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Optimization of Culture Parameters for Improved Production of Bioactive Metabolite by Endophytic *Geosmithia pallida* (KU693285) Isolated from *Brucea mollis* Wall ex. Kurz, An Endangered Medicinal Plant

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The present study envisaged to optimise the culture parameters for improved bioactive secondary metabolite production by *Geosmithia pallida* (KU693285), a potent endophytic fungi, isolated from endangered medicinal plant *Brucea mollis* Wall ex. Kurz. *G. pallida* was isolated from healthy, surface sterilized leaf, bark and root tissues of *B. mollis*. Sporulation was induced in sterile *G. pallida* by growing it on modified PDA media. Identification of *G. Pallida* was confirmed by ITS r-DNA sequence analysis. The culture parameters were optimized for enhanced production of metabolites. Maximum metabolite production was observed at 25°C incubation temperature and 6.5 pH on 28th day of inoculation, when lactose and yeast extract were added in the basal medium respectively as carbon and nitrogen source along with 3.5 g/l NaCl. The inhibition zone showed by *G. pallida* was significantly higher during the optimum culture conditions than that of the control (basal media). From the present study, it is concluded that for the maximum production of bioactive metabolite the culture parameters need to be optimized. These results would steer the authors to carry out further studies on purification, characterization, and identification of bioactive metabolites produced by *G. pallida* (KU693285) for pharmaceutical as well as agricultural applications.

Keywords: Endophytic fungi; *Geosmithia pallida* (KU693285); bioactive secondary metabolite; optimization; cultural parameters.

Endophytes are the microbes that colonize living internal tissues of plants without causing any immediate, overt negative effects[1]. Endophytic fungi are proven source of bioactive metabolites in today's world[2]. They are the nature's hidden resources of antimicrobials, antioxidants, insecticides, herbicides etc.[3,4]. The endophytic fungal diversity is more or less unexplored. Proper exploration of medicinal, endemic, rare, endangered etc. plants is required for the enumeration of novel endophytic fungi residing inside them for further study.

Appearance of multi-drug resistant strains of pathogens has guided researchers to search for new noble antimicrobial compounds. The endophytic microorganisms are one of the potential biological resources of potent active molecules because of the pristine specialized niche they have occupied[5]. It is observed that these organisms may produce metabolites identical to the one produced by their hosts[6]. The production of bioactive metabolites by fungi is affected by the culture parameters like, pH, temperature, incubation period, different nitrogen and carbon sources, NaCl concentration etc. of the medium

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and the availability of the metal ions in the media used[7,8]. Optimization and maintenance of suitable culture parameters are necessary criteria to achieve maximum production of bioactive metabolites by microbial strains.

B. mollis of family Simaroubaceae, is a small tree and is recorded as endangered species in North East India by CAMP survey (Annonymous 2003). It is traditionally used as antimalarial and antimicrobial agent but has antiplasmodial, cytotoxic, anticancer, diuretic, cardiovascular properties as well[9]. Being endangered and growing in biodiversity hotspot region of the world, *B. mollis* may have noble endophytic fungi having antimicrobial, antimalarial, anticancer etc. properties like that of the host. Use of endophytic fungi in place of the plants for the production of the compounds having antimicrobial, antimalarial, anticancer etc. properties, may help in conserving the host plants as their use will be lessen. Keeping this hypothesis in mind, an attempt was made to conserve endangered medicinal *B. mollis* through exploration of endophytic fungi associated with it, for antimicrobial compound production for pharmaceutical purposes in place of the plant.

The present investigation envisaged to optimise the medium for optimum production of metabolite by endophytic *G. pallida* (KU693284) isolated from *B. mollis.*

MATERIALS AND METHODS

Isolation and identification of endophytic fungi

Leaf, bark and root samples were washed and cut into about 2 cm long and 0.5 cm broad segments with sterile knife. Segments were sequentially immersed in 70% ethanol for 3 minutes, 4% aqueous solution of sodium hypochlorite (NaClO) for 5 minutes and 70% ethanol for 1 minute and finally in 0.1% Mercuric chloride ($HgCl_2$) for 3 minutes[10]. The segments thereafter were rinsed with sterile de-ionized water to remove the traces of $HgCl_2$ and then surface-dried. The effectiveness of surface sterilization was tested for every segment using imprint method[11]. The segments were then inoculated in Petri plates (90×15 mm) containing different media viz., Czapeck-Dox-Agar (CDA), Potato-Dextrose-Agar (PDA), Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), V8 juice agar (V8JA), nutrient agar (NA) and water agar media. All the media were procured from Himedia, India. Streptomycin (50 µg/ml) was used to prevent bacterial contamination.

As the endophyte *Geosmithia pallida* didn't sporulate, sporulation was induced using different media viz., potato dextrose agar, czapeck dox agar and malt extract agar amended with different concentrations of dextrose, peptone, yeast extract and sucrose. Modified media were ME-YEA (Malt extract-yeast extract agar), PDA-PS-YEA (Potato dextrose-peptone sucrose-yeast extract agar) and YE-RB-SA (Yeast extract-rose bengal-sucrose agar). In ME-YEA yeast-extract (0.4 g/l) was amended in malt extract agar media; in PDA-PS-YEA peptone (2 g/l), yeast-extract (2 g/l) and sucrose (2 g/l) were amended in potato dextrose media; in YE-RB-SA sucrose (15 g/l) and rose-bengal (2 g/l) were amended in yeast extract agar. After sporulation it was identified based on morphological characters using manuals of Nagamani *et al*. (2006)[12]. Taxonomical identity of the *G. pallida* was confirmed using molecular tools. The sequence was submitted to the GeneBank.

Optimization of in vitro physico-chemical parameters for optimum bioactive metabolite production

Standardization of the basal media

To standardise the basal media for optimum metabolite production by *G. pallida*, different media namely Czapeck-Dox-broth (CDB), Potato-Dextrose-Broth (PDB), Malt Extract Broth (MEB), Sabouraud Dextrose Broth (SDB), V8 juice Broth and Nutrient broth media were used[7]. All the media were procured from HiMedia Laboratories, Mumbai, India. Conical flasks containing 50 ml broth media having initial pH 6.6±0.2 was inoculated with 7 days old inoculum of the isolate and flasks were then incubated at stationary condition at 27 ± 2 °C for 20 days. The media in which the fungus produced highest metabolite was used as basal media for further work.

Effect of carbon and nitrogen sources

Different Carbon and Nitrogen sources at 10 g/l (w/v) were added in the 50 ml basal media[13]. Glucose, Glycerol, D-mannitol, Lactose, Sucrose, Galactose, Xylose, Starch, Maltose, Sorbitol, Na-citrate as carbon sources and Beef extract, Yeast extract, Peptone, Ammonium sulphate, Ammonium

chloride, Sodium nitrate as nitrogen sources were used. After inoculation the conical flasks were incubated at stationary condition for 20 days, at 27±2 °C temperature and pH 6.6±0.2.

Effect of NaCl concentration

The effect of NaCl concentration on the bioactive metabolite production by *G. pallida* was carried out by incubating the fungus in 50 ml basal media containing different concentrations of NaCl ranging from 1 g/l to 10 g/l, for 20 days, at 27±2°C temperature and pH 6.6±0.2 at stationary condition[14].

Effect of temperature

The fungal strain *G. pallida* was inoculated into the 50-ml basal medium and incubated at different temperatures ranging from 10 °C to 55 °C, at stationary condition for 20 days, at pH 6.6±0.2. Fungal metabolite production at each temperature was determined[15].

Effect of pH

To study the effect of pH on the bioactive metabolite production by *G. pallida* was tested using 50 ml basal media containing different pH levels ranging from 3.5 to 8.5. The pH of the basal medium was adjusted by adding 0.1N NaOH or 0.1N HCl and incubated at 27±2 °C temperatures for 20 days at stationary condition[16].

Effect of incubation period and shaking condition

Incubation periods ranging from 2 to 40 days were used to determine the effect of incubation period on the active metabolite production by *G. pallida* and other conditions were maintained as like that of the basal media[7].

To determine the effect of shaking condition on active metabolite production, culture flasks with basal media were incubated at 27±2 °C in an orbital shaker at 150 rpm at for 20 days, at pH 6.6±0.2.

Determination of production of bioactive metabolite

Production of bioactive metabolite by *G. pallida* was determined by antimicrobial activity assay of the metabolite against some microbes.

Extraction of the metabolite

Fungal mycelial mat was separated by filtration. After that bioactive secondary metabolite was extracted three times from the filtrate using ethyl acetate[17]. The ethyl acetate extract was concentrated to dryness by using rotary vacuum evaporator (Model: EYELA/NVC-2100) at 40 °C according to the protocols of Phongpaichit *et al.* (2007)[17]. The resulting extracts of the isolate was diluted with dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. The solution was sterilized by filtration through 0.4 µm Cellulose Acetate (hydrophilic) filter and kept for antimicrobial activity assay.

Antagonistic activity assay

Antimicrobial activity of the ethyl acetate extract of the metabolite produced by *G. pallida* was tested against two gram-negative, viz., *Escherichia coli* (MTCC 443) and *Klebsiella pneumoniae* (MTCC 619), two gram-positive, viz., *Bacillus subtilis* (MTCC 441) and *Staphylococcus epidermidis* (MTCC 435) bacteria and two fungi i.e., *Candida albicans* (MTCC 183) and *C. Tropicalis* (MTCC 1000). The test organisms except *S. epidermidis* and *Candida* sp. were collected from the Institute of Microbial Technology (IMTECH), Chandigarh, India. *S. epidermidis* was collected from Regional Institute of Medical Sciences (RIMS), Imphal, India and *Candida albicans* (MTCC 183) was collected from Defense Research Laboratory (DRL), Tezpur, India. Kirby-Bauer disc diffusion method was followed for determination of antimicrobial activity of the metabolite[18]. For this, plates with PDA and NA medium were inoculated with 0.2 ml of cultured test microorganisms[7]. The inoculum in the plates was evenly spread out with the help of a sterile cotton swab. A fixed volume of 20 µl of the extracted metabolite solution of the isolate was then applied to the sterile discs (6.0 mm diameter, Whatman antibiotic assay discs). The diameters of the inhibition zones formed surrounding the discs after 48-72 h at 25±2 ºC were recorded. Three replicates were maintained in each case. Larger was the zone diameter, more was the metabolite production[7,13,16].

Statistical analysis

All experiments were carried out in triplicates and results are expressed as mean ± SD (n=3). To analyse the differences among mean values of different assays one-way analysis of variance (ANOVA) was conducted followed by least significant difference (LSD) test in Microsoft office excel 2016. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Identification of *Geosmithia pallida*

G. pallida was identified based on morphological and reproductive characteristics. It was sterile in nature and creamy white in appearance when isolated and did not sporulate in unamended media. It sporulated when cultured on MEYEA and PD-PS-YEA media. Sporulation was the best in PD-PS-YEA media. The isolate, however, did not grow in YERBSA media. The ITS region of rDNA of *G. pallida* was sequenced to confirm its identification. The sequence was submitted to GeneBank-NCBI (Accession number KU693285).

Optimization of in vitro physico-chemical parameters for bioactive metabolite production

Standardization of Basal media

Maximum zone of inhibition was recorded in Potato dextrose broth (PDB) medium (Table 1). The zone of inhibition was significantly higher when PDB was used for metabolite production by the fungal strain than that of the other media used ($p<0.05$) (Table 1). This indicated that the ingredients of potato dextrose media induced bioactive metabolite production by the fungal strain. Therefore, PDB was selected as the basal medium for further experiments. Variations in the nutritional constituents of the culture media used affect the bioactive metabolite production by the strain. Photo-plates of antimicrobial activity shown by bioactive metabolite produced by *G. pallida* (KU693285) against *Bacillus subtilis* (Fig.1a) and *Candida albicans* (Fig.1b), co-assayed with antibiotic discs- Tetracycline (standard antibacterial) and Fluconazole (standard antifungal) as well as disc dissolved in 100% DMSO, have been shown in Fig.1.

Effect of Carbon and Nitrogen Sources

During the present investigation *G. pallida* was grown in the basal media by replacing the carbon sources as mentioned in material and methods. Lactose had maximum influence on antimicrobial metabolite production followed by starch (Fig.2). The zone of inhibition against *C. albicans* using lactose and starch respectively were 25.47±0.63 mm and 24.69± 0.25 mm. Among the N-sources used, Yeast extract was most effective followed by peptone for maximum antimicrobial metabolite production in terms of zone of inhibition against *C. albicans*, which were 25.38±0.51 mm and 24.91±0.44 mm respectively in yeast extract and peptone amended media (Fig.2). The inhibition was significantly higher in the basal media treated with lactose and yeast extract as C- and N-sources respectively than that of the control (basal media) ($p<0.05$).

Effect of NaCl concentrations

At 3.5 g/l concentration of NaCl, *G. pallida* produced maximum antimicrobial metabolite (zone

Table 1. Effect of different culture media on the bioactive metabolite production by *G. pallida* (KU693285) against test microbes

Media	Zone of inhibition (mm)					
	Gram- positive bacteria		Gram- negative bacteria		Fungi	
	Se	Bs	Kp	Ec	Ca	Ct
PDB	13±0.43[a]	14.07±0.12[a]	18.17±0.76[a]	11±0.09[a]	23.93±0.12[a]	17.72±0.21[a]
CDB	13.07±0.51[a]	12.14±0.39[b]	10.31±0.29[b]	9.19±0.23[b]	21.18 ± 0.5[b]	18.23±0.45[b]
MEB	11.91±0.23[b]	10.19±0.3[c]	11.29±0.41[c]	9±0.86[b]	16.45 ± 02[c]	17.28±0.34[a]
SDB	12.63±0.19[b]	12.38±0.11[b]	10.09±0.52[b]	11.33±0.17[a]	20.28 ± 0.48[b]	13.83±0.12[c]
V8JB	9.17±0.27[c]	7.18±0.28[d]	7.21±0.37[d]	6.81±0.18[c]	18.24 ± 0.25[d]	11.21±0.63[d]
NB	3.61±0.33[d]	4.58±0.42[e]	3.31±0.47[e]	0	7.89 ± 0.19[e]	6.43±0.35[e]
Antibiotic disc	16.67±0.58[e]	25.27±0.25[f]	10.91±0.36[b]	20.0±0.29[d]	15±0.64[c]	19.0±0.39[b]
Negative control	0	0	0	0	0	0

PDB=Potato dextrose broth, CDB=Czapek dox broth, MEB=Malt extract broth, SDB=Sabouraud dextrose broth, V8JB=V8 juice broth, NB=Nutrient broth. Se=*S. epidermidis,* Bs=*B. subtilis,* Kp= *K. pneumoniae,* Ec=*E. coli,* Ca=*C. albicans,* Ct= *C. tropicalis.* Positive control: Co-assayed antibiotics (Tetracycline-30 µg/disc, Fluconazole-10 µg/disc). Negative control: Sterile disc (5mm diameter) immersed in Dimethyl sulphoxide (DMSO). Data mean of three replicates ±SE (n=3). Means with different letters within a column were significantly different ($p < 0.05$).

of inhibition 25.34±0.19 mm) (Fig.2). After that, the metabolite production gradually decreased with the increase in salt concentration in the basal media. The inhibition was significantly higher in the media treated with 3.5 g/L NaCl than that of the control ($p<0.05$).

Effect of temperature

A varied range of incubation temperatures 10 to 55 °C was used to assess its impact on the bioactive metabolite production by *G. pallida*. The increase of the incubation temperatures from 25 to 30 ºC enhanced the production of bioactive metabolite (Fig.3). At 25 °C *G. pallida* produced maximum metabolite (inhibition zone 25.33 ± 0.61 mm). However, no antagonistic activity was observed at ≤ 15 °C and ≥ 50 °C temperatures. The inhibition was significantly higher when incubation temperature was 25 °C than the incubation temperature of the control ($p<0.05$).

Effect of pH

The antagonistic activities of *G. pallida* were also influenced by pH of the medium. Maximum antimicrobial metabolite production (zone of inhibition 25.79±0.44 mm against *C.*

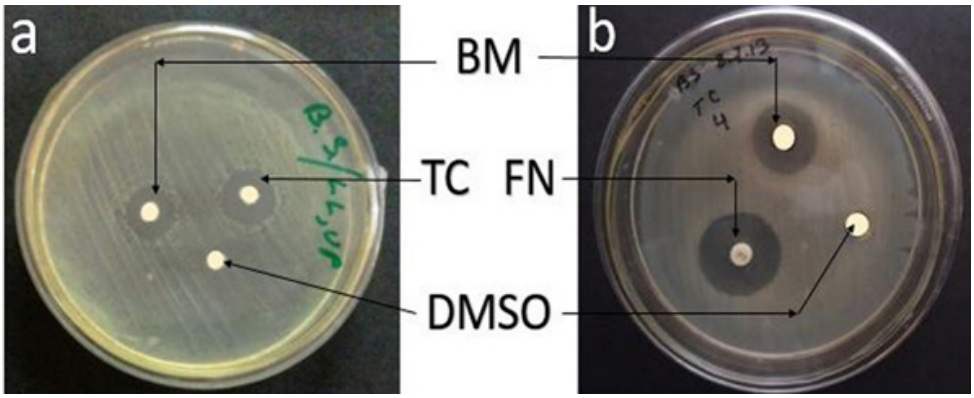

Fig. 1. Antimicrobial activity of bioactive metabolite (BM) produced by *G. pallida* (KU693285) against (a) *Bacillus subtilis* and (b) *Candida albicans* (MTCC183), Positive Control-(a) Tetracycline (TC-30 µg/dsc) and (b), Fluconazole (FN-10 µg/disc), Negative Control-Dimethyl sulfoxide (DMSO)

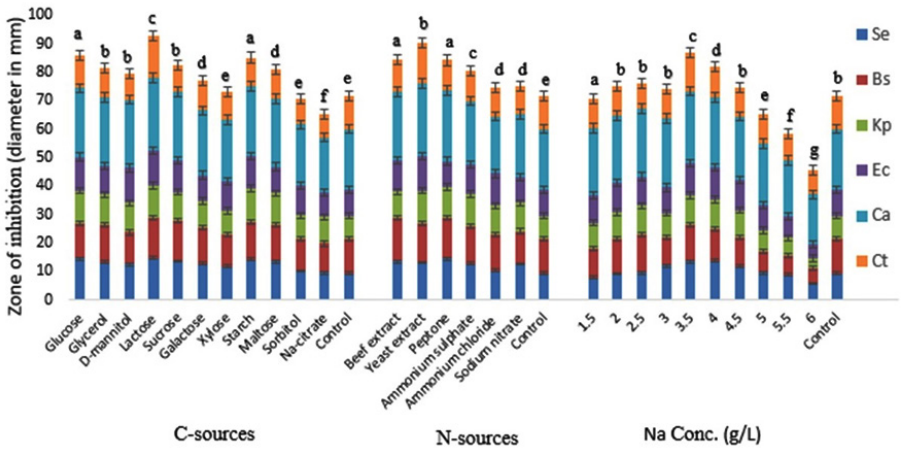

Fig. 2. Effect of different C-source, N-sources and NaCl concentration on the bioactive metabolite production by *G. pallida* (KU693285) against test microbes. Se=*S. epidermidis,* Bs=*B. subtilis,* Kp= *K. pneumoniae,* Ec=*E. coli,* Ca=*C. albicans,* Ct= *C. tropicalis.* Vertical bars represent standard deviation (±SD, n=3). Means with different letters within an assay were significantly different ($p < 0.05$)

albicans) was recorded at pH 6.5 (Fig.3). The inhibition was significantly higher when pH of the media was 6.5 than the pH of the control ($p<0.05$).

Effect of incubation period and shaking conditions

The isolate required incubation period of 28 days for maximum production of antimicrobial metabolite (zone of inhibition 25.71 ± 0.13 mm against *C. albicans*) under stationary conditions (Fig.3). The antimicrobial activity of bioactive secondary metabolite reduced when the conditions changed from stationary to shaking. In terms of zone of inhibition, the zone of inhibition decreased to 16.89±0.32 mm from 23.93±0.12 mm, while the culture broth (basal medium) was incubated at shaking conditions from stationary condition. The inhibition was significantly higher on 28th of incubation than the incubation period of the control ($p<0.05$).

DISCUSSION

Advances in technology have sparked a renaissance in the discovery of natural antimicrobial product from microbial sources, like actinomycetes, cyanobacteria, bacteria and fungi[19]. More recently, researches on endophytic fungi, has resulted in several newly discovered antibiotics as they are the source of a wide range of bioactive compounds fighting against various pathogens[20]. However, different researchers have proved that the culture parameters, *i.e*., the nutrient supplements, nutrient concentration and the culture conditions of the media effects the metabolite production by the fungi.

In the present study, maximum antimicrobial metabolite production by *G. pallida* (KU693285) was observed on Potato dextrose broth (PDB) medium which was used as basal media for further experiment (Table 1). This result supports the findings of Rabbani *et al*. (2011) and Bhattacharya and Jha (2011)[7,21]. While Kalyani *et al*. (2016) found maximum growth and metabolite production by *Aspergillus niger* (MTTC 961) using sabouraud dextrose broth[19].

Miao and Qian (2005) studied the effect of culture medium on mycelial growth, metabolite profile and antimicrobial compound yield by a marine derived fungus *Arthrinium* c.f. *saccharicola* suggesting the need of optimal culture parameters to achieve maximal bioactivity by the fungus[22]. Merlin *et al*. (2013) observed that the endophytic fungi *Fusarium solani* isolated from *Tylophoraindica*, had optimum growth and metabolite production when cultured for 9 days in the media containing 4% dextrose, 0.05% yeast extract and aspartic acid (0.01%) as carbon, nitrogen and amino acid sources respectively[23].

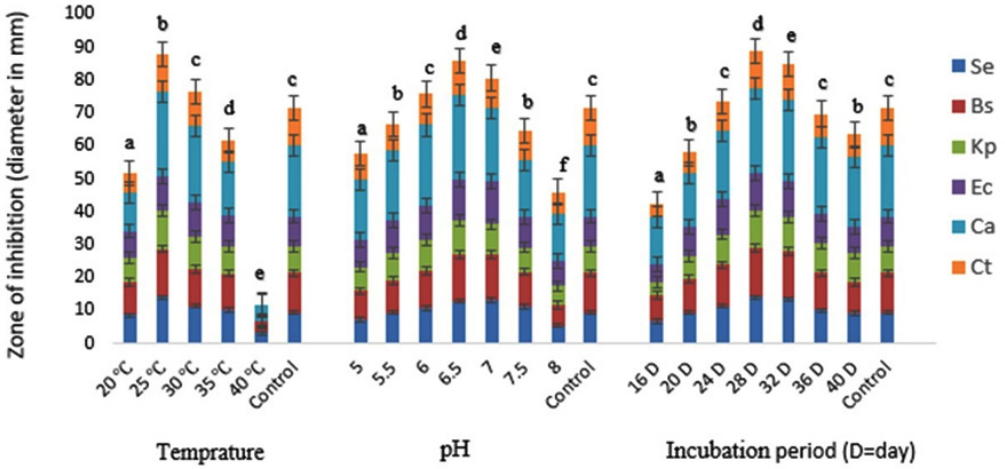

Fig. 3. Effect of different temperature, pH and incubation period on the bioactive metabolite production by *G. pallida* (KU693285) against test microbes. Se=*S. epidermidis,* Bs=*B. subtilis,* Kp= *K. pneumoniae,* Ec=*E. coli,* Ca=*C. albicans,* Ct= *C. tropicalis.* Vertical bars represent standard deviation (±SD, n=3). Means with different letters within an assay were significantly different ($p < 0.05$)

Bhattachryya and Jha (2011) observed that the most effective C- and N- sources for maximum bioactive metabolite production by *Aspergillus* strain TSF 146 respectively was sucrose and asparagine[7]. Merlin *et al*. (2013) optimized the growth and metabolite production by *Fusarium solani* using dextrose as C-source and yeast extract as N-source in the basal medium[23]. But in the present study lactose was the most effective followed by sucrose as C-source, while yeast-extract was the most effective followed by peptone as N-source for bioactive metabolite production by *G. pallida* (KU693285) (Fig.2). This result supports the findings of Gogoi *et al.* (2008) and Mathan *et al.* (2013)[16,24].

Gogoi *et al.* (2008) found that the NaCl concentration 2.5–3.0 % was the optimum for maximum production of bioactive metabolite by an antagonist fungus, *Fusarium* sp.[16]. Bhattachryya and Jha (2011) observed that the NaCl concentration of 5 g/l was optimal for maximum production of bioactive metabolite[7]. In the present study, optimal NaCl concentration was 3.5 g/l for *G. pallida* (KU693285) and the values gradually decreased with the increase in NaCl concentration in the basal media (Fig.2). This result supports the findings of Bhattachryya and Jha (2011) and Gogoi *et al.* (2008)[7,16].

Temperature is one of the major parameters influencing the growth rate of antagonist[25]. Jain and Pundir (2011) also reported 25 ºC temperature as optimal for maximum production of antimicrobial metabolite by *Aspergillus terreus*[26]. Maximum growth and metabolite production by *Aspergillus niger* (MTTC-961) was observed at 30 ^{o}C[19]. During the present study, *G. pallida* (KU693285) produced maximum bioactive metabolite at incubation temperature of 25 ºC supporting the findings of Ritchie *et al*. (2009), Jain and Pundir (2011) and Mathan *et al.* (2013)[24, 26,27].

According to Thongwai and Kunopakarn (2007) microorganisms can synthesize antimicrobial compounds at pH ranging from 5.5 to 8.5[28]. Mathan *et al.* (2013) and Jain and Pundir (2011) observed the maximum production of antibacterial metabolite by *Aspergillus terreus* respectively at pH 6.0. and 5.5[24,26]. During the present study, the antagonistic activity of ethyl acetate extract of metabolite produced by *G. pallida* was also influenced by pH of the medium and 6.5 was the optimum pH for maximum antimicrobial metabolite production (Fig.3).

Alberts *et al*. (1990) observed that the production of metabolite occurred after 12 days, reached maximum at 91st day and gradually decreased after 91 days of incubation when effect of incubation period was investigated on its production by *Fusarium moniliforme*[29]. *Aspergillus* TSF 146 required 16 days for optimum production of antimicrobial metabolite[7]. Optimum incubation period for maximum metabolite production by *Aspergillus niger* (MTTC-961) was 144 hr[19]. However, *G. pallida* showed maximum antimicrobial metabolite production on 28th day of incubation followed by 30 days of incubation period and gradually it decreased.

During the present study, shaking condition of incubation did not enhance the antimicrobial metabolite production by *G. pallida* supporting the findings of Kalyani *et al.* (2016), Alberts *et al.* (1990) and Bundale *et al.* (2015), as compared to the shaking condition[19,29,30]. From the results obtained during the present study, it can be drawn that to obtain maximum production of bioactive metabolite, the physico-chemical properties have to be optimized.

CONCLUSION

The results of this study represent that endophytic fungi may serve as a potential natural source of antimicrobial agents. Endophytic fungi are recommended as an organism of pharmaceutical importance with high medicinal value. Therefore, optimization of culture conditions for maximum production of bioactive metabolite by endophytic fungi is very important. *Geosmithia pallida* (KU693285) produced maximum bioactive metabolite in 25°C incubation temperature and 6.5 pH, on 28th day of inoculation, when lactose and yeast extract were used respectively as carbon and nitrogen source along with 3.5 g/l NaCl. However, the toxicity profiling, structural elucidation of the bioactive compounds produced by *G. pallida* are yet to be done for pharmaceutical as well as agricultural uses.

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