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Research Article

OPTIMIZATION OF GROWTH AND BIOACTIVE METABOLITE PRODUCTION: *FUSARIUM SOLANI*

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ABSTRACT

The present study aimed to evaluate optimize the growth and antibacterial activity of endophytic fungi isolated from *Tylophora indic*a. Based on the morphological and molecular characteristic, the isolate was identified as *Fusarium solani*. Modified liquid medium (M_2D) was used as a basal medium for growth and antibacterial activity. The growth and metabolite production were optimized with 4% dextrose, 0.05% yeast extract and aspartic acid (0.01%) as carbon, nitrogen and amino acid respectively. The optimum pH and temperature of the strain were 6.0, 25±2 °C observed for growth and secondary metabolite production. The 9th day was found to be optimum incubation period of growth and secondary metabolite production. The metabolite showed maximum inhibition against *Enterococcus faecalis*, lowest inhibition zone was against *Yersinia enterocolitica*. The ESI-MS analysis of bio active compound shows the peak at 301m/z.

Keywords: Endophytic fungus, Tylophora indica, antibacterial activity, Medium Optimization, ESI -MS

INTRODUCTION

Endophytes are microorganisms that reside in living plant tissues, apparently without inflicting negative effects [2]. Endophytes are presumably ubiquitous in the plant kingdom, some of which can improve the ecological adaptability of hosts [23, 27, 30]. Moreover, certain endophytic fungi capable to synthesizing the medicinal products produced similarly in plants [38]. At present, much research has focused on isolation of endophytic fungi from pharmaceutical plants, such as *Camptotheca acuminate* [21], pine [7], and *Taxus* plants [3, 13, 40], discovering a vast number of undescribed endophytic fungi species, some of which have potential to be used in the production of medicine. Recently investigations have been intensified due to the potentialities of endophytic microorganisms in production of bioactive metabolites, immune-suppressants, anticancer compounds and bio-control agents [36]. Although the active constituents may occur in lower concentrations, endophytic fungal pigments may be a better source of antimicrobial compounds than synthetic drugs. Several microorganisms such as *Monascus*, *Peacilomyces, Serratia, Cordyceps, Streptomyces* and *Penicillium* have the ability to produce pigments with high yield [33] which have been developed as a drug and used to treat the wound infections and skin diseases caused by the pathogens. Many of the endophytic fungal strains have attracted special attention because they have the capability of producing different colour pigments with high chemical stability [38]. The physical and chemical parameters like pH, temperature, incubation period, carbon and nitrogen sources and amino acid plays a major role on production of bioactive compounds and antimicrobial agents [11]. Since, *T. indica*is an endemic plant an attempt is made to conserve the medicinal plant through exploration of endophytic fungi for growth and bioactive compound production.

Tylophora indica (Burm. f) Merrill (Asclepiadaceae), an indigenous medicinal plant of Asian origin, is known to host several metabolites having insecticidal property [17] and medicinal property[29].Leaf and stem extracts have shown anti-asthmatic [35] anti-leukemic [8]anti-inflammatory [10] and anti-tumor [5], activities.Therefore, the present investigation was aimed to studythe medium optimization and antibacterial activity of *Fusarium strain* isolated from *Tylophora indica*.

Materials and Methods

Chemicals

All the analytical grade chemicals and solvents were purchased from Sigma chemical co., USA. SD fine chem.Ltd, Biosar, India and Hi-Media Pvt. Ltd. Mumbai, India.

Isolation and identification

The *Fusarium* strain was isolated from *T. indica* as we described previously [26], and strain was deposited in the GenBank - JN786598.

Culture medium and extraction

The medium adjusted to pH 5.5 was composed of (g/L): Potato 200; glucose 40.0; Yeast extract 0.8; Peptone 0.5; Soytone 1.0; $KH_2PO_4$2.0; $MgSO_4$ 0.5; $(NH_4)_2SO_4$3.0;phenyl alanine 0.01. Erlenmeyer flasks (500 ml) containing 200 ml of medium were incubated at 28°C in a static way for 21 days in dark. After incubation the culture was passed through four layers of cheese cloth to remove solids and extractedwith dichloromethane (DCM).

Antibacterial activity

Test Organisms

The human pathogenic bacteria used for the test included gram-positive and gram negative organisms were: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 15380, *Enterococcus faecalis* ATCC 29212, *Yersinia enterocolitica* MTCC 840, *Erwinia. sp* MTCC 2760, *Vibrio parahaemolyticus* MTCC 451, *Enterobacter aerogenes* MTCC 111, *Escherichia coli* ATCC 25922 and *Proteus vulgaris* MTCC 1771. All cultures were procured from IMTECH, chandigarh, India.

Disc-diffusion method

The crude extracts were dissolved in the DMSO (Dimethyl sulphoxide) to a final concentration of 20 mg/ml. Antibacterial tests were carried out by disc-diffusion method [24] using 100 µl of suspension containing 108 CFU/ml of test bacteria, spread on MHA (Muller Hinton Agar) medium, respectively. The discs (6 mm) were

impregnated with 20 µl of the extracts (1.25, 2.5, 5.0 mg/disc) with the concentration of 20 mg/ml and placed on the inoculated agar. Negative controls were prepared using the same solvent. Streptomycin (10 µg/disc) was used as positive control. The inoculated plates were incubated at 37 °C for 24 h for clinical bacterial strains. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms.

Isolation of active fraction

The crude extract was purified by thin layer chromatography (TLC) (Merk Ltd.) using dichloromethane - acetone (80: 20) as running solvent system.All the fractions were screened for antibacterial activity against Gram positive and gram negative bacteria by disc diffusion method [24]. The each disc were impregnated with 20 µl of each TLC fraction (1mg/ml) and placed on the medium and incubated at 37 °C for 24 h. Streptomycin, 10 µg/disc was used as positive control. The MIC of active fraction was determined using micro dilution bioassay by Eloff [6]. Further identification of active fraction was using the Electron Spray Ionization (ESI-MS) technique with an Agilent 1100 LC/ MSD trap. The nebulizer gas flow rate of the sample was 2 µL min-1 and the capillary voltage was 2.2 kV.

Medium Optimization

Standardization of Basal Medium

Standardization of basal medium for optimum growth and bioactive metabolite production consists of (g/l) CzapekDox Broth (CDB) containing: $NaNO_3$ 3.0g, K_2HPO_4 1.0g, $MgSO_4$ 0.5g, KCl 0.5g, $FeSO_4$ 0.01g, Sucrose 30.0g, Modified liquid medium-1 (M_1D); KNO_3 80.0mg, $CaNO_3$ 0.5g, Kcl 60.0mg, $MgSO_4$ 360.0mg, NaH_2PO_4 20.0mg, Sucrose 30g, Ammonium tartrate 5.0g, $FeCl_3$ 2.0mg, $MnSO_4$ 5.0mg, $ZnSO_4$ 3.0mg, H_3BO_3 1.4mg, KI 0.7mg, Yeast extract 0.25g, Peptone 1.0g, Soytone 1.0g, Modified liquid medium-2 (M_2D) Potato 200g, Glucose 40g, Peptone 0.5g, Yeast extract 0.8g, $(NH_4)_2SO_4$ 3.0g, $KH_2PO_4$2.0g, $MgSO_4$ 0.5g, Phenylalanine 0.01g,Potato dextrose broth (PDB) Potato 200.0g, Dextrose 20.0g, Potato dextrose yeast extract broth (PDYEB) Potato 200.0g, Dextrose 20.0g, Yeast extract 2.0g and Malt extract (ME) Malt extract 20.0 g, Peptone 1.0 g, glucose 20.0 g media were used. After 21 days of incubation at 28 ± 2 °C with pH 6.4, the biomass and secondary metabolite production were recorded. Biomass was determined by drying the mycelial mat at 70 °C until a constant weight was obtained.

Effect ofcarbon and nitrogensources

Various carbon sources such as dextrose, sucrose, starch, galactose, mannose, fructose, lactose and maltose.The various nitrogen sourceslike beef extract, yeast extract, peptone, malt extract, ammonium tartarate, ammonium nitrite, and ammonium sulphate, respectively were amended separately into thebasal medium (M2D) at a concentration of carbon source (4%) and nitrogen source (0.05%). Thestrain LCPANCF01 was inoculated to the respective medium andincubated at 28 ± 2 °C in dark for 21 days in static condition and their respective biomass vis-a`-vis metabolite production were recorded.

Effect of amino acid

The effect of various amino acids on growth and bioactive metabolite production was compared in basal medium in combination with dextrose and yeast extract. The medium amended with amino acids (0.001%), carbon source (4%) and nitrogen source 0.05% respectively. The strain LCPANCF01 was inoculated to the medium and incubated for 21 days in dark at 28 ± 2 °C in stationary condition. The biomass accumulation and bioactive metabolite production were estimated.

Effect of pH

Initial pH ranges were adjusted from 3 to 11 at a difference of one to the basal medium amended with dextrose and yeast extract, were incubated for 21 days in dark at 28 ± 2 °C under still condition. The biomass and bioactive metabolite production were estimated by comparing the dry weight of mycelial mat and the UV ƛ-max of the clear supernatant.

Effect of temperature

The strain LCPANCF01 was inoculated into basal medium amended with Dextrose and yeast extract, were grown in various range of temperature from 15 to 50 °C at a difference of 5 °C for 21 days in dark under stationary condition and the growth and secondary metabolite production were recorded.

Determination of incubation period

The strain LCPANCF01 was inoculated into the basal medium (M2D) amended with dextrose and yeast extract, incubated up to 21 days in stationary condition in dark at 28 ± 2 °C. Their growth and the secondary metabolite production was determined everyday by comparing mycelial weight and UV ƛ-max of the clear supernatant.

Effect of NaCl Concentration

The effect of salinity on growth and bioactive metabolite production was carried out by incubating the fungus in various NaCl concentrations (1% to 10%) into the basal mediumamended with 4% dextrose, 0.05% yeast extract as carbon and nitrogensource respectively, while keeping other parameters at optimum level. The biomass and bioactive metabolite production for each NaCl concentration were estimated.

Specific rate of product formation (qp)

The specific rate of active metabolite production (qp) was calculated according to the following equation:

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

Where X is the biomass concentration (µg/ml), P is antibacterial agent concentration and t is time respectively. The derivative dP/dt was calculated according to the method proposed by [20] with software for graphical analysis Origin PRO-7.5.

Results and Discussion

Antibacterial activity and MIC

Fusarium spp. has been reported as endophytes from several plants with diverse biological activity [34, 19, 3]. This suggests their ubiquity as endophytes within the plant kingdom and provides an opportunity to discover novel bioactive metabolites. Table 1and 2 shows antibacterial activity of isolate, MIC of bioactive compound was defined as the lowest concentration at which 100% inhibition of growth compared to antibiotic free control. Lowest MIC value of 62.5 µg/ml was found against *Enterococcus faecalis* (Table 2). Active compound revealed highest inhibition zone of 25 mm against *Enterococcus faecalis* and lowest inhibition zone was against *Yersinia enterocolitica* (15 mm). Similar MIC (25 µg/ml to 100 µg/ml), as well as inhibition zone, produced by some endophytes was reported by Lu, et al., [22].

Table 1: *In vitro* antibacterial activity of TLC fractionation against the test organisms

Test Organism	R_f Value (100 µg/ml) (Inhibition of Zone diameter in mm)						
	0.126	0.247	0.345	0.572	0.761	0.872	0.912
Staphylococcus aureus	-	-	-	-	-	20 (±2)	-
Klebsiella pneumonia	-	-	-	-	-	17 (±2)	-
Enterococcus faecalis	15	-	-	-	-	22(±2)	-
Yersinia enterocolitica	-	-	-	-	-	15 (±2)	-
Erwinia sp.	-	-	-	-	-	25 (±2)	-
Vibrio parahaemolyticus	12	-	-	-	-	19 (±2)	-
Enterobacter aerogenes	-	-	-	-	-	24 (±2)	-
Escherichia coli	-	-	-	-	-	21 (±2)	-

Proteus vulgaris	-	-	-	-	-	16 (±2)	-

Table 2: Minimum inhibitory concentration of TLC active fractionate (R_f - 0.872) isolated from the strain *Fusarium solani* LCPANCF01

Test Organisms	MIC of 0.872	Std
Staphylococcus aureus	500	100
Klebsiella pneumonia	1000	100
Enterococcus faecalis	62.5	25
Yersinia enterocolitica	500	50
Erwinia sp	250	<12.5
Vibrio parahaemolyticus	1000	100
Enterobacter aerogenes	500	100
Escherichia coli	250	100
Proteus vulgaris	1000	100

MIC; Minimum Inhibitory Concentration, Std; Standard.

Characterization of the bioactive metabolite

Approximately ~ 500 mg/l crude extract was obtained from the culture broth in dichloromethane solvent extraction. In the preparative TLC seven major fractions from the crude compound were recovered with different R_f values (Table2). Each of the major fractions from the TLC plates with different R_f values was bioassayed to determine the active fraction. The pure compound was soluble in methanol. The active fraction with R_f value 0.87 showed UV ƛ-max in methanol at 223 nm (Fig 1). ESI-MS spectrum of the red solid active fraction showed the peak at 301, m/z. Jittra, [15] also reported the peak at 303 m/z, hence the compound identified was javanicin derivatives (Fig 2).

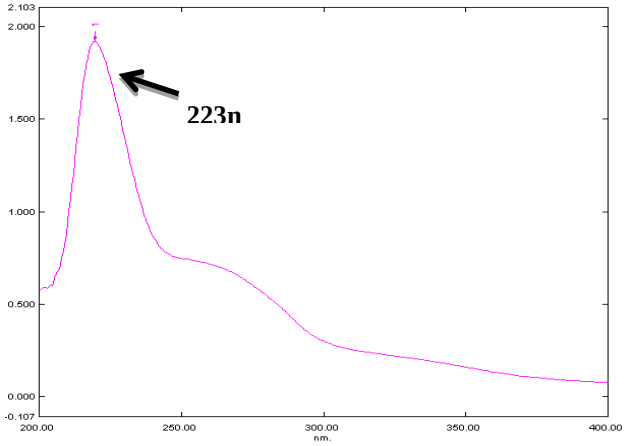

Fig 1: UV ƛ-max of bioactive active fractionation isolated from dichloromethane extracts of *Fusarium solani* LCPANCF01.

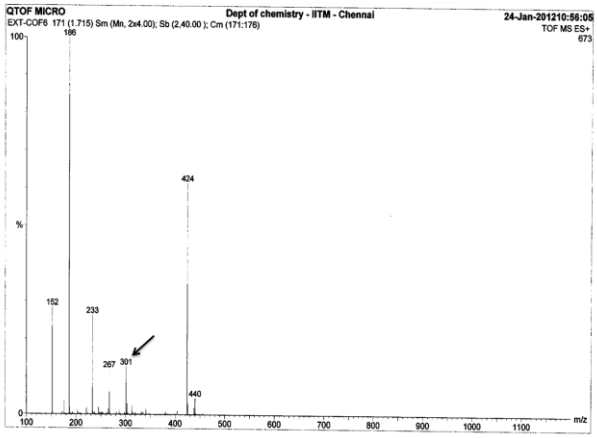

Fig 2: ESI- MS analysis of TLC active fraction shows the corresponding peak at 301m/z

MediumOptimization

Standardization of basal medium

Even though water agar medium was used for the isolation of *Fusarium solani* LCPANCF01, further standardization of medium showed that M_2D was suitable to use as basal medium (Fig 3a). Comparative study of growth vis-a`-vis secondary metabolite production indicated a statistically significant higher biomass and bioactive metabolite production in M_2D by *Fusarium solani* LCPANCF01. Hence, M_2D medium was used to optimize different cultural and environmental parameters for growth and bioactive secondary metabolite production.

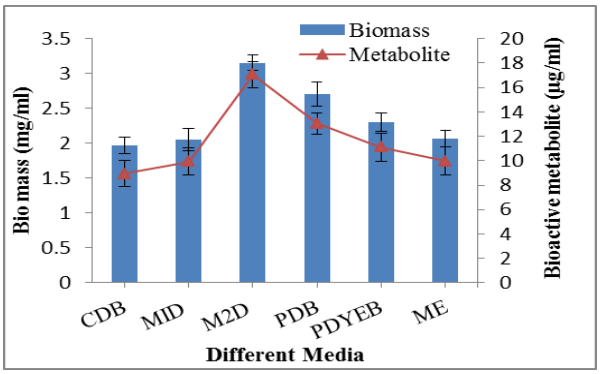

Fig 3a: Standardization of basal media on growth and bioactive metabolite production, Czapek Dox Broth (CDB), Modified liquid medium 1 (M1D), Modified liquid medium 2 (M2D), Potato dextrose broth (PDB), Potato dextrose yeast extract broth (PDYEB), Malt extract (ME).

Effect of carbon and nitrogen source

Fig. 3b shows the effect of carbon and nitrogen source on the production of biomass and bioactive metabolites. A statistically significant higher biomass (3.37 mg/ml) and bioactive metabolite (17.65 µg/ml) were produced by the LCPANCF01 in dextrose amended medium andit was followed by lactose (15.32 µg/ml), respectively in comparison to control (13.97 µg/ml) basal medium. Sucrose (11.45 µg/ml), starch (11.38 µg/ml), fructose (10.24 µg/ml), galactose (10.19 µg/ml), mannose (10.16 µg/ml) and glycerol (10.17 µg/ml) showed moderate amounts of biomass and bioactive metabolite production. This finding suggested that dextrose was a stable carbon source used by *F. Solani* LCPANCF01 which was also accordant with former research of Strobel, [37]. Amendment of yeast extract (18 µg/ml) enhanced the secondary metabolite production while peptone (2.36 mg/ml), Soytone (2.01 mg/ml) and beef extract (2.26 mg/ml) increase biomass production but not bioactive metabolite (Fig. 3b).

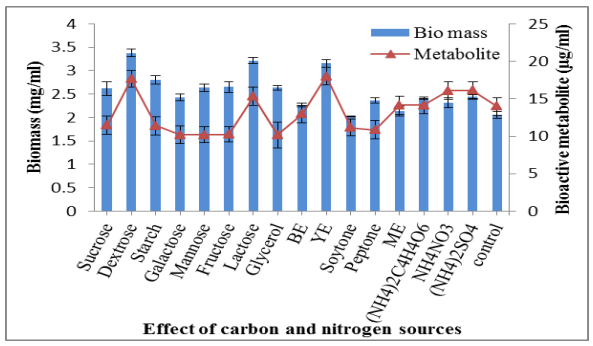

Fig 3b: Effect of Different carbon and nitrogen sources on growth and bioactive metabolite production, Beef extract (BE), Yeast extract (YE), Malt extract (ME), Ammonium tartarate $(NH_4)_2C_4H_4O_6$, Ammonium nitrite NH_4NO_3, Ammonium sulphate $(NH_4)_2SO_4$.

Radu, [28] also reported the maximum production of antifungal and antitumor compound from endophytic fungi grown in glycerol (4.0%) and yeast extract (0.5%) amended medium. The simple carbohydrates like glucose, dextrose through metabolic pathway affects the production of intermediates leading to primary as well as secondary metabolites in addition to CO_2, water and energy [39]. Addition of glucose resulted highest growth of the fungus, but significantly less bioactive metabolite production. In many fermentation processes higher concentration of glucose has a suppressive effect on production of bioactive metabolites [14].

Effect of amino acid

Amino acid along with dextrose enhanced the growth and secondary metabolite production of strain LCPANF01 (2.8 mg/ml, 17.69 µg/ml) (Fig 3c). It reflects the aminoacid supplement may have some role by sharing their carbon ring or both carbon and nitrogen skeleton in to the primary or secondary metabolism processes of microorganisms [25]. Kim [18] reported the importance of rice oil for improving the production of cephalosporin-C production by *Cephalosporium acremonium* M25.

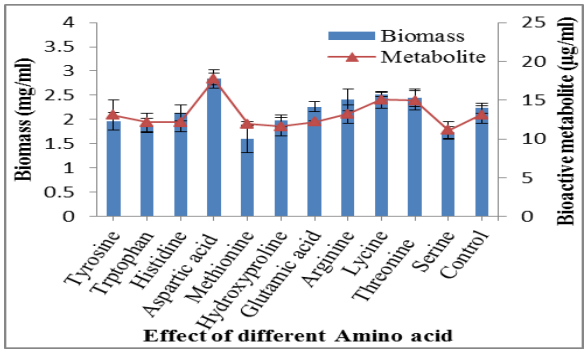

Fig 3c: Effect of different amino acids on growth and bioactive metabolite production.

Effect of pH

Medium with initial pH 6 was found to be optimal for growth (3.5 mg/ml) and bioactive metabolites production (14.72 µg/ml) by the isolate (Fig 3d), pH-5 and pH-7 also supports the growth and bioactive metabolite production of the strain. No growth was observed at pH < 3 and pH >11. Digrak, [4] reported that highest production of biomass by *F. equiseti* was at pH 8, whereas maximum toxic metabolite was produced at the pH 5. The pH of culture medium is one of the determining factor for the metabolism and hence for the biosynthesis of secondary metabolites. The pH is related to permeability characteristics of the cell wall and membrane and thus has got effect on either ion uptake or loss to the nutrient medium [12]. Although literature exists on the growth of fungus in acidic conditions, in our study it was found LCPANCF01 grows in between pH 5-7. Rubini, [31] also reported the growth and antibacterial agent production at neutral pH.

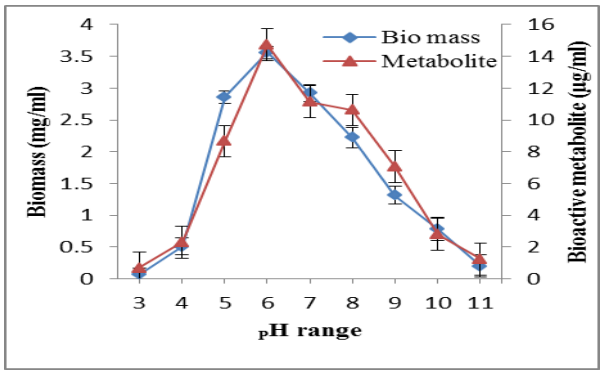

Fig 3d: Effect of pH on growth and bioactive metabolite production

Effect of temperature

Maximum growth (2.45 mg/ml) and bioactive metabolite production (13.06 µg/ml) by *Fusarium solani* LCPANCF01 was recorded at 25 °C and it was followed by 2.10 mg/ml growth and (11.46 µg/ml) bioactive metabolite production recorded at 30 °C (Fig 3e). Less growth and bioactive metabolite production was found at low (15 °C) as well as in high temperature (35 °C). An exponential growth pattern was recorded at temperature between 15 °C to 35 °C, while growth ceased at <40 °C and >50 °C. Study proved that low temperature may cease the metabolic activity of the fungus and high temperature kills the cell of the fungus. Similarly, Huang, [13] also reported the isolation of antifungal and antitumour agent from endophytic fungi at 25 °C and 7-9 days of incubation period.

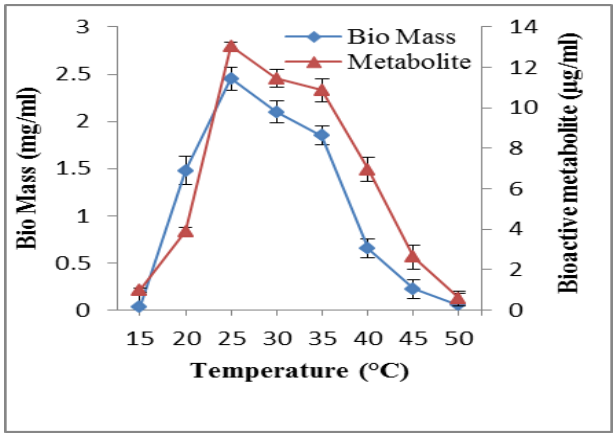

Fig 3e. Effect of temperature on growth and bioactive metabolite production

Effect of incubation period

The incubation period for 9 days was observed to be optimum for maximum biomass (3.42 mg/ml) and bioactive metabolite (14.08 µg/ml) production (Fig 3f). Moreover the growth (3.39 mg/ml) and secondary metabolite production (13.89µg/ml) was slightly lowered at 10 day of incubation period. Maximum growth and production of antibacterial agent was recorded after the fungus reached its stationary phase and remaining almost constant upto 15 days of incubation. After 15 days the growth and secondary metabolite production was significantly very low. Stinson, [36] also reported similar results in the case of endophyte *Gliocladium sp.* Many studies on isolation and characterization of endophytic diversity from different medicinally important plant species have been done or are in progress around the world [16]. Some workers have given a brief record on antifungal and antibacterial activity of endophytes isolated from different gymnosperms of North East India [1, 32].

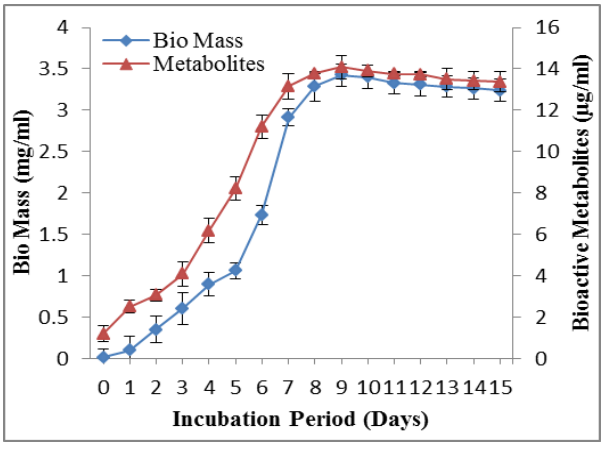

Fig 3f: Effect of incubation period on growth and bioactive metabolite production

Effect of NaCl

Regarding the effect of NaCl concentration, 3.0% was found to be optimum for growth (4.9 mg/ml) and production of the antibacterial agent (11.90 µg/ml) while the concentration above 6% reduces the growth as well as the metabolite production (Fig 3g).

CONCLUSION

Our study reports the optimization of still culture conditions for the high growth and production of bioactive secondary metabolite compound from *F. solani*. Using the statistical procedures, the optimized culture conditions were successfully established. These findings will assist in formulating still culture medium which suitable for producing the antibacterial compound from *Fusarium* strain.

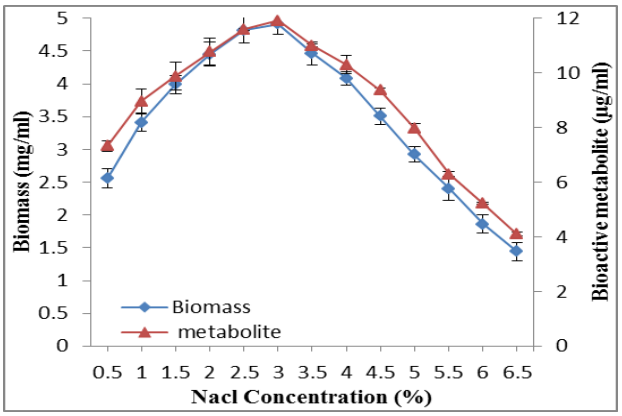

Fig 3g: Effect of NaCl on growth and bioactive metabolite production in *Fusarium solani* LCPANF01

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