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# Piperine Production by Fungal Endophytes Isolated from *Piper longum* L.: Effect of Carbon Sources

**Atmaja Elina Mishra[1], Nibha Gupta[1,*], Uday Chand Basak[1], Birendra Kumar Bindhani[2]**

[1]Regional Plant Resource Centre, Bhubaneswar, Odisha, India

[2]Kalinga Institute of Industrial Technology, Bhubaneswar, Odisha, India

Correspondence Author: **Dr. (Mrs.) Nibha Gupta**

**Abstract:** Bioactive secondary metabolites are becoming important due to their high biological activities and they have the potential to be used as lead compounds in drug discovery. *Piper longum* L. belongs to the family Piperaceae and have diverse pharmacological properties with fewer side effects in biological systems. It contains a pungent alkaloid 'Piperine' which have lots of pharmacological activities. In the present study, total 37nos. of fungi have been isolated from *Piper longum* grown locally. All fungi belongs to *Piper longum* were screened for piperine production by following the solvent extraction and UV spectrophotometric analysis protocols. Subsequently, these fungi *Trichoderma citrinoviride*, *Aspergillus sydowii* and *Fusarium proliferatum* have been selected for further experiments carried out on effect of carbon sources on piperine production in liquid culture conditions. The screening experiment carried out in different incubation period of fungal growth revealed variation in production of piperine at different growth period by different fungal species. Most of them preferred 21 days for better production. The three selected fungi exhibited production of piperine in 7 days of growth period. Further experiment exhibited the vital role of type of carbon sources used. *Trichoderma citrinoviride* preferred dextrose and *Fusarium proliferatum*, mannitol whereas *Aspergillus sydowii* did not perform well in any of these experimental conditions. Decline in the piperine content on fungal culture due to longer incubation period has been observed in these fungi studied. Observation and estimation of piperine content made in the present study is at preliminary stage. Further clarification for the presence of piperine content demands higher advanced instrumentation analysis. Hence, this is the first new report on piperine production by fungal endophytes *Aspergillus sp.* (17nos.), *Penicillium sp.* (5nos.), *Fusarium sp.* (4nos.), *Gibberella sp.*, *Trichoderma sp.*, *Paecilomyces sp.*, *Mycelia sp.* (5nos.), *Cladosporium sp.* (2nos.), *Piptocephalis sp.* isolated from *Piper longum*.

**Keywords:** *Piper longum*, Fungi, Endophyte, Piperine, Secondary metabolites, Carbon sources.

## Introduction

Fungi are known for production of bioactive secondary metabolites and enzymes. These extracellular microbial products are being used as important component in agriculture, food and healthcare industries. These microbes are cosmopolitan and occurred freely in air, soil, water. Many times they reside in host tissue and implement the phenomenon of mutualism, symbiosis and pathogenesis. Many a times, they reside in the host tissue without doing any harm to its living shelter, hence, known as endophytes.[1,2] Researchers and their findings exhibited their role in production of such metabolites which were produced by host plants. In other words, endophytes mimic the metabolism of host plants and capable to produce similar kind of metabolic content or compounds as their host plants does.[3]Medicinal plants are known for their therapeutic value, produces many organic compounds or metabolites useful for curing of different diseases and ailments. They have been studied extensively for pharmacological benefits like analgesic, antipyretic, anti-inflammatory and anticancer effects.[4]

*Piper longum* is an important medicinal plant belongs to Piperaceae family and being used as a traditional remedy for its pharmacological benefits. It is known for production of piperine, an alkaloid notably found in *Piper longum* and *Piper nigrum*. It is the primary active component of *Piper longum* and has been extensively studied for its analgesic, antipyretic, anti-inflammatory and anticancer activity.[5]Reports mentioned on presence of endophytic fungi in various plant parts of *Piper longum*. Verma et al., (2011) reported the presence of *Periconia sp*., in *P. longum* which has also been studied for the production of piperine. Comparable results have been reported for other *Piper nigrum* L. endophytes like *Colletotrichum sp*., *Phomopsis sp*., and *Mycosphaerella sp*., which have also synthesized piperine under liquid culture conditions.[6]Moreover, Chithra et al., (2014) isolated *Colletotrichum gleosporioides*, another piperine-producing fungus, from *Piper nigrum* L.[7] Additionally, *Ulocladium sp*., also derived from *Piper nigrum* L., has been documented for its piperine content, although this research did not explore the biological and ecological traits of the fungus.[8]However the yield and consistency of secondary metabolite production in laboratory conditions can be highly variable and is influenced by numerous factors including the culture medium, temperature, pH, light, and the concentration of available nutrients.[9]Optimizing these conditions is crucial not only for maximizing the production but also for the discovery of novel metabolites with potential therapeutic applications.

With this view, a systematic isolation of endophytic fungi for *P. longum* and their screening for piperine production has been carried out in the present study. As carbon component of nutrient has been vital for microbial growth, metabolism and biosynthesis of precursors and regulators for various metabolic pathways are important. An experiment has also been carried out to observe the effect of piperine production by isolated endophytic fungi from *P. longum*.

## Materials and methods

### Isolation of Fungal endophytes

Fungal endophytes were isolated from *Piper longum*, a well-known medicinal plant, for which the plant samples were collected locally and rinsed thoroughly in running tap water to clear dusts and debris. After proper washing, all the plant parts were air dried and cut into pieces in aseptic conditions. Surface sterilization of all cut pieces was done in 70% ethanol for 1min. Each set of plant material was then treated with 2.5% Sodium hypochlorite for 1min. They were finally rinsed with sterile distilled water to remove extra amount of sterilants. All the plant parts were then placed in petridishes containing Sabouraud dextrose agar medium (SD) and plates were incubated at 30˚C for 7 days. The grown fungal cultures were maintained in Sabouraud dextrose agar medium slants. Slants were incubated at 30°C for 4 days in static condition and then stored at 4°C.[10-12]

### Screening of endophytes for piperine production

Fungal cultures were transferred to Sabouraud dextrose broth (SDB) (culture medium containing dextrose 20g/l, peptone 10g/l, pH-5.5) by punching 6mm (4nos) plate culture discs into 50ml of media sterilized in 150ml Erlenmeyer flasks and incubated at 30°C for 7days, 14days, 21days and 28 days in an interval of 7 days. The extraction of piperine was carried out by collecting the culture filtrate through Whatman no. 1 filter paper. Ethyl acetate was added to the culture filtrate (1:2 v/v) and kept for 48 hours in room temperature. Ethyl acetate layer was separated and kept for evaporation. Crude sample was dissolved in methanol and estimation was done using spectrophotometric method.[13]Finally, three fungi were selected for further experiment.

### Effect of Carbon sources on Piperine production

Impact of different carbon sources on piperine production by three nos. of selected fungi were observed by inoculating them (4days old culture, 6mm disc/flask 50ml) in media at pH 5.5 supplemented individually with different carbon sources at the rate of 2% along with peptone 1% as nitrogen source.[14-15]

### Spectrophotometric analysis of piperine content

For the estimation process, 1ml of methanolic sample was taken and 0.1ml of Gallic acid was added to it followed by 5ml of conc. $H_2SO_4$. The mixture was incubated in water bath at 45°C for 5 minutes.[16]The standard stock solution (1mg/ml) prepared in HPLC grade methanol, stored at 4˚C in amber vials was used as standard purpose.[13]Then absorbance was measured at 650nm through spectrophotometer (Antech, Model No-AN-UV-7000N). The data obtained in the present study was treated with ANOVA and data interpreted in terms of significance.

## Results

A systematic study on 37nos. of fungi belonging to *Aspergillus sp., Penicillium sp., Fusarium sp., Gibberella sp., Trichoderma sp., Mycelliasterilia, Cladosporium sp.* and *Piptocephalis sp.* isolated from *P. longum*, screened for piperine production in general and in the presence of different carbon sources was investigated in the present work. Piperine content extracted and estimated at different incubation and growth period of all fungi exhibited the presence of piperine content in their culture filtrate. This study revealed the synthesis and extracellular production of piperine content in higher amount at 21 days of growth by *A. niger*, *P. italicum*, *A. fumigatus*, *G. pulicaris*, *Aspergillus sp*. 7, *Aspergillus sp.* 10 and *Cladosporium sp*.1 which was gradually declined at 28 days of growth. Comparatively higher estimated value of piperine was recorded in culture filtrate of *Cladosporium sp*.1 (6.85±0.35 µg/ml) and *Aspergillus sp*. 7 (5.50±0.14 µg/ml) at the age of 21days (Figures 1-9). One way repeated measures ANOVA calculated. The F ratio value is 5.86 and the P value is 0.000947. The result is significant at *P<0.05*. The two way ANOVA ordinary calculated. The F ratio is 4.41 and the P value is 0.0063. The result is significant at *P<0.05*.

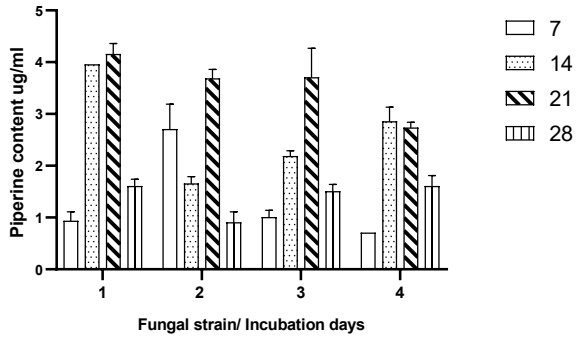


Figure 1: Piperine production by 1= *Aspergillus niger*, 2= *Penicillium italicum*, 3=*Aspergillus fumigatus* and 4= *Penicillium roquiforti* under different incubation period.

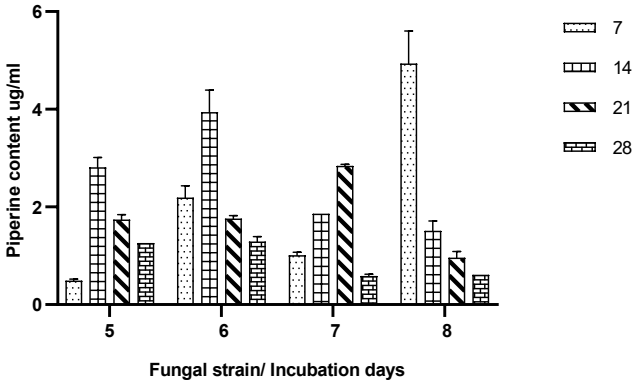

Figure 2: Piperine production by 5= *Penicillium glabrum*, 6= *Fusarium graminearum*, 7= *Gibberella pulicaris* and 8= *Trichoderma citrinoviride* under different incubation period.

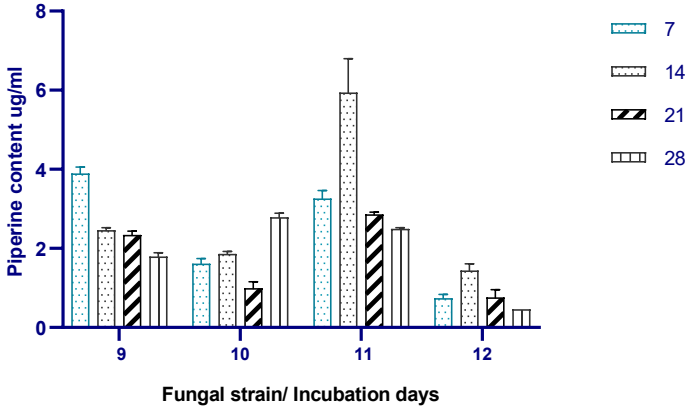


Figure 3: Piperine production by 9= *Aspergillus fumigatus*, 10= *Penicillum oxalicum*, 11= *Penicillium diversum*, 12= *Paecilomyces sp.* under different incubation period.

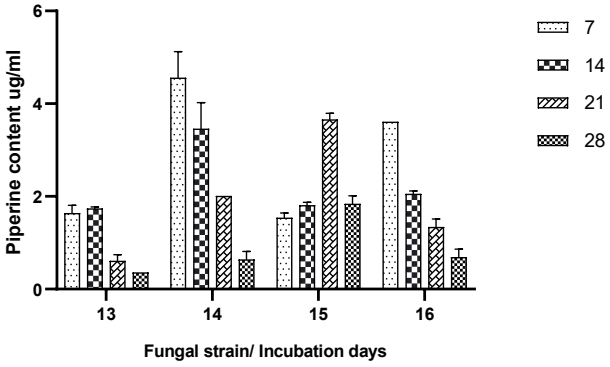


Figure 4: Piperine production by 13= *Aspergillus sp*. 1, 14= *Aspergillus sydowii*, 15= *Aspergillus sp*. 2, 16= *Aspergillus sp*. 3 under different incubation period.

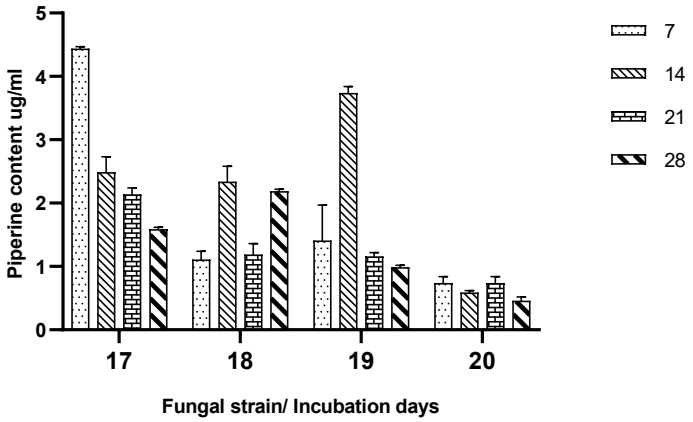

Figure 5: Piperine production by 17= *Fusarium proliferatum*, 18= *Fusarium dimerum*, 19= *Mycelia sterilia*1, 20= *Mycelia sterilia* 2 under different incubation period.

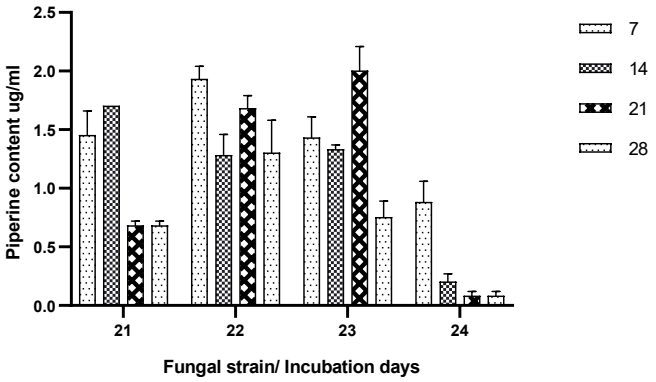


Figure 6: Piperine production by 21= *Mycelia sterilia* 3, 22= *Aspergillus sp*.4, 23= *Aspergillus sp*. 5, 24= *Aspergillus sydowii* under different incubation period.

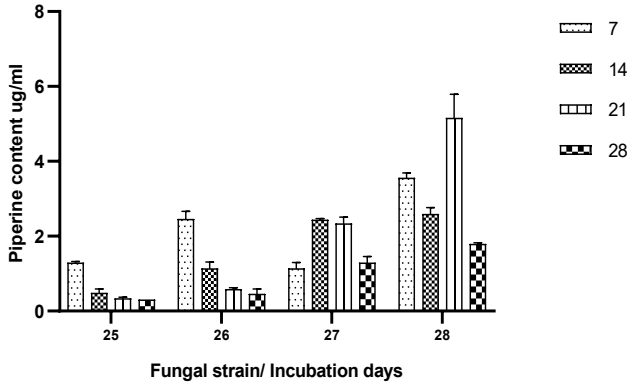


Figure 7: Piperine production by 25= *Mycelia sterilia* 4, 26= *Mycelia sterilia* 5, 27=*Aspergillus sp*. 6, 28= *Aspergillus sp. 7* under different incubation period.

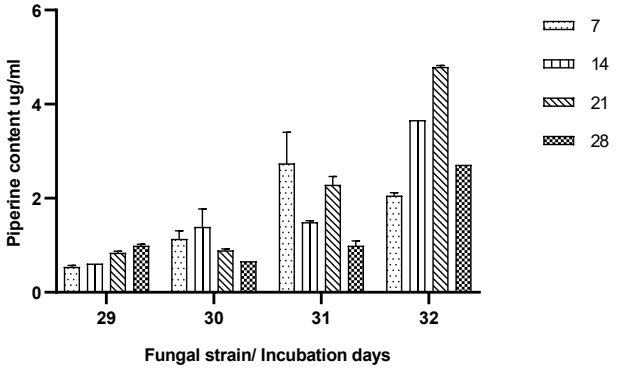


Figure 8: Piperine production by 29= *Aspergillus sp.* 8, 30=*Aspergillus sp.* 9, 31= *Fusarium sp*., 32= *Aspergillus sp.* 10 under different incubation period.

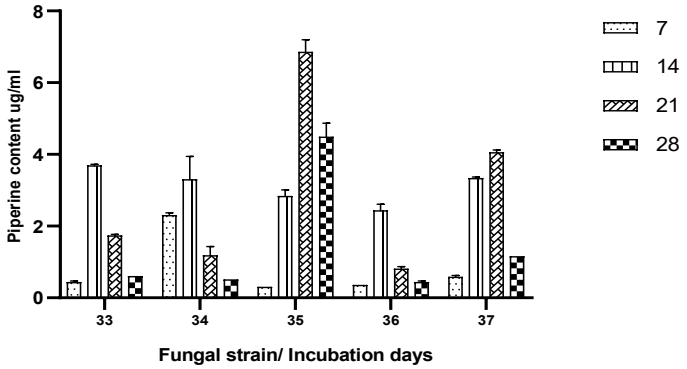


Figure9: Piperine production by 33= *Aspergillus sp.* 11, 34= *Cladosporium sp.*1, 35= *Cladosporium sp*.2, 36= *Piptocephalis sp*., 37= *Aspergillus ustus* under different incubation period.

Addition of dextrose, glucose and galactose in media used for *Trichoderma citrinoviride* clearly indicated the higher production of piperine i.e.7.80 ± 0.21µg/ml, 6.63 ± 0.53µg/ml and 5.48 ± 0.18µg/ml respectively (Figure 10). The calculated value of piperine in terms of biomass mg/gm showed the dextrose (3.23±0.24mg/gm) as better carbon source in this regards. This was followed by glucose and galactose (Table 1). However, carbon sources were contributed less in piperine production as far as *Aspergillus sydowii* is concern (Figure 11). Very different observation was recorded with *Fusarium proliferatum* where mannitol and fructose helped the fungus to produce good amount of piperine (4.38 ± 0.28 µg/ml and 4.20 ± 0.28 µg/ml) (Figure 12). The estimated value of piperine in terms of mg per gram biomass was lower in this organism. One way repeated measures ANOVA calculated for carbon sources. The F ratio value is 39.868 and the P value is 0.0001. The result is significant at *P<0.05, P<0.01, P<0.10*. The two way ANOVA ordinary is also calculated. The F ratio is 44.6 and the P value is 0.00001. The result is significant at *P<0.05, P<0.01, P<0.10*.

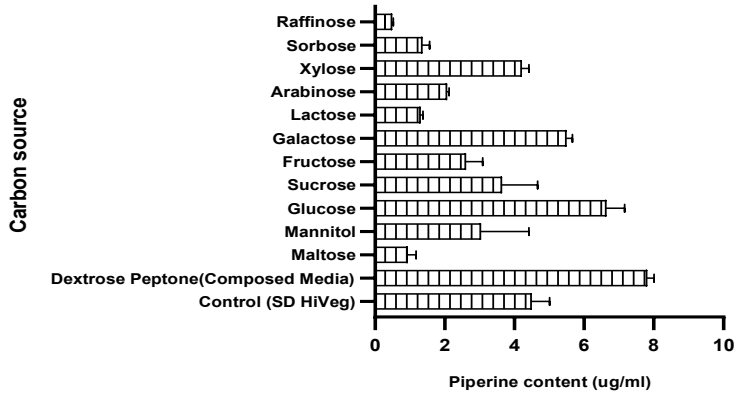


Figure 10: Piperine production by *Trichoderma citrinoviride* in media supplemented with different carbon sources.

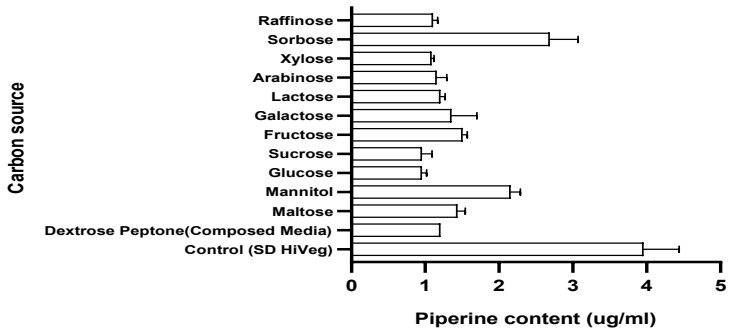


Figure 11: Piperine production by *Aspergillus sydowii* in media supplemented with different carbon sources.

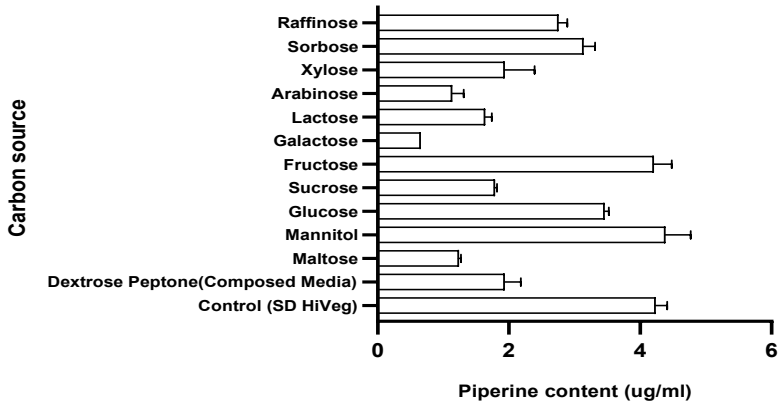


Figure 12: Piperine production by *Fusarium proliferatum* in media supplemented with different carbon sources.

**Table 1: Estimated value of piperine content produced by selected fungi (in terms of fungal biomass mg/gm)**

| S.No. | Carbon sources | Piperine content in per gram Biomass (mg/gm) | | |
|---|---|---|---|---|
| | | *Trichoderma citrinoviride* | *Aspergillus sydowii* | *Fusarium proliferatum* |
| 1 | Control (SD Hi Veg) | 1.07 ± 0.12 | 0.58 ± 0.12 | 0.85 ± 0.13 |
| 2 | Dextrose | 3.23 ± 0.24 | 0.12 ± 0.01 | 0.31 ± 0.01 |
| 3 | Maltose | 0.32 ± 0.06 | 0.14 ± 0.01 | 0.17 ± 0.03 |
| 4 | Mannitol | 0.56 ± 0.21 | 0.11 ± 0.01 | 0.72 ± 0.07 |
| 5 | Glucose | 2.69 ± 0.14 | 0.10 ± 0.01 | 0.39 ± 0.07 |
| 6 | Sucrose | 2.05 ± 0.82 | 0.09 ± 0.02 | 0.50 ± 0.13 |
| 7 | Fructose | 0.68 ± 0.28 | 0.14 ± 0.00 | 0.71 ± 0.03 |
| 8 | Galactose | 2.36 ± 0.06 | 0.17 ± 0.04 | 0.10 ± 0.01 |
| 9 | Lactose | 0.22 ± 0.00 | 0.15 ± 0.01 | 0.57 ± 0.03 |
| 10 | Arabinose | 1.18 ± 0.08 | 0.13 ± 0.02 | 0.16 ± 0.02 |

| | | | | |
|---|---|---|---|---|
| 11 | Xylose | 2.07 ± 0.31 | 0.11 ± 0.00 | 0.29 ± 0.05 |
| 12 | Sorbose | 0.51 ± 0.11 | 0.38 ± 0.06 | 0.44 ± 0.04 |
| 13 | Raffinose | 0.43 ± 0.01 | 0.12 ± 0.00 | 0.37 ± 0.02 |

± values are taken in triplicates.

## Discussion

Fungi are known for useful secondary metabolite production and its type, concentration depends upon the produced fungal species.[10]Present study revealed the potential of isolated fungal strains for piperine production. Several studies were augmented on isolation of endophytic fungi from *Piper longum*, few studies performed regarding the piperine production by these fungi. [17, 18] An endophytic fungus *Periconia sp.* isolated from *P. longum* has the ability to produce piperine.[6]Moreover, Chithra et al., (2014) isolated *Colletotrichum gleosporioides*, another piperine-producing fungus, from *Piper nigrum* L.[7] Additionally, *Ulocladium sp*., also derived from *Piper nigrum* L., has been documented for its piperine content.[8]

Carbon sources like glucose, sucrose, and other sugars are vital component and cellular building blocks required for growth, development and biosynthesis of other metabolites like antibiotics, pigments, and toxins known as bioprotective agents.[19, 20] Hence, supplementation of carbon sources can thus be a key strategy in maximizing the production of desired secondary metabolites in industrial and pharmaceutical applications.[21]Present study revealed the utilization of dextrose by *Trichoderma citrinoviride* and mannitol by *Fusarium proliferatum* for better production of piperine in liquid culture condition. This study corroborated with the other investigation on role of various carbon sources on enzyme and metabolite production.[21-23]*Fusarium solani*, a fungus isolated from *Chonemorpha fragrans* that is able to produce camptothecin (CPT), a secondary metabolite in which glucose was observed to be the best inducer (1% w/v), followed by sucrose.[24] Information was given by Mao et al., 2005 that a Chinese traditional medicinal mushroom *Cordyceps militaris* was investigated for cordyceplin production in shake flasks. The carbon sources examined were lactose, sucrose, glucose, fructose, galactose, maltose and xylose, and glucose was found to be most favourable to cordycepin production, whereas cells grew best in galactose medium.[25] Experiments carried out on screening and utilization of carbon sources exhibited the potential of fungal endophytes for piperine production. This may be one kind of novel report as few researchers reported their observation on piperine production by fungal endophytes.[6-8] Observation and estimation of piperine content made in the present study is at preliminary stage. Further clarification for the presence of piperine content demands higher advanced instrumentation analysis.

## Conclusion

Data recorded on piperine production by fungal endophytes isolated from *Piper longum* revealed the importance of these fungi as an alternate source of piperine production, as this alkaloid has been earlier reported and sourced from plants of Piperaceae especially *Piper longum* and *Piper nigrum*. The study carried out on screening of fungi for this purpose, provided a good numbers of fungal strains endowed the potential of this alkaloid production. Further experiments on effect of various carbon sources on metabolite production expressed the usefulness of dextrose and mannitol for enhanced production in in-vitro condition.

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**Conflict of interest:** The authors declare no conflict of interest.

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