguchi and Gaucher, 1977; John et al., 1999; Schulz et al., 2002; Keller et al., 2005), suggesting the outstanding potentiality of this microbial community as an important source of bioactive molecules.

Aspergillus, a fungi represented by large number of species, is known to produce anti-*hilicobacter pylori* secondary metabolites like helvolic acid, monomethylsulochrin, ergosterol and 3β-hydroxy-5□ (Gao et al., 2007) and cytotoxin, Brefeldin A (Wang et al., 2002). There have been several studies on the antimicrobial potentiality of *Aspergillus* spp. against a panel of bacterial and fungal pathogen (Maria et al., 2005; Kumar et al., 2010). Antimicrobial activities of an endophyte *Aspergillus* sp. against some clinically significant human pathogens have been reported (Tayung and Jha, 2007). Ability of antagonistic fungal strains to produce bioactive metabolites, however, is not a fixed property (Vinale et al., 2006).

Variation in the type of carbon and nitrogen sources besides changes in pH, temperature, incubation period, shaking and inoculum size of the antagonistic fungal strain can greatly influence antibiotic biosynthesis (Thakur et al., 2009). Antibiotic productivity can also decrease when media deficient in metal ions are used and culture vessels are incubated at high temperatures for long periods. Therefore, optimization and maintenance of proper culture conditions are necessary criteria to achieve maximum production of bioactive metabolites by an antagonistic microbial strain. There are, however, fewer reports on effect of nutritional and cultural conditions on mycelial growth and antimicrobial metabolite production by the antagonistic fungal strains (Vahidi et al., 2004; Gogoi et al., 2008; Ritchie et al., 2009; Jain and Pundir, 2011). There is also an increasing need of alternative novel drugs to control the dominant infectious diseases and multidrug resistant microorganisms. Besides, there are almost no investigations as regards to optimization of cultural conditions for better growth and production of bioactive metabolite by antagonistic fungal strains of subsurface soil horizons of Brahmaputra plains, a part of Indo-Burma Mega biodiversity hot spot. The subsurface system may harbour some microbes with some novel activities as they populate a habitat characterized by stressed conditions. The study of unique subsurface biosphere would provide valuable information about the ecology of soil fungi, the physical and chemical factors driving the soil fungal community composition and the energetics of fungal metabolism (Ekelund et al., 2001). Therefore, the present study was undertaken to screen a number of fungal strains isolated from the subsurface soil and find out the effect of different culture media, temperature, pH, incubation period, shaking and inoculum size along with various carbon and nitrogen sources on the growth and bioactive metabolite production by strain TSF 146 of *Aspergillus in vitro*.

MATERIALS AND METHODS

Sample collection, isolation and characterization of fungi

Soil was sampled from three different spots from a depth of 101-200 cm from subsurface regions of Brahmaputra plains (26 °40′N latitude and 92 °58′E longitude), Assam, India, using a sterilized hand auger during December 2008 to October 2010. Randomly collected soil samples were mixed thoroughly and the composite sample was brought to the laboratory. One gram of the soil was then suspended in 100 ml sterillized water and incubated in an orbital shaking incubator at 28 °C with periodic shaking at 200 rpm for 30min. Mixtures were allowed to settle and serial dilutions upto 10^5 were prepared using sterile distilled water. Isolation of fungi from this mixture was done using dilution plate technique (Johnson and Curl, 1972) in Potato dextrose agar medium. Fungi were characterized based on their cultural, morphological and spore characteristics and identified by consulting various taxonomic monographs (Subramanian, 1971; Watanabe, 1993; Domsch et al., 2007). Amongst the fungal isolates, TSF 146 exhibited broad spectrum antagonistic activity *in vitro* so, it was selected for further studies.

Microbial target organisms

The organism, *Bacillus subtilis* (MTCC441) was procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India and *Candida albicans* was obtained from Microbiology Division of Defence Research Laboratory (DRL), Tezpur, India and maintained on freshly prepared nutrient agar and sabouraud's agar medium respectively. The organisms were preserved at - 20 ºC in the presence of glycerol (15 %, v/v) for longer periods.

Selection of culture media

Different microbiological media namely Czapek-Dox Yeast Extract medium, Czepak-Dox broth medium, Potato dextrose broth medium, V8 juice medium, Nutrient broth medium, Malt Extract broth medium, Sabouraud broth medium, Sabouraud Glucose broth medium and Emmon's Culture medium were used to observe the effect of different media on the growth of *Aspergillus* strain TSF 146. The growth and production of extracellular bioactive metabolite in each medium was determined after seven days of incubation. All the media were procured from HiMedia Laboratories, Mumbai, India.

***In vitro* screening of antimicrobial activity and fungal growth**

To examine the antimicrobial activity of the metabolite produced by *Aspergillus* strain TSF 146, the culture broth was centrifuged and filtered through sterile cheesecloth to remove the mycelial mats and then bioassayed against the target organisms by agar-based disk diffusion assay (Barry and Brown, 1996). For this, nutrient agar plates were inoculated with 0.2 ml of overnight grown culture of target bacteria containing 1.0 x 10^9 cells. Similarly, sabouraud's agar plates were inoculated with 0.2 ml of cultured pathogenic fungi containing 1.0 x 10^9 cells. The plates were evenly spread out with the help of a sterile cotton swab. A fixed volume of 20 µl of the culture broth of our antagonist was then applied to the sterile discs (6.0 mm diameter, Whatman antibiotic assay discs) and subsequently placed on the agar surface. The diameters of the inhibition zones were recorded after 24 h of incubation at 37º C for *Bacillus subtilis* and after 48-72 h at 25 ºC for *Candida albicans*. Three replicates were maintained in each case. Growth of the antagonistic strain was determined as dry mycelial weight in 25 ml of culture medium by drying the cell mass in an oven at 70 ºC overnight and expressed as dry weight of mycelia (mg/ 25ml).

Optimization of culture conditions

Standardization of the basal media

Potato dextrose broth medium with or without carbon and nitrogen sources was used as the basal medium to determine the optimum nutritional and cultural conditions for maximum growth and antimicrobial compound production by the isolate, TSF 146. Conical flasks containing 25 ml culture broth was inoculated with twelve-day-old slant culture of TSF 146. Flasks were then incubated in stationary condition at 30 ºC for six days. Growth and antimicrobial bioassay were carried out at the end of the incubation period.

Effect of temperature, pH, incubation period, shaking and inoculum size

The fungal strain, TSF 146 was inoculated into Potato dextrose agar medium and grown at range of temperatures varying from 10 °C to 55 °C. Fungal growth and antibiotic production at each temperature was determined (Suetsuna and Osajima, 1990). To study the effect of pH, different pH values ranging from 3.5 to 8.0 were used after adjusting pH of the basal medium. Incubation periods ranging from 4 to 26 days were used to determine the effect of incubation period on the growth and active metabolite production by TSF 146. To determine the effect of shaking on growth and active metabolite production, culture flasks were incubated at 25 ºC in an orbital shaker at 150 rpm. Culture flasks were also inoculated separately with spores ranging from $2x10^4$ to $2x10^{10}$ (spores/ml) to determine the effect of size of inocula on the growth and production of active metabolite by TSF 146.

Carbon and nitrogen supplement

Glucose, glycerol, D-mannitol, lactose, Na-citrate, trehalose, sucrose, galactose, xylose, starch, L-arabinose, D-fructose, maltose and D-sorbitol as carbon sources and ammonium chloride, sodium nitrate, calcium nitrate, arginine, glutathione, asparagine, theronine, glutamine, glycine, tyrosine, bovine serum albumen and asparatic acid were used as nitrogen sources. Carbon and nitrogen sources were added separately into the basal medium at 10 g/l (w/v) and growth and antimicrobial metabolite production by TSF 146 was determined.

Effect of NaCl concentration

The effect of salinity on the mycelial growth and bioactive metabolite production was carried out by incubating the test fungus in various concentrations of NaCl ranging from 1 g/l to 10 g/l, into the basal media amended with sucrose (0.5 g/l).

Specific rate of product formation (qp)

The specific rate of bioactive metabolite production (qp) was calculated by using the following formula:

$qp= 1/X (dp/dt)$,

Where X, P and t represents mycelial growth (mg/ml), concentration of antimicrobial agent and time respectively. dP/dt was calculated as Le Duy and Zajic (Le Duy and Zajic, 1973).

Data analysis

The statistical processing of the data was conducted in completely randomized block design and computer programme Excel.

RESULTS AND DISCUSSION

In the present investigation, isolate TSF 146, exhibited maximum growth and antagonistic activity against the bacterial pathogen, *B. subtilis*, *in vitro*. Therefore, further experiments on optimization of cultural conditions for maximum growth and improved bioactive metabolite production was carried on with this bacterial pathogen.

Optimization of culture media

Results of the present study revealed that different microbiological culture media had significant effect on the growth and bioactive metabolite production by *Aspergillus* strain TSF 146 (Table 1). Maximum mycelial dry weight (71 mg/25 ml) was recorded in Potato dextrose broth medium with maximum zone of inhibition (25 mm) against *B. subtilis*. Therefore, this medium with or without carbon and nitrogen sources was selected as the basal medium for further experiments. The strain also showed significant growth (55 mg/25 ml and 52 mg/25 ml) and bioactivity (21 mm and 19 mm) in Czepak-Dox broth and Malt Extract broth medium respectively. Emmon's Culture medium exhibited minimum mycelial dry weight (22 mg/25 ml) and production of antimicrobials (6 mm zone of inhibition) out of all the media used. Alterations of some of the nutrients in the culture media used might have some promotional effects on the growth of the strain. Our results are in good agreement with Rabbani et al., (2011), who too reported Potato dextrose broth medium as the best medium for maximum growth and sporulation of *Drechslera hawaiiensis*, the foliar blight pathogen of *Marsilea minuta* L. Similarly, the effect of culture medium on mycelial growth, metabolite profile and antimicrobial compound yield by a marine-derived fungus *Arthrinium* c.f. *saccharicola* was investigated by Miao et al., (2006), suggesting the need of optimal culture composition to achieve maximal mycelial growth and bioactivity of the fungus.

Table 1: Effect of different culture media on the growth and bioactive metabolite production by *Aspergillus* strain TSF 146 (bioassayed against *B. subtilis*)

Culture media	Dry weight of mycelia (mg/25ml)	Diameter of inhibition zones (mm)[a]
Czapek-Dox Yeast Extract medium	31	12
Czepak-Dox broth	55	21
Potato dextrose broth	71	25
V8 juice medium	48	17
Nutrient broth	34	14
Malt Extract broth	52	19
Sabouraud broth	26	8
Sabouraud Glucose broth	37	9
Emmon's Culture medium	22	6

[a] **Assayed against *Bacillus subtilis*.**

Effect of temperature and pH

The *Aspergillus* strain TSF 146, showed a narrow range of incubation temperatures for effective growth and antibiotic production (Fig. 1). The increase of the incubation temperatures from 25 to 30 ºC enhanced the growth of the cells and production of bioactive metabolite. Maximum cell growth (70 mg /25 ml medium) and inhibition zone (25 mm) was recorded at 30 ºC. However, lowest growth and inhibition zone was observed at low temperature of 15 ºC as well as at high temperature (55ºC). No growth and antagonistic activity was recorded at ≤10 ºC. Different studies proved that temperature is one of the major conditions affecting the growth rate of antagonist (Kok and Papert, 2002). Incubation temperature ranging between 20 and 25 °C was detected to be optimum for mycelial growth of *Rhizoctonia solani* (Ritchie et al., 2009). Jain and Pundir (2011) also obtained maximum production of antimicrobial metabolite by *Aspergillus terreus* at 25 ºC.

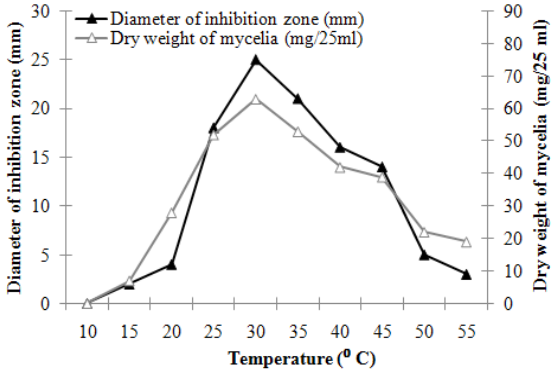

Fig. 1 Effect of temperature on the growth and production of bioactive metabolite by *Aspergillus* strain TSF 146, in broth culture (bioassayed against *B. subtilis*)

The growth and antagonistic activity of TSF 146 were also influenced by pH of the medium (Fig. 2). The test fungus grew well at low (pH 3.5) as well as at high pH (7.5). Optimum antimicrobial metabolite production (zone of inhibition 25 mm) as well as mycelial growth (68 mg /25 ml medium), however, was recorded maximum at pH 5.5. No growth and antagonistic activity was observed at pH ≤ 3.5 and ≥ 7.5. Similar observations were also made by Thongwai and Kunopakarn (2007). They pointed out that most of the microorganisms have the ability to synthesize antimicrobial compounds at pH ranging from 5.5 to 8.5. Maximum production of antibacterial metabolite by *Aspergillus terreus* in Potato dextrose broth medium at pH 6.0 was noted by Jain and Pudir (2011). Influence of pH on the growth and production of bioactive metabolite by an endophyte *Hypocrea* spp. NSF-08 isolated from *Dillenia indica* Linn. in North-East India, was well investigated by Gogoi et al., (2008).

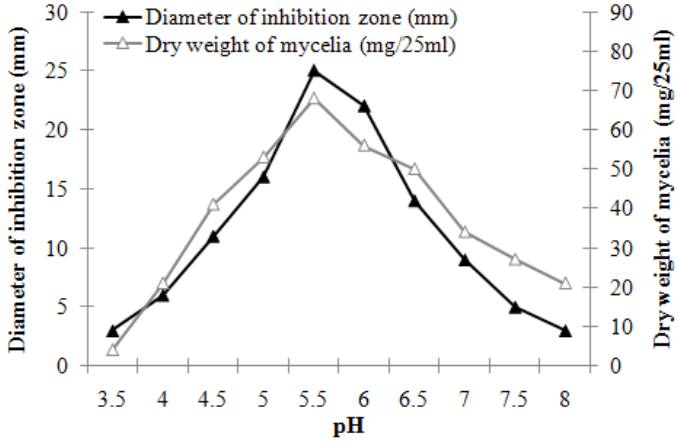

Fig. 2 Effect of pH of the medium on the growth and production of bioactive metabolite by *Aspergillus* strain TSF 146 (bioassayed against *B. subtilis*)

Effect of carbon and nitrogen sources

The strain grew on all the carbon sources tested during the present investigation though maximum growth (74 mg/25 ml) and antibiotic production (zone of inhibition 25 mm) was obtained when sucrose was used as sole carbon source (Table 2). Sucrose at a concentration of 2.0 g/l was found to be optimum for maximum growth and bioactive metabolite production by the test fungus (Fig. 3). The results are in good agreement with Thakur et al., (2009). The effects of various carbon sources like lactose, sucrose, glucose, fructose, galactose, maltose and xylose and glucose on the production of cordycepin (3′-deoxyadenosine) by *Cordyceps militaris* was earlier investigated (Mao et al., 2005). They observed that an increase in the initial concentration of glucose was most effective for mycelial growth.

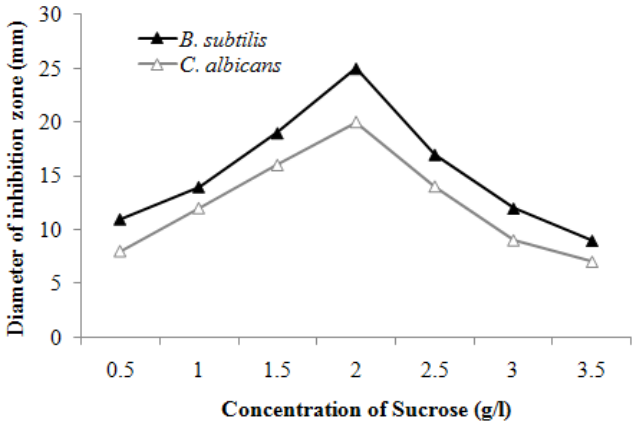

Fig. 3 Effect of different concentrations of Sucrose on the production of bioactive metabolite by *Aspergillus* strain TSF 146 in broth culture

Table 2: Effect of different carbon sources on growth and bioactive metabolite production by *Aspergillus* strain TSF 146 assayed against *B. subtilis*

Carbon source 1% (w/v)	Dry weight of mycelia (mg/25ml)	Diameter of inhibition zones (mm)[a]
Lactose	39	9
Trehalose	46	14
Glycerol	28	10
Glucose	59	19
Na-citrate	24	-
D-mannitol	52	17
Xylose	35	11
Maltose	27	7
D-sorbitol	47	14
L-arabinose	22	8
Sucrose	74	25
Galactose	21	-
Starch	16	-
D-fructose	36	7

-: indicates no zone of inhibition or growth.

[a] **Assayed against *Bacillus subtilis*.**

Different types of nitrogen sources affected the growth and production of secondary metabolite by *Aspergillus* strain TSF 146 (Table 3). Maximum antimicrobial activity was obtained when media was supplemented with asparagine followed by glycine, threonine and ammonium chloride. Nitrogen sources like sodium nitrate, aspartic acid and bovine serum albumine even though supported growth of the test fungus but were not suitable for the production of active metabolite. Supplementation of the medium with glutathione, however, inhibited both growth and antagonistic activity of the test strain (Table 3). The optimum concentration of asparagine for optimal mycelial growth and bioactive metabolite synthesis was recorded as 1.0 g/l (Fig. 4). Peighamy-Ashnaei et al., (2007) have also described the importance of various nitrogen sources in maximizing the growth of strains of *Pseudomonas fluorescens* and *Bacillus subtilis*. Similar observations were also reported by other workers (Jackson, 1997; Gao et al., 2007).

Table 3: Effect of different nitrogen sources on growth and bioactive metabolite production by *Aspergillus* strain TSF 146 assayed against *B. subtilis*

Nitrogen source 1% (w/v)	Dry weight of mycelia (mg/25ml)	Diameter of inhibition zones (mm)[a]
Ammonium chloride	34	12
Sodium nitrate	28	-
Asparagine	64	25
Tyrosine	39	7
Threonine	51	14
Glutamine	47	9
Calcium nitrate	32	8
Glycine	55	16
Aspartic acid	44	-
bovine serum albumen	13	-
glutathione	-	-
Arginine	8	5

-: indicates no zone of inhibition or growth.

[a] Assayed against *Bacillus subtilis*.

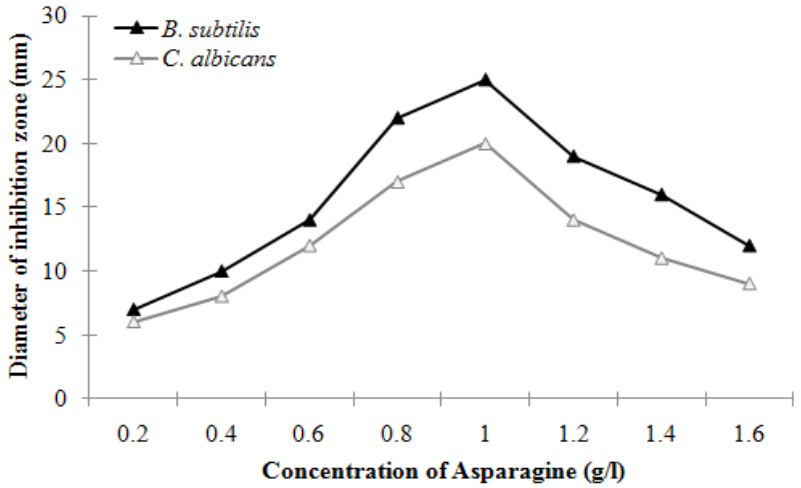

Fig. 4 Effect of different concentrations of Asparagine on the production of bioactive metabolite by *Aspergillus* strain TSF 146 in broth culture

Effect of incubation period, shaking, NaCl concentration and inoculum size

The strain required 14 days of incubation for maximum growth (65 mg/25 ml) while it required 16 days for optimum production of antimicrobial metabolite (zone of inhibition 25 mm) (Fig. 5) under shaking conditions. The growth and production of secondary metabolite get decreased after 14 and 16 days of incubation. However, the highest value of specific rate of formation of bioactive metabolite (0.0071 day^{-1}) was observed on 10^{th} day of incubation followed by 12^{th} day (0.0063 day^{-1}). Effect of incubation period on the production of bioactive compound (Fumonisin B_1) by *Fusarium moniliforme* was investigated by Alberts et al., (1990). They observed that the production of metabolite commenced after 12 days. The concentration of the metabolite in the medium continued to increase during the stationary phase and decreased after 91 days of incubation. Abbas et al., (2010) observed that periodic shaking of culture flask is an essential parameter for optimum biosynthesis of antibiotic by an antagonistic actinomyces strain.

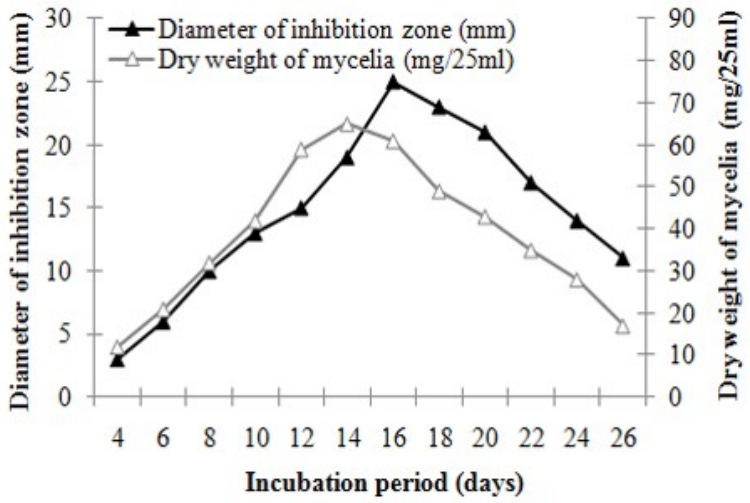

Fig. 5 Effect of incubation period on the growth and production of bioactive metabolite by *Aspergillus* strain TSF 146 (bioassayed against *B. subtilis*)

NaCl concentration of 5 g/l was recorded as optimal for maximum mycelial growth (49 mg/25 ml) and improved active metabolite production (zone of inhibition 23 mm) (Fig. 6) by the antagonistic strain TSF 146, whose values gradually decreased with increase in salt concentration in the basal media. NaCl concentration of 2.5– 3.0 % was found to be optimum for maximum growth (4.3 mg/ml, 3.8 mg/ml) and production of bioactive metabolite (10.6 lg/ml, 10.1 lg/ml) by an antagonist fungus, *Fusarium* sp. (Gogoi et al., 2008).

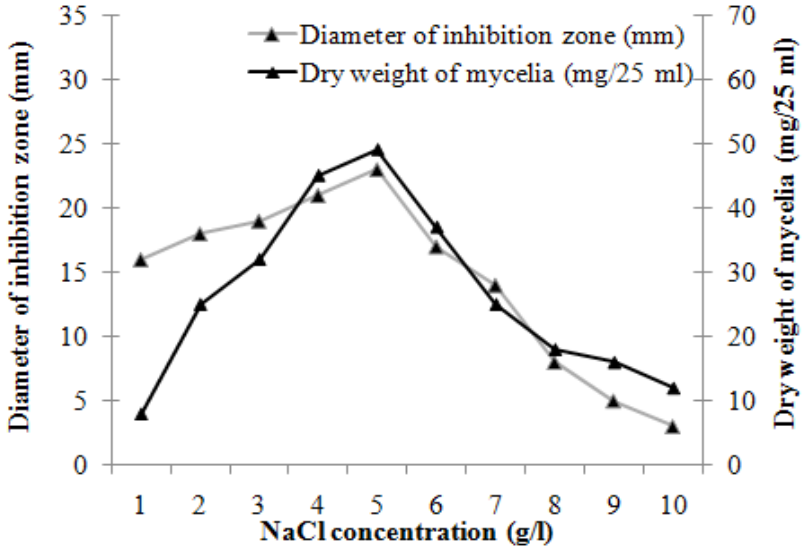

Fig. 6 Effect of NaCl concentration on the mycelial growth and production of bioactive metabolite by *Aspergillus* strain TSF 146 (bioassayed against *B. subtilis*)

The size of the inoculum also influenced the ability of the strain to produce antimicrobial metabolite. An inoculum size of $2x10^{10}$ spores /ml was adjusted as optimal inoculum for maximum growth and yield of antibiotics by the strain TSF 146 (Table 4). The present result is in accordance with Thakur et al., (2009), who also reported the importance of inoculum density in increasing mycelial growth and metabolite production by *Streptomyces* sp.

Table 4: Effect of inoculum size on the mycelial growth and bioactive metabolite production by *Aspergillus* strain TSF 146

No. of spores/ml	Dry weight of mycelia (mg/25 ml)	Diameter of inhibition zones (mm)[a]
$2x10^4$	14	7
$2x10^5$	32	10
$2x10^6$	41	13
$2x10^7$	47	18
$2x10^8$	50	21
$2x10^9$	56	23
$2x10^{10}$	58	25

[a] **Assayed against *Bacillus subtilis*.**

CONCLUSION

From the above investigation it is evident that the mycelial growth and subsequent production of antimicrobial metabolite by the antagonistic strain, *Aspergillus* sp. TSF 146, isolated from the subsurface soils of Brahmaputra plains, Assam, India, is mainly influenced by various nutrient supplements in the culture media. The maximum production of the antimicrobial metabolites by our antagonist, TSF 146, could be achieved in Potato dextrose broth medium supplemented with sucrose at 2.0 g/l as carbon and asparagine at 1.0 g/l as nitrogen source *in vitro*. Further process parameters, like incubation temperature at 25 ºC, pH 5.5, NaCl at 5.0 g/l, inoculum size of 2 x 10^9 spores/ml and 16 days of incubation under shaking condition are found to be optimum for the maximal production of bioactive metabolite.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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