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RESEARCH ARTICLE

ISOLATE ENDOPHYTIC FUNGI FROM LOCAL CATHARANTHUS ROSEUS AND ANALYZE THEIR EXTRACELLULAR ENZYME ACTIVITIES

Nguyen Chi Mai[1], Pham Thi Hoe[1], Vu Huong Giang[1], Le Quynh Lien[1], Nguyen Tuong Van[2] and Tran My Linh[1]

1. Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST).
2. Institute of Biotechnology, VAST.

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Abstract

Endophytic microbia are known as natural sources for producing valuable enzymes. In this study, four endophytic fungi were isolated from roots of local *Catharanthus roseus* (L.) G. Don var. *roseus* (purple flower) and C. roseus var. *ocellatus* (red stamens white flower) widely grown in Nha Trang. They were identified as *Fusarium solani* RN1, *Chaetomium funicola* RN3, *Penicillium rugulosum* RN4 and *Chaetomium homopilatum* WN1 based on morphologies colonies and spores. The activity analysis of their extracellular enzymes indicted all isolated endophytic fungi are able to produce protease, cellulose, xylanase as well as amylase. This is the first report on the endophytic fungi inhabited in *C. roseus* plant growing in the coastal regions of Vietnam, which could provide an attractive source for bioactive enzyme exploitation.

Introduction:-

Endophytes are microorganisms living inside plants for at least a part of their life cycle without causing any disease symptoms to the host plant (Petrini, 1991), in which, endophytic fungi are the most common groups found in plants and present in most their organs and tissues (Wang and Dai, 2011). One million of 1.5 million species of existing fungi in nature are reported to be endophytic. It is well-known that they play a vital role in enhancing the growth of plants and well known for their capability to produce bioactive compounds. Bioactive compounds synthesized by endophytic fungi have diverse of natural properties, such as antibiotics, anticancers, antioxidants, biopesticides, antiparasitics and agrochemicals (Pinheiro *et al.*, 2013). In addition, several highly stable and commercially valuable extracellular enzymes had been exploited from endophytic fungi. Among 40 endophytic fungi isolated from *Ocimum* species (Tulsi), Pavithra et al. (2012) found 50% of isolates to produce amylase and protease and more than 27% to produce tyrosinase. Uzma et al. (2016) reported 29% of 112 endophytic fungi isolated from six wild medicinal plants growing in Bisle region (Western Ghats, Karnatakwas) were positive for amylase, 28% for cellulase, 18% for pectinase and 40% for asparaginase activity. Alberto et al. (2016) identified 62% of endophytic fungal isolates of four medicinal plant species exhibited amylase, approximately 93% cellulase, 50% pectinase, and 64% protease activity.

Cantharanthus roseus is a herbaceous plant of the Apocynaceae family and native to the subtropics. It can produce various secondary compounds with diverse medicinal activities, for example treatments of cancer, diabetes, high blood pressure, antibacterial, antifungal etc… (Singh *et al.*, 2001; Ferreres *et al.*, 2008; Gajalakshmi and Rajeswari,

Corresponding Author:- Tran My Linh
Address:- Institute of Marine Biochemistry, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.

2013; Das and Sharangi, 2017). Recent studies have shown that a number of endophytes of *C. roseus* also play an important role in the biosynthesis of host-specific secondary metabolites. From the endophytic *Fusarium oxysporum*, Kumar et al. (2013) extracted an adequate amount of vinblastine and vincristine – two important anticancer drugs. Palem et al. (2016) also reported another endophytic fungus; *Talaromyces radicus* produced vinblastine and vincristine, which stimulated apoptosis in some human cancer cell lines. Similarly, Kuriakose et al. (2016) also found vincristine synthesized by an endophytic fungus, *Eutypella* spp-CrP14. Beside the synthesis of secondary compounds, endophytic fungi of *C. roseus* also produce a number of valuable extracellular enzymes. From 5 endophytic fungi isolated from root and leaf samples of *C. roseus* collected in the north of Peninsular, Malaysia, Ayob and Simarani (2016) observed that all isolates exhibited cellulase activity; amylase activity from *Colletotrichum s*p. and *F. solani* and protease activity only from *F. solani*.

In this study, the endophytic fungi isolated from *C. roseus* wildly growing in the central coast of Vietnam (Nha Trang) were identified and screened for their extracellular enzyme production. These findings might provide a considerable source of useful bioactive compounds.

Materials and Methods:-

Isolation and identification of endophytic fungi of C. *roseus*:

Roots of white and purple flower *C. roseus* plants collected in the coast of Nha Trang, Khanh Hoa, Vietnam were washed thoroughly with running tap water first. The samples was cut into 1 cm pieces, then surface sterilized by dipping them in 0.1% Tween 20 for 10 min, followed by 70% ethanol for 1 min, then rinsed in sterile distilled water thrice. After sterilization, samples were ground in the ceramic pestle and mortar and placed into a flask containing 8 ml of sterilized water. The flask was shaken for 15 min at room temperature. The extract solution (100 µl) was spread on humic acid plates supplemented with 100 mg/l ampicillin to suppress bacterial growth. Plates were incubated at 25°C for at least 7 days in darkness. After incubation, the colony diameters were measured. The production of soluble pigments was noted. Colonies were photographed with a Canon EOS 400D. Morphological characteristics such as growth pattern, hyphae, colony color, surface texture, aerial mycelium, sporulation using standard manuals and taxonomically to the genus level on the basis of macroscopic and microscopic morphological characters under Scanning Electron Microscope FESEM S4800.

Detection of extracellular enzyme activities:

The activities of extracellular enzymes produced by fungal endophytes was assessed by growing them on Hansen pH 6 (20 g/l glucose, 10 g/l tryptone, 2 g/l K_2HPO_4, 2 g/l $MgSO_4$, 18 g/l agar) at 30^oC for 7 days. Mycelial plugs (5 mm in diameter) were placed on the solid Czapek plates (3 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.5 g/l KCl, 0.01 g/l $FeSO_4.7H_2O$, 20 g/l agar) supplemented with 1% dissolved substrates, including casein, CMC, cellulose, starch, xylan to assess protease, cellulase, amylase and xylanase activities. After incubation for 3-7 days at 25^oC, the zone of enzyme activity surrounding the fungal colony was measured. Proteolytic activity was detected by casein hydrolysis on agar plates via the formation of a clear zone around colonies after covering the plate with 10% acid trichloacetic for 30 min. The degradation zones of cellulase, amylase and xylanase activities were detected by flooding the plates with Lugol dye. The diameter (D, mm) of degradation zone was used to assess enzyme activity. D values ≥ 25, ≥ 20, ≥ 15 and ≤ 10 mm were considered as very strong, strong, medium and weak enzyme production, respectively. All the experiments were performed in triplicates and the means were analyzed statistically with the SPSS program version 20. The significant differences between the means were determined through Duncan's multiple range test.

Result and Discussion:-

Isolation, morphological identification and characterization of endophytic fungi:

A total of 04 endophytic fungi isolates were obtained from the roots of local *C. roseus*, in which RN1, RN3, RN4 isolated from pink flower and WN1 from white flower *C. roseus* plants. Fungi were identified to the species level based on macroscopic and microscopic morphological and cultural characteristics.

Isolates RN1 culture has rapid growing and white-greyish aerial mycelia (Figure 1A). The colonies with wooly or cottony surface reached 3.5 to 4.0 cm after 7 days at room temperature of 24-26 °C on Czapek medium. Conidiosphores, 45-80 x 2.5-3.0 µm (length x width), are in mono or polyphyalidics (Figure 1B). Microconidia 8-16 x 2-4 µm with 0-1 septa and thick wall formed after 2-3 day culture of a conidiosphore. Macroconidia 35-55 x 4.5-6.0 µm with 3-5-7 septa, cylinder to rhombus with slightly curved at other ends were formed at 4-7th day of

culture (Figure 1C). Clamydospores 8-12 x 8-10 µm in globose and ovoid shape, smooth or rough surface also appeared after 7-14th day of culture. According to the keys proposed by Domsch and Gams (1980) RN1 is identified as *Fusarium solani* (Mart.) Sacc.

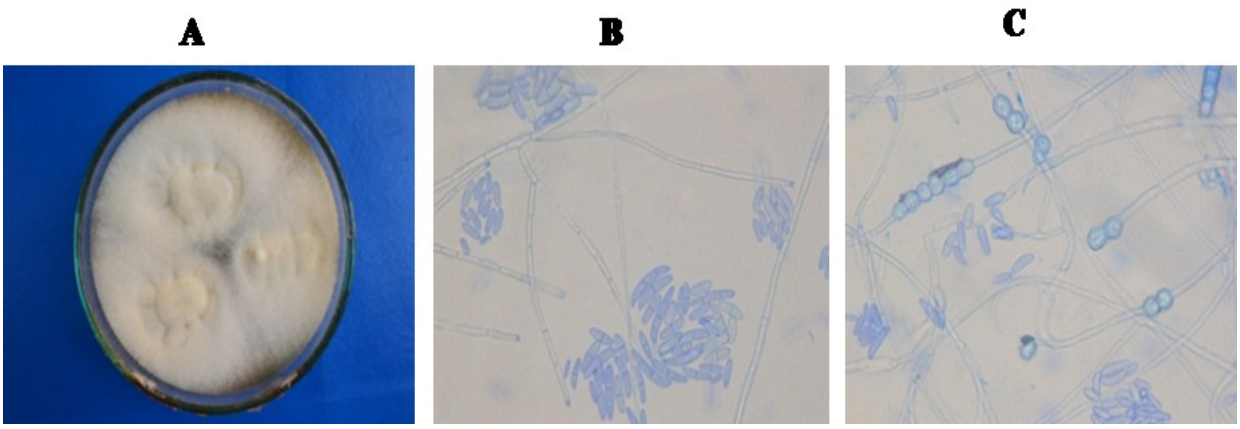

Figure 1:- Cultural and morphological characteristics of *Fusarium solani* RN1. (A) Colonies on Czapek medium at 25^0C, 7 days ; (B) Conidiosphores and conidia X 1000 magnification ; (C) Macroconidia and clamydospores X 1000 magnification.

For RN3 isolate, it grew as fast as 3.5-5.0 mm/day and the colony was covered by white or light gray aerial mycelia in the front and yellow color in the backside (Figure 2A). Ascomata 120-200 µm of RN3 appeared after two weeks of culture, which had dark brown or light gray color, ovoid to spherical (Figure 2B). The lateral hairs were straight or slightly curved, septate, rough, up to 4–5 µm wide at base. Clavate asci contained 8 ascospores, light red at early stage, 22-30 x 8-11 µm in size. Ascospores were in limoniform, slightly curved pointed to one or two ends, 6.0-7.5 x 4-4.5 µm with a brown germ pore and thin-walled at mature (Figure 2C). This isolate is matched with morphology of *Chaetomium funicola* Cooke offered by Axr et al. (1986).

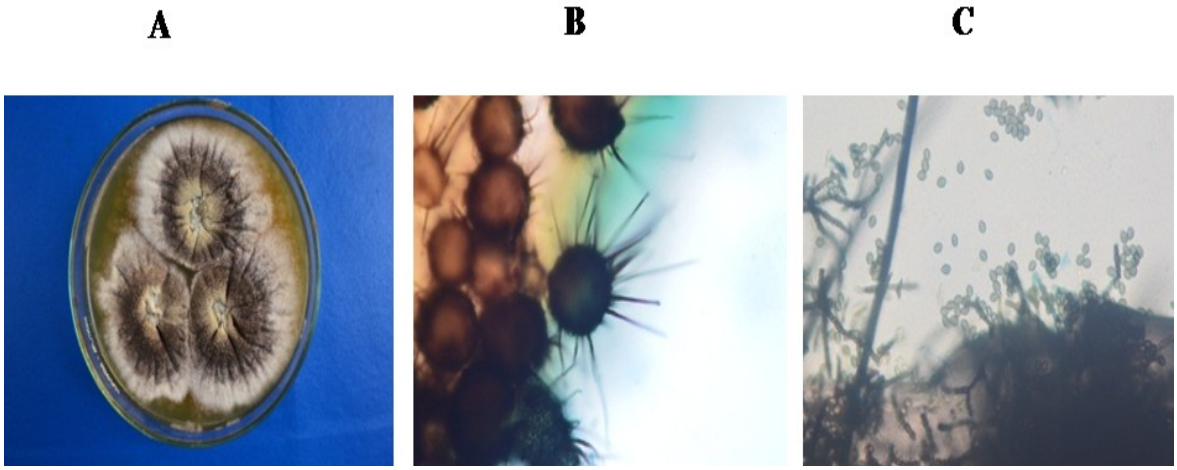

Figure 2:- Cultural and morphological characteristics of *Chaetomium funicola* RN3. (A) Colonies on Czapek medium at 25°C, 14th day; (B) Ascoma X 400 magnification; (C) Ascospores X 1000 magnification.

Strain RN4 was identified as *Penicillium rugulosum* Thom (Raper and Thom, 1949). The isolate developed as low as 1.5-2.5 cm in 14 days on Czapek medium, flat or partly partitioned colony surface (Figure 3A) and exhibited mycellium hyaline, yellow-green background with alternating red-orange strands holding conidiophores ; less secretion and soluble pigments ; light red or burgundy backside. Conidiophores 100-150 x 3.0-3.5 µm were biverticillate, symmetry; 5, 7 or 8 metulae 10-14 x 2.5-3.0 µm. Phialide 10-12 x 2.2-2.5 µm exhibited somewhat pointed apex. Conidia 3.0-3.5 x 2.5-3.0 µm were globose or ellipsoidal, smooth or rough-thick walled (Figure 3B).

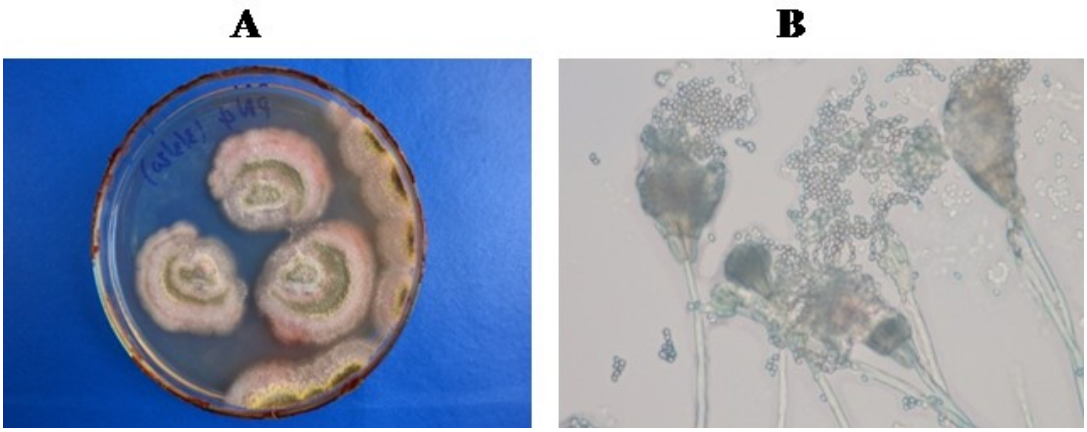

Figure 3:- Cultural and morphological characteristics of *Penicillium rugulosum* RN4: (A) Colonies on Czapek medium at 25°C, 14th day; (B) Conidiophore and conidia X 1000 magnification.

Similarly, WN1 colonies developed as fast as 2.5-3.5 mm/day at 25°C, white or gray-brown in color with white aerial mycelia (Figure 4A). Ascomata were formed after 2-3 weeks, olive brown or greyish, or globose, 100-170 µm; lateral hairs wider at base (Figure 4B). Clavate asci contained 8 ascospores. Ascospores 6.5-8.5 µm x 6.0-7.5 µm x 5-6 µm were discharged in pyriform with apiculate at both ends and a germ pore (Figure 4C). Chlamydospores 7-14 µm x 6-9 µm were globose, colorless or light brown formed on the top or beside aerial mycelia. According to von Axr et al. (1986) WN1 belongs to *Chaetomium homopilatum* Omvik.

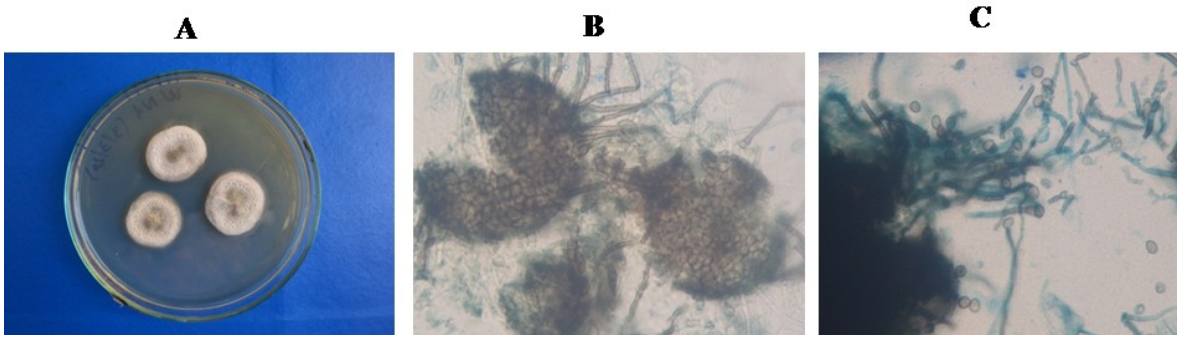

Figure 4:- Cultural and morphological characteristics of *Chaetomium homopilatum* WN1 : (A) Colonies on Czapek medium at 25°C, 7th day ; (B) Ascomata X 1000 magnification; (C) Asci X 1000 magnification.

The endophytic fungi have been isolated from the different medicinal plants. So far, this study is the first report involving the endophytic fungi from *C. roseus* inhabiting the central coastal area of Vietnam. Three isolates are from Sodariomycetes class, only *Penicillium rugulosum* belongs to Eurotiomycetes class. Among endophytic fungi isolated from 29 medicinal plants, Huang et al. (2008) found total of 45 endophytic fungi from different tissues of *C. roseus* grown in five locations of China, however no *Furasium*, *Chaetomium* and *Pelicilium* genera were recorded. In India, most of the indetified fungal species from *C. roseus* belonged to Hyphomycetes (Kharwar *et al.,* 2008). The difference in region from where plants are isolated could be one possible reason for different isolated microbial communities.

Extracellular enzyme production and activity of *C. roseus* endophytic fungi:

Extracellular enzyme activities of the studied fungal strains were screened based on their performances on culture medium supplemented with specific substrates. The larger zone of enzyme activity surrounding the fungal colony showed the stronger enzyme activities.

Table 1:- Analysis of extracellular enzyme activities from isolated endophytic fungi.

Host plants	Endophytic fungal strain	Diameter of degradation substrate zone (mm)				
		Casein	Cellulose	CMC	Starch	Xylan
While flower	*C. homopilatum* WN1	32±0.55	25±0.38	29±0.43	0	25±0.78

Pink flower	*F. solani* RN1	32±0.48	35±0.68	41±0.54	30±0.41	37±0.43
	C. funicola RN3	41±0.44	40±0.56	35±0.73	36±0.29	28±0.65
	P. rugulosum RN4	21±0.37	20±0.47	13±0.23	23±0.18	28±0.37

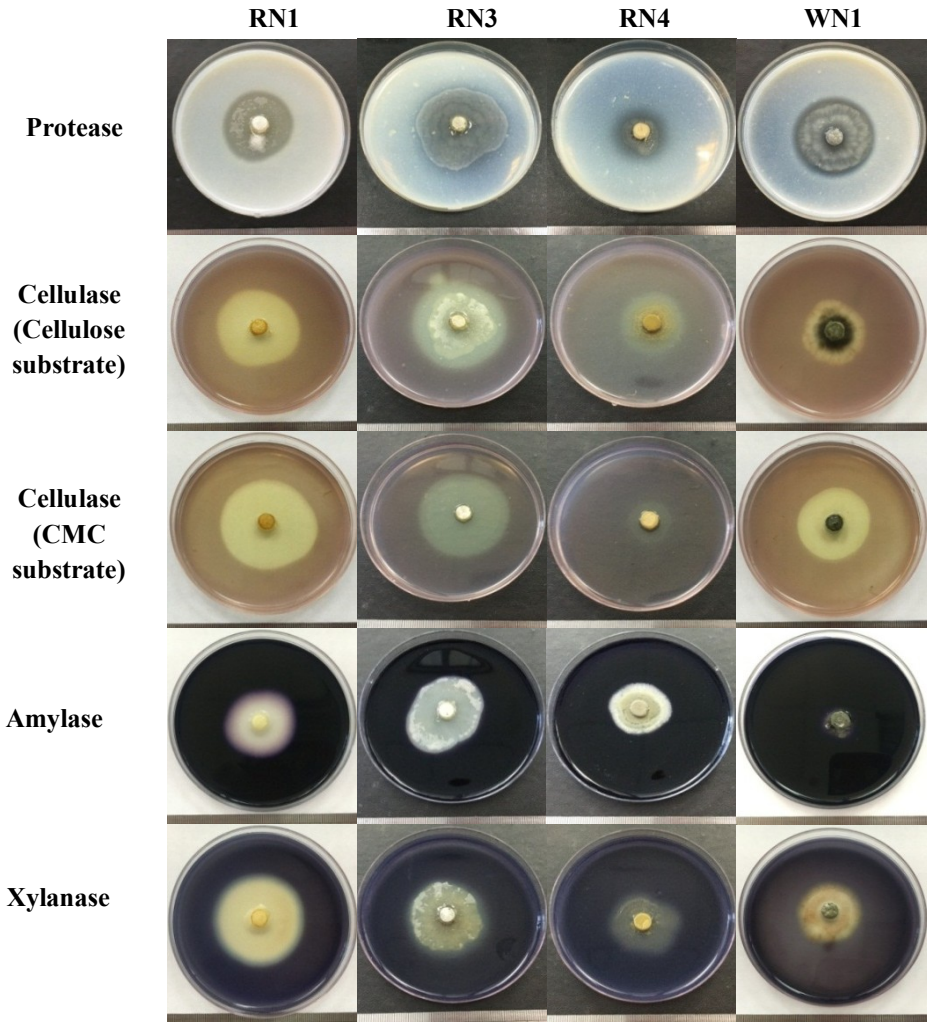

Figure 5:- Enzymes produced by isolated endophytic fungi based on substrate degraded zones (RN1: *F. solani*, RN3: *C. funicola*, RN4: *P. rugulosum*, WN1: C. *homopilatum*).

As shown in Figure 5, all endophytic fungi isolated from local *C. roseus* in Nha Trang were able to produce extracellular protease, cellulase, xylanase and amylase; except no amylase acitivity for *C. homopilatum* WN1. *C. funicola* RN3 exhibited the strongest protease activity with the diameter of substrate degradation zone to be 41 mm. Cellulase activities were strongly expressed in two isolates of *F. solani* RN1 and *C. funicola* RN3 on both culture medium supplemented with cellulose and CMC substrates. Amylase activity was found in 3 strains of *F. solani* RN1, *C. funicola* RN3 and *P. rugulosum* RN4, with substrate degradation diameters of 30 mm, 36 mm and 23 mm, respectively. The xylanase activity was also expressed in four isolates, in which the strongest was from *F. solani* RN1 (37 mm), followed by *C. funicola* RN3, *P. rugulosum* RN4 (28 mm) and *C. homopilatum* WN1 (25 mm).

These obtained results were in agreement with the previous reports on extracellular enzyme activity of these fungal strains isolated from different plant species. According to Ayob and Simarani (2016) *F. solani* isolates from *C. roseus* exhibited cellulase and amylase activities. Darwish and Abdel-Azeem (2020) recorded endophytic *Chaetomium* genus was able to produce several enzymes including amylase, dextranase, laccase, protease, chitinase, β-1, 3-glucanse, which were applied in agriculture, beer and wine fermentation, detergents and food production.

In this study, we determined that the *P. rugulosum* RN4 isolated from C. *roseus* varieties in Nha Trang (Vietnam) showed strong xylanase activity, relatively strong protease and amylase activities, and moderate cellulase activity. It also exhibited β-glucosidase and xylanase activities. *Penicillium* genus was known as the producer of several enzymes for degradating proteins, xylans, starches, lipids, etc. of agricultural wastes (Techapun *et al*., 2003). Yoon et al. (2007) reported β-glucosidase was strongly expressed in 106 *Penicillium* strains of and pectinase and xylanase activities were detected in 24 and 84 species, respectively. Two endophytic *Penicillium* strains isolated from medicinal plants, *Camellia caduca* and *Schima khasiana,* exhibited strong protease activity; and moderate activities of lipase, cellulose and xylanse (Bhagobaty and Joshi, 2012).

Conclusion:-

In the present study, four endophytic fungi were isolated from root samples of white and purple flower C. *roseus* varieties in Nha Trang coastal area (Khanh Hoa, Vietnam) and identified as *F. solani* RN1, *C. funicola* RN3, *P. rugulosum* RN4 and *C. homopilatum* WN1. This is the first report on the endophytic fungi inhabited in C. *roseus* plant growing in a coastal region of Vietnam. The analysis of extracellular enzyme activities indicated the sufficient production of protease, cellulase, and xylanase from all of them and amylase from *F. solani* RN1, *C. funicola* RN3, *P. rugulosum* RN4. In the coming time, further investigation will be carried out to confirm the practical application of obtained endophytic fungi isolates.

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