Nat. Volatiles & Essent. Oils, 2021; 8(6): 3353-3368

NVEO
Natural Volatiles &
Essential Oils

Optimization Of Fermentation Conditions For Enhanced Production Of Secondary Metabolite From Endophytic Fungi Of Berberis Aristata

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Abstract

Purpose: Berberis aristata, an excellent herb mentioned in Ayurveda since ancient times, is found in the Himalayan regions of India. Almost every part of the herb is used in treating various diseases since ancient times. The roots and leaves of Berberis aristata have high medicinal value and are used to treat eye-diseases, osteoporosis, diarrhoea, diabetes, cancer, gynaecological disorders, etc. Any organism (bacterial or fungal endophytes) isolated from this species can be of immense interest due to its potential of producing novel bioactive compounds.

Methods: In this study, we isolated 10 fungal endophytes which were identified through morphological and internal transcribed spacer (ITS) sequence analysis. Diversification of fungal endophytes and species richness was higher in leaf tissues as compared to root segments sampled for isolation. Antibacterial activity was observed by the ethyl extract of 80 % fungal isolates against one or more bacterial pathogens. Further the fungal endophytes were optimized for growth and production of antimicrobial compound in batch culture system. PDA media supplemented with 0.1g chloramphenicol favoured the growth at temperature of 30℃ and incubation period of 6-7 days.

Results: We are reporting for the first time Talaromyces flavus as fungal endophyte of Berberisaristata producing berberine.

Conclusion: This study can prove possible capability of endophytes for the production of plant metabolites having antimicrobial potential for which medicinal plants are regularly exploited and becoming endangered thus by such studies we can explore new antimicrobial agent from endophytes and at the same time conserving our biodiversity.

Keywords Berbersis aristata, bioactive compounds, endophytes, pathogens

Introduction

Endophytes are the intracellular microorganisms which reside inside the plant tissues in a symbiotic association for atleast a part of their life cycle [1].The endophytic fungi help the host in producing hormones which promote the growth of the host plants and also help them in relieving abiotic stress. Moreover, endophytes also produce some bioactive secondary metabolites similar to those

produced by the host plants [2,3].Taxol, the first ever biosynthesized anticancer drug, was originally isolated from the bark of Taxus brevifolia, and now, it has been reported to being isolated from various endophytic fungi isolated from different host plants [4,5]. From the last few decades, many endophytes have been reported for the production of bioactive secondary metabolites like diosgenin [6], camptothecin [7], hypericin [8], peniphenone [9], vinblastine [10], etc.

Medicinal plants have been constantly explored for obtaining novel secondary metabolites, since it is proved to be easy, cost effective and faster method of production. Berberis aristata is an important medicinal herb mentioned in Ayurveda. It is widely distributed in northern Himalayan region.The plant is widely used in Indian system of medicine as a traditional antibacterial, antiperiodic, antidiarrhoeal and anticancer and it is also used in the treatment of ophthalmic infections. It is very effective with anticancer properties which fights against human colon cancer [11]. Keeping in view the medicinal importance of the host and scientific investigations on B. aristata for the production of various metabolites by the resident endophytes, we carried out the present study to isolate potential fungal endophytes from various tissues of B. aristata and assessing their antimicrobial potential.

Methodology

Selection and collection of plant material:

The plant material for the present study was collected from the healthy and symptomless plants growing in the fields of Chamba district, H.P., India. Plants parts like roots and leaves were collected from disease free plants and kept in zip lock bags at 4 °C till further processing.

Isolation and maintenance of fungal endophytes:

The leaves and roots were cut into small pieces and washed under running tap water for 15 min. This was followed by drying the plant material between the folds of sterile filter paper. It was then rinsed with sterile glass beads and water in a conical flask for 15 min. This was followed by washing the plant material thrice with autoclaved water (each washing of 5 min), followed by subsequent washings with 70 % alcohol (1-2 min), autoclaved water (2-3 min) and 0.5 % NaOCl sol. (30 sec.-1 min) and finally three rinses of autoclaved water were given. The surface sterilized leaves and roots were placed on PDA plates supplemented with 0.5 µg /ml chloramphenicol at 28 ± 2 °C for isolation of the fungal endophytes. Final washing of the surface sterilized segments spread on potato dextrose agar plates served as control. Effectiveness of the surface sterilization method was also validated by Schultz imprinting method [12]. Fungal colonies were selected on morphological basis and maintained on PDA slants, stored at 4℃ intended for succeeding studies.

Characterization of endophytic fungi

Fungal isolates which showed positive results during primary and secondary screening were selected for morphological and molecular characterization.

Morphological identification

Morphological studies were carried out by plating the fungi on PDA and then incubating it for 7 days. The growth patterns and appearances were observed on both the sides of the culture plates. Tease mount method was used to prepare microscopic slides using lactophenol cotton blue and observed under microscope in bright day light conditions [13].

Molecular identification

Molecular identification was performed by following ITS-PCR. Firstly, fungal DNA was isolated by using CTAB method [14]. The isolated DNA was then air dried. The air dried DNA was dissolved in TE buffer and stored at −20 °C for further experimentation. PCR was performed using the primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS-4 (5'TCCTCCGCTTATTGATATGC3'). One micro liter of each purified DNA sample (50 ng/µl) was used as a template and added to the PCR master mixture which consisted of 1X PCR buffer, 2 mM $MgCl_2$, 10 mM dNTPs, 1 U/µl Taq polymerase, and 0.1 pM of each primer. The PCR thermal cycle parameters used were 94 °C for 5 min, 30 cycles of 1 min at 94 °C annealing at 55 °C, 1 min at 72 °C, and a final extension at 72 °C for 10 min. AMastercycler gradient machine (Eppendorf, Hamburg, Germany) was used to carry out the PCR reaction. The amplified products were checked on 2% agarose gel, which were then eluted and sequenced. The sequences thus obtained were compared with NCBI databases using the FASTA algorithm.

Production and extraction of secondary metabolites from isolated fungal endophytes

Pure cultures were inoculated in 250 ml flasks containing 125 ml of PDB for fungal endophytes which were then kept in shaking incubator at 28°c for 4-6 days at 100 rpm. Submerged fermentation of the endophytic fungi was followed for the production and extraction of secondary metabolites from the selected endophytes [15]. After incubating the flasks for 5 days, they were taken out the next day from the incubator shaker. The fungal biomass was collected, and then filtered by using autoclaved glass funnel, filter paper and muslin cloth. Secondary metabolites from these endophytes were extracted by solvent fractionation and Soxhlet extraction methods using ethyl acetate.

Optimization of fermentation conditions for metabolites production

Selection of the culture media

To select the suitable growth medium, the isolate was grown on different culture media such as Czapek's Dox broth,Sabouraud Dextrose Agar, Potato Dextrose broth and Malt extract broth. After 7 days of incubation at 30℃ and pH 7, the biomass accumulation and secondary metabolite production was recorded. Following incubation antimicrobial activity of each culture broths were tested by means of agar well diffusion method against pathogenic bacterial microorganisms [16]. The optimal medium achieved by this step for positive isolates was chosen as the basal media for further experiments.

Effect of incubation temperature

The positive cultures were inoculated into the basal medium and grown in various ranges of temperature (22, 25, 27 and 30℃) for 7 days at 180 rpm. After incubation, the screening of active metabolite was done with the same agar well diffusion method. Optimum temperature was kept as standard temperature for future experiments.

Effect of initial pH

The initial pH was adjusted from 3 to 10 at difference of one to the basal media and inoculated for 10 days.After incubation estimation of antibiotic production was checked by Agar well diffusion method. Optimum pH for positive isolates achieved by this step was fixed for succeeding experiments.

Effect of inoculum size

To determine the effect of inoculum size on antibiotic production, different cell concentration of positive isolates (0.5%, 1%, 1.5%, 2%, and 2.5%) were added to different flask containing basal media. The fermentation was carried out by keeping all other conditions at their optimum level and then assayed for antibiotic production. The optimum inoculum size for antibiotic production was kept as standard for next experiments.

Statistics

By using SPSS 16 version software the collected and recorded data were subjected to One way ANOVA ranked with Duncan's multiple range tests with explanatory examination type on different isolates against various MTCC Cultures. All the results (Inhibition zone) were measured in triplicate. The results having $P<0.05$ were measured to be statistically significant.

Screening of antibacterial activity

The antibacterial screening of fungal isolates was performed against some common pathogenic microorganisms using agar well diffusion method [17]. The bacterial cultures were obtained from MTCC. Table 1 exhibits the details of the test bacterial microorganisms;

Table 1: List of bacterial cultures used as test organism

Microorganism	Nature of Microorganism	Accession number
Bacillus cereus	Gram positive	MTCC 430
Bacillus subtilis	Gram positive	MTCC 441
Escherichia coli	Gram negative	MTCC 443
Staphylococcus aureus	Gram positive	MTCC 3160
Staphylococcus epidermidis	Gram positive	MTCC 1228
Klebsiella pneumonia	Gram negative	MTCC 109
Pseudomonas aeruginosa	Gram negative	MTCC 1934
Pseudomonas alcaligenes	Gram negative	MTCC 493

In each petri plate, particular number of wells were punched using a sterile cork borer (6 mm diameter). 100 µl of various concentrations of the test samples and control (positive and negative) were added separately into these wells. Dimethyl sulphoxide (DMSO) was used as a negative control and standard antibiotic chloramphenicol (10 µg/ml) was used as the positive control. Before incubation, the plates were allowed to stand for 1 h for diffusion of the extracts and then incubated for 24 h at 37 ± 2 °C. The antagonistic potential of different extracts of endophytic isolates was determined in terms of diameter of clear bactericidal zone around the well. The crude extracts which showed clear zone around the wells were considered as positive whereas those not showing clear zone around the well were considered as negative.

Thin Layer Chromatographic screening of the isolated endophytes

TLC based screening of the endophytic fungal isolates was done to check the presence of secondary metabolite, berberine. The active secondary metabolites were fractionated using ethyl acetate: methanol: formic acid: water (8:2:1:1) and ethyl acetate: acetone: formic acid: water (8:2:1:1) solvent systems in a pre- coated silica gel TLC plates. The eluted plates were developed under saturated conditions with solvent systems and dried completely that gave good separations. The chromatograms were visualized under UV at 365 nm and the movement of the metabolites along with the solvents was measured (Rf values).

Results

Isolation and identification of endophytic fungi

In our study, a total of 240 segments from various parts of plant (120 from leaves and 120 from root) were sampled for the isolation of endophytic fungi. One hundred and twelve isolates were isolated from these plant segments which include 58 from leaves and 54 from root (Table 2).

Fig 1. Berberis aristata growing in its natural habitat

Table 2: Isolation of endophytic fungi from different part of B. aristata

Plant part used for isolation	Segments	Fungal isolates obtained
Leaves	120	58
Roots	120	54
Total	240	112

Both leaf and root segments were found to inhabit good number of fungal endophytes. These fungal endophytes were identified primarily by morphological characteristics. The endophytes thus isolated were further identified on the molecular basis using internal transcribed spacer regions and 5.8S rRNA gene sequencing and NCBI-FASTA algorithm. Molecular identification of the selected endophytes disclosed that endophytic fungi belonged to 8 genera and 10 different species (Table 3). In leaf tissues, Alternaria alternata was found to have highest colonization rate (20 %), followed by Colletotrichum acutatum (10 %). Whereas, in roots, Alternaria tenuissima (11.6 %) had the highest colonization frequency (Table 3). There can be numerous reasons for the species diversification. Among those,one possible reason can be the structure and substrate which effects the colonization rate and distribution of endophytic fungi.

Table 3: Identified fungi and colonization frequency

Fungal activity Number	Identified Fungal Isolates	Plant Part	Total no. of segments incubated	No. of isolates obtain	Colonization frequency (%)
BL-1	Alternaria macrospora	Leaves	120	8	6.6
BL-2	Penicillium verrucosum	Leaves	120	4	3.3
BL-3	Microdeplodiahawaiiensis	Leaves	120	2	1.6
BL-4	Alternaria alternata	Leaves	120	24	20
BL-5	Talaromyces flavus	Leaves	120	12	10
BL-6	Phomopsis longicolla	Leaves	120	8	6.6
BR-1	Fusarium solani	Root	120	26	0.8
BR-2	Alternaria tenuissima	Root	120	14	11.6
BR-3	Paecilomycesvariotii	Root	120	8	6.6
BR-4	Trichoderma longibrachiatum	Root	120	6	5

Where, CF (%) = (N/Nt)100

as N= no. of isolates obtained N*100, Nt= total no. of segments incubated

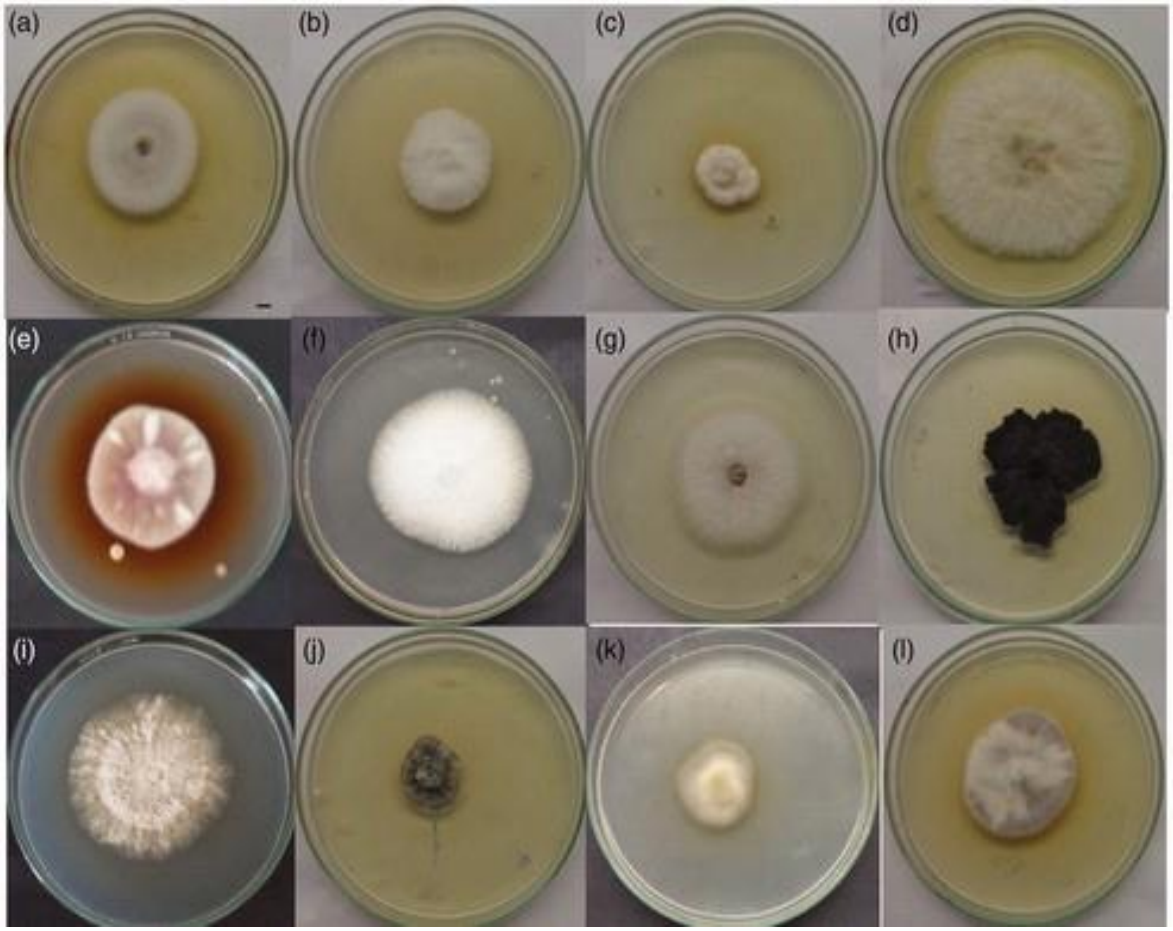

Fig 2: Colony features of seven days old various endophytic fungi isolated from B. aristata: a) Fusarium solani b) Colletotrichum acutatum c) Fusarium nematophilumd) Aspergillus niger e) Phomopsis longicollaf) Colletotrichum gleosporides g) Alternaria alternata h) Phyllostictacapitalensisi) Talaromyces flavus j) Microdiplodiahawaiiensis

Antibacterial activity

The antibacterial screening was carried out using agar well diffusion method as described by Lino and Deogracious (2008) with slight modifications [17]. Most endophytic fungi out of the total 10 endophytic fungi gave high positive results on S. aureus, B. cereus and P. alcaligenes respectively (Table 4).

Table 4: Antibacterial activity of the endophytic fungal isolates against various bacterial strains.

Test Microorganism	Fungal Isolates									
	BL- 1	BL- 2	BL- 3	BL- 4	BL- 5	BL- 6	BR- 1	BR- 2	BR- 3	BR- 4
	Zone of inhibition in mm									
Bacillus subtilis	14	12	11	11	20	14	8	13	7	12
Bacillus cereus	11	15	12	14	12	8	12	13	9	10
Escherichia coli	3	2	1	4	1	1	2	1	1	2

Pseudomonas alcaligenes	10	14	11	15	11	20	15	18	8	10
Pseudomonas aeruginosa	11	9	10	10	8	13	15	11	9	7
Klebsiella pneumonia	13	4	12	14	12	9	5	8	11	5
Staphylococcus aureus	18	14	12	24	14	19	20	12	12	20
Staphylococuus epidermidis	15	21	11	14	12	17	13	11	7	8

Table 4 shows that the maximum inhibitory activity was recorded against S. aureus by BL-4 (Alternaria alternata) extracts. The diameter of zone of inhibition was recorded as 24 mm followed by BL-5 (20mm) (Talaromyces flavus) and BR-4 (20 mm) (Trichoderma longibrachiatum). The least antibacterial activity was recorded against Escherichia coli. The diameter of zone of inhibition was 1 mm by isolates: BL-3, BL-5, BL-6, BR-2 and BR-3 (Table 3).

Optimization conditions

Effect of Optimized media

Figure 3 explains the consequences of various medium on antibiotic production by BL-5 Various culture mediums like Czapek's Dox, Sabourdard dextrose broth, Potato dextrose agar and malt extract were tried for the production of preferred antibiotic for BL-5 isolate. BL-5 showed maximum metabolite production with PDA.Therefore, PDA was confirmed to be the best antibiotic production media medium

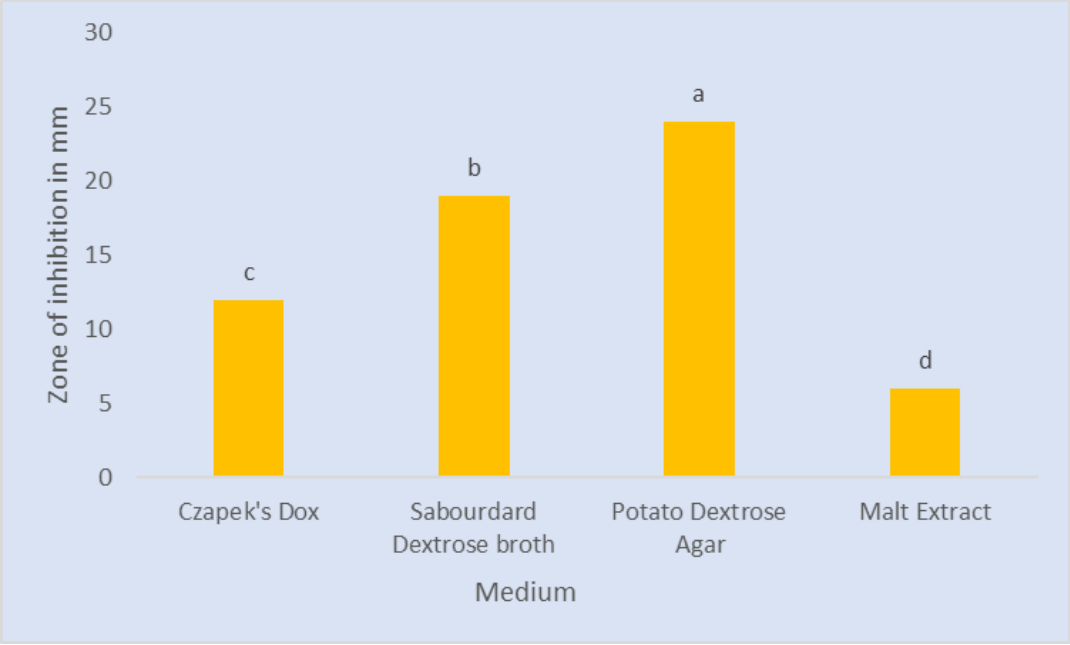

Figure 3. Outcome of various production medium over antibiotic production via BL-5 isolate.

Effect of temperature on antibiotic production

Temperature played an important role in the production of antibiotic. Effect of temperature on antibiotic production was examined for BL-5 strain. PDA medium with selected BL-5 isolate was inoculated at different temperature (22, 25, 27, 28, 30, 31, 34℃) on rotary shaker. For antibiotic production from BL-5, optimum temperature was recorded as 30℃. Figure 4 depicts the outcome of temperature upon antibiotic production.

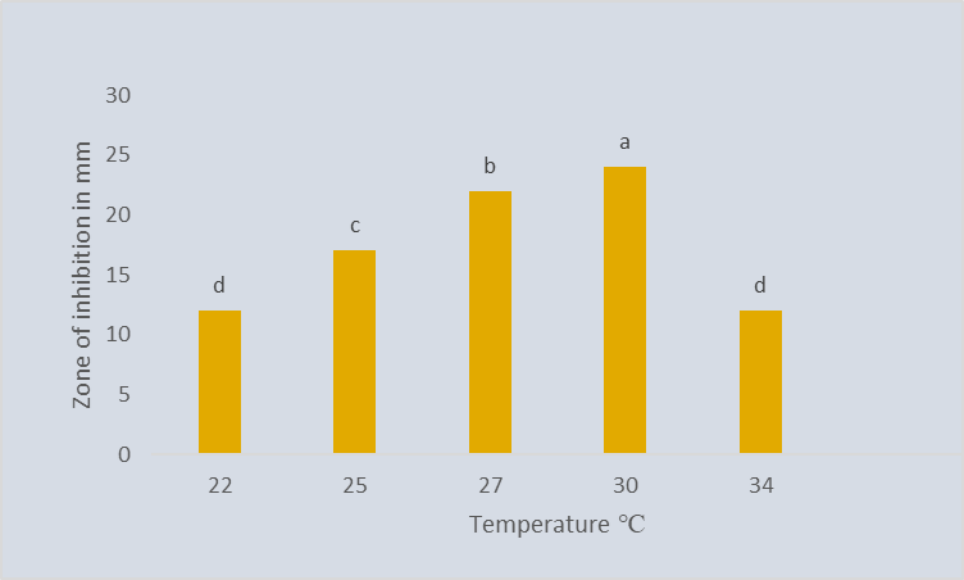

Figure 4: Outcome of various Temperature over antibiotic production via BL-5 isolate.

Effect of pH on antibiotic production

Production of secondary metabolites by endophytes also gets affected by pH. The pH values of PDA medium were adjusted from 3 to 10 individually. The antibiotic production was maximal at pH 7 for the selected BL-5 isolate. Figure 5 represents the effect of pH upon antibiotic production by isolate BL-5.

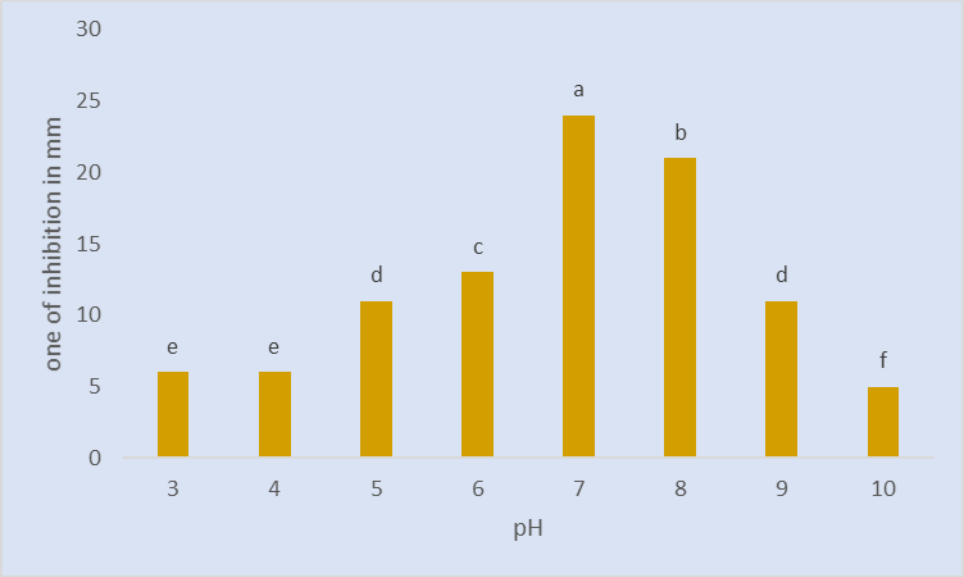

Figure 5: Outcome of various pH over antibiotic production via BL-5 isolate

Effect of Inoculum size on antibiotic production

Inoculum size showed direct effect on the secondary metabolite production. Antibiotic production varied with inoculum size percentage. Inoculum size consequence upon antibiotic production was studied by varying inoculum concentration in the optimized media for BL-5 isolate. 0.5 %, 1 %, 1.5 %, 2% and 2.5 % concentrations were used. The results verified that BL-5 showed maximal antibiotic production at 2.5 % (Fig. 6).

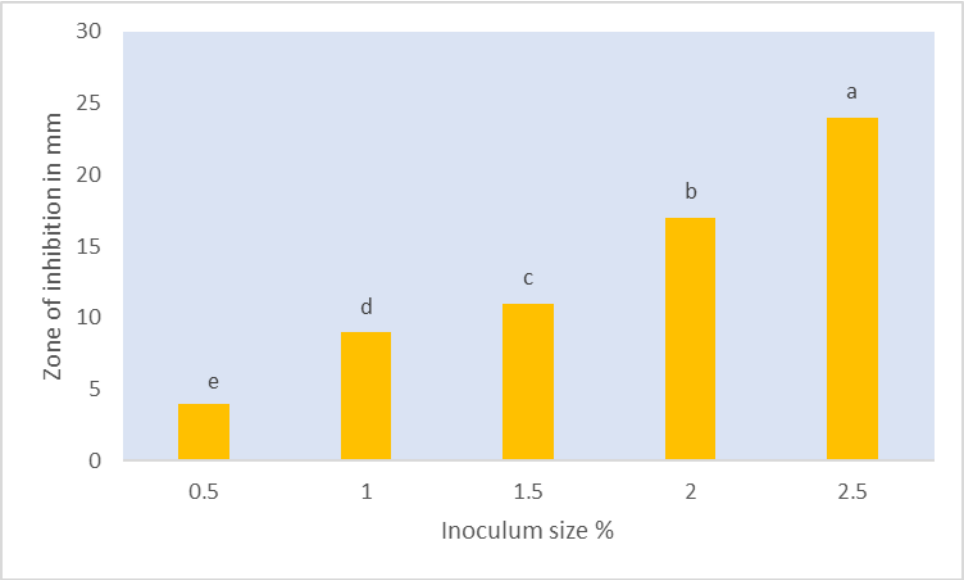

Figure 6: Outcome of various Inoculum sizes over antibiotic production via BL-5 isolate.

TLC analysis of the positive isolates

TLC analysis of the isolated fungal metabolites produced by the endophytes from leaves and root of the plant showed that the relative factor (Rf) values ranged from 0.58 to 0.84 whereas the Rf value for the standard berberine was 0.61when developed with solution of ethyl acetate: methanol: formic acid: water (8:2:1:1). Table 5 depicts that only two of the samples i.e, BL-5 and BL-4 showed almost similar Rf values as 0.59 and 0.58 respectively compared to that of standard berberine. When solution of ethyl acetate: acetone: formic acid: water (8:2:1:1) was used as developing solvent system (Fig 5b), the Rf value for the standard berberine was 0.43 and for the fungal metabolites these values ranged from 0.38 to 0.72. Only BL-5 and BR-4 showed almost the similar values i.e, 0.41 and 0.42 respectively (Table 5).

Table 5: Rf values of various isolates and the standard Berberine

Sample/Isolate	Rf values with developing system A	Rf values with developing system B
Berberine	0.61	0.43
BL-1	0.84	0.57
BL-2	0.81	0.59
BL-3	0.81	0.72
BL-4	0.68	0.58
BL-5	0.59	0.41
BL-6	0.80	0.63
BR-1	0.72	0.54
BR-2	0.81	0.38
BR-3	0.68	0.61
BR-4	0.58	0.42

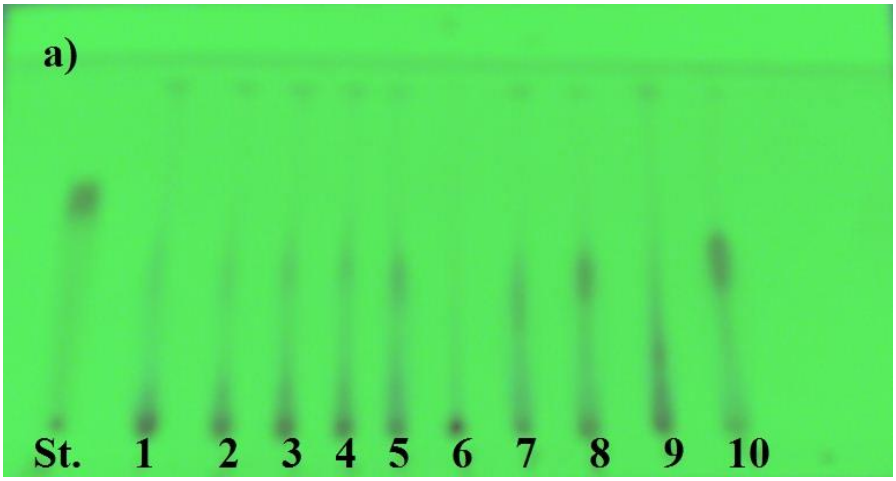

Fig. 7a): TLC profile with ethyl acetate: methanol: formic acid: water (8:2:1:1) as developing solvent, where St. is Standard, lane 1 -6 is BL-1 to BL-6, lane 7-10 is BR-1 to BR-4.

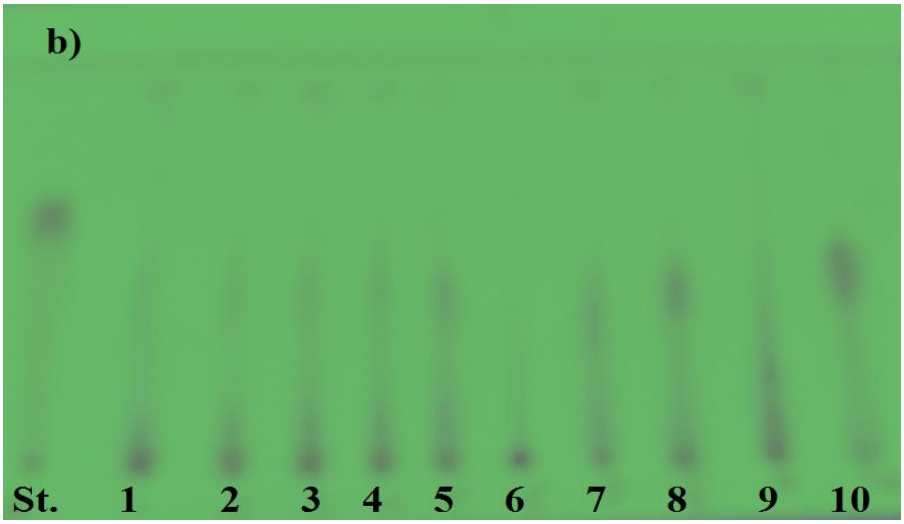

Fig. 7b): TLC profile with ethyl acetate: acetone: formic acid: water (8:2:1:1) as developing solvent where St. is Standard, lane 1 -6 is BL-1 to BL, lane 7-10 is BR-1 to BR-4.

Discussion

Endophytic fungi are polyphyletic microorganisms which are still under exploration for their potential in different areas. They constitute a community that reside inside the plant tissues without causing any disease symptoms, and ultimately building a mutual relationship with their host plants [18].Endophytes which harbour the medicinal plants are exploited more as compared to any other plants because of their possible consideration as the deep-pockected source of secondary bioactive metabolites [19].Investigations on the biosynthetic potential of endophytes have gained impetus owing to the ongoing discovery of strains capable to synthesize plant compounds, a property which may reflect an adaptative functional role in biocenosis. The interpretation of such connections will increase the understanding of evolution of complex defence mechanisms in plants and their associated organisms, which will further help in the exploitation of these microorganisms for a sustained production of a new valuable compounds beneficial for human race[20]. The hunt for isolating and identifying novel bio-actives from the endophytic fungi has resulted in the sampling of

host plants such as herbs, shrubs, tree species, and vines in unique places of ecological adaptations around the world. Such niches harbour great species diversity which are still untouched by human activities [21,22].The isolation and identification of endophytic mycobiota is necessary, since the medicinal properties of a plant can be a consequence of the capacity of its endophytic microorganisms to produce biologically active secondary metabolites [23,24]. This was the case in the classic example of taxol, an anticancer agent produced by Taxus brevifolia Nutt., and its endophyte Taxomycesandreanae [25].

The plant Berberis aristata has not been explored extensively for the endophytes. There, is a single report cited till date regarding the exploration of endophytes from this plant [26].However, few species of this particular plant have been reported to contain endophytes. In our study, we isolated 10 fungal endophytes which were tested for their antimicrobial potential and screened for secondary metabolite production. Talaromyces flavus (BL-5) was found to be the most potent one and hence selected for further studies. Moreover Talaromyces flavus has not been isolated from any of the species of this plant. To the best of our knowledge, our report is the first report of Talaromyces flavus as an isolated endophyte from this species. Alternaria sp., Colletotrichum sps., Aspergillus sps., Fusarium sps., and Phomposissps. have been isolated from other species of Berberis genera [26, 27, 28].T. flavus is reported to be the most common species of Talaromyces with a worldwide distribution. It is frequently isolated from soil, plants and other organic substrates [29].

Dethoup et. al studied the morphology and distribution of T. flavus by growing it in PDA media and its potential use as a biological control agent plant pathogenic fungi [30]. Our studies revealed that the optimum medium for the production of secondary metabolites from the selected strain was PDA at an optimal temperature of 30℃. 20 isolates of T. flavus have been isolated from 45 soil samples from various locations of Bangkok. The optimum temperature for growth has been recorded from 28℃ to 30℃ [31]. This species has been reported as a heat resistant fungus in food products [32] and a biological control agent of soil- borne fungal diseases [33]. When inoculum size was varied and checked for maximum antibiotic production, 2.5 % was found to be appropriate inoculum size at pH 7. Few studies revealed that pH and inoculum size for this species can vary from 2 – 7 % at pH 4-10 when isolated from soil samples [31].

Our study revealed a high phylogenetic diversity among endophytic fungi isolated from this plant.All the isolated exhibited good antibacterial activity against the tested pathogenic microorganisms. Anand et al. revealed that the extract isolated from T. flavus SP5 from marine sediment samples acted as a potent antibacterial, antifungal and anticancer agent against Pseudomonas aeruginosa, E. coli and Candida tropicalis [34]. Moreover, the TLC results showed that the fungal isolate BL-5 (Talaromyces flavus) showed almost similar values that to the standard Berberine when developed with two different systems.Some strains of T. flavus can produce novel bioactive compounds, such as actofunicone, deoxyfunicone and vermistatin, which reinforce the antimicrobial activity againstCandida albicans activity[35]. Funicone compound isolated from T. flavus showed inhibition against a human pathogenic fungus, Aspergillus fumigatus [36]. However, there is no report of production of berberine from this particular species. The finding of this work clearly indicates that endophytes isolated from this medicinal shrub has great antimicrobial properties which can be further used to synthesize new novel drugs.

Conclusion

Our results revealed that the isolated endophytes produce bioactive molecules which might be a good source of broad range antibacterial molecules.Their production can be enhanced by varying one single production component and keeping rest of the components same at a single time. Fungal endophytes are the future research of microbial science. They are able to produce secondary metabolites as novel compounds. In the years to come, these can be used to increase the soil fertility and crop yield. They can be used to cure a number of diseases like cancer, tuberculosis, diabetes, etc.

Acknowledgement

The authors are thankful to the management of Arni University for the keen support.

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