



Optimization of Culture Conditions for Growth and Production of Bioactive Metabolites by Endophytic Fungus – *Aspergillus tamarii*

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Abstract

Aim: The aim of this study is to optimise the culture conditions of the fungal endophyte *Aspergillus tamarii* to enhance the growth and production of bioactive compounds (production of phenol, flavonoid and IAA) and its antifungal activity. **Method:** The potent fungus is subjected to stepwise optimisation in various physico-chemical parameters like culture medium, carbon and nitrogen sources, NaCl, pH and temperature. After each optimising step dry weight of mycelial biomass was recorded, and fungal culture filtrate was extracted using ethyl acetate solvent. Concentration of phenol, flavonoid and IAA were estimated. Further, zone of inhibition against *Fusarium oxysporum* was studied to select the optimised parameter for enhancing the production of bioactive compounds. **Result:** The optimised culture media was determined as Potato Dextrose Broth (PDB) using dextrose as its carbon source, yeast extract as its nitrogen source along with 5g/l NaCl concentration at 30°C in pH 5, as the various optimised condition for the maximum growth and production of bioactive compounds of *Aspergillus tamarii*. **Conclusion:** *Aspergillus tamarii*, isolated from the root of *Casuarina junghuhniana* Miq., a potent fungi is a rich source of polyphenolic compounds. *Casuarina junghuhniana* is a multipurpose forest tree species belonging to family *Casuarinaceae*. This tree species can grow in extreme climatic and edaphic conditions. Endophytes growing within host plants in adverse condition are far more potential than the endophytes isolated from host plant residing in healthy conditions. These secondary metabolites are effectively utilised in agriculture, medicinal and pharmaceutical industry.

Keywords

Aspergillus tamarii, *Casuarina junghuhniana*, endophyte, optimization.

INTRODUCTION

Endophytes are microorganisms which reside asymptotically within the living tissue of its host plant [1]. Endophytes shares symbiotic to slightly pathogenic relationship with their host [2]. Several studies revealed that endophytic fungus produces secondary metabolites similar to that of its host [3]. In recent past, endophytic fungus is considered to be a promising source of many novel bioactive compounds which can be utilised as biocontrol agent, antibiotics, immunosuppressant, anti-cancer drugs etc. Endophytes also produce indole acetic acid (IAA), a phytohormone, which is one of the most physiologically active auxin. IAA promotes root elongation, increase in number of root hairs and lateral root production which influences in higher uptake of nutrition [4]. These biologically important compounds hold great importance in medicinal, pharmaceutical and agriculture research [5, 2].

Several microbes are able to sustain in extreme environmental conditions such as high temperature, low pH, and high salt concentration. These physical conditions plays vital role in its growth and metabolite production. Growth media also strongly influence in production of secondary metabolites. Secondary metabolism are known to be regulated by carbon and nitrogen sources, trace elements, precursors, phosphate, catabolic repression and inhibition, feedback repression and inhibition [6]. Muturu and Konda (2007) reported that IAA production by bacteria varies not only among different species but also it largely depends on culture conditions, substrate availability and growth stage. Therefore, it is essential to optimise the culture conditions to attain maximum growth and for enhancing metabolite production which can be effectively used in research and industries [7].

The principle elements required for fungi for its synthesis of cellular constituents and functional processes depends on its nutritional sources [8]. To optimise growth and production of secondary metabolite, nutrient requirement and other growth conditions requires standardisation at various culture conditions [9-12]. Growth and production of secondary metabolites of fungi varies significantly not only within its genera but also with strains of same isolates. *Aspergillus*, a fungi associated with many species is known to produce various secondary metabolites having antimicrobial activity against a wide range of fungal and bacterial pathogen [13].

Casuarina junghuhniana Miq. is an exotic, woody, nitrogen fixing tree species belonging to *Casuarinaceae* family. These are non-leguminous and fast growing tree which can sustain in nutrient

deficient soil and can easily adapt to extreme climatic conditions [14]. Casuarinas are known to be rich in various phytoconstituents like alkaloids, carbohydrate, phenols, flavonoids and tannins. Further, *Casuarina* is a multipurpose tree with wide range of application. In our previous study, *Aspergillus tamaraii* (CJN5) was isolated and identified using 18S rRNA molecular sequencing, from the root of *Casuarina junghuhniana* Miq. This isolate proved to a promising source of phenolic compounds like gallic acid and salicylic acid, also are considered as plant growth promoting fungus [15].

The objective of the present study is to optimise the culture conditions of the fungal endophyte *Aspergillus tamaraii* to enhance the growth and production of bioactive compounds (production of phenol, flavonoid and IAA and its antifungal activity.

MATERIALS AND METHODS

As mentioned before, *Aspergillus tamaraii*, a potent fungal endophyte which was isolated from the root of *Casuarina junghuhniana* Miq. was identified using morphological analysis and molecular sequencing. Data obtained after sequencing have been submitted in the NCBI GenBank database to attain accession number. *Aspergillus tamaraii*, was maintained in Potato Dextrose Agar medium. Potato dextrose broth medium (PDB) was used as a basal medium. The growth and production of bioactive compounds was optimised on the basis of culture media, carbon and nitrogen sources, NaCl, pH and temperature. After each optimising step, the best condition was selected for further optimising study.

Selection of culture media:

The effect of different culture medium on growth and production of bioactive compounds was studied. The fungal isolate was grown in different media namely, Czapek's Dox broth (CDB), Yeast extract broth (YEB), Potato dextrose broth (PDB), Sabourod's broth (SB), Potato carrot broth (PCB), for selection of suitable growth medium for the accumulation of biomass and production of secondary metabolites. The media in which the fungal isolates exhibits maximum growth, bioactive metabolite production and antifungal activity has been selected as optimised medium for further study.

Effect of carbon sources on biomass and bioactive metabolite production:

Effect of carbon sources such as fructose, starch, dextrose and sucrose on growth of fungal isolates was studied. 1g/100ml of each carbon sources was individually added to the selected culture media. Each flask containing different carbon source was inoculated with five days old fungal isolate and was

incubated for ten days. After the incubation period, mycelial dry weight (biomass), production of biological active compounds and antifungal activity were recorded [16].

Effect of nitrogen source on biomass and bioactive metabolite production:

Effect of different nitrogen sources namely, Sodium nitrate, Beef extract, Ammonium chloride, Yeast extract and Peptone was studied. 1g/100ml of each nitrogen source was individually added to the culture media and optimised source of carbon in all the treatments. These flasks were inoculated with five days old *Aspergillus tamaris* under aseptic condition and were incubated for ten days. The mycelial dry weight, bioactive compound production and antifungal activity were recorded at the end of the incubation period [16].

Effect of NaCl concentration on biomass and bioactive metabolite production:

The effect of salinity on fungal growth and production of bioactive metabolites were carried out by amending the basal medium in optimised culture conditions along with different concentration of NaCl ranging from 1-11 g/100ml concentration. The biomass production, antifungal activity and production of metabolites was estimated.

Effect of pH on biomass and bioactive metabolite production:

The effect of pH on fungal growth and production of bioactive compounds was assessed using basal culture medium containing optimised conditions along with different pH level, ranging between pH 3-11 in 100ml of basal medium. The pH of the medium was adjusted using 0.1 N NaOH or 0.1 N HCl [17]. After inoculation, each flask was incubated for 10 days and dry mycelia weight, bioactive metabolite production and antifungal assay was recorded.

Effect of temperature on biomass and bioactive metabolite production:

The fungal isolate was subjected to different temperature ranging between 15 °C to 45 °C for determining the optimum temperature required for the growth and biologically active metabolite yield. The basal media was sterilized and was inoculated with fungal isolates under aseptic condition and incubated for the period of ten days at its specific temperature with optimised culture conditions. Dry mycelial weight, production of bioactive compounds and antifungal activity were recorded after incubation period [18]. Triplicates were maintained for each tested parameters.

Determination of biomass and quantification of bioactive compounds:

After each of the optimising step, fungal mycelia were separated from the culture medium and were dried at 70°C until a constant weight was obtained. The weight of the fungal biomass was expressed in mg/100ml [16].

The extraction of secondary metabolite of the culture filtrate was carried out using ethyl acetate solvent for determining the effect of optimizing parameters responsible for bioactive metabolite production. These extracts were used for estimation of phenol, flavonoid and IAA. Total phenolic content, total flavonoid content and IAA concentration of these extracts were estimated spectrophotometrically (UV 1650 PC Shimadzu) using gallic acid, quercetin and IAA as standards respectively [19- 20].

Antifungal Assay:

In previous study, two different concentrations of the extracts (50µg & 100µg) was initially assayed against fungal phytopathogens such as *Fusarium solani*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Alternaria alternata*, *Curvularia lunata* and *Rhizoctonia solani*. Maximum zone of inhibition was found against *Fusarium oxysporum*. Therefore, for the various optimised parameters, antifungal assay were carried out against *Fusarium oxysporum* following well diffusion method using PDA medium. Carbendazim (100 µg) was used as positive control whereas Ethyl acetate solvent was used as negative control. Triplicates were maintained for all the samples. The plates were incubated at room temperature up to 72hrs. Zone of inhibition was observed and recorded [21].

STATISTICAL ANALYSIS

Each experiment was conducted in triplicates. Results are shown as mean \pm standard error. Experimental results were analysed following appropriate methods such as Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using statistical software SPSS 20.0.

RESULTS AND DISCUSSION

The growth and yield of bioactive compounds of microbes can be increased substantially by optimising its physical and chemical factors. This is due to the fact that antibiotic biosynthesis is a specific microbial property which depends significantly on culture condition. Thus, enhancement in growth and antibiotic production can be achieved by manipulating the nutritional and physical parameters of the culture condition [22]. In

this study, *Aspergillus tamarii* (GenBank accession number: MH762146), was maintained in Potato Dextrose Agar medium.

Selection of culture media:

Fig 1, table 1 shows that PDB is the ideal medium for the growth of fungal endophyte *Aspergillus tamarii* in terms of biomass (1600mg/100ml) with the production of maximum concentration of IAA ($90.03 \pm 0.29 \mu\text{g/ml}$), total phenol ($208.62 \pm 0.16 \text{ mg/g}$) and flavonoid content ($25.37 \pm 0.13 \text{ mg/g}$) and produces maximum zone of inhibition in PDB, however minimum growth and production of secondary metabolites was observed in PCB. Bhattacharya and Jha expressed similar results where PDB is considered as best culture media for the growth of *Aspergillus* sp and maximum zone of inhibition was obtained against *Bacillus* sp [23]. Rabbani *et al*, (2011) also reported PDB medium as best medium for growth and sporulation of *Drechslera hawaiiensis* [24].

The growth of *Aspergillus tamarii*, antifungal activity and concentration of total phenol, flavonoid and IAA were found to be highly significant ($p < 0.001$) and based on Duncan Multiple Range Test (DMRT) all the culture media studied significantly differs at 5% level showing maximum production and antifungal activity.

Effect on carbon sources on biomass and bioactive metabolite production:

The effect of different sources of carbon on growth and production of metabolites by the fungal isolates is shown in Fig 2, table 2. The fungal isolate was able to grow in all the tested carbon sources, however, maximum growth and production of bioactive compounds were observed when dextrose was used as the carbon source. Monosaccharides were found to be more effective carbon source for growth of isolates and for the production of biologically active compounds but not for the biosynthesis of antibiotics [25], in contrary to this report, the present study revealed that biomass, bioactive compound production (IAA $91.30 \pm 0.21 \mu\text{g/ml}$, total phenol content $195.63 \pm 0.15 \text{ mg/g}$, total flavonoid content $78.58 \pm 0.16 \text{ mg/g}$) and antifungal activity is enhanced significantly using dextrose as carbon source.

The growth of *Aspergillus tamarii*, antifungal activity and concentration of total phenol, flavonoid and IAA were found to be highly significant ($p < 0.001$) and based on Duncan Multiple Range Test (DMRT) all the carbon sources studied significantly differs at 5% level showing maximum production and antifungal activity.

Effect of nitrogen source on biomass and bioactive metabolite production:

Effect of nitrogen source on growth and bioactive metabolite production was found maximum in yeast extract (fig 3, table 3). It was found that organic nitrogen source induces higher growth (1000mg/100ml) and production of bioactive compound (IAA $54.43 \pm 0.13 \mu\text{g/ml}$, total phenol content $189.43 \pm 0.07 \text{ mg/g}$, total flavonoid content $41.32 \pm 0.13 \text{ mg/g}$) than inorganic nitrogen sources. Narayana, K. and Vijayalakshmi, M. (2008); Sunita *et al.*, (2015) stated the importance of various nitrogen sources in maximizing the growth of *Pseudomonas fluorescense* and *Bacillus subtilis* [26-27].

The growth of *Aspergillus tamarii*, antifungal activity and concentration of total phenol, flavonoid and IAA were found to be highly significant ($p < 0.001$) and based on Duncan Multiple Range Test (DMRT) all the nitrogen sources studied significantly differs at 5% level showing maximum production and antifungal activity with different nitrogen sources.

Effect of NaCl concentration on biomass and bioactive metabolite production:

Effect of NaCl (salinity) on growth and bioactive metabolite production is shown in Fig 4, table 4. Fungal isolate grew in all tested concentration of NaCl, however maximum growth (1300mg/100ml) and metabolite production (IAA $92.80 \pm 0.65 \mu\text{g/ml}$, total phenol content $125.58 \pm 0.18 \text{ mg/g}$, total flavonoid content $5.57 \pm 0.09 \text{ mg/g}$) was observed in 5g/l concentration. Thus, the endophyte can be considered as halotolerant fungus. Halotolerant organisms are those which are able to grow in a wide range of salt concentration. *Fusarium* sp is reported as a halotolerant species. The ability of fungus to tolerate high salt concentration is due to accumulation of arabitol (a five atom noncyclic polyol), an increased level of phosphalidic acids in the phospholipids, depletion of sterols/phospholipid ratio and elevated level of unsaturated phospholipids [28]. Similar conditions were reported by Suja *et al* (2013) for growth [29] and biologically active metabolite production of *Aspergillus terreus*. Bhattacharya and Jha (2011) also reported 5g/l concentration of NaCl to be optimum for maximum mycelial growth of *Aspergillus* sp (49mg/25ml) and maximum zone of inhibition was obtained against *Bacillus* sp. A gradual decrease in mycelial growth was observed with increase in salt concentration in basal media [23].

The growth of *Aspergillus tamarii*, antifungal activity and concentration of total phenol, flavonoid and IAA were found to be highly significant ($p < 0.001$) and based on Duncan Multiple Range Test (DMRT) all the

NaCl concentration studied significantly differs at 5% level showing maximum production and antifungal activity.

Effect of temperature on biomass and bioactive metabolite production:

The effect of temperature on fungal biomass and metabolite production is shown in fig 5, table 5. It revealed highest growth and maximum bioactive metabolite was obtained for fungal isolate growing in temperature between 25-30°C. Decrease in biomass and metabolite production was observed at maximum and minimum studied temperature. Increase in temperature from 25-30°C has been reported to enhance the growth of cells (1300mg/100ml) and bioactive metabolite production (IAA 84.07±0.03 µg/ml, total phenol content 178.37±0.20 mg/g, total flavonoid content 47.48±0.17 mg/g) in *Aspergillus* strain. Maximum fungal biomass (70mg/25ml medium) and zone of inhibition against *Bacillus* sp was obtained at 30°C has been reported by Bhattacharya and Jha [23]. Similarly, 30°C is reported to be optimum for mycelial growth of *Rhizoctonia solani* [30].

The growth of *Aspergillus tamarii*, antifungal activity and concentration of total phenol, flavonoid and IAA were found to be highly significant ($p < 0.001$) and based on Duncan Multiple Range Test (DMRT) all the temperature studied significantly differs at 5% level showing maximum production and antifungal activity.

Effect of pH on biomass and bioactive metabolite production:

In the present study, maximum growth (1700 mg/100ml) and production of bioactive metabolite, total phenol content 163.59±0.09 mg/g, total flavonoid content 78.73±0.14 mg/g) was observed at pH 5, however, maximum production of IAA of 90.43±0.03 µg/ml was obtained at pH 7. Minimum growth and metabolite production was observed at pH 3 and pH 11(Fig 6, table 6). Similar to this result, Gogoi *et al* (2008) reported that growth and production of bioactive metabolites was found to be maximum at pH 5 by endophyte *Hypoceria* sp isolated from *Dilline indica* [31]. pH of the culture medium is reported to be one of the most important factor due to the fact that it induces activity of several enzymes that catalyses metabolic reaction, also influence in complex physiological phenomenon such as cell morphology and membrane permeability [32]. It is reported that changes in pH affects many cellular processes. Maximum antibacterial activity by *Aspergillus terreus* was found at pH 6 by Jain and Pudir (2011) [33].

The growth of *Aspergillus tamarii*, antifungal activity and concentration of total phenol, flavonoid and IAA were found to be highly significant ($p < 0.001$) and based on Duncan Multiple Range Test (DMRT) all the pH studied significantly differs at 5% level showing maximum production and antifungal activity (Table 30 a and b).

Table 1: Estimation of total phenol, flavonoid and IAA content using different culture medium

<i>Aspergillus tamarii</i>			
Basal medium	Total phenol content (mg/g)	Total flavonoid content(mg/g)	IAA concentration (µg/mL)
YEB	18.73±0.68 ^b	2.17±0.12 ^b	6.97±0.12 ^b
CDB	0.00±0.00 ^a	9.48±0.10 ^d	18.47±0.26 ^c
PDB	208.62±0.16 ^d	25.37±0.13 ^e	90.03±0.29 ^e
PCB	0.00±0.00 ^a	0.00±0.00 ^a	5.53±0.19 ^a
SB	26.49±0.18 ^c	8.54±0.11 ^c	42.73±0.18 ^d
F value	6607.456	37131.721	26863.213
P value	<0.001**	<0.001**	<0.001**

- Note:
1. The value refers to Mean ± SE
 2. ** denotes significant at 1% level.
 3. Different alphabet among basal medium denotes significant at 5% level using Duncan Multiple Range Test (DMRT)

Table 2: Estimation of total phenol, flavonoid and IAA content using different carbon sources

<i>Aspergillus tamarii</i>			
Carbon Source	Total phenol content (mg/g)	Total flavonoid Content (mg/g)	IAA concentration (µg/mL)
Starch	27.58±0.14 ^a	0.00±00 ^a	11.87±0.07 ^a
Fructose	63.63±0.09 ^c	0.00±00 ^a	20.43±0.09 ^b
Sucrose	32.51±0.16 ^b	11.56±0.17 ^b	21.76±0.04 ^c
Dextrose	195.63±0.15 ^d	78.58±0.16 ^c	91.30±0.21 ^d
F value	323761.800	102640.760	94840.193
P value	<0.001**	<0.001**	<0.001**

Note: 1. The value refers to Mean ± SE
 2. ** denotes significant at 1% level.
 3. Different alphabet among carbon sources denotes significant at 5% level using Duncan Multiple Range Test (DMRT)

Table 3: Estimation of total phenol, flavonoid and IAA content using different nitrogen sources

<i>Aspergillus tamarii</i>			
Nitrogen sources	Total phenol content (mg/g)	Total flavonoid content (mg/g)	IAA concentration (µg/mL)
Sodium nitrate	18.60±0.15 ^b	9.50±0.03 ^b	36.63±0.07 ^c
Beef extract	0.00±00 ^a	0.00±00 ^a	30.47±0.03 ^b
Yeast extract	189.43±0.07 ^c	41.32±0.13 ^c	54.43±0.13 ^e
Peptone	0.00±00 ^a	0.00±00 ^a	40.43±0.09 ^d
Ammonium chloride	0.00±00 ^a	0.00±00 ^a	5.63±0.18 ^a
F value	1240891.840	89197.329	25751.321
P value	<0.001**	<0.001**	<0.001**

Note: 1. The value refers to Mean ± SE
 2. ** denotes significant at 1% level.
 3. Different alphabet among nitrogen sources denote significant at 5% level using Duncan Multiple Range Test (DMRT).

Table 4: Estimation of total phenol, flavonoid and IAA content using different NaCl concentration

<i>Aspergillus tamarii</i>			
NaCl(g/L)	Total phenol content (mg/g)	Total flavonoid content (mg/g)	IAA concentration (µg/mL)
1	3.70±0.07 ^a	5.47±0.12 ^a	46.53±0.19 ^c
3	60.37±0.15 ^c	8.40±0.06 ^b	13.57±0.12 ^b
5	125.58±0.18 ^e	5.57±0.09 ^a	92.80±0.65 ^e
7	78.45±0.13 ^d	30.62±0.03 ^d	87.33±0.03 ^d
9	7.73±0.12 ^b	17.37±0.09 ^c	3.17±0.12 ^a
F value	148734.515	16801.870	16852.942
P value	<0.001**	<0.001**	<0.001**

Note: 1. The value refers to Mean ± SE
 2. ** denotes significant at 1% level.
 3. Different alphabet among NaCl concentrations denote significant at 5% level using Duncan Multiple Range Test (DMRT)

Table 5: Estimation of total phenol, flavonoid and IAA content using different temperature

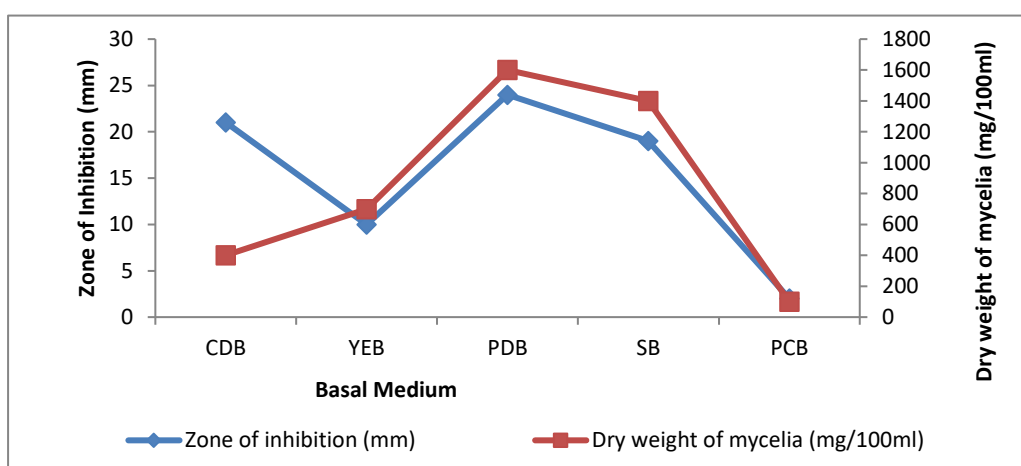
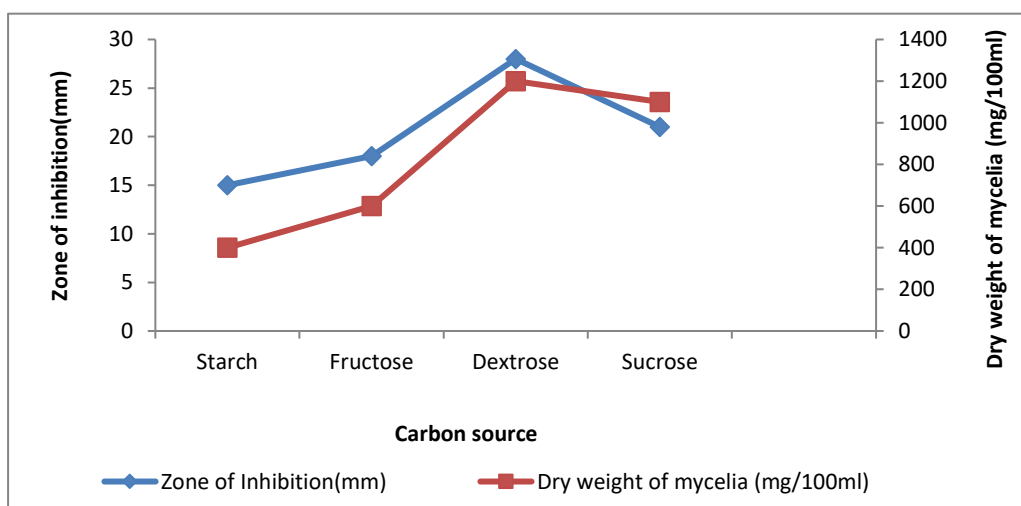
<i>Aspergillus tamarii</i>			
Temperature (°C)	Total phenol content (mg/g)	Total flavonoid content (mg/g)	IAA concentration (µg/mL)
15	11.29±0.5 ^b	0.00±00 ^a	1.83±0.09 ^b
25	133.22±0.13 ^c	9.57±0.06 ^b	80.33±0.09 ^c
35	178.37±0.20 ^d	47.48±0.17 ^c	84.07±0.03 ^d
45	0.00±00 ^a	0.00±00 ^a	0.00±00 ^a
F value	516246.947	61340.139	529250.378
P value	<0.001**	<0.001**	<0.001**

- Note: 1. The value refers to Mean \pm SE
 2. ** denotes significant at 1% level.
 3. Different alphabet among different temperature denotes significant at 5% level using Duncan Multiple Range Test (DMRT)

Table 6: Estimation of total phenol, flavonoid and IAA concentration using different pH

<i>Aspergillus tamarii</i>			
pH	Total phenol content (mg/g)	Total flavonoid content (mg/g)	IAA concentraion (μ g/mL)
pH3	0.00 \pm 0.00 ^a	0.00 \pm 0.00 ^a	0.00 \pm 00 ^a
pH5	163.59 \pm 0.09 ^e	78.73 \pm 0.14 ^c	89.40 \pm 0.06 ^d
pH7	85.68 \pm 0.15 ^d	0.00 \pm 0.00 ^a	90.43 \pm 0.03 ^e
pH9	5.76 \pm 0.09 ^c	3.52 \pm 0.16 ^b	34.13 \pm 0.03 ^c
pH11	5.40 \pm 0.12 ^b	0.00 \pm 0.00 ^a	4.27 \pm 0.12 ^b
F value	489753.182	131508.223	489292.444
P value	<0.001**	<0.001**	<0.001**

- Note: 1. The value refers to Mean \pm SE
 2. ** denotes significant at 1% level.
 3. Different alphabet among different pH denotes significant at 5% level using Duncan Multiple Range Test (DMRT)


Fig 1: Effect of different culture medium on biomass and bioactive metabolite production

Fig 2: Effect on carbon sources on biomass and bioactive metabolite production

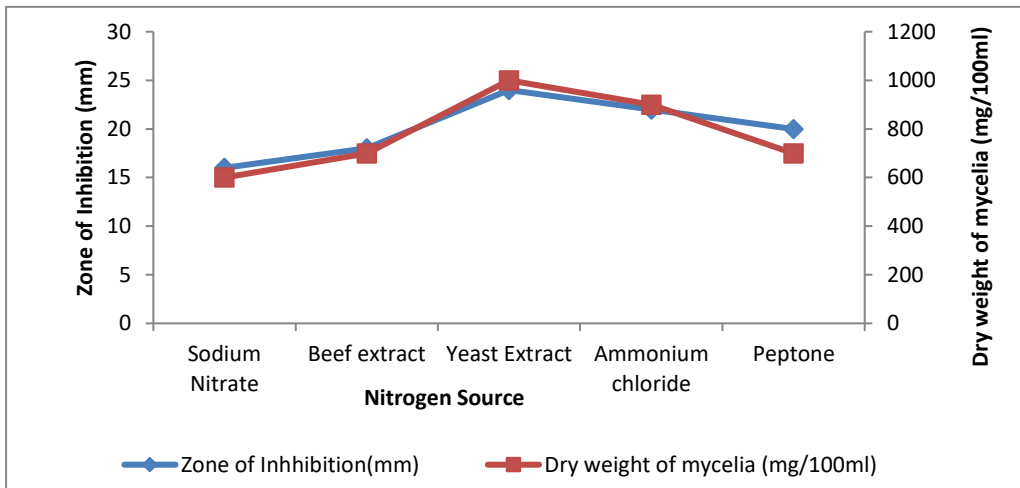


Fig 3: Effect of nitrogen source on biomass and bioactive metabolite production

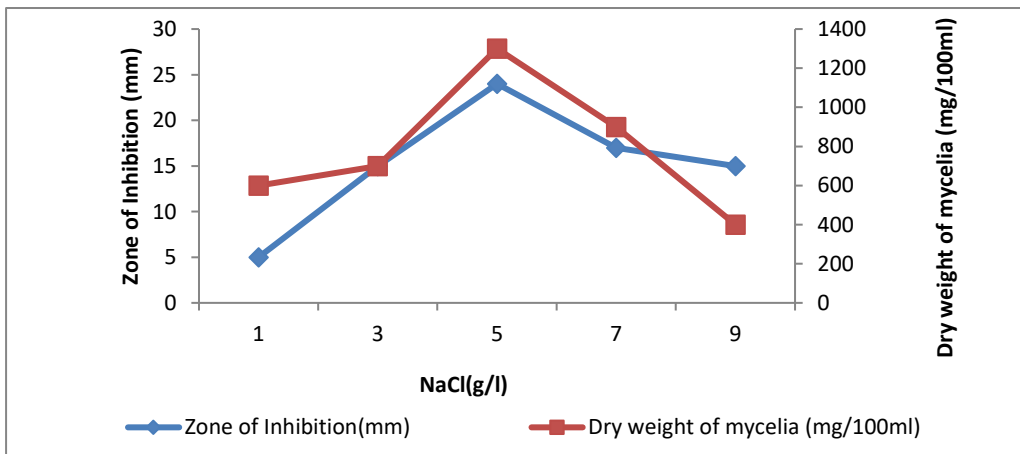


Fig 4: Effect of NaCl concentration on biomass and bioactive metabolite production

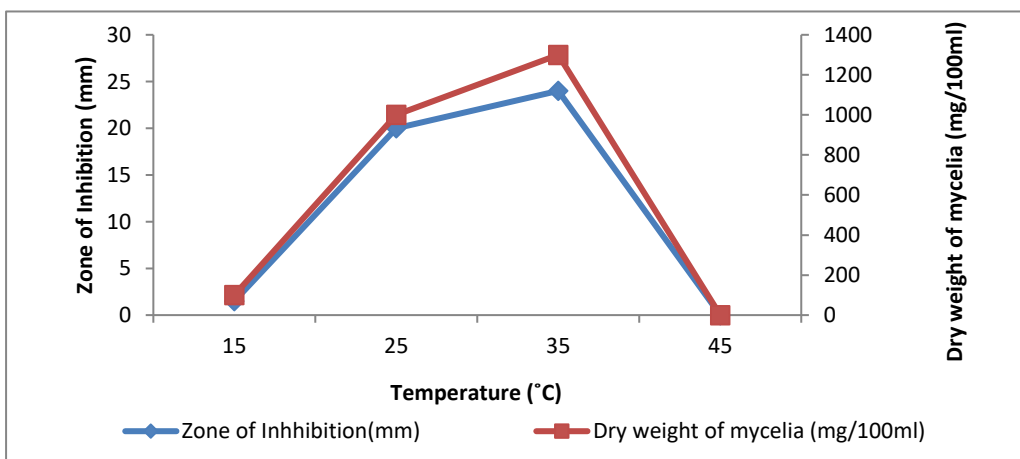


Fig 5: Effect of temperature on biomass and bioactive metabolite production

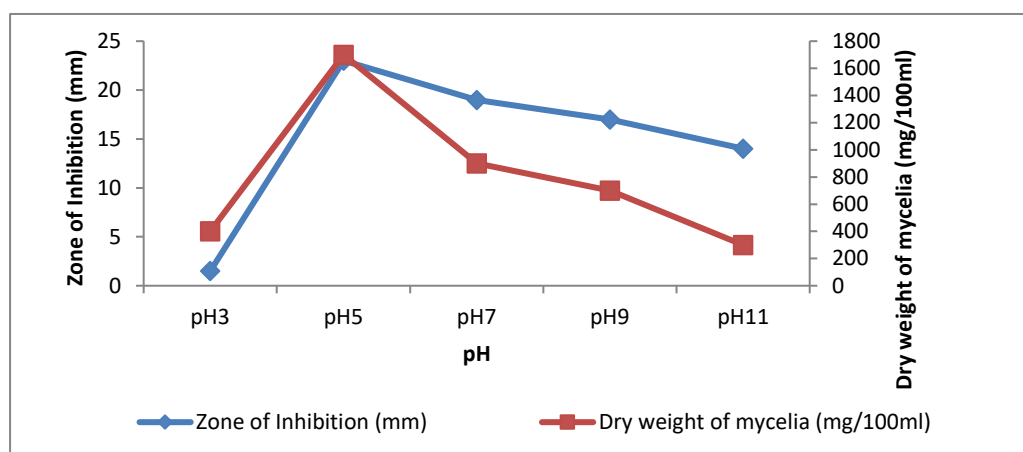


Fig6: Effect of pH on biomass and bioactive metabolite production

CONCLUSION

The present study infers that the optimised endophyte was found to be on par with the native endophyte isolated from the tree species, hence the endophyte can be effectively applied in the formulation of bio protectants and for the enhancement of commercially important agricultural crops, as well as the tree species. This endophyte can be used as an agroforestry model, where the intercrop will be benefitted by the endophytes inhabiting the tree species.

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