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# Isolation and identification with different enzyme production from marine associated plants

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**Key words:** Endophytic fungi, *Avicennia marina*, *Suaeda maritima, Salicornia brachiata*, Enzyme production

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## Abstract

The current research focuses on the diversity and screening of endophytic fungi found within three distinct marine-associated plants, which reside inside the host plant without causing any observable negative impact on them like *Avicennia marina*, *Suaeda maritima, Salicornia brachiata*. The maximum number of colonies was isolated from *Avicennia marina,* and *Salicornia brachiata* followed by *Suaeda maritima*. The eighteen fungi were identified such as *Aspergillus conicus, A. fumigatus, A. niger, A. luchuensis, A. ochraceus, A. terreus, A. ustus, Alternaria geophylla, Alternaria tenuis, Choanephora cucurbitarum, Curvularia geniculata, Fusarium falcatum, Helminthosporium sativum, Neonectria ranularia, Nigrospora sphaerica, Penicillium janthenellum, Pyricularia oryzae, Rhizopus stolonifer* by morphological characters were significantly resulted. The fungal strains were screened by amylase, cellulase, lipase, and protease production. Among them only fungal strain were maximum (*A. niger*) and minimum zone of inhibition (*Choanephora cucurbitarum*) were observed in protease production followed by amylase. Maximum produced cellulase (*A. ochraceus*) and minimum zone of inhibition (*Rhizopus stolonifer*) followed by lipase. Totally these (*Aspergillus conicus, A. ochraceus)* two fungi are present in all enzyme production. However, these endophytic fungi were excellent biological activities for future endeavor.

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## Introduction

Endophytic fungi that live inside the tissues of living plants are under - explored group of microorganisms (Akinloye *et al.*, 2002). There are believed to be at least a million of various kinds of endophytic fungi. Recently, there has been a surge in interest surrounding them due to their ability to safeguard their host against infections, insect pests, and even domestic herbivores.

(Arnold *et al.*, 2003 and Arnold *et al.*, 2001) almost all the plant species (400,000) harbor one or more endophytic organisms, (Choedon *et al.*, 2006) Only a select group of plants have been extensively examined in terms of endophytic biodiversity and potential for generating bioactive secondary metabolites. Endophytic fungi usually exist slowly with their host just under certain conditions they can turn into facultative pathogens. Endophytic fungi are naturally found within plants' tissues and cause no detectable disease symptoms in the plant. However, it is believed to possess supporting ecological and physiological benefits for the plant (Iyabo *et al.*, 2023). One of the most important functions of endophytic fungus is to start the biological degradation of a host plant that's dead or dying, which is essential to nutrient recycling (Clay and Schardl, 2002). The coast protection, storage of carbon, and buffering of seawater from terrestrial pollutants are only some of the biological benefits that marshes, which are transitional places between terrestrial and aquatic ecosystems provide. They ecological services additionally serve to improve the health of water. Because these are able to absorb a lot of wind and wave energy, salt marshes along the coast contribute to reducing storm damage (Traut, 2005). All plants in nature have a symbiotic relationship with fungi, that is essential for their capacity to fight off numerous illnesses and biotic and abiotic stresses in order survive and grow (Selim *et al.*, 2012; Evans, 2007).

Ecosystems that inhabit mangrove forests are fascinating and complex (Feller *et al.*, 2010). Mangrove plants are salt-tolerant plants that act as important sources in the marine food chain. In addition, they create novel metabolites that are native to the environment and have many of important economic and ecological functions (Bandarnayake, 2002). The *Salicornia* species are small, succulent-like plants with erect lateral branches and a jointed horizontal main stem that grows to be usually less than 30 cm tall. The plant may appear to be lacking leaves due to the small, scale-like leaves (Schulz *et al.*, 2002; Strobel and Daisy, 2003). Endophytic fungi have been discovered as possible sources of bioactive secondary metabolites. Fungal endophytes are a polyphyletic category of predominantly ascomycetous fungi that dwell within wholesome host tissues during at least one phase of their life cycle and without affecting any visible symptoms of disease or negative effects on their hosts (Sandrawati *et al.*, 2020). In this context, microorganisms of unique and unexplored ecological niches such as endophytes that inhabit such biotopes, containing marine plants like algae, sea-grass, driftwood, and mangrove plants (Esraa *et al.*, 2021).

## Materials and methods

*Study site*

Kodiyakarai coast the present study area is located in the north western part of coastal zone of Tamilnadu, India. It lies between 10. 17 ° N and 16.08° N latitudes and 79. 51° E and 54.36° E longitudes (Fig. 1).


**Fig. 1.** Location for Kodiyakkarai

*Collection of plant samples*

The samples were collected seasonally from coastal areas of Kodiyakarai. The plant samples were collected at

depth of 10cm, by using metal spatula, and sterilized every time with 70% alcohol. At each station 5 to 7 samples were collected randomly and were pooled together. The samples were kept in sterilized polythene bags, sealed and transported to the laboratory.

*Isolation of endophytic fungi*

Asymptomatic healthy leaf materials were thoroughly washed in running tap water, then surface sterilized by a modified method of (Raviraja, 2005). The selected leaf segments were immersed in 95% ethanol for 30 sec, 45% sodium hypochlorite solution for 15sec and 95% ethanol for 30sec followed by rinsing with sterile distilled water three times for 10sec and allowed to surface dry under sterile conditions. After drying, each leaf segment was cut into approximately 0.5cm squares and placed on petri plates containing potato dextrose agar medium (PDA). The Streptomycin sulphate (100mg/L) was added to prevent the growth of bacteria. Then it was monitored every day for growth of endophytic fungal colonies. Fungi growing out from the samples were subsequently transferred to fresh PDA plates.

*Identification of endophytic fungi*

The identification of fungal taxa followed as the standard manual of endophytic fungi such as A Manual of Penicillia (Raper and Fennell, 1965), A Manual of endophytic fungi (Gillman, 1957), Manual of Aspergilli (Smith 1946), Hyphomycetes (Subramanian, 1971), Dematiaceous Hyphomycetes (Ellis, 1971).

*Presentation of data*

Percentage of contribution and percentage of frequency of fungal isolates were calculated by using the following formula.

Percent contribution = {(No of fungal colonies in a sample)/ (Total number all colonies of all the species in a sample)} × 100

Percent frequency = {(Number of all samples in which a particular fungus occurred)/ (Total number of samples examined)} × 100

Based on the frequency occurrences, the fungi were grouped as rare (0-25% frequency), occasional (26-50 frequency), frequent (51-75% frequency) and common (76 – 100% frequency) species.

*Lacto phenol cotton blue mountin***g**

A loopful culture was picked up with the help of a sterile inoculation loop and semi – permanent slides were prepared using lacto phenol cotton blue. The slides were gently heated in a spirit lamp so as to release the air bubbles if any present inside the cover glass. The excess stain was removed by using tissue paper and the cover glass was sealed with white nail polish.

*Screening (Migahed, 2003)*

The amylolytic fungal isolates were screened following the method for their best enzymatic starch hydrolysis. The isolate with maximum clearance of zone was further studied and selected as the potential three fungal strains. Culture maintenance and preparation of pure isolates were done.

*Cellulase (Subramanian, 1971)*

The cellulase substrate used in the agar plate medium of clearing zone test was prepared according to the procedure as recommended. Cellulase activities of the highly active fungal filtrates were determined by using a carboxymethyl cellulase activity assay (CMC). Basal medium containing (g L-1 ): CMC (10), $NaNO_3$ (6.5), $K_2HPO_4$(6.5), yeast extract (0.3), KCl (6.5), $MgSO_4 \cdot 7H_2O$ (3.0) and agar (17.5), was used for plate screening. In addition, conidia from one-week-old PDA plate's cultures were suspended in sterile water. A small well created in the middle of the screening agar plates and same number of conidia of each strain (~105) was inoculated into the wells. Plates were incubated at 28 °C for three to five days followed by 18h in the same conditions. Cellulolytic strains were selected based on the diameter of the cellulase hydrolysis and zone of surrounding the colonies were observed. For observations, plates were stained with 1% Congo red dye (0.5-1 h), followed by distaining with 1M NaCl solution for 15-20 min.

*Protease*

Production of proteolytic enzymes by fungal isolates was detected by using the Plate assay method. Which

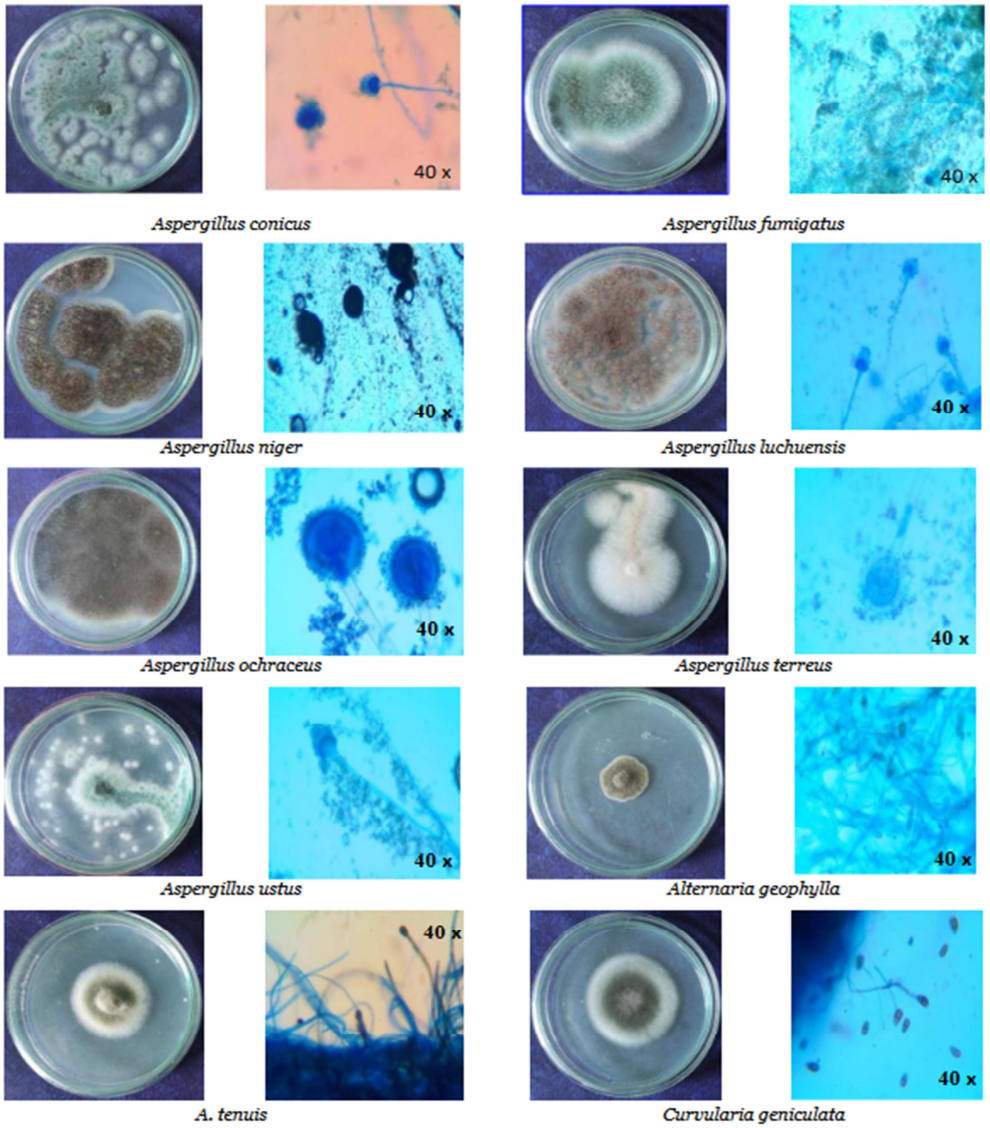
gelatin is the protein source of that growth medium. The fungal isolates were spot inoculated in Petri dishes and supplemented with 1% gelatin (Peptone, 5g; Beef extract, 3g; NaCl, 5g; Agar, 15g; Distilled water of 1 liter, pH 7). The Petri dishes were incubated at 28 ± 1°C for 3 days. After a week of incubation, gelatin degradation was observed as a clearing zone around fungal colony.

*Lipase*

The esterasic activity is observed by growing on the peptone agar media (10g peptone, 5g Nacl, 0.1g $CaCl_2$ $2H_20$, 16g agar, 1000ml distilled water, pH 6) described by Sierra (1957). The sterilized peptone agar culture media, previously sterile the Tween 20 is added in a final concentration of 1% (v/v) is added. This media was inoculated with the isolates and incubated. The presence of halos was observed around the colony formation.

**Results and discussion**

Study of marine fungal diversity plays a vital role to the understanding of the different process of the marine environment which will help to identify potential fungal organisms with novel bioactive compounds. These previous results are agreement with the finding of who reported that 25 species belong to (10) genera are identified (APHA, 1989). In the present study, totally 18 species of fungi belong to 11 genera were isolated by plating techniques were identified and enumerated from costal area of medicinal plant (Fig. 2).



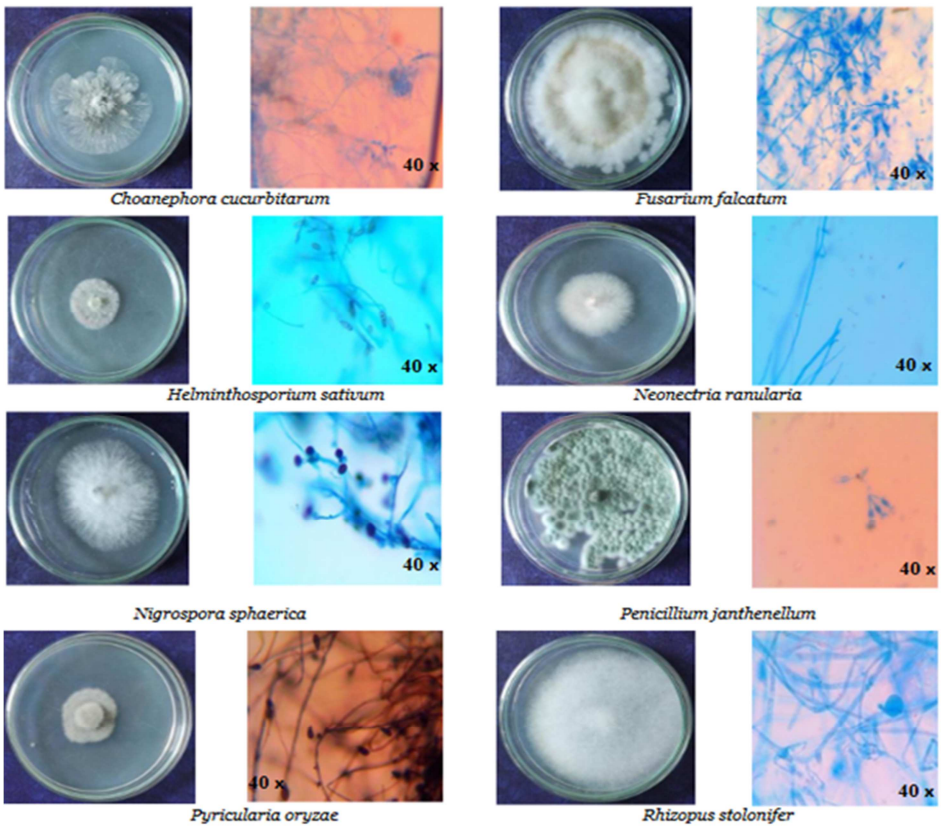



**Fig. 2.** Pure culture and microphotography of endophytic fungi

**Table 1.** Isolation and identification of endophytic fungi from mangrove associated medicinal plants

| SL | Endophytic fungi | *Avicennia marina* | *Suaeda maritima* | *Salicornia brachiata* |
|---|---|---|---|---|
| 1. | *Aspergillus conicus* | - | 1 | 2 |
| 2. | *A. fumigatus* | - | - | 1 |
| 3. | *A. niger* | - | 2 | - |
| 4. | *A. luchuensis* | - | 1 | - |
| 5. | *A. ochraceus* | 1 | - | - |
| 6. | *A. terreus* | 1 | - | - |
| 7. | *A. ustus* | - | - | 1 |
| 8. | *Alternaria geophylla* | - | 1 | - |
| 9. | *Alternaria tenuis* | 2 | - | - |
| 10. | *Choanephora cucurbitarum* | 1 | - | - |
| 11. | *Curvularia geniculata* | - | - | 1 |
| 12. | *Fusarium falcatum* | 2 | - | 1 |
| 13. | *Helminthosporium sativum* | - | - | 1 |
| 14. | *Neonectria ranularia* | 1 | 1 | 2 |
| 15. | *Nigrospora sphaerica* | 2 | 1 | - |
| 16. | *Penicillium janthenellum* | 1 | - | 2 |
| 17. | *Pyricularia oryzae* | - | - | 1 |
| 18. | *Rhizopus stolonifer* | 1 | 1 | - |
| Total number of colonies | | 12 | 8 | 12 |
| Total number of fungi | | 9 | 7 | 9 |

In the previous report *Aspergillus* sp. were seems to be the predominant genera with 21 species. *Fusarium* sp. was represented by four species followed by *Curvularia* sp, *Penicillium*, and *Aspergillus niger* which were represented by twenty-three species (Swathi *et al.*, 2013).

*Aspergillus* and *Penicillium* were reported to be the dominant genera together India's south-east coast. *Aspergillus* was also reported to be the dominant genera among the 23 colonies noticed in the Ramanathapuram District of Tamil Nadu (Madhanraj *et al.*, 2010).

In current study the marine plants like *Avicennia marina, Suaeda maritima, and Salicornia brachiata* produced fungal isolates in significant quantities. The maximum number of fungal colonies were found in *Salicornia brachiata* and *Suaeda maritima* when compared with *Avicennia marina*. In percentage of contribution was found with *Avicennia marina* (66.6%), *Suaeda maritima* (44.4%), *Salicornia brachiata* (66.6%) were seems to be the predominant genera with 9 species. *Aspergillus* sp, (38.8 %), *Alternaria* sp, (11.1%), *Choanephora cucurbitarum*, *curvularia* sp, *Fusarium falcatum*, *Helminthosporium sativum*, *Neonectria ranularia*, *Nigrospora sphaerica*, *Penicillum* sp, *Pyricularia oryzae*, and *Rhizopus stolonifer* sp were (5.55%),was also reported to be the dominant genera noticed from kodiyakarai, Nagapattinam district of Tamil Nadu (Table 1).

**Table 2.** Screening of endophytic fungi by using different enzyme indicators by in vitro methods

| Endophytic fungi | Zone of clearance (mm) | | | |
|---|---|---|---|---|
| | Amylase | Cellulase | Lipase | Protease |
| *Aspergillus conicus* | 6.16±0.03 | 1.09±0.13 | 0.56±0.00 | 0.83±0.03 |
| *A. fumigatus* | 1.76±0.08 | 3.14±0.18 | - | 0.95±0.07 |
| *A. niger* | 6.95±0.16 | - | 0.62±0.05 | 3.13±0.03 |
| *A. luchuensis* | 6.26±0.13 | 1.12±0.01 | - | - |
| *A. ochraceus* | 1.46±0.18 | 3.87±0.25 | 0.93±0.21 | 2.36±0.08 |
| *A. terreus* | 1.11±0.11 | 1.98±0.27 | - | 1.81±0.05 |
| *A. ustus* | 3.12±0.17 | - | 1.00±0.12 | - |
| *Alternaria geophylla* | 1.09±0.05 | 0.80±0.15 | 0.67±0.11 | - |
| *A. tenuis* | - | - | 1.05±0.04 | 0.95±0.01 |
| *Choanephora cucurbitarum* | 2.24±0.13 | 3.14±0.24 | - | 0.35±0.00 |
| *Curvularia geniculata* | - | 1.20±0.12 | 1.40±0.02 | 0.76±0.32 |
| *Fusarium falcatum* | - | - | - | 1.29±0.05 |
| *Helminthosporium sativum* | - | - | 1.23±0.04 | 2.00±0.06 |
| *Neonectria ranularia* | 1.56±0.21 | 0.90±0.10 | - | 0.47±0.00 |
| *Nigrospora sphaerica* | - | - | - | 1.87±0.14 |
| *Penicillium janthenellum* | 2.23±0.33 | - | 0.56±0.00 | - |
| *Pyricularia oryzae* | - | 1.72±0.05 | 2.12±0.43 | 1.53±0.03 |
| *Rhizopus stolonifer* | 2.73±0.08 | 0.67±0.01 | - | 0.72±0.01 |

*Enzyme assay*

In previous investigation, 11 endophytic fungi isolates were screened for the presence of extra cellular enzymes such as amylase, cellulase, laccase, lipase and protease. It was developed on a particular medium described before in a part on materials and processes. After the plant-host dies, endophytes may consume plant material as a source of starch (Choi *et al.*, 2005). Most of the selected endophytic fungi in which eight showed amylase activity. While cellulase has only two endophytes (i.e., *Cladosporium cladosposides*, *Curvularia verruiformis*) and laccase activity (i.e., *Curvularia brachyspora, xylariales* sp). The amylase of fungal origin was stable than bacterial amylase enzyme (Duochuan *et al.*, 1997). Approximately 4000 secondary metabolites, mainly from the *Penicillium, Aspergillus,* and *Cremonium* genera, have been isolated from a number fungal species as biologically active chemicals (Dreyfuss and Chapela, 1994; Onifade, 2007). Many terrestrial fungus produce the extracellular degradative enzyme cellulase, which uses in the paper industry (Eriksson, 1993), but many marine fungi produce the laccase enzyme, which is used for breaking up cellulose (Raghukumar *et al.*, 1994; Pointing *et al.,* 1998; Bucher *et al.*, 2004) degradation. The increase in lipase activity indicates that cholesterol can be used as a energy source (Maria *et al.*, 2005). *Colletitrichum gloeosporioides* was found be the best of producing alkaline lipase and also hydrolase wide range of oils (Choedon *et al.*, 2006). The endophytes lack certain active enzyme for some reason it prevents the host plants from damage (Gessner, 1979). The protease activity was observed *in Curvularia vermiformis, Drechslera hawaiensis, Colletotrichum gloeosporioides, Colletotrichum carssipes, Colletotrichum falctum* and *xylariales* indicated by formation of clear zone around the colony).

The 20 higher marine fungi from salty marshes found to produce lipase and protease activity was seen in 13 marine fungi (Pisano *et al*., 1964).

In present investigation, 18 endophytic fungal isolates were screened for the presence of extra cellular enzyme such as amylase, protease, cellulase, and lipase which was grown on a specific medium discussed earlier in materials and methods (Table 2) . The maximum zone of present in protease enzyme *A. niger* (3.13±0.03mm), *A. ochraceus* (2.36±0.08mm), *Helminthosporium sativum* (2.00±0.06mm), *Nigrospora sphaerica* (1.87±0.14mm) and minimum zone of clearness *A. terreus* (1.81±0.05mm), *Pyricularia oryzae* (1.53±0.03 mm), *Fusarium falcatum* (1.29±0.05mm), *A. tenuis* (0.95±0.01mm), *A. fumigatus* (0.95±0.07mm) at followed by amylase activity maximum zone in *A. niger* (6.95±0.16mm), *Aspergillus conicus* (6.16±0.03mm), *A. luchuensis* (6.26±0.13mm), *A. ustus* (3.12±0.17mm) and minimum zone of clearness *Rhizopus stolonifer* (2.73±0.08mm), *Penicillium janthenellum* (2.23±0.33mm), *Choanephora cucurbitarum* (2.24±0.13 mm), *A. fumigates* (1.76±0.08 mm) (Table 2).

In cellulase enzyme maximum zone present in *A. ochraceus* (3.87±0.25mm), *Choanephora cucurbitarum* (3.14±0.24 mm), *A. fumigatus* (3.14±0.18 mm) and minimum zone in *A. terreus* (1.98±0.27mm), *Curvularia geniculata* (1.20±0.12mm), *Pyricularia oryzae* (1.72±0.05 mm) at followed by lipase enzyme maximum zone of clearness *Pyricularia oryzae* (2.12±0.43 mm), *Curvularia geniculata* (1.40±0.02mm) and minimum zone in *Helminthosporium sativum* (1.23±0.04mm), *A. tenuis* (1.05±0.04 mm), *A. ustus* (1.00±0.12mm) respectively (Table 2). There are 14 higher marine fungi from salty marshes found to produce protease activity was seen in 12 marine fungi at amylase activity finally 10 marine fungi are present in other two cellulase and lipase activity.

## Conclusion

The findings of this study indicated that *Aspergillus* and P*enicillium* as possible important fungal species to future ecological and evolutionary studies as well as research into the mechanisms through which microorganisms can adapt to extreme conditions. It was discovered that 25 species represented three medicinal plants throughout the year round. The colonization rate was higher in *Suaeda maritima* and *Salicornia brachiata* **,** The isolation frequencies of the fungus in leaves were high in both plants. These results showed tissue specificity of endophytic fungi.

However, because of the small sampling size, more sampling in different geographic localities is needed to make further assumption about the organ specificity of endophytic fungi with *Avicennia marina, Suaeda maritima* and *Salicornia brachiate*. The result suggested that these associations might be valuable for plants acclimatizing to tropical environments. Fungal enzymes are more stable than enzymes obtained from plants and animals. It is used in food processing industries, production of beverage, textile and leather industries. Screening of endophytic fungi for then several metabolities like enzymes, antibiotics, anti-cancer drugs which will be useful for our future generation that leads to eco- friendly technological improvement for the better life on earth.

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