only include species *Ostraea belcheri, Echinolittorina reticulata, Echinolittorina tuberculata, Littoraria intermedia*…; the habitat in the beach area has 63 species of benthic animals. Crustacea (*Diogenes mixtus Diogenes violaceus, Acetes japonicus, Paradorippe granulata, Ashtoret lunaris*…). Bivalvia (*Anadara subcrenata, Saccostrea glomerata, Ostraea belcheri*…). Gastropoda (*Babylonia areolata, Umbonium vestiarium, Littoraria intermedia*…).

Currently, in the coastal area of Cua Lo (Nghe An), there is no research data on benthic groups in this area. Therefore, there has been no process of comparing the benthic species composition with previous data. Along with the short benthic research time, the number of species is still limited. Research shows that the Cua Lo benthic ecosystem is experiencing large-scale loss of benthic habitat, receives less attention than other ecosystems, and is under direct threat, due to exploitation, indirectly due to environmental pollution, swimming, garbage and other activities taking place on land. Therefore, there needs to be some solutions to preserve and protect benthic groups such as: Limit exploitation of benthic animals and coastal tidal flats along with educating the community to protect the ecosystem; Limit and prevent the exploitation of species at risk of biodiversity loss; build a strict conservation area for benthic animals at home and offshore; build and develop valuable domestic and international benthic groups ▪

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1. INTRODUCTION

Currently, the circular economy as well as the green economy and green growth are economic models that aim to effectively use and save resources and recycle waste, contributing to economic efficiency and environmental sustainability. Circular economy is considered an inevitable trend of the times and the green industrial revolution of the 21st century. Accordingly, developing green and sustainable agriculture and processing industry is being focused on by the Government (Decision No. 687/QD-TTg dated June 7th, 2022 of the Prime Minister approving the Circular Economy Development Project in Vietnam and Decision No. 882/QD-TTg dated July 22nd, 2022 of the Prime Minister approving the National Action Plan on green growth for the period of 2021-2030). The application of useful microbial strains (bacteria, fungi, actinomycetes, yeast...) as well as their secondary products in the circular, green and sustainable production chain of livestock, crop cultivation and food processing industry, is an application direction that is being widely developed (increasing health, productivity, quality of crops, livestock, post-harvest products; treating waste water, agricultural and industrial by-products into useful products such as fertilizer, animal feed, natural materials, irrigation water...). In particular, microbial strains have the ability to biosynthesize extracellular enzymes (cellulose, proteinase, lipase, amylase...) and resist pathogenic bacteria (such as *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Pseudomonas aerigunosa*, *Bacillus subtillis*, *B. megaterium, Lactobacillus casei, L. plantarums, Rhodopseudomonas, Azotobacter, Azospirillum, Enterobacter*…), *Actinomycetes Streptomyces, Actinomyces*...; microfungi such as *Trichoderma harzianum, Aspergilus tubingensis*... have been commonly used to replace and reduce the amount of food, fertilizer, antibiotics and other chemicals (Sindhu et al., 2018; Inamuddin et al., 2022).

However, to increase the effectiveness of practical applications, research and selection of multi-active strains is necessary. Plant endophytic fungi (especially in herbal plants) represent one of the potential alternatives as they have

Biological activity research of endophytic fungi on Huperzia javanica plant with application orientation in the circular economy

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Abstract
The application of beneficial microorganisms as well as their secondary products in the livestock, agriculture, and food industry supply chains for sustainable green production is a developing trend. The research direction of selecting multi-activity strains to enhance the effectiveness of practical applications has been receiving attention. This study aims to select endophytic fungal strains on Huperzia javanica plant in Vietnam that have the ability to produce multi-extracellular enzymes and multi-resistance to pathogenic microorganisms by determining enzyme activity and testing antimicrobial activity. The results showed the following: (1) All 9 strains have the ability to produce 1 to 5 types of enzymes and inhibit 1 to 5 pathogenic microorganisms with potential activities; Strains TLC11 and TLC9 produce 4÷5 enzymes (cellulase, lipase, protease, phosphatase and β-galactosidase) with the highest hydrolysis zone diameters of 22÷25 mm (protease) and 20÷23 mm (lipase); (2) Strain TLC13 inhibits all 5 tested microbial strains Escherichia coli, Staphylococcus aureus, Candida albican, Bacillus cereus, Pseudomonas aerigunosa) with the highest activity against 3 species B. cereus (24±1.2 mm), P. aerigunosa (26±1.1 mm) and C. albican (36±1.5 mm); Strains TLC10 and TLC19 are resistant against 4/5 tested microorganisms except S. aureus (TLC10) and E. coli (TLC19). These strains could be the potential sources for further in-depth research aiming to expand their applications in sustainable agriculture, aquaculture, and industry production fields.

Keywords**:** *Biological activity, Huperzia javanica, endophytic fungi, extracellular enzyme, antimicrobial.*
JEL Classification: *N50, N53, N57, O13.*
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demonstrated high efficiency in the production of active metabolites with new biological properties, not only antibacterial properties but also other wide-range biological activities. These species inhabit various tissues and organs of healthy plants at certain or all stages of their life cycle, in addition to being able to biosynthesize biologically active compounds corresponding to the host plant, they can also produce other active substances (enzymes, antibacterial substances, protein, alkaloids, polyketides ...) that help the host plant increase growth, inhibit disease, and withstand saltwater - drought - temperature as well as can be applied in the food, agriculture - fishery, environmental and pharmaceutical industries (Daniel et al., 2022; Fatima et al., 2022; Cripwell et al., 2021; Lu et al., 2022; et al., 2021; Jouda et al., 2014).

H. javanica is a precious medicinal plant (currently under conservation) belonging to the Lycopodiaceae family, known for supporting the treatment of some neurological diseases, rheumatism, and hepatitis, diarrhea... *H. javanica* likes moisture and shade, grows on moist soil with a thick layer and lots of humus, at an altitude of 1,000-1,500m; distributed in China, India, Japan and Vietnam (Sun et al., 2015). There have been many published studies on the biological activities of plant endophytic fungi strains in other medicinal plants, however, studies on *H. javanica* are almost non-existent. This study focuses on surveying and selecting plant endophytic fungi strains in Vietnam. *H. javanica* has the ability to produce multi-enzymes and multi-resistance to pathogenic microorganisms with the goal of further research and application in circular and sustainable food, agricultural - industrial - fishery - pharmaceutical production which will contribute to reducing environmental pollution.

2. MATERIALS AND METHODS

2.1. Research materials

In previous research, 9 plant endophytic fungi strains were isolated and selected from the *H. javanica* tree distributed in Ha Giang (Vietnam) with the ability to biosynthesize the active pharmaceutical ingredient huperzine (an alkaloid that supports treatment of dementia, especially Alzheimer's disease). In this study, the strains were further researched on other biological characteristics in order to survey and select strains that are multi-enzyme-synthesizing and multi-resistant to pathogenic microorganisms towards other applications in circular and sustainable production of agriculture, industry, fishery and pharmaceuticals. Strains include: Neurospora calospora TLC9, N. calospora TLC10, N. calospora TLC11, Schizophyllum commune TLC12, Epicoccum sorghinum TLC13, Alternaria tenuissima TLC14, Daldinia sp. TLC19, Cephalotrichum sp. TLC20, Schizophyllum sp. TLC22. Tested microbial strains: *E. coli* (ATCC 25922), *S. aureus* (ATCC 33591), *C. albican* (ATCC 10231), *B. cereus* (ATCC 11778), *P. aerigunosa* (ATCC 27853) were provided by the Center for Breeding and Preserving microbial genetic resources, provided by the Institute of Biotechnology.

2.2. Research Methods

2.2.1. Method for determining the ability of fungal strains to produce extracellular enzymes

Determination of amylase production ability

Fungal strains were grown on PDB liquid medium at 28^{o}C for 5-7 days. Prepare a substrate medium plate containing 20 g/l agar supplemented with 1% starch, drill wells with a diameter of 8 mm, each well add 100 μl of fungal extracellular fluid. The negative control is PDB medium without fungal strains. Keep the plate at 4^{o}C overnight to allow the enzyme to diffuse into the medium. Continue incubation at 37^{o}C for about 24 hours for the enzyme to activate. The relative activity of the enzyme was determined based on the difference D-d (mm). Where D is the resolution ring diameter (mm), d is the agar hole diameter (mm). D-d > 25 mm: a very strong enzyme activity; D-d = 20-25 mm: a strong enzyme activity; D-d = 10-20 mm: an average enzyme activity; D-d < 10 mm: a weak enzyme activity. The experiment was repeated 3 times.

Determine the ability to produce protease, cellulase, lipase and phosphatase

Carry out the same method to determine the amylase production ability of fungal strains with substrate medium supplemented with 1% casein, carboxyl methyl cellulose (CMC), Tributylin and $Ca_3(PO4)_2$ to determine the ability to produce corresponding enzymes including protease, cellulase, lipase and phosphatase.

Determination of β-galactosidase enzyme activity

X-gal was dissolved in dimethyl sulphoxide reached a concentration of 20μg/mL, stored in the dark at -20^{0}C. After sterile autoclaving, the PDA medium was poured into a plate, then 50 μl of X-gal indicator was spread evenly on the surface of the PDA agar plate medium. Inoculate and score plant endophytic fungi strains on agar plate medium with X-gal indicator. Cultured in a 28°C incubator for 3-10 days, endophytic fungal strains that produce a blue color on the indicator plate are strains capable of synthesizing the enzyme β-galactosidase. The experiment was repeated 3 times.

2.2.2. Agar plate diffusion method

The anti-microbial activity was determined by the diffusion method on agar plates. The researched fungal strains were cultured in PDB medium for 5-7 days and the mushroom extract was collected. Drop 100μl of fungal extract into each well created on an LBA medium plate that has been inoculated with control microorganisms. A well of PDB environment was used as a negative control, and a well of the antibiotic ampicillin at a concentration of 1 mg/ml was the positive control. The plate is kept at 4^{0}C for 2 - 4 hours to allow the enzyme to diffuse into the medium, then the plate is incubated at 37^{0}C for 24 hours. Antibacterial activity is determined by the diameter of the sterile ring D-d (mm), in which: D is the diameter of the sterile ring, d is the diameter of the well. The experiment was repeated 3 times.

3. RESULTS AND DISCUSSIONS

3.1. Enzyme-producing ability of plant endophytic fungi strains

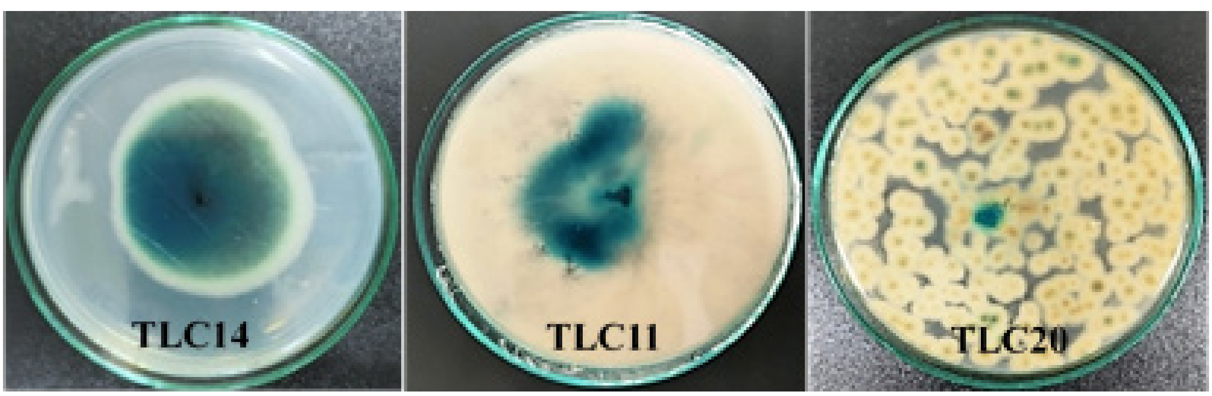
9 plant endophytic fungi strains were tested for their ability to produce 6 extracellular enzymes, including cellulose, lipase, protease, amylase, phosphatase and β - galactosidase.

The ability to produce extracellular enzymes of the 9 studied fungal strains is shown in Table 1, Figure 1 and Figure 2, showing that all 9 strains are capable of producing from 1 to 5 types of tested enzymes; among them, the number of strains capable of producing lipase and protease accounts for the highest proportion with 7 strains (77.77%), followed by the number of strains producing cellulose (6 strains; 66.66%), the number of strains producing phosphatase accounts for the highest proportion with rate of 33.33% (3 strains), 2 strains produced β -galactosidase (rate 22.22%) and only 1 strain TLC10 produced amylase (11.11%) with low activity. Among the enzymes produced by the studied strains, protease have the strongest activity expressed in fungal strains, 3 strains (TLC9, TLC11 and TLC12) have hydrolysis circle diameters from 20 ÷ 25 mm, 4 strains (TLC13, TLC14, TLC20 and TLC22) hydrolysis ring diameter over 25 mm. Of the 7 lipase-producing strains, 6 strains (TLC9, TLC10, TLC11, TLC14, TLC19 and TLC22) have strong lipase activity (dialysis circle diameter from 20 ÷ 25 mm).

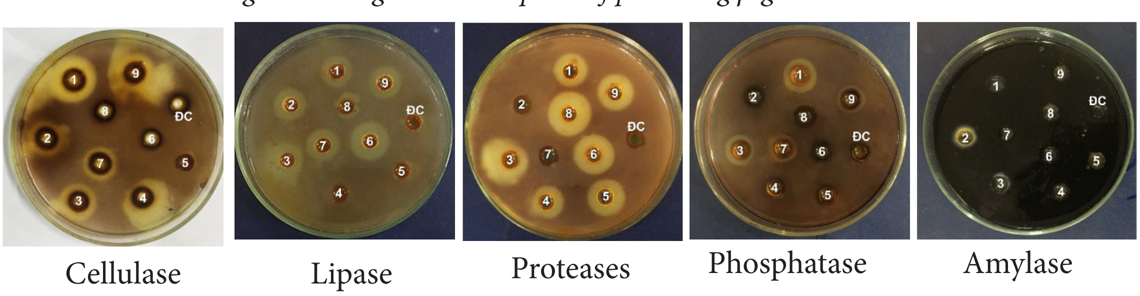
When cultivating the fungus on a medium containing X-gal indicator, the strains stain blue, showing the ability to produce β-galactosidase. Strains with faster staining times and darker colors will have a higher ability to produce β-galactosidase. Among the 3 strains with β-galactosidase-producing activity, strain TLC14 has colonies that turn green in a very short time of colony formation (after 2 days of culture), and its strong staining ability proves its ability to produce β-galactosidase highly. Meanwhile, the remaining 2 strains (TLC11 and TLC20) have colonies that turn green more slowly (after 3 days of culture) and the color staining of the colonies is not uniform, so they may not have a high ability to produce β-galactosidase.

Table 1. Enzyme-producing ability of the studied fungal strains

No.	Strains	Enzyme activity (mm)					
		Cellulase	Lipase	Proteases	Phosphatase	Amylase	β - galactosidase
1	TLC9	12±0.9	20±1.4	25±1.5	8±0.7	-	-
2	TLC10	4±0.6	23±1.2	-	-	5±0.6	-
3	TLC11	9±0.8	23±1.1	22±1.2	6±0.5	-	+
4	TLC12	7±0.5	-	22.5±1.2	-	-	-
5	TLC13	-	-	31±1.6	-	-	-
6	TLC14	-	28±1.7	32±1.5	-	-	+
7	TLC19	3±0.3	25±1.5	-	3±0.3	-	-
8	TLC20	-	10±0.9	34±1.7	-	-	+
9	TLC22	11±0.9	24.5±1.2	28±1.3	-	-	-
10	Control (-)	-	-	-	-	-	



▲ *Figure 1. Fungal strains capable of producing β-galactosidase*



▲ *Figure 2. Enzyme biosynthesis ability of fungal strains (1. TLC9, 2. TLC10, 3. TLC11, 4. TLC12, 5. TLC13, 6. TLC14, 7. TLC19, 8. TLC20, 9. TLC22, Control (-): negative control)*

Among the 6 enzymes tested, strain TLC11 showed the ability to produce 5/6 types except amylase enzyme; then is strain TLC9, which produces 4/5 types of enzymes; 4 strains produce 3 types of enzymes including: TLC10, TLC19, TLC20 and TLC22 and the only strain TLC13 only strongly produces 1 type of protease enzyme with an active circle of 31±1.6 mm. Notably, the three strains TLC9, TLC10 and TLC11 are the same *N. calospora* species but have different enzyme-producing abilities.

According to previous studies, enzymes are biological catalysts of more than 5,000 types of biochemical reactions that help promote rapid metabolism in cells. Microbial metabolism produces different types of enzymes and is a large source of natural enzymes. Proteases are one of the three largest groups of industrial enzymes, accounting for about 60% of total global enzyme sales; hundreds of proteases have been commercialized and used in detergents, food processing, animal feed additives, leather processing, waste treatment, pharmacology and drug production (Sindhu et al., 2018). Cellulases are important enzymes both industrially and naturally, playing a key role in the global carbon cycle. Cellulase hydrolysis can serve a "dual" purpose: reducing plant waste, converting biofuels to fuel, and narrowing the growing dependence on fossil fuels and for other industrial purpose such as in pulp, food, wine productions... Some cellulose-producing bacteria such as Pestalotiopsis sp., Microsphaeropsis sp., Sclerocystis sp., Cephalosporium sp., Penicillium sp., Fusarium oxysporum, Aspergillus sp., Penicillium chrysogenum, Xylaria sp... have been isolated from *Acanthus ilicifolius*, *Zea mays, Sabina chinensis, Taxus chinensis, Keteleeria evelyniana, Pinus massoniana...* (Fatima et al., 2022; Sindhu et al., 2018). Amylase is an enzyme that hydrolyzes the alpha bond of polysaccharides to create glucose and maltose, that is used in food, beverages, and medicine and produced naturally by many different species of fungi; including the plant endophytic fungi species such as plants of *P. microspore, A. oryzae and P. chrysogenum*, *Rhizophora mucronata, Avicennia ofcinalis, A. marina and Asclepias sinaica* (Fatima et al., 2022; Cripwell et al., 2021). Lipase is an enzyme that decomposes triglycerides into free fatty acids and glycerol, with great applications in the food industry: increasing vegetable oil processing productivity and increasing aroma in the baking and dairy industries. The best source of lipase is exploited from many fungal species such as Rhizopus, Mucor, Geotrichum, Pencillium, Aspergillus, Humicola; In addition, there are plant endophytic fungi species such as *R. oryzae, Cercospora kikuchii, Lasiodiplodia theobromae* from Tithonia diversifolia and Cocos nucifera trees (Fatima et al., 2022; Sindhu et al., 2018). The ability to utilize insoluble phosphate in soil can be improved by using phosphatase enzymes to help plants grow and develop better; Fitriyana and Ainy (2019) isolated plant endophytic fungi strain from *R. mucronate* roots with phosphatase activity. β-Galactosidase is an exoglycosidase that hydrolyzes the β-glycosidic bond which formed between galactose and its organic part; β-galactosidase is used in dairy products such as yogurt, sour cream, and some cheeses that are enzymatically treated to break down any lactose before human can consume (Eriana et al., 2000).

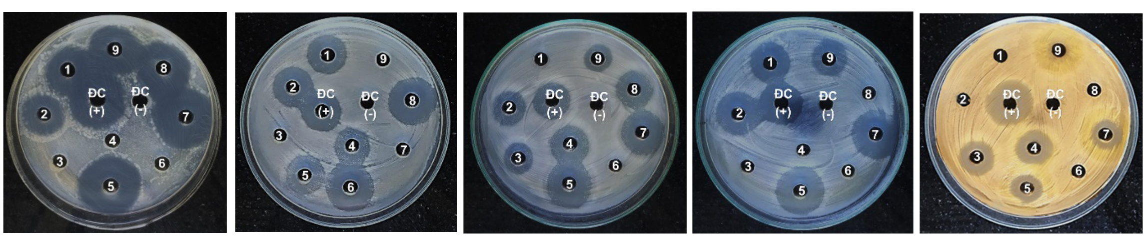
Research results on *H. javanica* plant species show that the species has ability to produce the above enzymes is almost unpublished. These positive results show that this species is a source of raw materials for further research to obtain high yields of natural enzymes aimed at applications in sustainable agro-industrial-fishery production such as applications in creating animal feed, processing agricultural, industrial and fishery waste into organic fertilizer for farming and replacing chemical fertilizers for soil improvement...

3.2. Resistance to pathogenic microorganisms of the studied fungal strains

Evaluate the resistance to pathogenic microorganisms of 9 strains of studied fungi with 5 strains of tested microorganisms, including: 2 strains of gram-positive bacteria (B. cereus, S. aureus), 2 strains of gram-negative bacteria (*E. coli, P. aerigunosa*) and 1 yeast strain *C. albicans*. The results shown in Table 2 and Figure 3 show that 7 fungal strains (77.77%) are resistant to *B. cereus* bacteria; 6 strains (66.66%) were resistant to *E. coli* bacteria as well as to *C. albican* yeast; 5 strains (55.55%) were resistant to *P. aerigunosa*; 4 strains were resistant to *S. aureus*. All strains have potential activity against tested pathogenic microorganisms with inhibition zone diameters ranging from 9±0.4 to 37±1.4 mm. In particular, strain TLC13 has the ability to inhibit all 5 strains of tested microorganisms with almost the highest activity with 3 species of tested microorganisms ranging from 24±1.2 mm (*B. cereus*), 26±1.1 mm (*P. aerigunosa*) and 36±1.5 mm (*C. albican*); The two strains TLC10 and TLC19 are both resistant to 4/5 tested microorganisms. Strain TLC19 is strongly resistant to *B. cereus, P. aerigunosa* and *C. albican*; the 4 strains were resistant to 3/5 of the tested microorganisms and the strain was resistant to only 1 type of E. coli bacteria, strain TLC14.

Table 2. Resistance to pathogenic microorganisms of the studied fungal strains

No.	Fungal strains	Inhibition zone for the growth of tested pathogenic microorganisms (mm)				
		G (+) bacteria		Bacteria G (-)		Yeast
		B. cereus	*S. aureus*	*E. coli*	*P. aerigunosa*	*C. albicans*
1	TLC9	-	-	16±0.9	20±1.1	28±1.4
2	TLC10	15±0.5	-	17±0.4	23±0.9	17.5±0.3
3	TLC11	13±0.4	15±0.6	-	-	-
4	TLC12	17±0.5	16±0.7	14±0.4	-	-
5	TLC13	24±1.2	13±0.6	16±0.5	26±1.1	36±1.5
6	TLC14	-	-	26±0.9	-	-
7	TLC19	22±0.9	12±0.3	-	25.5±1.2	37±1.4
8	TLC20	18±0.8	-	25±1.2	-	33±1.3
9	TLC22	9±0.4	-	-	18±0.9	28±1.2
10	Control (+)	18±0.8	24±1.1	16±0.5	23±1.2	36±1.3
11	Control (-)	-	-	-	-	-



▲ *Figure 3. Resistance to pathogenic microorganisms tested against C. albican, E. coli, B. cereus, P. aerigunosa, S. aureus of fungal strains (1. TLC9, 2. TLC10, 3. TLC11, 4. TLC12 , 5. TLC13, 6. TLC14, 7. TLC19, 8. TLC20, 9. TLC22, Control (-): negative control, Control (+): positive control)*

For a long time, antibiotics derived from filamentous fungi have been known and used effectively in treating diseases of humans, animals, and plants. The search for new antibiotics of natural origin from microorganisms is of interest. According to Balick and Cox, in 1996, out of 119 chemical compounds, at least 90 were of plant origin. These are drugs that are being used in more and more countries. The fungal strain Nigrospora sphaerica URM-6060 isolated from the leaves of the medicinal plant Indigofera suffruticosa produces biologically active substances with pharmaceutical potential such as hydrolyzed tannins, alkaloids, cinnamic derivatives with antibacterial activity against both positive Gram (+) and negative Gram (-) bacteria (Santos et al., 2015). Jouda et al (2014) isolated three polypeptides, penealidins AC (134-136), with activity against *Acinetobacter sp.* and *E. coli* from the endophytic fungus Penicillium sp. CAMMC64 isolated from the leaves of Garcinia nobilis (Clusiaceae) distributed in Cameroon. Extracts of plant endophytic fungi strains *E. nigrum, F. Tricinctum* and Phoma sp. isolated from *Dendrobium devonianum* and *D. thyrsiflorum* plants that are resistant to bacteria *B. subtilis, C. albicans, E. coli* and *S. aureus*; strains Alternaria sp., Bjerkandera sp., Diaporthe sp., Penicillium sp. and Xylaria sp. isolated from *Schinus terebinthifolius* have the ability to resist *C. albicans, P. aeruginosa* and *S. Aureus* (Daniel et al., 2022).

Studies on plant endophytic fungi species resistant to pathogenic microorganisms in *H. javanica* plants have not been published. Plant endophytic fungis trains with a broad resistance spectrum and potential antibacterial activity such as TLC13, TLC19 and TLC10 may be a potential source of raw materials for the search and discovery of new anti-bacterial active ingredients that can be applied in human life such as in the field of animal husbandry, preservation and post-harvest processing...

4. CONCLUSION

Research and application of plant endophytic fungi strains (especially plant endophytic fungi in herbal plants) in circular economy is a potential research direction. Studies on the plant's ability to produce enzymes and resist pathogenic microorganisms have not been published yet. This study has screened and selected a number of plant endophytic fungi strains of the *H. javanica* plant that have the ability to biosynthesize multiple extracellular enzymes and are multi-resistant to pathogenic microorganisms. Among the 6 enzymes tested, strain TLC11 showed the ability to produce 5/6 types except amylase enzyme; then is strain TLC9, which produces 4/5 types of enzymes; 4 strains produce 3 types of enzymes include: TLC10, TLC19, TLC20 and TLC22. Protease and lipase are the two activities that are most strongly expressed in active strains, with hydrolysis ring diameters from 22 ÷ 34 mm (protease) and 20 ÷ 28 mm (lipase). Among the 5 types of pathogenic microorganisms tested, strain TLC13 inhibited all 5 tested strains of microorganisms with almost the highest activity against 3 species B. cereus (24±1.2 mm), P. aerigunosa (26±1.1 mm) and C. albican (36±1.5 mm); 2 strains TLC10 and TLC19 were resistant to 4/5 tested microorganisms except S. aureus (TLC10) and E. coli (TLC19) ; 4 strains were resistant to 3/5 tested microorganisms. The research strains are capable of producing extracellular enzymes with strong activity, have a relatively broad spectrum of resistance to pathogenic microorganisms with potential inhibitory activity: TLC9, TLC11, TLC13, TLC10, TLC19. These strains will be a potential source of raw materials for application in the fields of sustainable agriculture - industry - fisheries – pharmaceuticals productions. However, further research is needed such as identifying anti-bacterial active ingredients, the activity of antibacterial substances and extracellular enzymes, fermentation conditions to increase productivity and the absorption of antibacterial enzymes, substances as well as as well as testing their application in practice, thereby providing specific application solutions for each industry such as livestock farming, cultivation, post-harvest processing and preservation, food industry and environmental treatment.

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