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Optimization of Culture Conditions for Enhanced Production of Bioactive Metabolites Rich in Antimicrobial and Antioxidant Activities Isolated from *Emericella quadrilineata* an Endophyte of *Pteris pellucida*

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An endophytic fungus was isolated from leaf of *Pteris pellucida* collected from Arunachal Pradesh, India during April, 2011. Microscopically it was identified as *Aspergillus* species but later authenticated as *Emericella quadrilineata* applying molecular technique. The bioassay profile of *E. qudrilineata* includes antibacterial, antifungal and antioxidant activities. The growth conditions were optimized for improved production of bioactive metabolites and enhanced bioactivity against pathogens. Potato dextrose broth (PDB) has been found to be the best medium for growth and production of metabolites amended with different carbon and nitrogen sources. PDB with -0.1% starch was found to increase the antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus* and *Aeromonas hydrophila.* Incubation period of 25 days coupled with 25ºC temperature were the most suitable for better production of bioactive compounds. *E. quadrilineata* had shown salt stress tolerance from 10 mM to 100 mM NaCl when incubated for 21 days at 26 ºC. The antimicrobial activity of *E. quadrilineata* was elevated only from 10 mM to 30 mM NaCl, whereas relatively it showed low activity above 40 mM. This endophyte had also shown antifungal activity against *Fusarium oxysporum*, *Corynespora* sp., *Aspergillus niger* and *Curvularia* sp. Apart from antimicrobial activity, the culture also exhibited remarkable antioxidant activity. After exploring the culture condition such as media formulation, temperature, incubation time and salt stress, it is evident that different culture conditions affect the production of biomass and activity of bioactive compounds.

Key words: Antibacterial, Antifungal, Fungal endophyte, Inhibition zones, Natural products, Salt stress.

Endophytes are the microbes that colonize internal tissue of the plants without causing any negative effect to their host plants (Bacon and White *et al*. 2000). Often endophytes produce secondary metabolites that are endowed with multitude of biological activities such as antibacterial, antifungal, antioxidant etc. Keeping this fact in view, various ethno medicinal plants of selected locations were exploited time to time for endophytic fungal isolation. Fungal endophytes properties such as antimicrobial, anticancer (Pacilitaxel), antidiabetic (Pestacin and Isopestacin), anti-viral (Cytonic acid), immunosuppressant (Subglutinol A, B and Colutellin A), and antimycotic (Cryptocandin A and Oocydin) were earlier described (Strobel *et al*. 2003;

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Ren *et al*. 2008). Myriads of medicinal attributes of Pteridophytes (fern) found in North-East India are discussed (Benzamine *et al*. 2011). They are being used by tribal people of Arunachal Pradesh against various ailments such as stomach disorders, asthma, poisonous bite, rheumatic problems, cough and diabetes etc. Several endophytic fungi of indigenous medicinal plants have been evaluated for their bioactive compounds (Kharwar *et al*. 2009; Swetha *et al* 2010; Kusari *et al*. 2012). Endophytic fungi have been found to be associated as prolific producer of many biologically active compounds such as taxol, piperine, azadirachtin, and quercetin etc (Strobel *et al*. 2004; Kusari *et al*. 2012), which may serve as leading molecules in drug industries. In this study, endophytic fungus *Emericella quadrilineata* isolated from *Pteris pellucida* has been targeted for extraction of natural bioactive compounds, which could be a potential producer of certain compounds isolated earlier, such as antiviral compound isoindolone (Zhang *et al*. 2011),varitriol (anticancer activity), varioxirane, dihydroterrian and varixanthone (antimicrobial activity) isolated from *Emericella variecolor* (Malmstrom *et al*.2002). Two new xanthone derivatives which have calmodulin inhibition activity were characterized from *Emericella* sp. (Figueroa *et al*. 2009).

Despite the compounds production property, *E. qaudrilineata* is also reported as an opportunistic pathogen for invasive aspergillosis (Verweij *et al*. 2008). Due to inadequate extraction of natural bioactive compounds from endophytic fungi, compelled us to focus the improved production and enhanced activity of bioactive compounds present in crude extract against target bacterial and fungal pathogens. Hence fermentation techniques were used to provide a magnificent improvement in the biomass, yield and bioactivity of *E. quadrilineata*. Effect of chemical and physical factors were examined by Bhattacharya *et al* (2011) on the regulatory mechanism of fungal bioactive compound production such as carbon, nitrogen sources, incubation period, pH, temperature and extraction solvents. Optimization on cultural conditions of induced endophytic fungus *Phomopsis* sp., produces a potent anticancer compound mycoepoxydiene, a novel metabolite which have been reported previously by Narukjapora *et al*. (2010). Here an endeavour has been made to enhance the production of bioactive compound, which is highly active against target organisms such as *Aeromonas hydrophila* and *staphylococcus aureus* by optimizing the fermentation techniques. The batch fermentation method was adopted to optimize the culture conditions with simple strategies. Addition to incubation period, temperatures range, salt stress and solvent selection, an adequate value to starch as carbon source to increase the yield of crude compounds and their activity was given.

MATERIALS AND METHODS

Isolation and characterization of fungal isolate

The endophytic fungus was isolated from medicinal fern *Pteris pellucida* collected during April 2011, at Ganga Lake, Arunachal Pradesh (27 º 06' 00N93 º 37' 12E) situated in region of North-East India. It has been highly used as medicine folklore. Isolation was done following the standard protocol with minor modification (Petrini *et al*. 1991). For surface sterilization, leaf and stem were treated with 70% w/v ethanol followed by 2% sodium hypochlorite. Treated leaf and stem were cut with sterile blade into small pieces (0.5×0.5×0.5cm) and placed on to PDA plates at 26ºC in stationary condition for 21 days. The culture has been maintained on PDA slant. Pure culture was identified, using the microscopic details and manuals (Ainsworth 1973: Hawksworth *et al*. 1997). Molecular identification of culture was performed after isolating DNA using the method (Sim *et al*. 2010). The Internal Transcribed Spacer (ITS) regions (ITS1-5.8S-ITS2) were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplified PCR product was resolved by electrophoresis in a 1.5 % (w/v) agarose gel stained with ethidium bromide (0.5¼g/ml) for visual examination. PCR product was sent to Chromas Biotech (Sahakara Nagar, Bangalore, India) for sequencing. The ITS rDNA sequences obtained were used to retrieve similar sequences using the BLAST program from the NCBI GenBank sequence database.

Test pathogens

Different bacterial and fungal pathogens were used as test organisms for antimicrobial assay.

The bacterial cultures used were, *Staphylococcus aureus* (IMS/GN7) *Aeromonas hydrophila*, (IMS/GN11) *Klebsiella pneumoniae* (IMS/GN9), *Salmonella typhi* (MTCC 3216), *Shigella flexneri* (IMS/GN1), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 25923) and *Morganella morganii* (IMS/GN6). The fungal pathogens used were *Fusarium oxysporum*, *Corynespora* sp., *Curvularia* sp., *Aspergillus flavus*, *Aspergillus niger* and *Alternaria alternata.* All bacterial pathogens were obtained from Institute of Medical Science (IMS), and fungal pathogens from Institute of Agricultural Sciences (IAS), Banaras Hindu University, Varanasi.

Selection of culture medium for biomass and bioactive compound

For selecting the best suitable growth medium, four different culture media, i.e. potato dextrose broth (PDB), Sabouraud dextrose broth (SDB), malt extract broth (MEB) and Czapek dox broth (CZB) were tested. The potential of bioactive compound extracted from *E. quadrilineata* was assessed by measuring fungal biomass and activity of crude extract against bacterial and fungal pathogens. The accumulated biomass and bioactive metabolites were measured by drying at 70°C until a constant weight was obtained. The activity of desired compound was assessed by measuring zone of inhibition against targeted bacterial pathogen.

Effect of incubation period on biomass and bioactive compound

E. quadrilineata was incubated at difference of five days till 30 day to observe the biomass and activity of compound in the crude extract. Six Erlenmeyer flask of 250 ml volume containing 100 ml broth (PDB) were inoculated with equal diameter (5mm) of fungal spore cake. All flasks were incubated at 26°C for 5, 10,15,20,25 and 30 days respectively. Activity of compound against targeted bacterial pathogens was assessed by performing disk diffusion assay.

Effect of temperature on biomass and bioactive compound

The working culture had been grown on different temperature to observe the effect on biomass and bioactive metabolite production. Culture was inoculated in 250ml flask containing 100ml of PDB and incubated for 21days at three different temperatures 25°C, 30°C and 35°C respectively. Biomass and bioactive metabolite were measured by dry cell weight and disk diffusion. Activity of compound against targeted bacterial pathogens was assessed by performing disk diffusion assay.

Effect of carbon and nitrogen sources on biomass and bioactive compound

To enhance the activity of compound present in crude extract, our basal media had been supplemented with different carbon and nitrogen sources. Each 250 ml flask contains100 ml PDB supplemented with carbon and nitrogen sources. PDB without any supplement was used as control. The carbon sources were starch, sucrose, dextrose, mannitol and nitrogen sources were yeast extract, peptone, beef extract, NH_4Cl and NH_4SO_4. The effect of various concentrations (100mg/ml to 500mg/ml) of carbon source on the growth and bioactivity were also analysed.

Effect of different concentration of Starch in PDB media

PDB along with varying concentration of starch (100, 200,300,400,500 mg/100ml) has been tested for improved production of bioactive compounds. *E. quadrilineata* were inoculated in all the five flasks and incubated at 26ºC for 21 days. The biomass and activity of bioactive metabolite was measured using dry cell weight and disk diffusion method respectively.

Effect of salinity on the endophytic culture

The effect of salt concentration on the growth and metabolite production was carried out by incubating the fungus at different concentration of salt from 10 mM to 100 mM NaCl/100 ml. PDB without any additional salt was used as control. The culture flasks were incubated for 21 days at 26ºC. The biomass and activity of bioactive metabolite production was measured using dry cell weight and disk diffusion method respectively.

Effect of organic solvents on extraction and activity of compound

E. quadrilineata was grown at 26°C for 21days to complete its fermentation. Equal volume of culture broth was extracted with three different organic solvents such as hexane, chloroform and ethyl acetate. Concentrated crude extract was checked against different bacterial and fungal pathogens to observe its ability to inhibit the growth.

Specific rate of product formation (qp)

The specific rate of bioactive product (secondary metabolites) formation was calculated according to following equations:

qp = 1/X(dp/dt)

Where X is the biomass concentration (ug/ml), P is antimicrobial agent concentration and t is time respectively. The derivative dp/dt was calculated according to the method proposed by Le Duy and Zajic (1973) and software used for graphical analysis was origin PRO-8.0

In-vitro antimicrobial bioassay

E. qaudrilineata (RS-5) was screened for antibacterial and antifungal activities by performing disc diffusion method (Kirby and Bayer 1960) and dual culture method respectively. Bacterial suspension was made in autoclaved distilled water and lawn culture was prepared on Mueller Hinton agar plate with the help of sterile cotton swab. Sterile disc impregnated with crude extract at the concentration of 1mg/disc were placed on the lawn culture and observed for the zone of inhibition after 48 hours. In dual culture method both, organisms and fungal pathogens were kept opposite end of Petri plate in triplicate with a control. The percent inhibition was measured by scale.

Endophyte as source of antioxidant compound

In vitro antioxidant assay via DPPH free radical scavenging activity

Crude extract of *E. quadrilineata* culture broth was evaluated for antioxidant assay using DPPH (2, 2-diphyenyl-1-picrylhydrazyl) scavenging method. The free radical scavenging activity of crude extract was the ability to bleach the stable DPPH radical. A stock solution of DPPH was prepared (100mg/ml) with methanol. Stock solution was used to measure the antioxidant activity. Decrease in absorbance while increasing the concentration of crude extract was recorded along with IC -50 values. DPPH alone used as standard. Percentage inhibition activity was calculated by given formula;

[(A0–A1)/A0] x100,

Where A0 is the absorbance of the control, and A1 is the absorbance of the extract and DPPH. The inhibition curves were prepared and IC_{50} value was calculated. The free radical scavenging activity of the extract, based on the scavenging activity of the stable DPPH free radical was determined by the method described by (Marwah *et al*. 2007). Crude extract (0.1 ml) was added to 3ml of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min of incubation period.

Statistical analysis

All experiments were carried out in triplicate in order to obtain authentic data using above variables. Mycelia growth and bioactive metabolite production of the strain were statistically evaluated by one way analysis of variance (ANOVA). The level of significance considered was $P<0.05$. Means of above parameter were calculated and significant errors were calculated using 5 percent. The entire graphs were plotted using origin 8.0 PRO. Statistical analysis was performed in SPSS version 16.0 software packet.

RESULTS

Characterization of Endophytic fungus

Morphologically an unidentifiable endophytic fungus was cultured on PDA and coded as RS-5. The macroscopic appearance of the culture was initially white colour which turns to yellowish brown after a few days (fig 1). Based on spore arrangements the culture was identified as *Aspergillus* sp. (fig 2), but to authenticate the

Table 1. Antimicrobial profile of bioactive metabolite produced by Emericella quadrilineata

S. No	Test organism	Zone of inhibition(mm)
	Bacterial pathogens	
1	*Aeromonas hydrophila*	22
2	*Staphylococcus aureus*	18
3	*Shigella flexneri*	13
4	*E.coli*	7
5	*E. fecalis*	6
6	*P mirablis*	Nd
7	*K. pneumoniae*	Nd
	Fungal pathogens	Percentage of inhibition
8	*Fusarium oxysporum*	66.52%
9	*Curvularia* sp.	33.34%
10	*Alternaria* sp.	Nd
11	*Aspergillus flavus*	Nd
12	*Aspergillus niger*	30%
13	*Corynespora* sp.	60%

identification, ITS region of the isolate was amplified and got it sequenced. Then the sequence data was used for a nucleotide-nucleotide search using BLAST. The sequence of RS-5 had shown 99% sequence similarity to *Emericella qaudrilineata* and submitted to genbank with accession number KC662361. Hence, the endophytic fungus RS-5 was confirmed as *E. quadrilineata* which is known as perfect state (teliomorph) of *Aspergillus nidulans* belonging to class Ascomycetes and order Eurotiales.

Antibacterial and antifungal activity

Emericella quadrilineata isolated from *Pteris pellucida* was observed for antimicrobial and antifungal activities. *E. quadrilineata* culture had shown the impressive antibacterial activity against *Aeromonas hydrophila* (20mm) and *Staphylococcus aureus* (18mm) (fig 3a). It showed diminutive activity against *Salmonella typhi, Enterococcus faecalis, Escherichia coli* and *Shigella flexneri* by showing approximately 6-7 mm zone of inhibition while no activity observed against *Klebsiella pneumoniae* and *Proteus mirabilis.* This fungus had also shown antifungal activity against *Aspergillus niger* (30%) *Curvularia* sp (33.34%), *Corynespora* sp., (60%),

Fig.1. Pure culture plate of *Emericella quadrilineata*

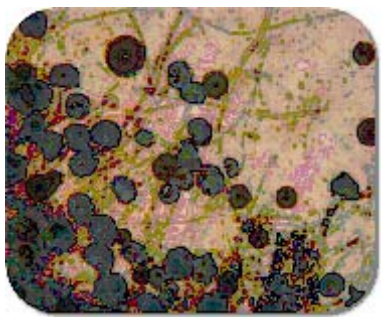


Fig.2. Microscopic view of *Emericella quadrilineata*

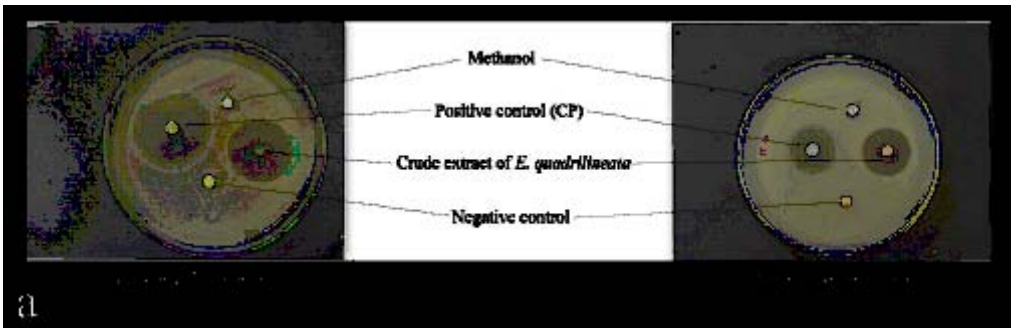


Fig. 3a. Antibacterial assay of *Emericella quadrilineata* crude extract against targeted bacterial pathogens

Fig. 3b. Antifungal dual culture assay of *Emericella quadrilineata* against *Aspergillus niger*, *Curvularia* sp., and *Fusarium oxysporum*

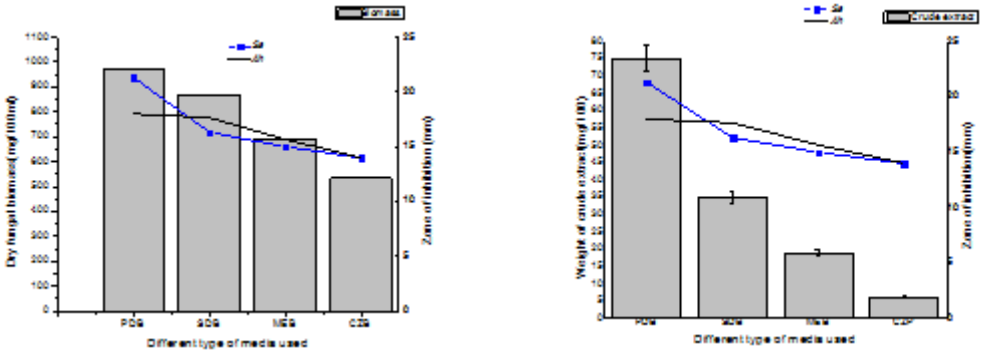
and *Fusarium oxysporum* (66.52%). No antagonism was observed against pathogenic fungi *Aspergillus flavus*, *Aspergillus terreus* and *Alternaria atlternata* (Table 1). Ciprofloxacin (CP) was used as positive control while testing antibacterial activity (Fig 3a and 3b).

Optimization of Media

Selecting the suitable media for fermentation always favour the production of secondary metabolites that may lead to extraction of desired compound. Potato dextrose agar (PDA) was used for the isolation and growth of fungal culture. Although, all four media used for the production of bioactive metabolites showed reasonable amount of production along with biomass however, PDB showed the best growth followed by Sabouraud dextrose broth (SDB), malt extract broth (MEB) and Czapek dox broth (CZB) (fig. 4a & 4b). Potato dextrose broth (PDB) had shown significantly higher biomass (9.7mg/ml) and bioactive metabolite (750µg/ml) production than to other media. CZB biomass produced lowest biomass (5.3mg/ml) and bioactive metabolites (63µg/ml) production. Hence potato dextrose broth was selected to optimize different culture conditions for the enhanced production of biomass and metabolites.

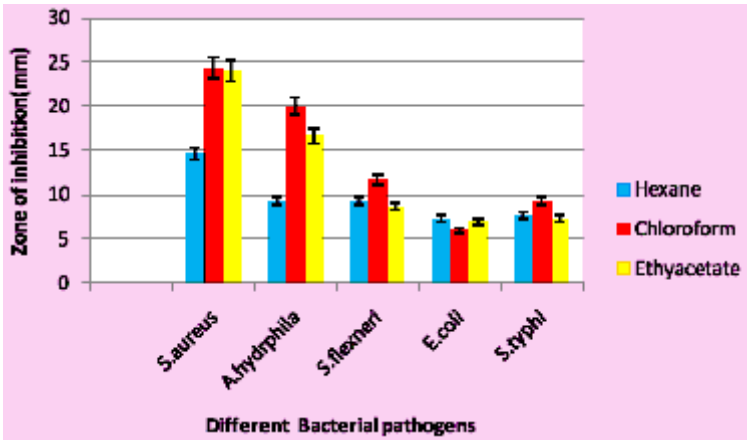
Effect of different solvents on the extraction

Among three organic solvents taken, ethyl acetate showed maximum extraction efficiency followed by chloroform and hexane. Natural products are organic in nature and all polar compounds dissolved in organic solvent on the basis of polarity. Maximum amount of crude extract



*Data on mycelial growth and bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

Fig. 4a & 4b. Growth pattern and production of bioactive metabolite by *Emericella quadrilineata* on different media



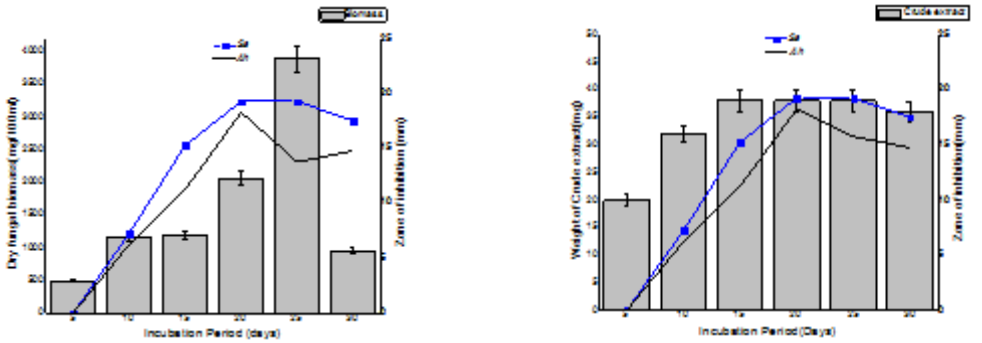
*Data of bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

Fig. 5. Different organic solvents extracted activity of bioactive metabolites of *Emericella quadrilineata* against different pathogenic bacteria

was obtained in chloroform (580µl/ml), followed by ethylacetate (443µl/ml) and hexane (140 µl/ml). The crude extract compound was dissolved in all solvent to a certain extent. The culture broth which extracted with chloroform has shown maximum activity against the bacterial pathogens *S. aureus* and *A. hydrophila*. Other culture broth extracted with ethyl acetate and hexane has shown comparatively lesser activity against *S. typhi*, *E. faecalis*, S. *aureus* and *A. hydrophila* (fig. 5)

Effect of incubation period on biomass and bioactive metabolite secretion

Emericella quadrilineata secreted maximum compounds at 25th day of incubation period. The biomass and metabolite production enhanced with an incubation period of 25 days, but it has declined after 25 days (fig. 6a and 6b). Both biomass and bioactive metabolite followed the principle of batch culture. Comparative analysis of fig 6a and 6b showed that biomass and bioactive metabolite production increased significantly from 1 to 25 days. At 25th day, production of fungal biomass and bioactive compound was recorded maximum when the culture reached its stationary phase and become constant. The highest value of specific rate of bioactive metabolite production was observed at 25th day i.e. 0.971µg/ml (fig. 6c).



*Data on mycelial growth and bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

Fig. 6a & 6b. Incubation periods effect on growth pattern and production of bioactive metabolites by *Emericella quadrilineata*

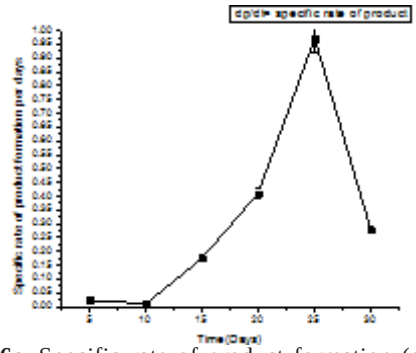
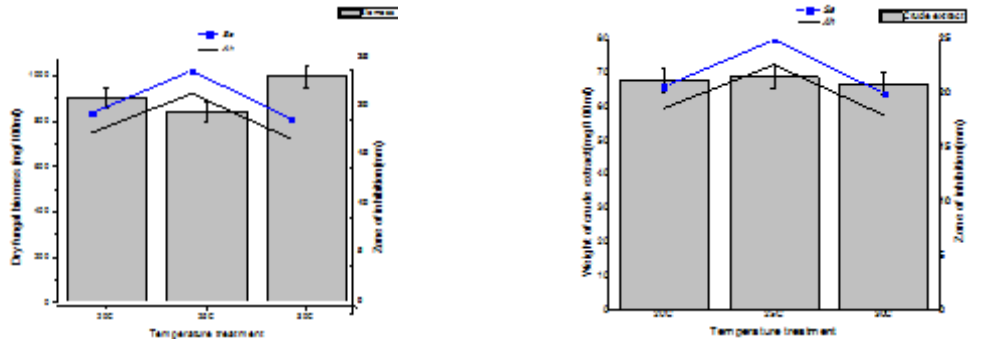


Fig. 6c. Specific rate of product formation (qp) by *Emericella quadrilineata*

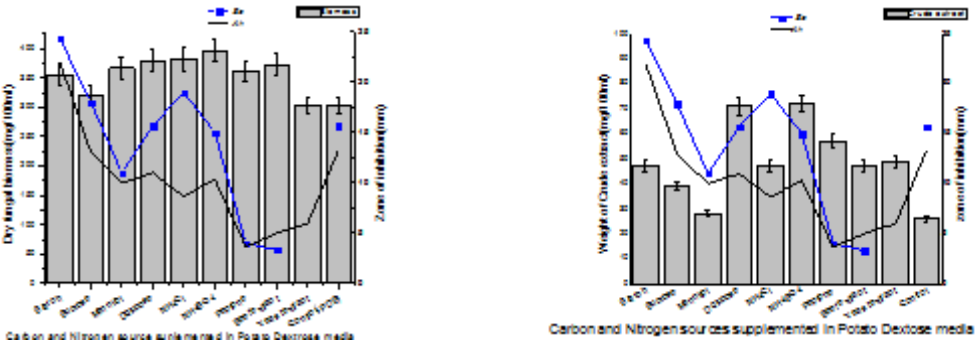


*Data on mycelial growth and bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

Fig. 7a & 7b. Growth pattern and production of bioactive metabolite by *Emericella quadrilineata* at different temperatures

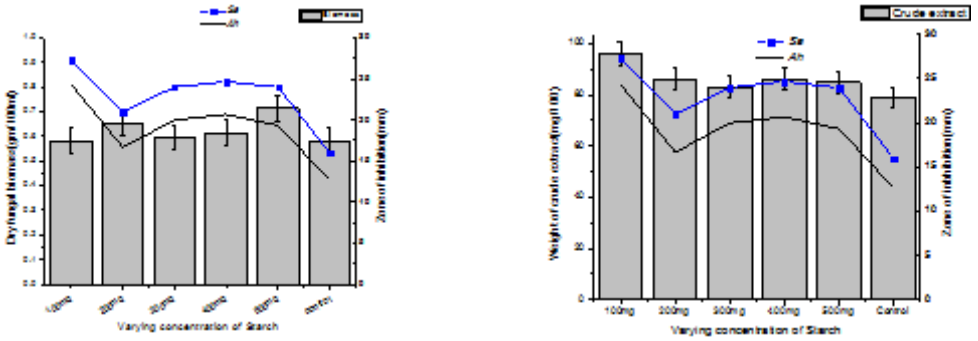
Effect of temperature on biomass and metabolite production

The culture of *E. qaudrilineata* grew on three different temperature, T1-20ºC, T2-25ºC and T3-30ºC. It has been found that maximum biomass production (9.9mg/ml) was obtained at 30ºC and maximum bioactivity against bacterial culture was recorded in the culture incubated at 25ºC followed by 30ºC and 20ºC (fig. 7a). Maximum amount of bioactive metabolite production (690µg/ml) was found at 25ºC, followed by (680µg/ml) at 20 ºC and lowest (676µg/ml) at 30 ºC (fig. 7b).



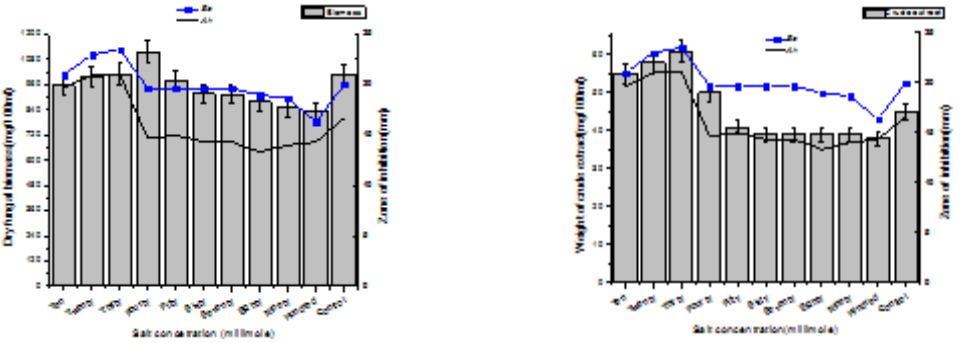
*Data on mycelial growth and bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

Fig.8a & 8b. Growth pattern and production of bioactive metabolite by *Emericella quadrilineata* on PDB



*Data on mycelial growth and bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

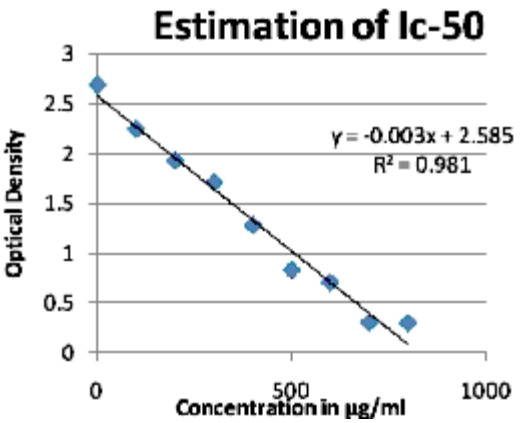
Fig. 9a & 9b. Growth spectrum and production of bioactive metabolite by *Emericella quadrilineata* at varying concentration of starch medium supplemented with carbon and nitrogen source



*Data on mycelial growth and bioactive metabolite yield were statistically analysed by one way ANOVA and found to be significant at 5%

Fig. 10a & 10b. Growth pattern and production of bioactive metabolite by *Emericella quadrilineata* at different concentrations of salt stress

Fig. 11a. Antioxidant activity of crude extract via DPPH assay



Absorption of control=2.697
Y=2.697/2=1.3485
According to graph analysis Ic-50 value should fall on 412µg/ml concentration.
1.3485= -0.003x+2.585=412.16

Fig. 11b. Estimation of Ic-50 value of antioxidant activity

Effect of supplemented carbon and nitrogen sources

PDB has been supplemented with different carbon sources such as glucose, dextrose, starch, mannitol and nitrogen sources beef extract, yeast extract, peptone, NH_4Cl and NH_4SO_4 at the concentration of 0.1%, while PDB without any supplement acted as control. The effect of carbon and nitrogen sources on biomass and metabolite production is apparent (fig. 8a and 8b), PDB supplemented with starch had enhanced the metabolite production and its activity against the targeted pathogenic bacteria *Staphylococcus aureus* and *Aeromonas hydrophila*. Among carbon source PDB supplemented with glucose showed best mycelial growth with the rate of 3.8 mg/ml (fig.8a), as well as maximum weight of crude extract 710µg/ml (fig.8b). Among nitrogen sources NH_4Cl supplemented with PDB, showed greater activity of compound against two more bacterial pathogens such as *S. typhi*, *E. faecalis* including *S. aureus* and *A. hydrophila*. PDB supplemented with NH_4SO_4 had produced maximum biomass (3.9mg/ml) and maximum crude extract (723µg/ml).

Effect of varying concentration of Starch from (0.1gm to 0.5gm/100ml)

Basal media supplemented with starch had showed maximum zone of inhibition against targeted bacterial pathogens. Taking this in to consideration, PDB supplemented with starch with varying concentrations was observed for biomass and antimicrobial activity of *E. qaudrilineata*. Although, PDB supplemented with 0.1% starch concentration was found to be the best for the yield of bioactive metabolites (960µg/ml) and for their activity against bacterial pathogens (Fig. 9b), while maximum biomass (7.1mg/ml) was observed with 0.5% starch (Fig. 9a).

Different salt concentrations and their effect

The culture showed significant tolerance potential of salt stress from 10 mM to 100 mM concentrations. The production of bioactive metabolites was increased continuously from 10 to 40 mM of NaCl per 100ml and became constant after 40 mM. Maximum biomass production was observed at 30 and 40 mM concentration (fig 10a), (approx. 1mg/ml) and least amount of biomass production was observed at 90 mM NaCl (850µg/ml). Maximum yield (610 µg/ml) of crude extract and maximum zone of inhibition were found at 30mM salt concentration whereas minimum (380µg/ml) at 100mM NaCl (fig. 10b).

Antioxidant activity

Crude extract of fungus was further evaluated for antioxidant activity of crude compound by calculating IC-50 value. For this, varying concentration of crude extract in methanol (100-1000 µg/ml) were taken, and assessed by DPPH radical scavenging activity. On calculation IC-50 value was found at 400µg. It has been found that absorbance is significantly correlated with the concentration of crude extract. Change in colour was observed in increasing order of the concentration from 100-1000µg/ml (fig 11a). According to calculation and graphical analysis (fig11b) it has been found that *E. quadrilineata* displayed moderate antioxidant activity.

DISCUSSION

Endophytic fungi have been the unexplored source of natural products that may be exploited as huge repertoire of unparallel chemical and structural diversity with potent biological activity (Mishra *et al*. 2012a,b; Kharwar *et al*. 2011: Verma *et al*. 2009; Strobel and Daisy, 2003). Endophytes produce secondary metabolites, which are biosynthetically derived from primary metabolite by genetically controlled method, enzymatic catalysed reaction that lead to formation of complex compounds (natural product). Generally, production and yield of these compounds are limited as they depend upon physiological and developmental stages of fungus. To circumvent this problems here an attempt has been made to optimize the cultural and physiological conditions in order to enhance the production of biomass, yield of products and their bioactivity. In present work, endophytic species *Emericella quadrilineata* has been targeted for optimizing the parameter of culture conditions for the production of bioactive metabolites. Although, in many earlier studies based on pharmacological importance endophytic fungus *Emericella* had been a great source of various compounds such as varitriol (anticancer activity), varioxirane, dihydroterrian and varixanthone for antimicrobial activity (Malmstrom *et al*., 2002). Zhang *et al*. (2011) had also discovered iso-indolone derivative having antiviral activity from endophytic fungus *Emericella* sp. associated with medicinal plant *Aegiceras corniculatum*. Different chemical structures were elucidated from cytotoxic extract of endophytic fungus *Aspergillus niger* from liverwort *Heteroscyphus tener* (Steph) (Li *et al*. 2013). In present study, it was observed that culture of *E. qaudrilineata* found to possesses many attributes of biological activity such as antibacterial, antifungal and antioxidant etc. (Table1, fig 3a, 3b and11a). Endophytic fungi *Alternaria alternata*, *Cladosporium cladosporioides*, *Microsporum gypseum*, *Fusarium udum* and *Trichophyton rubrum* have been reported from the leaves of *Eucalyptus citriodora*, which had shown antifungal activity against six pathogenic fungi (Kharwar *et al*. 2010). More than 75% endophytic fungi displayed antibacterial activity against several bacterial pathogens from *Nythanthus arbor*-tristis (Gond *et al*. 2012). *E. quadrilineata* exhibited an impressive antibacterial activity against *S. aureus* and *A. hydrophila* and antifungal activity against *Fusarium* sp. and *Curvularia* sp. Further, cultural parameters such as media amendments, incubation period, carbon and nitrogen sources, and temperature were optimized to increase the production of biomass and bioactive metabolites.

Among four different basal media tested, all the media have produced considerable amount of biomass and bioactive metabolites. However, potato dextrose broth was found to be the best amongst all that produced high amount of biomass and an impressive inhibition against bacterial pathogens (fig 4a and 4b). In the optimization process we observed that, biomass and bioactive metabolite productions were directly proportional to each other. The results obtained (fig 4a and 4b) illustrate the different parameters and its relationship to the biomass, crude extract yield and zone of inhibition, which show the conformity with the earlier studies (Bhattacharya *et al*. 2011; Xinanaung 2013). Some other studies also reported PDB as the best medium for the growth and enhanced production of secondary metabolites from *Aspergillus terreus* (Premlatha *et al*. 2012; Suja 2013). The PDB has also been reported as the best medium for biomass production and napthaquinone biosynthesis from *Fusarium moniliforme* (Premlatha *et al*. 2012). However, the same fungus *Fusarium* sp., has produced the contrary results from previous and found modified

liquid medium as the best for biomass and bioactive compounds production (Merlin *et al*. 2013).

In order to achieve the higher growth kinetics and optimum period of metabolite accumulation, incubation period must be optimized in batch fermentation. During citric acid production *Aspergillus niger* showed the growth disturbance due to perturbation in metabolism and exhaustion of carbon and nitrogen source in medium (Berry *et al*. 1977)). Generally secondary metabolites secreted in stationary phase, and they are supposed to be the target in which the bioactive compounds are present. So, it is important to determine the stationary phase of the particular fungus to get higher amount of natural compound of interest. In present study, six different incubation periods were maintained, and *E. quadrilineata* grew best at 25 days of incubation with enhanced bioactivity of metabolite in the same flask (fig 6a and 6b). This elucidates that 25 day incubation period found to be optimum for the growth of fungus and production of bioactive compounds. After that the culture starts going under decline phase which results decreased biomass and reduced bioactivity and that may be due to accumulation of some inhibitory/toxic residues against secreted bioactive compounds. Specific rate of bioactive metabolite product was observed at 25 day $0.971 day^{-1}$ (fig 6c). Kinetics of fermentation used by geometrical approach (Le and Zajic *et al*.1973) analysis leads to separation of period of mycelial growth and bioactive product formation. Using the same geometrical approach lower specific product rate 0.0058 day^{-1} at 9th day has been reported previously in *Fusarium* sp., which is contrary to present result (Gogoi *et al*. 2008; Merlin *et al*.2013). Since, this fungus has taken a bit long incubation period 25^{th} day for higher specific product rate of antimicrobial compounds (fig 6), hence we can say every fungus has its own production potential of secondary metabolites at difference incubation period (Xinuang *et al*. 2013). Incubation period explains the growth rate of fungi and its metabolite production.

Considering growth media and incubation time, temperature also plays a remarkable role on production of metabolites. In current study, out of 3 different temperature 25 ºC was recorded to be best for production of bioactive metabolites while increasing the temperature up to 30 ºC enhanced production of biomass and metabolites were observed (fig 7a and 7b). The results of present study closely correlate with results of some previous studies (Suja *et al*. 2013; Bhattacharya *et al*. 2011). But, another report found completely same results, where a marine derived fungus *Antirhinium c.f. sacchricola,* showed maximum biomass production at 30ºC whereas maximum antibacterial activity was recorded at 25 ºC (Miao *et al*. 2006). Bhattacharya *et al*. (2011) concluded that *Aspergillus strain TSF* showed best growth and production of bioactive metabolites at the same temperature (30 ºC).

Organic solvents also play important role in extraction of bioactive natural products in form of crude compounds from broth. Among three different solvent tested, all three extracted the bioactive metabolites, but maximum extraction was recorded in chloroform (fig 5). As a result we found that all solvent used were extracting adequate amount of crude, but maximum crude as well as activity was recorded in chloroform extract of *E. quadrilineata*. This result corroborates the finding of experiment with *Aspergillus* where chloroform was also reported as best extraction solvent (Jain and Pundir 2011). Whereas, mangrove associated endophytic fungi culture extracted with ethylacetate showed highest antagonistic activity against pathogenic fungi and bacteria (Thangaraj *et al*. 2013). Apart from this, it was found that dichloromethane extraction of broth culture of endophytic fungus *Pestalotiopsis* sp. had maximum zone of inhibition against *Bacillus subtilis*, *E. coli* and *Candida albicans* (Bagyalakshmi *et al*. 2012). The metabolites consist of numerous compounds that have varied degree of solubility in different solvents. These results indicate the importance of appropriate selection of solvent that certainly alleviate the problem of less extraction and activity of compound.

PDB media supplemented with starch improved the bioactivity of compound against targeted bacterial pathogens. Since**,** maximum biomass production was found in PDB, while maximum bioactivity was recorded in PDB supplemented with starch (fig 8a and 8b). This finding supports the previous report (Rajasekar *et al*. 2012), whereas others found quite variant with respect to our results (Bhattacharya *et al*.2006; Premlatha *et al*. 2012; Suja *et al*.2013). Gogoi *et al*.

(2008) found leaf extract media supplemented with dextrose as carbon sources and yeast as nitrogen sources resulted in higher production of bioactive metabolite from endophytic fungus (DF-2) isolated from *Taxus wallichiana*. According to Premalatha (2012) highest yield of Napthaquinone produced from *Fusarium moniliforme* had been observed in PDB supplemented with glucose as carbon source. Similarily, dextrose and sucrose were found suitable carbon sources respectively, while working with fermentation parameters with endophytic fungi *Fusarium* sp. and *Aspergillus terreus* (Merlin *et al*. 2013; Suja 2012). Yeast extract has been reported as the nitrogen sources among several experiments (Premlatha *et al*. 2012; Merlin *et al* 2013; Suja *et al*. 2012). From above observations and it can be concluded that a fungus primarily exploits simple sugar (rather than disaccharide and polysaccharide) for its survival and adaptation. However, here exclusively PDB supplemented with starch had shown promotional effect in antibacterial activity of crude compounds isolated from *E. quadrilineata* (fig 8a and 8b) and it may be due to specific preferences of carbohydrates utilizing capacity of fungal strain physiology. Earlier it has been reported that difference in growth of *A. nidulance* on different carbohydrates could be due to key catabolic enzyme or of specific system uptake (Ramano and Kornberg 1968; Kornberg 1973). Different species of *Aspergillus* found to posses variety of enzymes such as amyloglucosidase, which attack on alpha 1-4 and alpha 1-6 bond of amylose and amylopectin and hydrolysed starch completely to glucose (Barbesgaard 1977). Therefore, it can be predicted that this step may be playing remarkable role in biosynthesis of major precursor of bioactive compound present, in preference to other sources present in media.

After testing with different concentration of starch, 01mg/ml concentration of starch was found to be effective for maximum production of bioactive metabolite (Fig 9a and 9b).

Looking the ubiquitous occurrence of fungi, different natural environmental condition has led to exploit this property by giving varying salt concentration to *in vitro* conditions. Salt tolerance is a special adaptation of fungi which could be adapted further for biotechnological process in pharmaceutical work. Beside above modifications, fungi undergo salt stress to enhance the leading compound present in crude extract. In the present work, effect of salt concentration in PDB was evaluated from 10 mM to 100 mM of NaCl. Organism has adapted to the salinity for growth and production of metabolite, but only up to certain level (30 mM), after which metabolite production decreases. Growth of culture and metabolite production increases from 10 to 30 mM NaCl but decreased gradually after 40 mM (50 to 100 mM) (fig 10 and 10b). However, it was found in this experiment that metabolic product secretion was continued, at concentration of 100mM which was the highest in experiment (fig 10b). Salt mediated changes leads *de-novo* synthesis of enzyme in halotolerant fungus *Cladosporium sphaerospermum* (Karlekar *et al*. 1985). Here these enzymes can be exaggeratory source to increase production of fungal mycelia and bioactive compounds present in crude and leads to halotolerant fungus. Bhattacharyya *et al*. (2006) and Gogoi *et al*. (2008) experimented on fungus grown with different salt concentrations and found that increased salt concentration affected the production of bioactive metabolites. While working with a marine fungus, use of NaCl has promoted the growth of fungal cultures (91.5%) and a considerable increase (14.1%) in antimicrobial activity (Jinjing *et al*. 2011). Based on present experiment and previous examples salinity is one of influencing factor which enhance the fungal growth and bioactivity/or *de-novo* product. Hence, it can be concluded that fungi could tolerate only reasonable concentration of salt, but much higher concentration further will eventually affects the production of active metabolites.

Endophytic fungi have also been the good source of antioxidant compounds as pestacin and isopestacin are reported from *Pesalotiopsis microspora* (Harper *et al*. 2003; Strobel *et al*. 2002). Scavenging effect of fungal extract of *E. qaudrilineata* (RS-5) was revealed via DPPH radical scavenging assay. Varying concentration of fungal extract was taken from 100-1000µg/ml. DPPH was used as standard. Since IC_{50} value of metabolites falls at around 400µg/ml via mathematical analysis. Its evaluation by graphical analysis found that value of metabolite falls around 412µg/ml. Hence it can be concluded that *E. qaudrilineata* has exhibited moderate antioxidant

activity. In a couple of previous studies, antioxidant activity of endophytic fungi isolated from *Withania somnifera* whereas, flavipin (1,2-Benzenedicarboxaldehyde-3,4,5-trihydroxy-6-methyl) reported from the endophytic fungus *C. globosum* (CDW-7) with strong antioxidant and nematicidal activity (Madki *et al*. 2010; Ye *et al*. 2013). Zeng *et al* (2011) investigated the antioxidant activity, from endophytic fungi of *Scapania verucossa* that was evaluated by DPPH and ABTS. Bhagobaty and joshi (2012) had found antioxidant activity to endophytic fungi from ethno medicinal plant of the sacred grooves of Meghalaya.

From the above observations it is evident that *Emericella qaudrilineata*, an endophytic fungus of *Pteris pellucida* produced the good amount of antimicrobials with significant activity and reasonable level of antioxidant property. In this study an endeavour has been taken to produce high amount of natural compounds using different physiological parameters. It had been found that different physiological conditions affect both biomass and bioactive metabolite production. In future, these physiological conditions could be utilized in pharmaceutical and biotechnological industries to scale up the production of natural compounds. Apart from all the parameter tested and discussed in this paper, further purification, characterization and elucidation of bioactive principle from endophyte *Emericella quadrilineata* RS-5 is under progress.

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