OPTIMIZED GROWTH CONDITIONS FOR PRODUCTION OF BIO-ACTIVE METABOLITES OF ENDOPHYTIC FUNGI

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Abstract:

Endophytic fungi are a group of microorganisms that reside within the internal tissues of living plants, exhibiting a communalistic relationship with their host without causing any apparent harm to the latter. The dynamic nature of plant ecosystems is attributed to the modification of species' structure and composition in response to various circumstances across different plant tissues. Certain endophytic fungi have exhibited the potential to serve as a prolific reservoir of innovative bioactive compounds that hold promise for diverse fields such as pharmaceuticals, agriculture, and industrial applications. This research aimed to improve the metabolites generated by endophytic fungus by isolating and growing them under optimal physical and chemical conditions. The different sources of substrates like carbon, nitrogen, inorganic nitrogen, concentration of sodium chloride, pH and temperature levels were provided for the optimum growth condition of *Curvularia geniculate* to produce secondary metabolites at high yield.

Keywords:

Optimization, growth factor, secondary metabolites, *Curvularia geniculate*

Introduction

Research conducted has revealed that endophytic fungi, which inhabit macroalgae, possess a significant potential to synthesize diverse naturally-occurring bioactive compounds that exhibit unique structural characteristics (Baracaldo et al., 2019). According to Venkataraman et al. (2010), the emergence of treatments that are more precise in their targeting and less harmful to normal cells has introduced new paradigms in the field. Endosymbionts are often recognized by comparing morphological characteristics differentiating between closely, comparing theoretically similar species is typically a difficult undertaking (Kaddes et al., 2019). The fungi's reproductive and vegetative compatibility can be influenced by culture factors, and their morphological characteristics may vary depending on the growing medium. (Kang JG et al., 2004).

The majority of fungi derived from coastal algae exhibit a remarkable ability to flourish in a challenging and unique habitat, thereby enabling them to generate innovative

secondary metabolites. The aforementioned phenomenon can be attributed to the organisms' existence and acclimation to a particular ecological niche, as evidenced by the studies conducted by Flewelling et al. (2015) and Wahaibi et al. (2019). Marine endophytes continue to serve as a source of novel and medically relevant secondary metabolites, which can be manipulated to produce valuable products at a higher efficiency from their natural productome, as noted by Hussain et al. (2012). The significance of endophytic antimicrobial substances in safeguarding against pathogens has been suggested by Gowrish et al. (2016). Moreover, owing to the plant's inherent ability to act as a natural selection mechanism, these chemical compounds have the potential to mitigate cellular toxicity in superior organisms.

Over the last decade, there has been an increasing emphasis on the creation of innovative biological substitutes for the synthesis of safe pigments. Furthermore, the discovery of supplementary characteristics such as antibacterial properties and biodegradability has been reported in studies conducted by Babitha et al. (2009) and Kongruang et al. (2011). Endophytic fungi have been observed to occur ubiquitously across all plant species and have demonstrated the ability to produce bioactive agents with anti-cancer, anti-protozoal, and anti-malarial properties. Certain individuals have the ability to produce physiologically active compounds that are similar or indistinguishable from those produced by the host organism, as exemplified by Taxol (Stierle A et al., 1993 and Strobel G et al., 1996).

Curvularia species have been found to contain secondary metabolites such as alkaloids, terpenes, polyketides, and quinones. In a recent study conducted by Kalimuthu et al. (2022), an in vitro investigation was carried out to assess the antioxidant and anti-cancer potential of Curvularia geniculata, which was isolated from Phyllanthus niruri L. Furthermore, it has been observed that Curvularia geniculata exhibits noteworthy proficiency in the synthesis of Phytohormone and phosphate solubilization, as reported by Priyadarshini et al. The current manuscript aims to investigate diverse physicochemical parameters to determine the most favorable growth conditions and augment the yield of secondary metabolites derived from Curvularia geniculata.

Optimization Growth Conditions for Production of Bio-Active Metabolites:

The fungal isolate EF2’s growth medium for secondary metabolic synthesis was optimized. The carbon source, nitrogen source, NaCl content, incubation temperature, and pH were all examined for growth and metabolite formation associated with antibacterial activity. (Dasari et al., 2009).

The Outcome of Carbon Sources on Metabolite Production:

Glucose, Starch, sucrose, and lactose were chosen as carbon sources. Each carbon source was introduced at 1% to the basal

medium of potato dextrose broth separately. Each flask with a different carbon source was infected with a mycelial disc from a seven-day-old fungal culture and grown at 28℃ for ten days.

Following incubation, the optimization medium was subjected to filtration using filter paper to extract the endophytic fungus biomass. The process of liquid-liquid fractionation, also known as partition, was executed utilizing a 1:1 ratio of ethyl acetate solvent. The resultant extract was then concentrated through employment of a rotary evaporator, ultimately producing the ethyl acetate-based extract of endophytic fungi. The biomass that had been separated was subjected to further drying in an oven in order to attain its dry weight, as per the methodology described by Bhardwaj et al. (2015).

The Effect of Nitrogen Sources on Metabolite Production:

The study aimed to examine the impact of diverse nitrogen sources on the synthesis of antibacterial compounds and mycelium development. This was achieved by introducing organic nitrogen sources at a concentration of 1% and inorganic nitrogen sources at a concentration of 0.01% (w/v) into the optimization medium. Organic nitrogen sources such as casein hydrolysate, yeast extract, beef extract, and peptone were utilized in the experiment.

The inorganic sources of nitrogen include ammonium nitrate, ammonium sulfate, potassium nitrate, and sodium nitrate. Individual flasks containing PDB were provided with the selected nitrogen source in a separate manner. A mycelial disc from a fungal culture that was seven days old was administered to the subjects, who were then incubated for a period of ten days at a temperature of 28℃.

Following the incubation period, the optimization medium was subjected to filtration using filter paper to eliminate the organic matter of the endophytic fungus. The process of liquid-liquid fractionation, also known as partition, was executed utilizing a 1:1 ratio of ethyl acetate solvent. The resultant extract was then concentrated with a rotary evaporator, ultimately producing the ethyl acetate extract of endophytic fungi. Subsequently, the biomass that had been separated was subjected to drying in an oven to attain its dry weight, as per the methodology outlined by Bhardwaj et al. (2015).

The Effect of Sodium Chloride Concentration for Metabolic Production:

Although the endophytic fungus isolate was obtained from a marine source, the sodium chloride content is an essential factor in the formation of secondary metabolites. The following sodium chloride (NaCl) concentrations were investigated for maximal metabolite production: 0.25%, 0.5%, 0.75% and 1%. Each of the aforementioned concentrations was added to the medium with a 10% v/v inoculum and cultured for 10days at 28℃.

After the incubation period, the biomass of endophytic fungi was removed from the optimized medium using filter paper. The liquid-liquid fractionation (partition) was performed in a 1:1 ration via ethyl acetate solvent, and the extract recovered was concentrated with a rotary evaporator to yield the ethyl acetate extract of endophytic fungi. Following that, the separated biomass was dried in an oven to acquire the dry weight (Bhardwaj et al., 2015).

The Effect of Incubation Temperature for Metabolite Production:

Each basal medium with is selected carbon and nitrogen sources was inoculated with 2% (v/v) of seed culture and incubated at different temperatures, such as 20℃. 25℃, 30℃ and 35℃ for endophytic fungal isolates, and the incubation period was 10 days.

Post-incubation the by- products of endophytic fungus were filtered out of the optimal medium using filter paper. The liquid-liquid fractionation (partition) was performed in a 1:1 ration using ethyl acetate solvent, and the extract was further concentrated with a rotary evaporator to develop the ethyl acetate extract of endophytic fungi. The separated biomass was finally dried in an oven in order to get the dry weight (Bhardwaj et al., 2015).

The Effect of pH for Metabolite Production:

Using an alkaline and acidic pH range, each basal medium with its chosen carbon and nitrogen sources was inoculated with 2% (v/v) of seed culture and incubated at different pH values ranging from 5.0, 6.0, 7.0, 7.5 and 8.0 for endophytic fungal isolates.

Eventually, after the incubation process, the organic matter of the endophytic fungus was scooped out of the optimized medium using filter paper. The liquid-liquid fractionation(partition) had been carried out in a 1:1 ration using ethyl acetate solvent, and the extract was concentrated with a rotary evaporator, leading to the ethyl acetate extract of endophytic fungi. The separated biomass was later dried in an oven to get the dry weight (Bhardwaj et al., 2015)

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Tables showing Effect of each source for Metabolite production

Table.1 Effect of Carbon for Metabolite production

Carbon	Metabolites in mg/liter	Dry weight g/liter	Dry weight mg/liter
Glucose	964	2562	2.562
Sucrose	622	1872	1.872
Starch	562	1661	1.661
Lactose	277	876	0.876

Table.2 Effect of Organic Nitrogen for Metabolite production

Organic Nitrogen	Metabolites in mg/liter	Dry weight g/liter	Dry weight mg/liter
Peptone	554	1592	1.592
Yeast	550	1482	1.482

Table.3 Effect of Inorganic Nitrogen for Metabolite production

Inorganic Nitrogen	Metabolites in mg/liter	Dry weight g/liter	Dry weight mg/liter
AN	330	967	0.967
AS	593	1693	1.693
PN	363	1023	1.023
NaNO3	354	986	0.986

Table.4 Effect of Nacl concentrations for Metabolite production

NaCl concentration	Metabolites in mg/liter	Dry weight g/liter	Dry weight mg/liter
0.25%	1251	2234	2.234
0.50%	1229	2132	2.132
0.75%	900	2034	2.034
1%	466	1102	1.102

Table.5 Effect of Temperature for Metabolite production

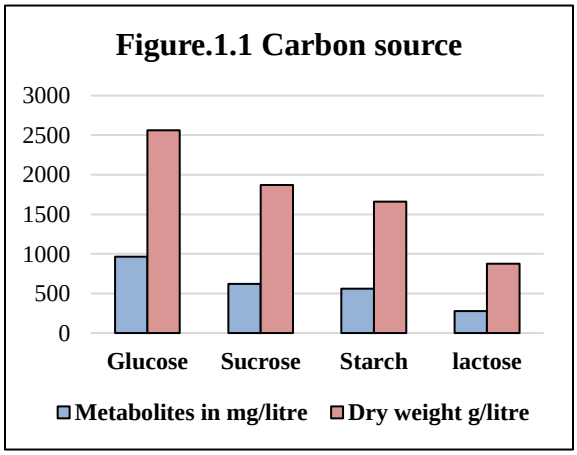
Temperature	Metabolites in mg/liter	Dry weight g/liter	Dry weight mg/liter
20°C	622	1589	1.589
25°C	913	2178	2.178
30°C	403	1034	1.034
35°C	200	674	6.74

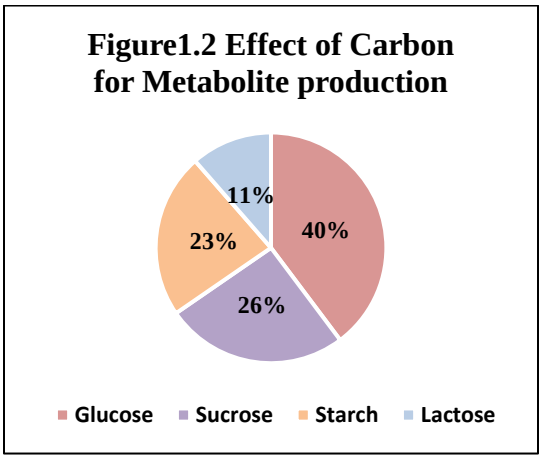
Table.6. Effect of pH for Metabolite production

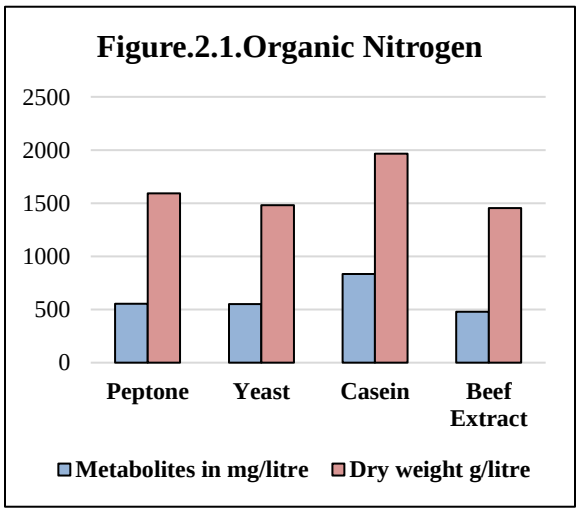
pH	Metabolites in mg/liter	Dry weight g/liter	Dry weight mg/liter
5pH	444	1254	1.254

6pH	902	2143	2.143
7pH	786	1723	1.723
7.5pH	830	1897	1.897
8pH	423	1111	1.111

Figures showing Effect of each source for Metabolite production







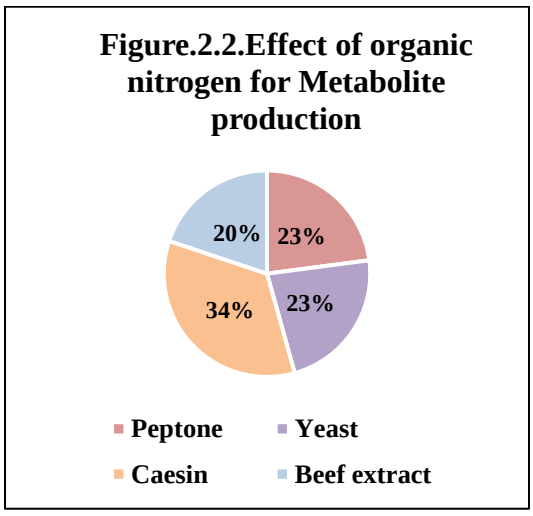


Figure.3.1.Inorganic Nitrogen

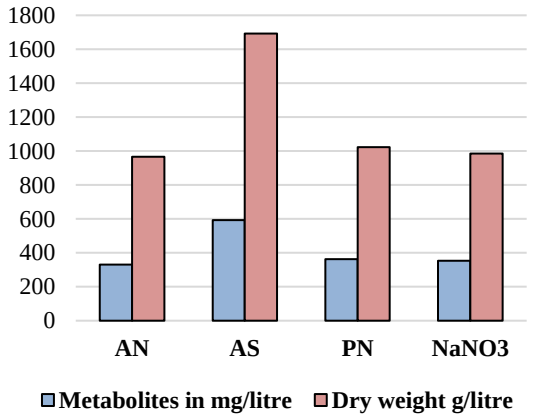


Figure.3.2.Effect of Inorganic nitrogen for Metabolite production

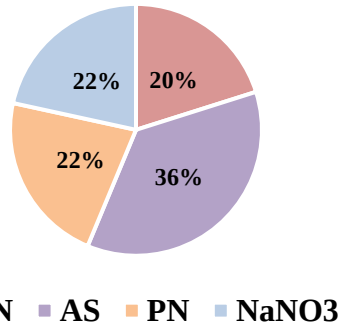


Figure.4.1.NACL Concentration

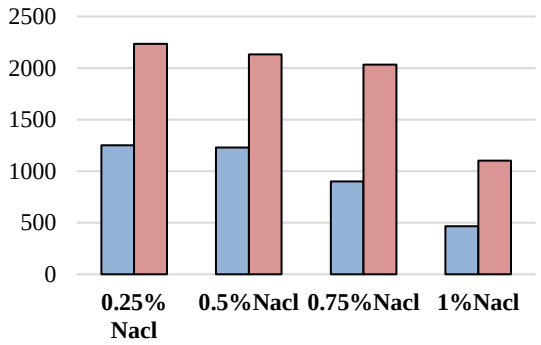


Figure.4.2.Effect of Nacl concentration for Metabolite production

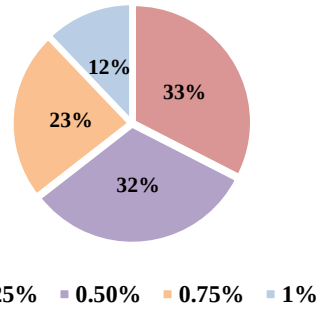


Figure.5.1.Temperature

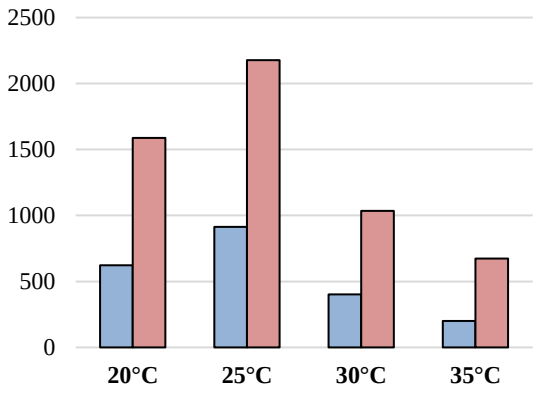
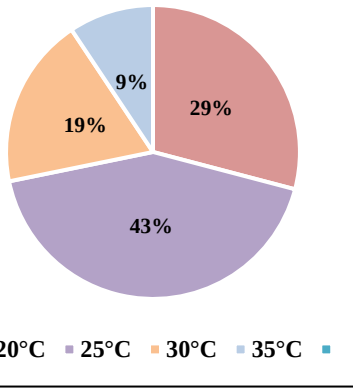
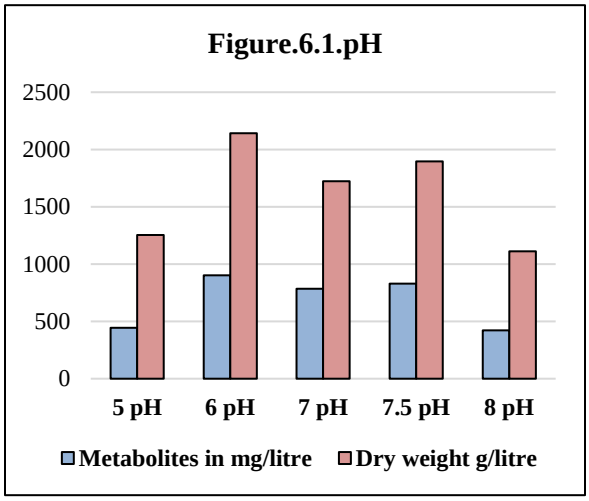
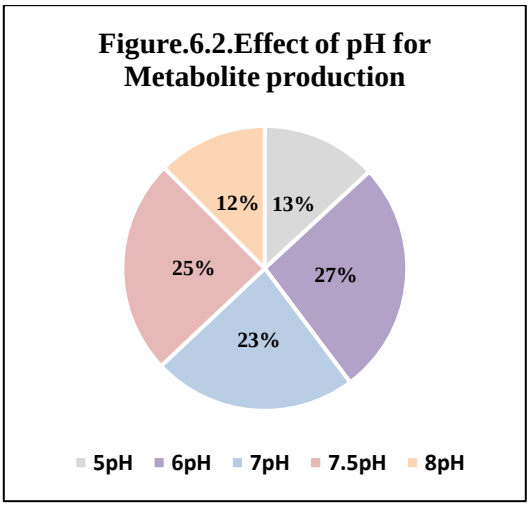


Figure.5.2.Effect of Temperature for Metabolite production







DISCUSSION

Studies conducted by Zhang et al. (2016) have demonstrated that endophytic fungi found in macroalgae possess an extensive ability to produce diverse bioactive natural compounds with unique structural characteristics. Their morphological characteristics can be growing medium specific and culture factors can influence the fungi's reproductive and vegetative compatibility (Myers et al., 2000)). The identification of a secondary metabolite exhibiting anti-cancer properties was accomplished through the utilization of GCMS, FTIR, and NMR techniques. To enhance the synthesis of secondary metabolites, Curvularia geniculata was subjected to diverse substrates such as carbon, NaCl, organic nitrogen, and inorganic nitrogen sources. The bacterial growth was observed under different temperature and pH conditions (Table. 1-6 and Figure. 1.1-6.2).

The study examined various sources of substrates, including carbon (glucose, sucrose, starch, and lactose), nitrogen (beef extract, casein, peptone, and yeast), and inorganic nitrogen (ammonium nitrate, ammonium sulphate, potassium nitrate, and sodium nitrate). A sequence of sodium chloride concentrations were applied at 0.25% and 0.5%. The experiment involved subjecting the organism Curvularia geniculata to varying pH levels of 5, 6, 7, 7.5, and 8, and incubation temperatures ranging from 20℃ to 35℃. The aim was to determine the optimal growth conditions for the organism to produce a secondary metabolite with a high yield. The pH levels used were 0.75% and 1%, respectively (Table 5 & Fig. 5.1, Fig. 5.2).

The concentrations of metabolites (mg/L) resulting from carbon sources, namely glucose, sucrose, starch, and lactose, were found to be 964, 622, 562, and 277 mg/L, respectively (Table1 & Fig. 1.1, Fig. 1.2). On the other hand, the concentrations of

metabolites (mg/L) resulting from NaCl sources were observed to be 1251, 900, and 466 mg/L for 0.25%, 0.75%, and 1% NaCl concentrations, respectively (Table 4& Fig. 4.1, Fig. 4.2).

In contrast, the quantities of organic nitrogen were found to be as follows: peptone at 554mg/l, yeast at 550mg/l, casein at 833mg/l, and beef extract at 480mg/l (Table 2 & Fig. 2.1, Fig. 2.2). Similarly, the amounts of inorganic nitrogen were determined to be ammonium nitrate at 330mg/l, ammonium sulphate at 593mg/l, potassium nitrate at 363mg/l, and sodium nitrate at 354mg/l (Table 3 & Fig. 3.1, Fig. 3.2). The obtained yields of secondary metabolites across different temperature ranges are presented. The concentration of the substance at different temperatures was measured and recorded as follows: at 200°C, the concentration was 622mg/l; at 250°C, it was 913mg/l (Table 6 & Fig. 6.1, Fig. 6.2).

Thus the aim of the present investigation was to isolate an endophytic microorganism from its host algae and optimize the production parameters for a metabolite with antibacterial properties is acheived.

CONCLUSION

In order to provide the optimal environment for the development of Curvularia geniculata, glucose was used as the carbon source, casein was used as the organic nitrogen source, ammonium nitrate was used as the inorganic nitrogen source, and a concentration of 0.25 percent sodium chloride was used. According to the findings of the research, the optimal levels of pH and temperature were found to be 6 and 250 degrees Celsius, respectively.

REFERENCES

1. Babitha S. 2009. Production of pigments, pp. 147–162. In: Nigam P. & Pandey A. (eds) Biotechnology for Agro-Industrial Residues Utilisation, Springer, Heidelberg.
2. Baracaldo, P., Bianchini, G., Cesare, A. D., Callieri, C., and Chrismas, N. A. M. (2019). Insights into the evolution of picocyanobacteria and phycoerythrin genes (mpeBA and cpeBA). Front. Microbiol. 10, 45. doi:10.3389/fmicb.2019.0004.
3. Deshmukh, S. K., Prakash, V., and Ranjan, N. (2018). Marine fungi: a source of potential anticancer compounds. Front. Microbiol. 8:2536. doi: 10.3389/fmicb.2017.02536.
4. Flewelling, A. J., Currie, A., Gray, C. A., and Johnson, J. A. (2015). Endophytes from marine macroalgae: promising sources of novel natural products. Curr. Sci. 10, 88–111. doi:10.1177/1934578x1501001003.

5. Gowrish A, Vagdevi HM, Rajashekar H. Phytochemical screening and antimicrobial activity of Leucas marrubioides desf. Root extracts. Int J Pharm Pharm Sci 2016;8(12):209-12.
6. Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z., and Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. J. Pharm. Bioall. Sci. 4:10. doi: 10.4103/0975-7406.92725.
7. Kaddes, A., Fauconnier, M.-L., Sassi, K., Nasraoui, B., and Jijakli, M. H. (2019). Endophytic fungal volatile compounds as solution for sustainable agriculture. Molecules 24, 1065. doi:10.3390/molecules24061065.
8. Kamat, S., Kumari, M., Sajna, K. V., and Jayabaskaran, C. (2020b). Endophytic fungus, Chaetomium globosum, associated with marine green alga, a new source of Chrysin. Endophytic fungi of marine alga from Konkan coast, India -a rich source of bioactive material. Sci. Rep.. 10, 18726. doi:10.1038/s41598-020-72497-3.
9. Kalimuthu A. K., Parasuraman P., Sivakumar P., Murugesan S., Arunachalam S., Pandian S. R. K., et al.. (2022). In silico, in vitro screening of antioxidant and anticancer potentials of bioactive secondary metabolites from an endophytic fungus (Curvularia sp.) from Phyllanthus niruri L. Environ. Sci. Pollut. Res. 29, 48908–48925. 10.1007/s11356-022-19249-0.
10. Kang JG, Shin SY, Kim MJ, Bajpai V, Maheshwari DK, Kang SC. Isolation and anti-fungal activities of 2-hydroxymethyl-chroman4-one Produced by Burkholderia sp. MSSP. J Antibiot (Tokyo) 2004;57(11):726-31.
11. Kongruang S. 2011. Growth kinetics of biopigment production by Thai isolated Monascus purpureus in a stirred tank bioreactor. J. Ind. Microbiol. Biotechnol. 38: 93–99.
12. Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J. Biodiversity hotspots for conservation priorities. Nature 2000;403(6772):853-8.
13. Stierle A, Strobel GA, Stierle D. Taxol and taxen production by Taxomyces Andreanna an endophytic Fungus of Pacific yew. Science 1993;260(5105):214-6.

14. Strobel G, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM. Taxol from Pestalotiopsis microspora, an endophytic fungus of Taxus wallachiana. Microbiology 1996;142:435-40.
15. Venkatraman, P. Specifcity in the interaction of natural products with their target proteins-a biochemical and structural insight. Mini-Rev Med Chem. 10, 540–549 (2010).
16. Wahaibi, A. S. M., Emilia, L., Nithyalakshmy, R., Sergey, D., Robert, U., and Grant, B. G. (2019). Secretion of Dnases by marine bacteria: a culture based and bioinformatics approach. Front. Microbiol. 10, 969. doi:10.3389/fmicb.2019.0096.
17. Zhang, P., Li, X., and Wang, B. G. (2016). Secondary metabolites from the marine algal-derived endophytic fungi: chemical diversity and biological activity. Planta Med. 82, 832–842. doi:10.1055/s-0042-103496