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Potential of *Trichoderma* spp. isolated in the rhizosphere to produce biofertilizer from organic materials

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Abstract. *Thuy NP, Nam NN, Trai NN, Thao NHX, Phong VT, Khang DT. 2022. Potential of* Trichoderma *spp. isolated in the rhizosphere to produce biofertilizer from organic materials. Biodiversitas 23: 6386-6396. Trichoderma* spp. are filamentous fungi present in nearly all soils and capable of secreting large amounts of cellulase enzymes that can degrade carbohydrate polymer. In the current study, eight isolates of *Trichoderma* spp. (TC10-RL11, CT11-VO11, CN2-DP11, CN1-DP11, TC9-RL11, CN4-VR11, CK6-VC11, and TC8-RL11) were isolated. Among the eight fungal strains, the CN4-VR11 had highly potent CMC degradation with a halo's diameter zone of 8.7 ± 1.5 cm. The BLAST result indicates that the CN4-VR11 strains and *Trichoderma reesei* had a similarity of 98.92% with a max score of 1158 and an E-value of 0.0. The *Trichoderma reesei* CN4-VR11 was selected for bioproduct production. The application of *Trichoderma reesei* CN4-VR11 bioproduct passively increased the efficient decomposition of the organic fertilizers, after only 30 days of incubation, the total organic matter content ≥ 20% was observed to be soft, spongy, and brown-back in color. According to the Government of Vietnam's Decree No. 108/2017/ND-CP on fertilizer management, dated September 20, 2017, the T5 and T7 treatments were chosen for the production of granular bio-compost, which has a more practical use in agricultural land.

Keywords: Biofertilizer, cellulose, organic materials, *Trichoderma*

INTRODUCTION

In the past decade, Vietnamese production of rice, fruits, and vegetables have grown substantially and steadily (Ngoc et al. 2021). The rice sub-sector in particular is the foundation for comprehensive development and the main contributor to Vietnam's economy. Rice straw, a residual by-product of the rice harvest, has been estimated at around 76 million tons for 4 million hectares of rice field. Residual rice straw has been used as cooking fuel, animal feed, roofing, and compost (Nguyen et al. 2019). Similarly, peanuts are distributed in the delta areas throughout the country. The whole production amounts to approximately 530,000 tons, accounting for 2% of the global production. According to the farmers' harvesting behavior, only the peanut seeds are collected; the other parts of the peanut tree, such as leaves and stems, are mainly used as firewood, fodder for livestock, and landfill waste. According to the Vietnamese Deputy Minister of Agriculture and Rural Development, 160 million tons of by-products are generated per year as waste, of which 90 million tons are from post-harvest crops and farm product processing (56.2%) and 62 million tons from cattle and poultry manure (38.7%). The huge amount of agricultural by-products creates environmental challenges, such as pollution, surface nutrient evaporation, and soil degradation (Dung et al. 2012). Recently, the way they handle issues of agricultural wastes and the model of compositing microbial organic fertilizer have attracted interest. The assisted microbial model can decompose organic wastes and residues into bioactive compounds such as biofertilizers. Microbial technology has been used effectively in the treatment of agricultural and municipal waste (Hidalgo et al. 2022).

The treatment of agricultural by-products by microbial technology, especially cellulase enzyme and extracellular peroxidase from microorganisms, have yielded several benefits (Thu et al. 2011). Fungi are one of the groups of microorganisms capable of decomposing organic materials containing cellulose and lignin. Generally, fungi have been considered the main degraders of lignocellulose due to their hyphal growth form and highly effective enzymatic system, which allows them to redistribute nutrients to the nutrients-poor substrate (Andlar et al. 2018). The previous reports prove that fungi assist agricultural waste decomposition. Some fungi, such as *Aspergillus* sp., *A. fumigatus* (PH-C5), *A. fumigatus* (PH-L4), *P. janthinellum* (PHL3), *Penicillium* sp., *Pseudomonas* sp., *Rhizomucor variabilis* (PH-L6), *Trichoderma* sp., and *T. viride*, significantly increase the decomposing rate and the content of NPK substances and reduce the C/N ratio (Cam et al. 2015). *Trichoderma* spp. have been found to have wide applications in agricultural production as bio-fungicides and biofertilizers. They are also well known as a bio-agent for controlling fungal pathogens (Yusnawan et al. 2019). The modes of action of *Trichoderma* have been proven to suppress pathogen growth by competition, antibiosis, synthesis of cellulase,

and hydrolytic enzymes such as protease, chitinase, and β-1,3 glucanase (Tyśkiewicz et al. 2022; Nakkeeran et al. 2018; Mukhopadhyay and Kumar 2020).

Moreover, *Trichoderma* spp. have been found to produce various plant growth-promoting compounds such as enzymes and phytohormones (Illescas et al. 2021). *Trichoderma* spp. have been utilized for the biodegradation of agricultural residues such as rice straw, coco peat, and peanut stem (Nayak and Mukherjee 2015). The cellulase enzymes produced by *Trichoderma* spp. hydrolyze the cellulose. Although several fungal strains could secrete cellulase, *Trichoderma* spp. produce higher amounts of cellulases (Marcella et al. 2018). In this regard, the use of *Trichoderma* for biofertilizer compost degradation is of high interest in research. The production of *Trichoderma*-assisted organic fertilizer using local agricultural by-products with low cost, environmentally friendly, and massive production is greatly meaningful to the development of organic and sustainable agriculture. Incubation of *Trichoderma* spp. are reported as the natural decomposition of bioremediation, which increases the rate of the decomposition process (Siddiquee et al. 2017). *Trichoderma* spp. are one of the most studied fungi to discover other potential strains and species. Recently, the identification of *Trichoderma asperellum* strain Ta13, which involved a combination of morphology and molecular analysis using genes cal, tef1, act, rpd2, and ITS was reported (Matas-Baca et al. 2017; Ming Xue et al. 2021; Pandian et al. 2016). Twelve strains of *Trichoderma reesei*, a species usually isolated from tropical regions, were surprisingly obtained from Austria (Hinterdobler et al. 2021).

It is reported that microbial communities from different ecosystems and soil types have similar functional properties and capacities for the degradation of a certain substrate (Koranda et al. 2014; Wertz et al. 2006). The microbial communities are affected by bottom-up and top-down interactions when determining microbial and fungal diversity and function (Crowther et al. 2015). The microbial communities in paddy fields are considered to be unique agroecosystems that consist of diverse microbial habitats. Tra Vinh, located in the Mekong Delta of Vietnam, has two agroecosystems comprising irrigated and rainfed areas. Agriculture is a primary source of livelihood in Tra Vinh, particularly rice, peanut, king orange, guava, custard apple, water spinach, and vegetable spinach. The cultivation behaviors of local farmers may affect the distinct microbial communities. *Trichoderma* has a diverse range of strains and species and impacts the functioning of the ecosystem, such as the decomposition rates. Depending on the strains, the use of *Trichoderma* spp. in agriculture provides numerous advantages (Sood et al. 2020; Vinale et al. 2008). Following these rationales, the aim of this study is to isolate and select *Trichoderma* spp. from different paddy fields in the Tra Vinh province of Vietnam. The high potential cellulose degrading *Trichoderma* was identified and used to produce biofertilizers, which have the ability to decompose agricultural wastes.

MATERIALS AND METHODS

Collection of soil samples and isolation of cellulose-degrading *Trichoderma*

Soil samples were collected from 12 different farms (5 samples per farm) of fruits, peanuts, and rice in the 4 districts, namely, Tra Cu, Cau Ngang, Cau Ke, and Chau Thanh of Tra Vinh province (Table 1). The soil samples were collected by digging at depths of 0-15 cm, and each sample contained 100g of soil. Five samples were mixed into one with labels distinguishing name, date of collection, place of collection, and the characteristic of the sample.

The soil samples were then mixed and diluted in sterile water by 10^{-1}, 10^{-2}, and 10^{-3} times. Then, 0.1 mL of each mixture sample was spread onto TSM (*Trichoderma* specific medium) Petri plates. The incubation was performed at room temperature (30°C-32°C). After 3-4 days, the clear, separate, and representative colonies of *Trichoderma* were picked and subcultured on TSM agar plates (Elad et al. 1981). The putative *Trichoderma* was selected and identified according to the taxonomic keys proposed by Dong and Van (2000). The purified single strain of *Trichoderma* was then transferred to the PDA (potato dextrose agar) medium.

Table 1. Details and pH characteristics of collection sites in Tra Vinh province of Vietnam

Item	Plant	Place	pH of soil
CN1-DP	Peanut	Ben Kinh hamlet, My Long commune, Cau Ngang district, Tra Vinh province.	6.8
CN2-DP	Peanut	Ben Kinh hamlet, My Long commune, Cau Ngang district, Tra Vinh province.	6.6
CN3-DP	Peanut	Ben Kinh hamlet, My Long commune, Cau Ngang district, Tra Vinh province.	6.8
CN4-VR	Water spinach and vegetable spinach	Ben Kinh hamlet, My Long commune, Cau Ngang district, Tra Vinh province.	6.9
CK5-VC	King orange	Two hamlet, Thanh Phu commune, Cau Ke district, Tra Vinh province.	6.8
CK6-VC	King orange	Two hamlet, Thanh Phu commune, Cau Ke district, Tra Vinh province.	6.5
CK7-VC	King orange	Two hamlet, Thanh Phu commune, Cau Ke district, Tra Vinh province.	6.5
TC8-RL	Rice	Leng hamlet, Tan Son commune, Tra Cu district, Tra Vinh province.	7.2
TC9-RL	Rice	Leng hamlet, Tan Son commune, Tra Cu district, Tra Vinh province.	6.4
TC10-RL	Rice	Leng hamlet, Tan Son commune, Tra Cu district, Tra Vinh province.	6.8
CT11-VO	Guava	Tri Phong hamlet, Hoa Loi commune, Chau Thanh district, Tra Vinh province.	7.1
CT12-VMC	Custard apple	Tri Phong hamlet, Hoa Loi commune, Chau Thanh district, Tra Vinh province.	6.8

Morphological and molecular identification

Morphological characteristics

Morphological characteristics of the colonies were examined after 3-7 days and a longer time for the slow-growing species. The morphology was observed for following characteristics: colony diameter, color and form of the colony, pigment secretion of the colony, and other unique characteristics. The microscopic features were obtained when the colony expressed color (containing fungal spore) and ~0.5-1 cm in diameter. One side of the colony was cut by a sterile scalpel to a rectangular form of 3-4 cm × 1.5-2 cm, incubated for 2-3 days, and observed under the microscope. The samples were also treated with blue cotton lactophenol to observe the reproductive structure of the fungi under the microscope under a resolution of 40x and 100x. The identification followed the taxonomic keys proposed by Rifai (1969), Barnett and Hunter (1972), and Bissett et al. (2015).

DNA extraction

The fungi were cultured on PDA for three days at 28 ± 2°C. Then, the fungal filaments were collected for DNA extraction based on the previous report (Sambrook et al. 1989). Typically, the putative biomass of *Trichoderma* was ground in liquid nitrogen, then placed into falcon 15 mL. To this, 3 mL of lysis buffer was added and incubated at 65°C for 1-2 h. The mixture was then added with 1 mL of phenol/ chloroform/ isoamyl alcohol (25: 24: 1) and centrifuged at 4000 rpm and 10°C for 10 min to collect the supernatant. One milliliter chloroform/ isoamyl alcohol (24: 1) was added and centrifuged at 4000 rpm and 10°C for 10 min to collect the supernatant. Isopropanol was mixed with the sample and aged overnight. The precipitated DNA was collected and washed with 70% ethanol 3-4 times. Finally, the DNA pellet was air dried for 2-3 h and suspended with 1 mL TE 1X.

Quantification of DNA and PCR amplification

The genomic DNA quality was then determined by electrophoresis on a 1% agarose gel in TAE 1X buffer and staining with SafeView DNA stain (ABM, USA). The result was observed under ultraviolet light by GelDoc Go system (BioRad, USA). DNA concentrations were determined by NanoDrop One spectrophotometer (ThermoFisher, USA).

The ITS region on fungal DNA was selected for amplification. The two primers of ITS1 (forward: 5′-TCC GTA GGT GAA CCT GCG G-3′) and primer ITS4 (reverse: 5′TCC TCC GCT TAT TGA TAT GC-3′) were used. The polymerase chain reaction (PCR) was performed in a total volume of 25 µL, which contained 15 µL dH_2O, 12 µL My Taq mix 2X, 1 µL primer, and 2 µL DNA. PCR was started with denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 sec, extension at 72°C for one min, and final elongation at 72°C for 7 min for amplification of ITS region (Hassan et al. 2019). PCR amplification was then separated by electrophoresis in 2% agarose gel in 1X TAE buffer and stained with 0.5 µg/mL SafeView DNA stain then visualized under a UV transilluminator.

DNA sequencing

PCR products were purified and the fungal DNA sequences were done by Next Gen Scientific Co., Ltd (Ho Chi Minh City). The sequences were then analyzed and compared with the NCBI GenBank to identify the isolated fungi.

Cellulose degradation

The test for cellulose degradation of the isolated *Trichoderma* was carried out as point culture on CzapekDox medium with 1% carboxyl methyl cellulose (Cz + 1% CMC) and incubated at room temperature for five days with three replications. The magnitude of cellulose degradation activity was estimated by measuring the halo zone diameters. The Lugol reagent was added to the agar surface to evaluate the cellulose degradation ability of the isolated *Trichoderma* sp. following the calculation of D - d (D: diameter of the zone, d: diameter of the colony).

Bioproduct of *Trichoderma*

Trichoderma proliferated in Erlen containing 50 g substrate, which composed of 60% corn starch and 40% coco peat with a 60% moisture for five days. The formulation of 1-kilogram bioproduct of *Trichoderma* powder: 60% rice bran, 30% corn starch, 10% of additives and micronutrients (peptone, K_2PO_4, magnesium sulfate, ferric ammonium citrate, and potassium chloride), and *Trichoderma* spore from the proliferation medium. The mixture was mixed and supplied with water to reach 60% moisture. The mixture was then spread on the sterilized stainless-steel trays and incubated at room temperature for seven days. The mixture was evaluated by observation test and dried at 45°C for 24h. The final bioproduct of *Trichoderma* was stored and sealed in an aluminum package. The density of *Trichoderma* in the bioproduct was determined by a number of *Trichoderma* colony-forming units, cultured on the selective growth medium, TSM (*Trichoderma* selective medium).

Production of organic fertilizer by isolated *Trichoderma*

The materials used in this study were cow manure, straw, peanut plant, coco peat, and bioproduct of *Trichoderma* sp. The manure was obtained from a cattle farm, the straw and the peanut plant from fields, and the coco peat from a wood peat factory. All raw materials were collected from Tra Vinh Province, Vietnam. The compost was prepared by a mixture of cowpat and substrate (straw, peanut plant, and coco peat), which were decomposed by an as-prepared bioproduct of *Trichoderma* sp. to biofertilizer (Table 2). The as-prepared *Trichoderma* bioproduct and the agricultural wastes are described above. The formulation was designed as a randomized complete block design (RCBD), one factor with seven treatments and three replications. Firstly, cow manure was spread into a layer thickness of 20-30 cm. A thin layer of CaO powder was then sprinkled on the manure surface. This was followed by a layer of substrate, molasses, and *Trichoderma* sp. bioproduct. The mixture pile was 0.7-1 m in diameter and 0.4-0.6 m in height. The compost was covered and incubated for 30 days, and the mixture of *Bacillus* sp. bioproduct with 1 liter of water was applied at date 20. The quality assessment was carried out following the standard of microbial organic fertilizer (No. 108/NĐ-CP 20/9/2017).

Table 2. The components of the compost treatment

Treatment no.	Cow manure (kg)	Straw (kg)	Peanut plant (kg)	Coco peat (kg)	Molasses (kg)	CaO powder (kg)	Bioproduct of *Trichoderma* (kg)	Bioproduct of *Bacillus sp.* (L)
T1	100				1	1	0.1	0.1
T2	75	25			1	1	0.1	0.1
T3	75		25		1	1	0.1	0.1
T4	75			25	1	1	0.1	0.1
T5	60	20	20		1	1	0.1	0.1
T6	60	20		20	1	1	0.1	0.1
T7	60		20	20	1	1	0.1	0.1

Note: *Straw and peanut plants were cut into small pieces of lengths of 1-2 cm.

The quality of biofertilizers were evaluated and compared according to the following criteria: Organic matter content (%), total N content (%), P available content (%), total P content (%), total K content (%), the density of aerobic microbes (CFU/g dried soil), the density of *Trichoderma* (CFU/g dried soil), the density of *E. coli* (CFU/g dried soil), the density of *Salmonella* (CFU/g dried soil), humic acid content (%), humidity (%), and pH.

Data analysis

The variance for various criteria was estimated following ANOVA as per randomized complete block design (RCBD) as outlined by Panse and Sukhatme (1954). Analysis was performed using the "Windostat" computer program. The level of significance was tested at 5% using the F-test. If a treatment test shows a significant effect between treatments for descriptive analysis, it is followed by Duncan's follow-up test (DMRT) to determine the effect between levels.

RESULTS AND DISCUSSION

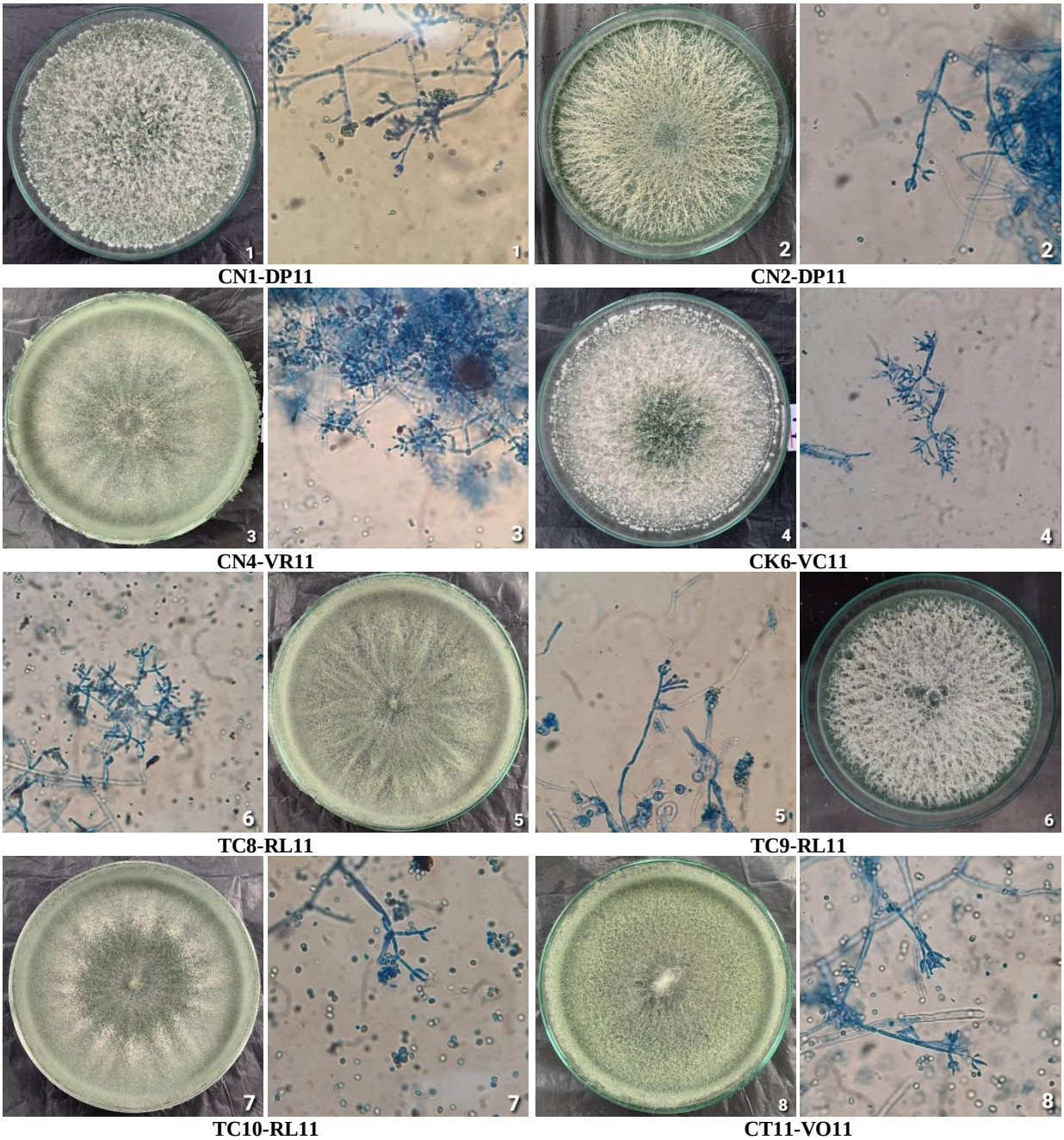
Isolation of *Trichoderma* spp.

A total of 8 *Trichoderma* strains were isolated on TMS agar from 14 soil samples collected from different farming models. *Trichoderma* generally could grow in a variety of soil with a pH range from 6.4 to 7.2 (Table 1). The morphological characteristics of the fungal colonies showed variation in color, shape, buoyancy, and edge of the colony as well as the properties of the colony on the upper and lower surfaces of the agar plate. The colonies were woolly and became dense in due course. The two colonies' forms were obtained, the irregular round shape accounted for 68.64 %, whereas the regular one accounted for 31.36%. Colors of the fungal colony differed from white, black, yellow, brown, gray, and blue. As shown in Figure 1, the white and black colored colonies comprised 29.15% and 14.72%, respectively. The yellow color was obtained to be 11.12%, while the blue color was 26.32%. Gray accounted for 12.48% and the brown one took up 6.21%. Eight isolates of *Trichoderma* were collected from the TSM plates, which signify soil from different regions of the Tra Vinh province of Vietnam. The isolation of *Trichoderma* was performed in the different soil samples in various plant farming models. Attitalla et al. (2012) recommended TMS as suitable for the isolation of *Trichoderma*. TSM is superior compared to Martin's medium (MT), potato dextrose agar (PDA), malt extract medium, glucose-Czapek's agar medium (CZ), and this medium recorded an efficiency ranging from 120%-140%, favouring TSM for further studies on *Trichoderma* to determine its survival and proliferation in soils. Recently, Alwadai et al. (2022) also reported that 48 *Trichoderma* spp. were successfully isolated on TMS from the soil samples collected at six locations in Abha, Saudi Arabia.

Most *Trichoderma* performed at a fast growth rate. The diameters of the growth ring reached 8-10 cm after three days of culture on the PDA medium. The *Trichoderma* conidiophores formed after 3-4 days of culture. Especially, the TC10-RL11 and CT11-VO11 strains formed spores after two days of culture. The TC9-RL11 strain witnessed a significant growth of hyphae, but the spore was formed late, after 8-10 days of culture. The color of the fungal spore of CN2-DP11 was observed to change from light blue to dark yellowish-green color. This color change of the growth media was caused by CN1-DP11 and CN4-VR11; however, this did not occur on the other strains. Most of the fungal strains formed a large population of conidiophores and spread, covering the entire surface of the culture plate but that of CK6-VC11 and TC8-RL11 slightly formed and distributed as scattered clusters. Under the observation at 40X, there was no significant difference in the structure of conidiophores of the as-isolated *Trichoderm*a (Figure 2). The representative morphology of the *Trichoderma* genus was obtained and compared to the Bui Xuan Dong report. It was observed that potato dextrose agar media was suitable for the massive growth of *Trichoderma* spp. (Jahan et al. 2013; Oszust et al. 2021). The isolated *Trichoderma* spp. were purified and microscopically identified. The hyphae, conidiophores, phialides, and conidia were clearly obtained. The conidiophores contained phialides and conidia. The conidiophores were hyaloid, branched, and arranged compactly on the culture plate. The phialides attached to the conidiophores may occasionally separate or distribute in clusters. The conidia were circle shaped, rough or smooth surfaces, located almost at the tips of the phialides. This is in agreement with a previous report (Jaklitsch et al. 2006; Awad et al. 2018; Mukhopadhyay and Kumar 2020).

CK6-VC11 **TC8-RL11** **CN4-VR11** **CT11-VO11**

Figure 1. The digital image of *Trichoderma* spp. cultured on TSM after 48 h



CN1-DP11 **CN2-DP11**

CN4-VR11 **CK6-VC11**

TC8-RL11 **TC9-RL11**

TC10-RL11 **CT11-VO11**

Figure 2. The morphology of the colony, filament, and spore of isolated *Trichoderma* spp.

Degradation of CMC

Degradation of cellulose of eight *Trichoderma* spp. were evaluated by culture on CzapekDox medium supplying 1% CMC. All eight isolated *Trichoderma* sp. had the ability of cellulase enzyme synthesis, followed by the halos with different diameters. The statistical analysis indicated that the halo rings produced by eight fungal strains were significantly different ($p<0.05$) with a diameter ranging from 3.3 ± 4.5 cm to 8.7 ± 1.53 cm. Three fungal strains of CN4-VR11, CT11-VO11, and CK6-VC11 had the larger diameter zones which were 8.7 ± 1.5 cm, 7.7 ± 1.5 cm, and 7.0 ± 4.4 cm, respectively (Figure 3). The other three strains of TC8-RL11, CN1-DP11, and CN2-DP11witnessed the diameter zone ranging from 6.9 ± 4.0 cm to 6.3 ± 1.2 cm. The diameter zone of the TC10-RL11 strain was 4.2 ± 3.5 cm and the smallest one of TC9-RL11 was 3.3 ± 4.5 cm. In the previous report (Ngoc et al. 2014), the fungal strain of no. 10 had a halo diameter reaching 11 mm after 4 days of culture. Whereas the halos diameters by eight isolated fungal strains in this study were significantly larger, the CN4-VR11 strain had a diameter zone reaching 8.7 ± 1.53 cm. In this regard, the CN4-VR11 strain with the largest halo ring was selected for further experiments that related to the bioproduction of the CN4-VR11 strain. The presence of CN4-VR11 may increase the efficiency of organic matter decomposition.

Molecular identification

DNA extraction was an important initial step that determined the success of the PCR assay. The DNA extraction of *Trichoderma* was difficult due to the very thin and soft cell wall made of chitin, unlike plant cell walls, which is made of cellulose. The *Trichoderma* cytostome contained several polysaccharides, proteins, and lipids (Pedersen et al. 2021). The as-extracted DNA protocol in this study was simple and effective in achieving high-purity DNA. The sample was ground in liquid nitrogen (which protects the cell wall) until the cells were separated to help the lysis buffer work effectively. Moreover, the use of phenol/chloroform/ isoamyl alcohol (25: 24: 1), followed by chloroform/isoamyl alcohol (24: 1) helped the DNA to separate from protein and removed all protein, polysaccharide, lipid, RNA, and the residual phenol. The centrifugation was carried out at a low speed (4000 rpm) for a long stretching time (10 min) to obtain the purified DNA and reduce DNA breakage. In addition, the sample was precipitated by cool isopropanol overnight, and a stick was used to collect the precipitated DNA, instead of centrifugation, to ensure the DNA was purified and avoid DNA breaking. In this regard, the as-extracted DNA had high purity with a concentration of 142 ng/µL (Figure 4).

The single product of 500-600 pb was obtained from PCR amplification with specific primers ITS1 and ITS4, which was also in agreement with the previous report (Figure 5). According to the study by Kullnig-Gradinger et al. (2002), the sequence and length of ITS regions were one of the most reliable loci for the identification of a strain at the species level. The result of the agarose gel electrophoresis of the ITS regions also indicates that the ITS PCR amplicons were suitable to sequence ITS regions of as-isolated *Trichoderma*.

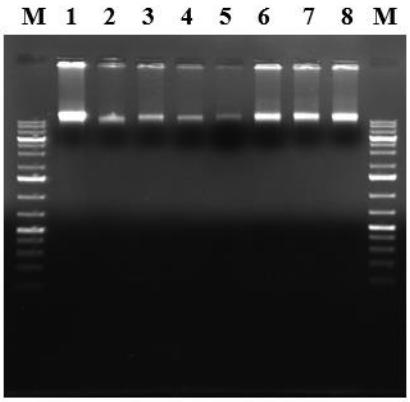


Figure 4. Agarose gel electrophoresis (1% agarose) of total DNA extraction of CN4-VR11; M: DNA marker; Lanes 1-8: total DNA of CNV-VR11

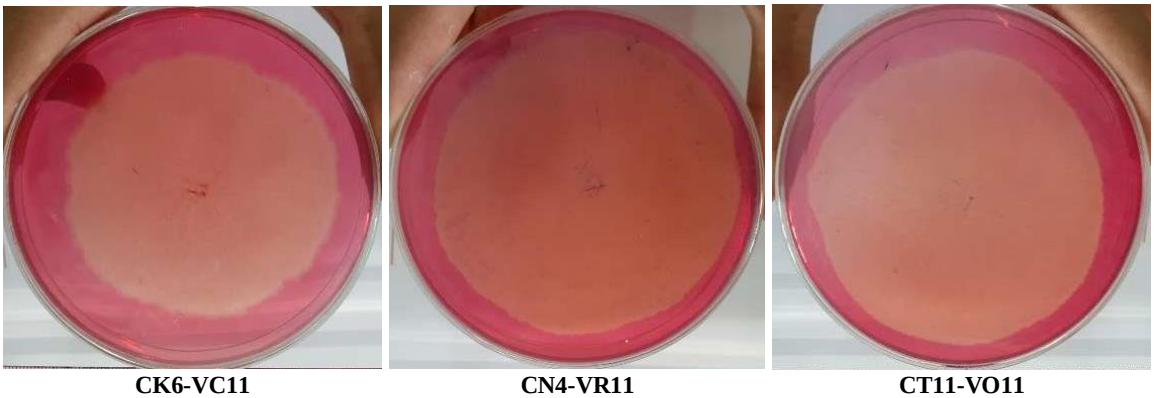


Figure 3. The efficiency of CMC degradation by isolated *Trichoderma* spp.

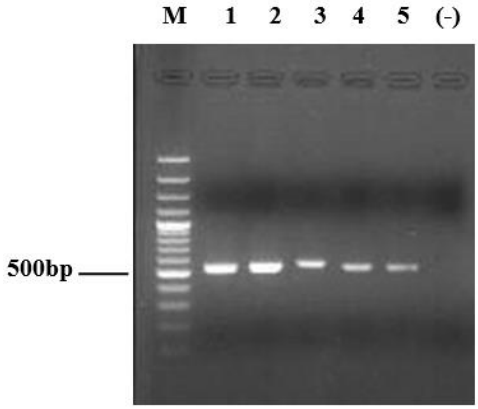


Figure 5. Amplification of DNA barcodes from CN4-VR11. Product of I*TS primer ranged from 500-600 bp on* 2% agarose gel with 1kb ladder; *M: DNA marker; Lanes 1-2: Trichoderma; Lanes 1-2: Samples; Lanes 3-5: Positive control;* NC: Negative control without DNA

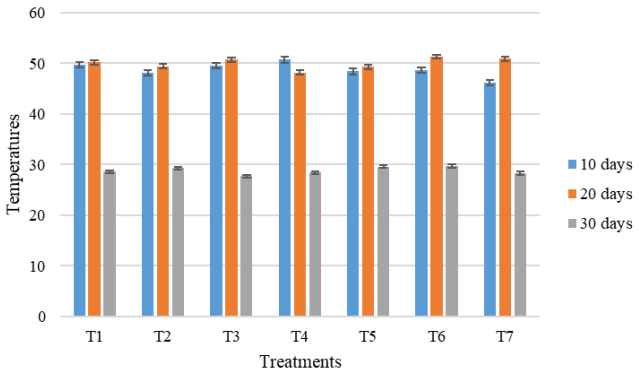


Figure 6. Progression temperature of the compost

PCR amplification of the 5.8S-ITS region was conducted using specific primers ITS1 and ITS4. The 5.8S-ITS DNA fragments were amplified from all *Trichoderma* isolates and PCR products were sequenced. All sequences were uploaded to the GenBank database and their accession number was OP420797. Then, the BLAST program was used to determine the species identity of *Trichoderma* isolates. The sequence alignment analysis indicates that 647 nucleotides were aligned with the similarity of 640/647 nucleotides. The BLAST result indicates that the CN4-VR11 strains and *Trichoderma reesei* had a similarity of 98.92 % with the Max score of 1158 and E-value of 0.0. This confirmed the isolated fungi in this study belonged to the *Trichoderma reesei* species and were named *Trichoderma reesei* CN4-VR11. Bellemain et al. (2010) used molecular markers, particularly those based on DNA sequence data, as an essential technique to decipher the genetic variability of any species. In addition, the interspecies identification from different isolates was carried out in this study. The results showed that although the rDNA ITS sequence was very conservative, there were variations in sequence and length among different isolates, and there was a genetic differentiation at various levels. Therefore, the ITS sequence analysis clearly differentiated between species. Similarly, Kuhls et al. (1997) used sequence analysis to differentiate between *T. reesei* and *T. longibrachiatum.* Many other researchers have used ITS sequences to identify *Trichoderma* spp. (Sharma et al. 2009; Shahid et al. 2013). Consequently, variation among individuals of the same species was noticed (Fahmi et al. 2016; Hassan et al. 2019; Mazrou et al. 2020); therefore, it is of great interest to identify the species at the molecular level.

Bioproduction of *Trichoderma*

The bioproduct of *Trichoderma* achieved a density of 3.2×10^8 CFU/g.

Production of organic fertilizer by isolated *Trichoderma*

Temperature

The temperature change of the compost pile during composting is presented in Figure 6. After 10 days of incubating, the assistance of *Trichoderma* sp. increased the temperature of the compost to 48.1°C-50.7°C. After 20 days of incubating, the temperature ranges between 48.2°C-51.3°C. After 30 days of incubating, the temperature was reduced to the environmental temperature of 27.7°C-29.7°C, indicating reduction in the microbiota activity.

The increase in temperature indicates that the growing microorganisms inside the bioreactor generate heat during the composting period (Lopez et al. 2014). According to Rongfei et al. (2017), the temperature of the composting process began to rise soon after the establishment of composting conditions. The temperature in the active stage at high-temperature treatment gradually increased to a maximum value of about 51°C on day 35. Meanwhile, in this study, the organic materials were treated with *Trichoderma* sp., which helps to speed up temperature up to 51°C by Day 10. Thus, *Trichoderma* sp. is considered a compost activator that shortens the rising temperature of the composting period.

Waszkielis et al. (2013) pointed out that the temperature stimulates the growth and metabolic activity of the microbial community within compost mass. It can directly affect the biodegradation rate of the organic matter during composting and treat pathogenic bacteria, protozoa, and helminths occurring in animal manure (Figure 7).

pH

Ameen et al. (2016) found that pH content is an important parameter to evaluate the maturity of compost prepared by using different types of organic waste. The alkaline pH is important to evaluate compost maturity and stability. The results of the experiment in terms of pH value were different (Table 3). All treatments showed alkaline pH throughout the composting process, in the range of 7.57-8.29. L. Zhang and Sun (2016) reported that for composting, ideal values for pH generally range from 5.5-8.0, but Bernal et al. (2009) opined that a pH value between 6.7-9.0 is effective to promote good microbial action. The

acidic pH affects the rate of respiration of microbes and decreases the rate of degradation. The pH of the compost should be alkaline throughout the composting process. The high activity of microbes at the thermophilic stage is because of the alkaline pH (Sundberg et al. 2004).

Moisture content

The moisture conditions essentially impinge microbial activity, oxygen uptake rate, temperature, and the porosity level within composting (Petric et al. 2015). In this study, the moisture content ranged between 26.0 (T7) and 30.45 (T2) (Table 3). The moisture content at the initial stage of the composting process is optimized and its value should not increase from 50%-60% (Pezzolla et al. 2021). Moisture is important for the activity of microbes because it increases the rate of metabolism. Microbial activity was minimum when low moisture was provided (Adhikari et al. 2009). The reduction in the value of moisture content at the end of composting is a positive sign of decomposition and results mature compost (Jain et al. 2019).

Microbial count in microbe organic fertilizer

Along the composting process, the microbial additive to a compost mix affects the temperature profile and promotes organic degradation within composting by releasing various substrate-based hydrolytic enzymes (Barthod et al. 2018; Rastogi et al. 2020).

Microbial count of fungi and bacteria in the compost after 30 days of composting are presented in Table 3. Among the treatments of the experiment, the range of variation for density of *Trichoderma* sp. was observed from 1.26×10^5 CFU/g dried soil (T1) to 1.02×10^6 CFU/g dried soil (T3). The density of aerobic microbes varied from 1.08×10^7 CFU/g dried soil (T1) to 7.21×10^7 CFU/g dried soil (T3). *Escherichia coli* and *Salmonella* were not found in all treatments. This indicates that the addition of inoculants leads to a higher number of *Trichoderma* within the composts, which helped to hasten the decomposition process (Organo et al. 2022). In Vietnam, according to Degree No 108/ND-CP, organic fertilizer should be free of *Salmonella* while the density of *E. coli* should be lower than 1.1×10^3 MPN/g. The density of beneficial microbes should be higher than 1×10^6 CFU/g.

C-organic content

The C-organic of bio-compost was significantly different ($p \leq 0.05$) (Table 3). Specifically, the highest C-organic was found at T3 (50.167 ± 0.674 %), the lowest C-organic was found at T5 (32.293 ± 1.713 %), and this value was no different from T1. Lantik et al. (2020) reported that the C-organic of the bio-compost from oil palm empty bunches (1 kg), given *Pleurotus ostreatus* (8 g) and *Trichoderma harzianum* (2 g) as decomposers, was found to be 21.63%. According to Decree No. 108/2017/ND-CP dated September 20, 2017, of the Government of Vietnam on fertilizer management, the minimum C-organic content should be higher than ≥20%. The aspect of C-organic content, the concentration of all treatments, as a choice substrate and supplement for the production of granular bio-compost was more practical on agricultural land.

C: N ratio

The C: N ratio of the composting experiment showed significant differences (Table 3). The lowest C: N was recorded in both T5 and T7 (11.553 ± 0.303 and 12.137 ± 0.857). The lowest C: N was shown in T6 (20.393 ± 0.192). This was due to higher initial nitrogen content in peanut plant litter than in other organic materials. The result also confirmed the study by Mohammadi Torkashvand et al. (2015).

Nitrogen content

The key factors to determine the quality of organic fertilizers are the nitrogen, phosphorus, and potassium contents. The result of this experiment indicated that analysis of nitrogen content in various treatments show a significantly different effect on nitrogen content in organic fertilizer. Nitrogen ranged from 0.367 ± 0.021% (T6) to 0.590 ± 0.026% (T3). The highest nitrogen was found at T3 (0.590 ± 0.026%) and this value was not significant ($p>0.05$) for treatments of T5 (0.543 ± 0.025%), T1 (0.533 ± 0.041%), T2 (0.530 ± 0.026%) and T7 (0.523 ± 0.032%). The highest total nitrogen in organic fertilizer was observed in all treatments with the addition of peanut plant, consistent with the reports of (Torkashvand et al. 2015). Thus, it can be concluded that peanut plant compost can increase the total nitrogen of the media, and *Trichoderma* can decompose nitrogen compounds into simple nitrogen elements including NO_2 (Halifu et al. 2019).

Humic acid content

Humic acids, a component of the soil's organic matter, are the soil fraction that is most resistant to microbial degradation. Composting of agroindustrial wastes may produce composts that are rich in humic substances and nutrients through humification and mineralization (Nguyen and Shindo 2011). In this study, humic acid content was significantly influenced by different treatments (Table 3). Humic acid content ranged from 1.597 ± 0.254 % (T6) to 2.363 ± 2.55 % (T5). Production of organic fertilizer produced good quality humic acid.

Phosphor and potassium content

The result of various treatments showed statistically significant differences ($P \leq 0.05$) (Table 3). The highest phosphorus was found at T6 (0.350 ± 0.002 %), the lowest at T1 (0.439 ± 0.003 %), while no significance for T2 (0.418 ± 0.002%) and T3 (0.411 ± 0.006 %) were found. The highest potassium was found at T7 (0.747 ± 0.042 %), while the lowest was found at T5 (0.443 ± 0.028 %). More recently Kusumawati et al. (2021) reported the effect of *Trichoderma* addition on *Sargassum* organic fertilizer and that the chemical characteristics of *Sargassum* liquid seaweed fertilizer were phosphorus (0.15 + 0.06 %) and potassium (1.13 + 0.01 %). In comparatively, our research shows better results in terms of nitrogen, phosphorus, and potassium content.

Table 3. The assessment criteria for microbe organic fertilizer

Treatment No.	Total N (%)	Total organic matter (%)	Total P (%)	Total K (%)	C/N ratio	Humic acid (%)	Humidity (%)	Density of aerobic microbes [CFU/g (10^7)]	Density of *Trichoderma* (CFU/g)	Density of *E. coli* (CFU/g)	Density of *Salmonella* (CFU/g)	pH
T1	0.533 ± 0.041^{ab}	34.958 ± 2.028^{d}	0.439 ± 0.003^{a}	0.550 ± 0.036^{b}	13.353 ± 0.186^{cd}	2.423 ± 0.144^{a}	28.55	1.08×10^7	1.26×10^5	0	0	8.03
T2	0.530 ± 0.026^{ab}	39.200 ± 1.632^{bc}	0.418 ± 0.002^{b}	0.533 ± 0.015^{bc}	13.723 ± 0.155^{cd}	2.233 ± 0.130^{abc}	30.45	2.16×10^7	1.78×10^5	0	0	8.17
T3	0.590 ± 0.026^{a}	50.167 ± 0.674^{a}	0.411 ± 0.006^{b}	0.453 ± 0.035^{cd}	14.390 ± 0.229^{c}	1.670 ± 0.356^{bc}	29.55	7.21×10^7	1.02×10^6	0	0	8.40
T4	0.457 ± 0.025^{b}	41.470 ± 1.454^{b}	0.283 ± 0.001^{e}	0.567 ± 0.012^{b}	17.553 ± 1.228^{b}	2.317 ± 0.268^{ab}	26.85	3.66×10^7	1.35×10^5	0	0	7.79
T5	0.543 ± 0.025^{a}	32.293 ± 1.713^{d}	0.121 ± 0.001^{f}	0.443 ± 0.028^{d}	11.553 ± 0.303^{e}	2.363 ± 2.550^{a}	28.55	4.90×10^7	3.42×10^5	0	0	8.29
T6	0.367 ± 0.021^{c}	41.558 ± 1.462^{b}	0.350 ± 0.002^{d}	0.527 ± 0.025^{bc}	20.393 ± 0.192^{a}	1.597 ± 0.254^{c}	27.95	6.01×10^7	3.33×10^5	0	0	7.57
T7	0.523 ± 0.032^{ab}	36.243 ± 0.456^{cd}	0.392 ± 0.001^{c}	0.747 ± 0.042^{a}	12.137 ± 0.857^{de}	1.823 ± 0.212^{abc}	26.20	4.52×10^7	4.48×10^5	0	0	7.76

Note: Means with different letters in the same column are not significant difference at 95% confidential level (Duncan's test).

Figure 7. The digital images of microbial organic fertilizers of T1, T2, T3, T4, T5, T6, and T7

In conclusion, organic fertilizer considers a great supplement for farming soil in the current conditions that have negative effects in agriculture, and it brings to a sustainable production. The isolated *Trichoderma reesei* CN4-VR11 was demonstrated to have potential applications in microbial organic fertilizer, which open the way for the use of local microbiota in agricultural research and sustainable development. According to the general standard, the T5 and T7 treatments were the best choices for the production of granular bio-compost, which has more practical use in agricultural land. These formulas will highly contribute to farmer's income as an extra profit from agricultural by-products.

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