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Isolation of *Trichoderma* strains from rhizospheric soil and assessment of their potential for biofertilizer from freshwater aquaculture pond sediment

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Abstract. *Thi QVC, Nhan TH, Hung NVK, Thuy NP. 2024. Isolation of* Trichoderma *strains from rhizospheric soil and assessment of their potential for biofertilizer from freshwater aquaculture pond sediment. Biodiversitas 25: 2866-2876. Trichoderma* spp. are filamentous fungi found in almost all soils and can synthesize massive quantities of cellulase enzymes which can simply break down cellulose polysaccharides. Freshwater water aquaculture pond sediment has caused some environmental pollution which is required to be properly dealt with. The objective of this study was to isolate *Trichoderma* strains from rhizosphere soil and to evaluate their potential for biofertilizer from freshwater aquaculture pond sediments. Twenty-six strains of *Trichoderma* spp. were isolated from soil. The Carboxymethyl Cellulose (CMC) degrading ability of the S25 strain was extremely strong, with a halo diameter zone measuring 8.9±3.8 cm. According to the BLAST result, there was 99.11% similarity between S25 strains and *Trichoderma asperelloides*, with a max score of 1011 and an E-value of 0.0. The *T. asperelloides* S25 was selected for bioproduct production. The use of *T. asperelloides* S25 bioproduct passively enhanced the efficient decomposition of organic fertilizers. After just 30 days of incubation, total organic matter content was ≥ 30% and material had a spongy, soft, and brown-black in color. According to the Government of Vietnam's Decree No. 14/2019/ND-CP on fertilizer management, dated September 14, 2019, all treatments were chosen for the production of granular bio-compost. These findings highlight the potential of bioproduct *T. asperelloides* S25 for sustainable aquaculture practices and organic waste management.

Keywords: Biofertilizer, coco peat, cow dung, shrimp pond, sludge, *Trichoderma*

INTRODUCTION

Vietnam is one of the top ten nations with the highest overall production of wild production and aquaculture farmed (FAO 2020). It is crucial for the agriculture sector's economic restructuring plan as well as for the accomplishment of efforts to combat hunger and lower poverty in various areas of the country (Nguyen et al. 2019; Tri et al. 2021). Aquaculture produced 4.49 million MT of the 8.27 million MT total fisheries productivity in 2019; 3.14 million MT came from cultured fish, and 899.84 thousand MT came from cultured shrimp (GSO 2021). However, the research has indicated that many aquaculture farms were established in an unsustainable way with little consideration for protecting the environment (Anh et al. 2010). An estimated roughly one million tonnes of agricultural by-products are produced from fisheries (Nam 2022). The water quality of aquatic habitats is compromised by the discharge of wastewater containing rich nutrients and organic matter from shrimp farms and snakehead fish (Puspaningsih et al. 2018; Bull et al. 2021**)**. Sludge with a 35-60 t/ha/shrimp manufacturing process can be produced by the cultivation method (Suwoyo et al. 2020). The research reported 50.12 g, 15.73 g, and 126.85 g of total N/kg, total P/kg, and C organic/kg shrimp, respectively, from whiteleg shrimp production with a stocking density of 500 ind./m^2 in 1000 m^2 ponds (Kraemer et al. 2019). Furthermore, Dróżdż et al. (2020a) reported that the characteristics of fish pond sediments included nitrogen (1.08-7.03 g/kg), carbon (18.3-92.3 g/kg), potassium (0.62-2.25 g/kg), and phosphorus (0.22-2.07 g/kg).

Considering its high concentrations of organic matter, nitrogen, and phosphorus, pond sediments are a potential organic material that can be utilized as fertilizer (Da et al. 2020, 2021; Dróżdż et al. 2020b). To reduce the waste disposal volume and environmental degradation as well as increase land productivity, organic fertilizer production from solid waste and subsequent use in agriculture and fisheries are highly recommended (Suwoyo et al. 2020). Recently, methods to solve the problem of agricultural wastes and model of compositing microbial organic fertilizer have attracted attention. Microbial technology has been applied effectively in the treatment of agricultural and municipal waste (Hidalgo et al. 2022). Previous studies have been reported in China, Poland, Vietnam, Thailand, and Bangladesh that have concerned on the wastewater and sludge/sediment recycling method from catfish pond farming and composting fish pond sludge/sediment mixed with agriculture waste residues as organic fertilizers for paddy rice, common vegetables, and fodder grass to assist to reduce water pollution, while still improving agricultural production and soil quality (Haque et al. 2016; Dróżdż et al. 2020b; Da et al. 2021).

Furthermore, it has been reported that *Trichoderma* spp. synthesize a variety of substances that promote plant development, including enzymes and phytohormones (Illescas et al. 2021). For the biodegradation of agricultural byproducts, such as coco peat, rice straw, and peanut stem,

Trichoderma spp. has been used due to its ability to synthesize cellulase enzymes (Nayak and Mukherjee 2015). While a variety of fungal strains can secrete cellulase, *Trichoderma* species produce larger quantities of cellulase enzymes (de Souza et al. 2018). Research on the utilization of *Trichoderma* for using in biofertilizer compost degradation is highly interesting. The development of organic and sustainable agriculture benefit enormously from the low-cost, ecologically harmless, large-scale manufacture of *Trichoderma*-assisted organic fertilizer derived from local agricultural byproducts (Korkom and Yildiz 2022, 2024). The organic decomposition of bioremediation has been reported to occur through incubation of *Trichoderma* spp., which accelerates the decomposition procedure (Siddaiah et al. 2017). *Trichoderma* spp. is one of the most extensively studied fungi in terms of isolating and characterizing other possible strains and species. Currently, *Trichoderma asperellum* strain Ta13 was identified by using morphology and molecular analysis (tef1, rpd2 act, genes cal, and ITS) (Xue et al. 2021; Matas-Baca et al. 2022). Furthermore, twelve *Trichoderma reesei* strains, which were previously exclusively found in tropical locations, were discovered in Austrian soil (Hinterdobler et al. 2021). Based on these reason, the objective of this study was to isolate *Trichoderma* strains from rhizosphere soil and to evaluate their potential for biofertilizer from freshwater aquaculture pond sediments.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from twenty-six rice, peanut, vegetable, and fruit crop farms in Tra Vinh Province's five districts of Tra Cu, Cau Ke, Cau Ngang, Cang Long, and Chau Thanh (Table 1). Soil samples were obtained from the rhizosphere by digging at depths ranging from 0 to 20 cm, following perpendicular lines within each sampling area. To ensure a representative sample of the microbial diversity in each area, five subsamples were taken from distinct locations within the region and thoroughly mixed to form a composite sample. This composite sample was labeled with the relevant collection details, including the name, date, location, and any notable properties of the sample.

Isolation and characterization of cellulose-degrading *Trichoderma*

90 mL of sterile distilled water was added to a 250 mL Erlenmeyer flask holding 10 g of sample. Each flask was incubated at 30°C for 60 minutes in a shaking incubator at 150 rpm. Soil samples were plated on *Trichoderma* Selected agar Medium (TSM) using serially diluted technique (Elad et al. 1981). The spread plate technique was used to inoculate 0.1 mL of each soil samples, and plates were incubated at 30°C for 5 days (Boczek et al. 2014). On TSM, colonies with a clear zone were picked and sub cultured three times. The pure isolates were cut into small plugs 2mm in diameter from fresh vigorously growing mother culture (TSM) and transferred to a sterile solution of 20% glycerol for long-term storage at -75°C for further analysis.

Morphological analysis

A morphological study of cellulose-degrading *Trichoderma* colonies was performed after 5 days, with slower growing species require long time. Morphology was examined for following characteristics: colony color, diameter, pigment secretion, colony shape, and other distinguishing traits. The microscopic features were recorded when the colony was matured and measured 0.5-1 cm in diameter. One side of the colony was cut into a rectangular shape measuring 3-4 cm 1.5-2 cm with a sterilized knife. It was then cultivated on Potato Dextrose Agar (PDA) for two to three days before being observed under a microscope. Furthermore, a small portion of fungus was kept on slide and then added 2-3 drops of lactophenol cotton blue so that reproductive structure could be observed at 40× and 100× magnification. The taxonomic keys proposed by Bissett et al. (2015) was followed for the identification.

Cellulose degradation analysis

Trichoderma was examined for cellulose degradation activity by using point culture technique on Czapek Dox Agar media containing carboxyl methyl cellulose (1%) (Cz + 1% CMC), and was cultured for five days at room temperature with three replications. The size of halo zones was used to assess the magnitude of cellulose degradation activity. Following the determination of D - d, the Lugol reagent was applied to agar surface to test the cellulose breakdown capacity of the isolated *Trichoderma* sp. (Nandini et al. 2021).

Table 1. Collection sites in Tra Vinh Province of Vietnam

Plants	Places
Chau Nghe Mango	Cang Long District, Tra Vinh Province
Chau Nghe Mango	Cang Long District, Tra Vinh Province
Chau Nghe Mango	Cang Long District, Tra Vinh Province
Water spinach	Tra Vinh City, Tra Vinh Province
Vegetable spinach	Tra Vinh City, Tra Vinh Province
King orange	Cau Ke District, Tra Vinh Province
King orange	Cau Ke District, Tra Vinh Province
King orange	Cau Ke District, Tra Vinh Province
Rice	Tra Cu District, Tra Vinh Province
Rice	Tra Cu District, Tra Vinh Province
Rice	Tra Cu District, Tra Vinh Province
Guava	Chau Thanh District, Tra Vinh Province
Custard apple	Chau Thanh District, Tra Vinh Province
Cat Chu Mango	Cau Ke District, Tra Vinh Province
Cat Chu Mango	Cau Ke District, Tra Vinh Province
Cat Chu Mango	Cau Ke District, Tra Vinh Province
Vegetable spinach	Cau Ke District, Tra Vinh Province
MD7 Peanut	Duyen Hai District, Tra Vinh Province
MD7 Peanut	Duyen Hai District, Tra Vinh Province
MD7 Peanut	Duyen Hai District, Tra Vinh Province
Vegetable spinach	Cau Ngan District, Tra Vinh Province
MD7 Peanut	Cau Ngan District, Tra Vinh Province
MD7 Peanut	Cau Ngan District, Tra Vinh Province
MD7 Peanut	Cau Ngan District, Tra Vinh Province
Leaf mustard	Chau Thanh District, Tra Vinh Province
Lady finger	Chau Thanh District, Tra Vinh Province

Table 2. The components of compost treatment

Treatments	Cow manure (kg)	Sludge from snake-head fish pond (kg)	Sludge from shrimp pond (kg)	Coco peat (kg)	Rice bran (kg)	Molasses (kg)	CaO powder (kg)	Bioproduct of *Trichoderma* (kg)
T1	60	10		30	5	1	1	0.1
T2	60	20		20	5	1	1	0.1
T3	60	30		10	5	1	1	0.1
T4	60		10	30	5	1	1	0.1
T5	60		20	20	5	1	1	0.1
T6	60		30	10	5	1	1	0.1

Molecular identification of *Trichoderma* strain

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). The Internal Transcribed Spacer (ITS) region on fungal DNA was selected for amplification. The universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used for analysis. 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 process consisted 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 (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. PCR products were purified and the fungal DNA sequences were done by Next Gen Scientific Co., Ltd (Ho Chi Minh City). The ITS region gene sequence was analyzed using the The National Center for Biotechnology Information (NCBI). The Basic Local Alignment Search Tool (BLAST) program according to E-value, % homology, and query coverage. The ITS region sequence acquired in the current study in the GenBank database of the NCBI database to receive a corresponding number (Benson et al. 2018).

Bioproduct of *Trichoderma*

Trichoderma was proliferated in Erlenmeyer flask containing 50 g substrate, which was composed of 60% corn starch and 40% coco peat with 60% moisture for five days. The formulation of 1-kilogram bioproduct of *Trichoderm*a powder was as follows: 60% rice bran, 30% corn starch, 10% 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).

Organic fertilizer produced by *Trichoderma* sp.

The cow manure, sludge from a snakehead fish pond, shrimp pond, coco peat, rice bran, and bioproduct of *Trichoderma* sp. were used in the present study. A mixture of cow dung and substrate (coco peat, sludge from snakehead fish and shrimp ponds) was used to make the compost, which was then decomposed by pre-prepared *Trichoderma* sp. powder to produce biofertilizer (Table 2). The experiment was conducted in Randomized Complete Block Design (RCBD) consisting of one factor, six treatments, and three replications. First, wet sludge from cultured snakehead fish and shrimp ponds were sundried for 7 days. In the next step, a layer of cow dung, 20 to 30 centimeters deep, was spread out. Next, a thin coating of rice bran and CaO powder was applied to the manure's surface. This was followed by a layer of substrate, molasses, and *Trichoderma* sp. bioproduct. The mixed pile had a height of 0.4-0.6 meters and a diameter of 0.7-1 meters. The compost was covered and incubated for 30 days. Microbial organic fertilizer standard followed in execution of quality evaluation (No. 14/NĐ-CP 14/11/2019).

The following criteria were used to analyze and compare the quality of bio fertilizer: Organic matter content P available content (%), total P content (%), total N content (%), total K content (%), density of *Trichoderma* spp. (CFU/g dried soil), the density of aerobic microbes (CFU/g dried soil), colony number of *Bacillus* spp. (CFU/g dried soil), colony number of *Salmonella* (CFU/g dried soil), colony number of *Escherichia coli* (CFU/g dried soil), pH, humidity (%), and humic acid content (%).

Statistical analysis

One-way ANOVA with Randomized Complete Block Design (RCBD) was used to examine the variance for various criteria (Panse and Pandurang 1954). For analysis, "Windostat" software was used. The significance criterion was set at 5% using the F-test. If a treatment test demonstrates a significant impact between treatments for descriptive analysis, Duncan's follow-up test (DMRT) was employed to evaluate the effect between levels.

RESULTS AND DISCUSSION

Isolation and characterization of cellulose-degrading *Trichoderma* spp.

A total of 26 strains of *Trichoderma* spp. were successfully isolated from 26 soil samples collected from various freshwater aquaculture pond farming models, utilizing *Trichoderma* Selective Medium (TSM). The soil pH across sampling sites ranged from 6.4 to 7.2 (Table 3), demonstrating the adaptability of *Trichoderma* spp. to a range of slightly acidic to neutral soil conditions. Morphological characterization of the isolated fungal colonies revealed considerable diversity. Colony color, texture, shape, and margin exhibited variations, as did characteristics observed on both the front and back surfaces of the agar plates. Notably, all colonies exhibited a woolly and dense appearance, a common trait of *Trichoderma* spp. Two distinct colony shapes were recorded: irregular round (59.8%) and regular (40.2%), highlighting morphological variability within the isolates. Colony color distribution was as follows: white (21.5%), yellow (24.2%), and blue (54.3%) (Figure 1). The predominance of blue-colored colonies was noteworthy, as it suggests potential dominance of specific *Trichoderma* species or strains known for this pigmentation.

All *Trichoderma* isolates displayed rapid growth on PDA media, reaching diameters between 7.8 and 9.9 cm within three days. Conidiophore development varied among strains. Strains S8, S13, S20, and S25 exhibited early sporulation, initiating within two days, while strain S4 displayed delayed sporulation, taking 8-10 days for conidiophore emergence. Interestingly, strain S7 displayed a unique color change, transforming from bright blue to dark yellowish-green spores during maturation. Strains S12 and S20 also possessed a distinct characteristic, altering the color of growth media, unlike other isolates. Colony morphology differed as well. While strains S27 and S9 displayed scattered growth patterns, most isolates produced abundant conidiophores that covered the entire culture plate. Microscopic examination of conidiophores of strains S8, S13, S20, and S25 at 40× magnification is presented in Figure 2. Conidiophores displayed either simple or complex branching patterns. Simple conidiophores possessed a single main axis with lateral branches, while complex structures exhibited multiple branching levels. Flask-shaped phialides were readily apparent, often clustered at the terminal ends of the conidiophores. Conidia, observed in clusters or chains, appeared as small, oval, or ellipsoidal structures with green or blue-green coloration.

Degradation of CMC

Twenty-six isolated *Trichoderma* strains demonstrated the ability to produce cellulase, an enzyme that breaks down cellulose, a major component of plant matter. This enzymatic activity was evident by the formation of clear zones (halos) around the fungal colonies on agar plates Containing Carboxymethyl Cellulose (CMC) (Table 3). The diameters of these halos varied significantly ($p<0.05$) among the strains, ranging from 2.6±1.2 cm to 8.9±3.8 cm. Notably, six strains (S25, S21, S19, S12, S2, and S6) exhibited particularly large halos, exceeding 7.0 cm in diameter. In contrast, strains S23, S10, and S15 produced the smallest halos, all under 3.0 cm. Given its exceptional cellulase activity, as evidenced by the largest halo of 8.9±3.8 cm, strain S25 was selected for further investigation into its potential as a biofertilizer component. The ability of S25 to rapidly degrade cellulose suggests that it could play a significant role in accelerating the decomposition of organic matter in aquaculture pond sediment.

Molecular identification

Among the 26 strains, strain S25 with the highest cellulose degradation activity was selected as a candidate. The 5.8S-ITS region of fungal was amplified by using the specific primers ITS1 and ITS4 and PCR amplification products were sequenced. The sequence information was submitted to the GenBank database and OR856642 was their accession number. Then, *Trichoderma* species identification was determined using the BLAST tool. According to the sequence alignment, 622 nucleotides were aligned with 557/562 nucleotide similarity. According to the BLAST result, there was a 99.11% similarity between the S25 strains and *Trichoderma asperelloides*, with an E-value of 0.0 and a Max score of 1011. This verified that the isolate identified in this research, which was identified as *T. asperelloides* S25, belonged to *T. asperelloides*.

Table 3. CMC degradation ability of isolated *Trichoderma* spp. strains

Items	pH of soil	Diameter of zones (cm)
S1	6.8	6.3±3.1
S2	6.6	7.2±1.5
S3	6.8	4.8±2.5
S4	6.9	6.1±1.8
S5	6.8	4.2±2.6
S6	6.8	7.0±2.4
S7	6.5	6.5±3.9
S8	6.5	5.2±1.3
S9	7.2	6.9±4.0
S10	6.4	2.9±4.5
S11	6.8	4.2±3.5
S12	7.1	7.5±1.5
S13	6.8	4.4±2.6
S14	6.6	6.2±3.9
S15	6.7	2.6±1.2
S16	6.6	6.2±2.6
S17	6.9	5.2±2.0
S18	6.8	4.7±4.9
S19	6.8	7.6±3.2
S20	6.9	6.6±1.7
S21	6.9	7.7±1.5
S22	6.8	6.7±4.9
S23	6.6	3.0±1.2
S24	6.8	6.6±1.5
S25	6.9	8.9±3.8
S26	6.9	6.5±2.3

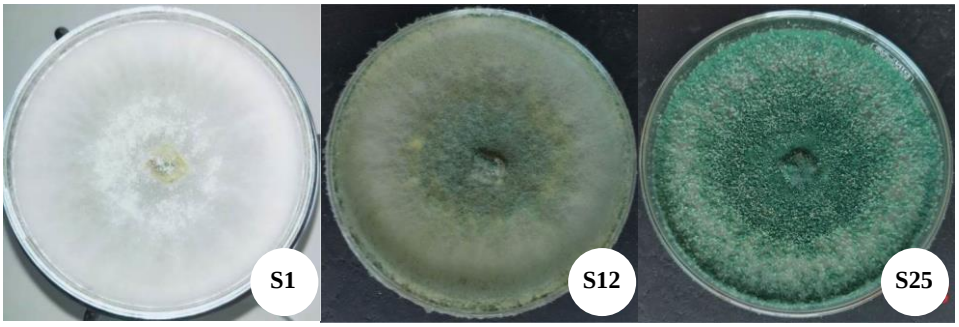

Figure 1. Colony appearance of *Trichoderma* spp. grown for 96 h at 28°C

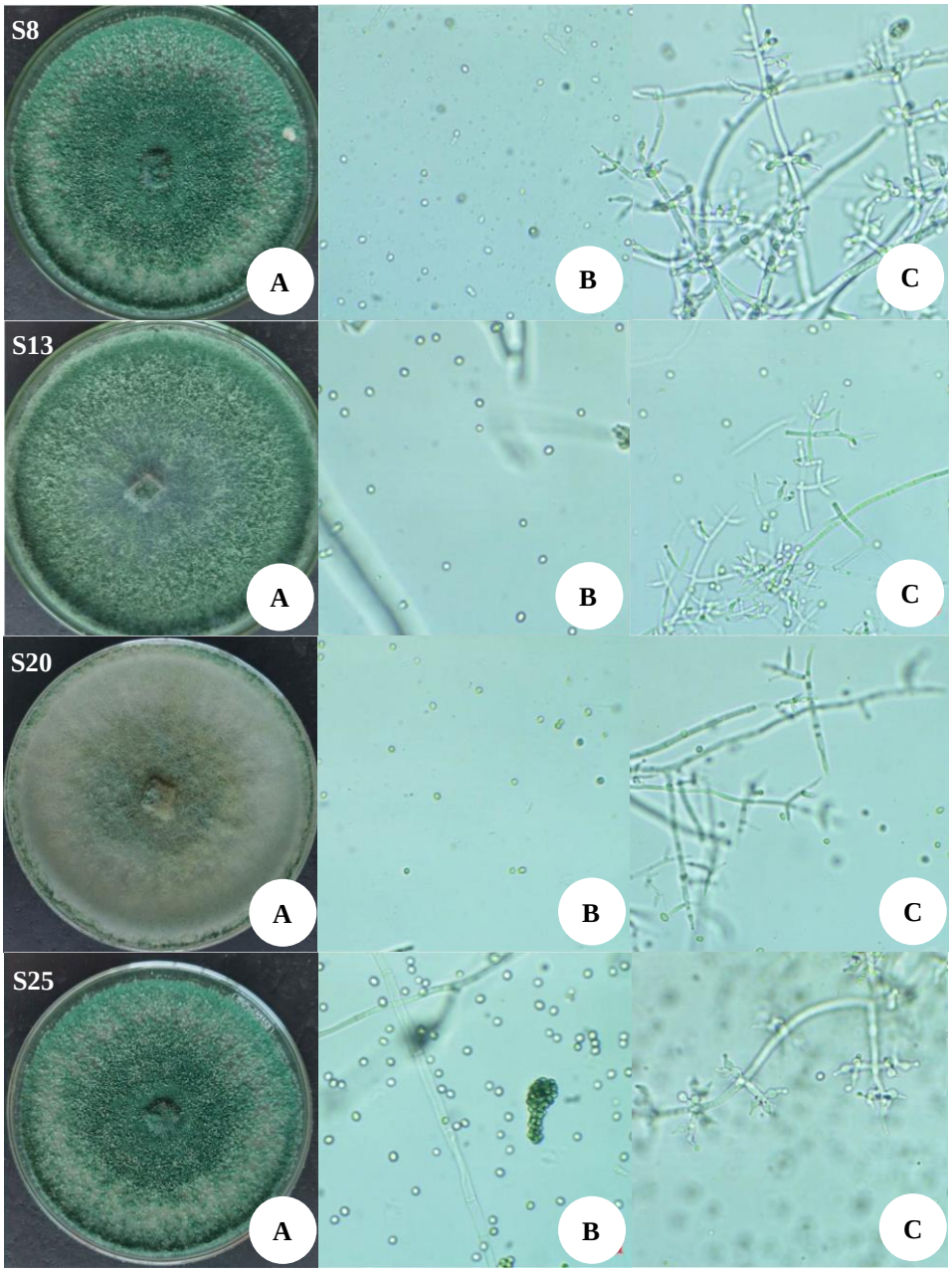

Figure 2. The morphology of isolated *Trichoderma* S8, S13, S20, and S25 strains colony appearance on: A. PDA; B. Conidia; C. Conidiophores

Bioproduction of *Trichoderma*

The final bioproduct of *T. asperelloides* S25 achieved a density of 4.7×10^8 CFU/g.

Production of organic fertilizer by isolated *Trichoderma*

Temperature, pH, and moisture content

The results of experiment change in compost pH over time for the different treatments (Table 4). All treatments showed alkaline pH throughout the composting process, in the range of 7.06 (T1)-7.99 (T6). Figure 3 shows the temperature fluctuation of the compost pile throughout the composting process. With the assistance of *Trichoderma* sp., the compost's temperature ascended two-fold after five days of incubation, from 32.00°C on day 0 to 60.40°C (T6)-62.00°C (T1). Temperature was lowered after incubation from 10 to 25 days. Following 30 days of incubation, the temperature decreased to 36.20°C (T6)-39.40°C (T5), which was the approximate ambient temperature. Changes in values of moisture content that increased on day 5 of the composting process after decreasing on the latter day for all treatments (Figure 4). The initial moisture content was 75%. The highest moisture level ranged from 81.27% (T2) to 84.47% (T5) on day 5, while the lowest moisture content ranged from 35.60% (T1) to 47.20% (T5) on day 30. The maximum moisture level for T5, T3, and T6 occurred on day 5, with 84.47%, 83.93%, and 83.07%, respectively. On day 10, the lowest moisture content for T6, T1, and T2 was 74.07% and 79.07, respectively. Finally, on day 30, the lowest moisture level for T1 and T2 were 35.60% and 40.00%, respectively.

Microbial density in organic microbe fertilizer

Table 4 shows the microbiological enumeration results of the compost. T4 had the highest density of *Trichoderma* sp. (6.0×10^6 CFU/g dried soil), whereas T5 had the lowest density (3.3×10^6 CFU/g dried soil). T3 had the highest density of *Bacillus* sp. (3.9×10^6 CFU/g dried soil), whereas T5 had the lowest density (2.3×10^6 CFU/g dried soil). The density of aerobic microbes diverged from 7.8×10^7 CFU/g dried soil (T1) to 9.3×10^7 CFU/g dried soil (T3). All treatments were not found *Salmonella* and *E. coli*.

C-organic, nitrogen, potassium, phosphor, phosphor available content and C/N ratio

The results of C-organic, potassium, nitrogen, phosphor, and phosphor available from all of the treatments revealed statistically significant differences ($P\leq0.05$) (Table 4). The freshwater aquaculture pond sediment samples showed significant changes ($p>0.05$) in C-organic, with the highest value at T4 (27.67±1.44%) and the lowest at T2 (13.38±0.23%). The nitrogen content was highest in T4 (1.20±0.05%), and T5 (1.14±0.04%), which was not significant ($p>0.05$). The percentage of nitrogen was lowest at T1 (0.90±0.00 %), and was not significant ($p>0.05$) with the values of T2 (0.91±0.10 %) and T3 (0.96±0.01 %). The potassium of composting experiment showed significant differences ($p>0.05$). T2 and T6 had the highest potassium levels (2.04±0.02 and 1.99±0.12), respectively. T3 and T1 had the lowest potassium levels (1.49±0.01 and 1.56±0.02). T2 had the highest percentage of phosphorus (1.42±0.10 %), while T1 had the lowest (1.02±0.01%). There was no significant difference T6 (1.05±0.03%), T5 (1.10±0.07%), T4 (1.10±0.09%) and T3 (1.15±1.15%). Phosphorus availability was found to be highest at T3 (0.88±0.01%), but not significantly different from T2 (0.86±0.04%), lowest at T4 (0.38±0.01%), and not significantly different from T1 (0.64±0.03%), T5 (0.67±0.02%), and T6 (0.67±0.02%). There were significant differences in composting experiment's C:N ratio. T4 had the highest percentage of C:N (23.05±0.27%), While T6 had the lowest (14.76±0.01%) C: N ratio.

Total organic matter, fulvic acid, humic acid content

Table 4 demonstrated the results of total organic matter, fulvic acid, and humic acid content in the treatment variation that indicated statistically significant changes ($P\leq0.05$). The ranges of concentration of total organic matter was 23.05±0.27%, 40.39±0.01%, 37,66±0.15%, 37.44±0.58%, 35.99±0.11%, and 32.48±0.02% in T4, T1, T5, T6, and T7, respectively. The minimal total organic matter (32.48±0.02%) was recorded in the T7 treatment, while maximal concentration (23.05±0.27%) was recorded in T4 treatment. The composting experiment's humic acid exhibited significant changes ($P\leq0.05$). The values of humic acid varied from 4.35±0.02%, 4.20±0.04%, 4.16±0.09%, 3.87±0.05%, 3.58±0.01%, and 2.99±0.02% in T4, T6, T3, T5, T2, and T1 treatment, respectively. The lowest value of humic acid was in T1 treatment (2.99±0.02%), while maximal value (4.35±0.02%) was recorded in T4. From these results, fulvic acid observed in T4 (4.38±0.08%) was better than T6 (4.25±0.05%), T5 (4.13±0.05%), T1 (3.67±0.12%), T3 (3.88±0.10%), and the lowest light fulvic acid was in the T2 treatment (1.78±0.03%).

The ease of isolating *Trichoderma* from various substrates, including soil and roots, is well-established, due to its rapid growth, prolific sporulation, and efficient colonization of organic matter (Gupta et al. 2014). Our study, yielding 26 *Trichoderma* isolates from diverse freshwater aquaculture pond sediments, aligns with global findings from various crops and soils. This reaffirms the widespread presence of *Trichoderma* in different environments, including groundnut fields in India (Jaisani and Pandey 2017), rice, okra, pepper, and tomato fields in Pakistan (Anees et al. 2018), and orange orchards in Argentina (Ferreira et al. 2020).

Trichoderma Specific Medium (TSM) has been shown to enhance *Trichoderma* isolation, outperforming other media with a 120-140% growth rate (Attitalla et al. 2012). This superior performance supports its use in future research on *Trichoderma* viability and soil growth. (Alwadai et al. 2022) further confirmed this, reporting varying fungal densities on PDA (16×10^2 to 45.3×10^2 CFU/g) and TSM (8.3×10^2 to 83×10^2 CFU/g) from different soil samples.

Morphological analysis of our isolates, consistent with (Dong and Nguyen 2000), confirmed the suitability of Potato Dextrose Agar (PDA) for *Trichoderma* spp. cultivation, as noted by Oszust et al. (2021). Microscopic examination revealed distinct hyphae, phialides,

conidiophores, and conidia, further validating previous findings (Awad et al. 2018; Mukhopadhyay and Kumar 2020). The conidiophores were compact, branched, and hyaline, with phialides occasionally dispersed or separated from them. The conidia, located near the phialide tips, exhibited round, smooth, or rough surfaces.

PCR amplification using ITS1 and ITS4 primers produced a single 500-600 bp product, consistent with previous studies (Kim et al. 2023) and confirming the reliability of ITS regions for species-level identification of fungi (El-Sobky et al. 2019). Electrophoresis results validated the suitability of ITS PCR products for sequencing the ITS regions of isolated *Trichoderma*.

Molecular markers, particularly those based on DNA sequence data, are invaluable for assessing genetic diversity within species (Bellemain et al. 2010). Our interspecies identification, utilizing multiple isolates, revealed variations in ITS sequence length and composition, indicating varying levels of genetic differentiation. This enabled us to differentiate distinct species, echoing previous work (Kuhls et al. 1997) that utilized sequence analysis to distinguish between *T. reesei* and *T. longibrachiatum*. Similar approaches have been employed in numerous studies for *Trichoderma* spp. identification using ITS sequences (Shahid et al. 2013). The observed intraspecies variation emphasizes the importance of molecular identification for accurate characterization of microorganisms, as also reported by others (Fahmi et al. 2016; Hassan et al. 2019; Mazrou et al. 2020).

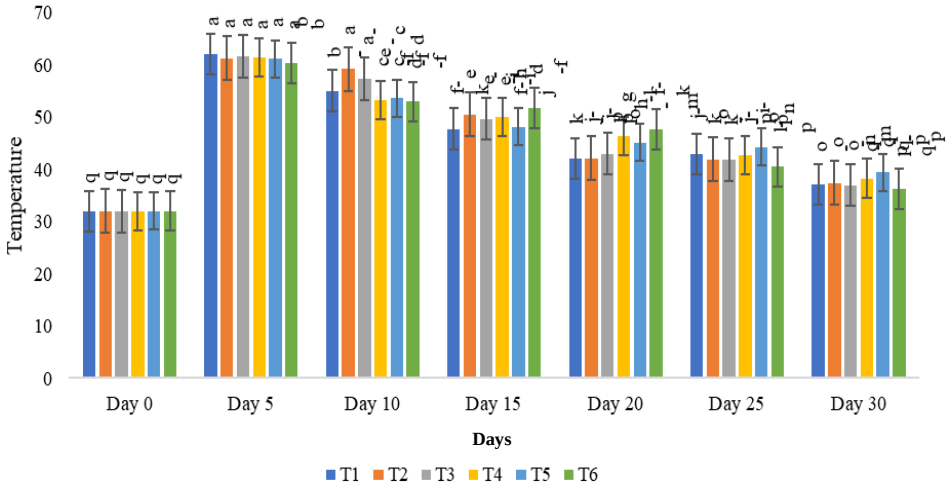

Figure 3. Temperature analysis during 30 days composting process. Note: Values followed by the same letter (s) were not significantly different at P≤0.05 according to the Duncan's multiple range test

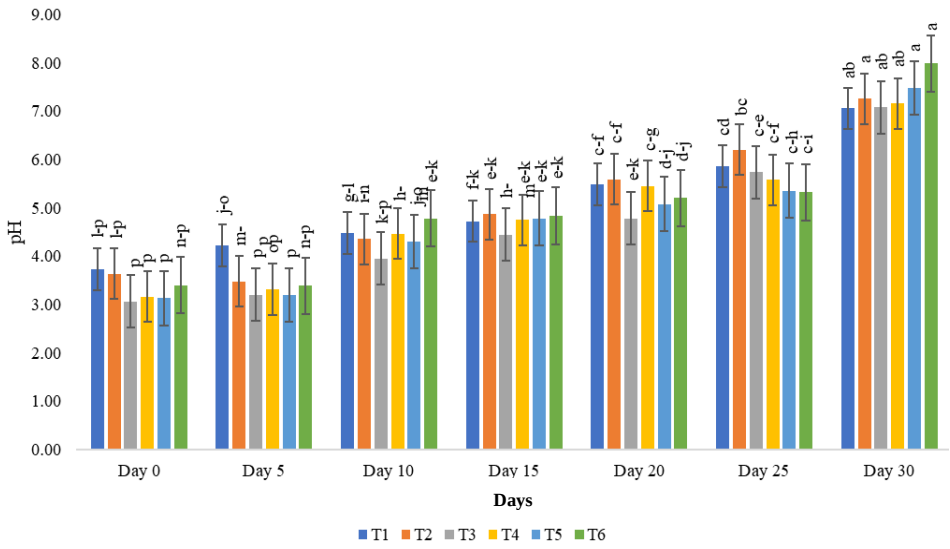

Figure 4. Moisture analysis during 30 days composting process. Note: Values followed by the same letter (s) were not significantly different at P≤0.05 according to the Duncan's multiple range test

Table 4. Microbe organic fertilizer evaluation criteria

Treatments	C-organic content	Total N (%)	Total K (%)	Total P (%)	Total P available (%)	C/N ratio	Total organic matter (%)	Humic acid (%)	Fulvic acid (%)	Density of aerobic microbes (CFU/g)	Density of *Trichoderma* (CFU/g)	Density of *Bacillus* sp. (CFU/g)	Density of *E. coli* (CFU/g)	Density of *Salmonella* (CFU/g)	pH
T1	16.52 ± 0.18 cd	0.90 ± 0.01 c	1.56 ± 0.02 cd	1.02 ± 0.01 c	0.64 ± 0.03 b	18.36 ± 0.00 b	40.39 ± 0.01 b	2.99 ± 0.02 e	3.67 ± 0.12 d	9.3×10^7	4.2×10^6	3.6×10^6	0	0	7.06
T2	13.38 ± 0.23 f	0.91 ± 0.02 c	2.04 ± 0.02 a	1.42 ± 0.10 a	0.86 ± 0.04 a	14.76 ± 0.01 e	32.48 ± 0.02 e	3.58 ± 0.01 d	1.78 ± 0.03 e	8.9×10^7	4.6×10^6	3.8×10^6	0	0	7.26
T3	16.34 ± 0.14 d	0.96 ± 0.01 c	1.49 ± 0.01 d	1.15 ± 0.05 b	0.88 ± 0.01 a	17.02 ± 0.26 c	37.44 ± 0.58 c	4.16 ± 0.09 b	3.88 ± 0.10 d	9.3×10^7	5.9×10^6	3.9×10^6	0	0	7.08
T4	27.67 ± 1.44 a	1.20 ± 0.05 a	1.66 ± 0.05 c	1.10 ± 0.09 bc	0.38 ± 0.01 c	23.05 ± 0.27 a	50.72 ± 0.60 a	4.35 ± 0.02 a	4.38 ± 0.08 a	7.8×10^7	6.0×10^6	3.2×10^6	0	0	7.16
T5	19.57 ± 0.71 b	1.14 ± 0.04 a	1.80 ± 0.05 b	1.10 ± 0.07 bc	0.67 ± 0.02 b	17.12 ± 0.07 c	37.66 ± 0.15 c	3.87 ± 0.05 c	4.13 ± 0.05 c	9.0×10^7	3.3×10^6	2.3×10^6	0	0	7.48
T6	17.67 ± 1.15 c	1.08 ± 0.07 b	1.99 ± 0.12 a	1.05 ± 0.03 bc	0.67 ± 0.02 b	16.36 ± 0.05 d	35.99 ± 0.11 d	4.20 ± 0.04 b	4.25 ± 0.05 b	8.9×10^7	5.4×10^6	3.4×10^6	0	0	7.99

Note: Means with different letters in the same column did not differ significantly at the 95% confidence level (Duncan's test)

Extensive research has explored the impact of additives on composting, revealing their effects to be multifaceted and influenced by factors such as dosage, substrate composition, oxygen availability, pH, C/N ratio, and moisture content (Reyes-Torres et al. 2018). Temperature is a key driver of microbial activity during composting, though other factors like C/N ratio, moisture, aeration, and pH are crucial for microbial adaptation (Ugak et al. 2022). (Koul et al. 2022) delineated the composting process into four distinct temperature phases: latent, growth, thermophilic, and maturation. The degree of temperature fluctuation reflects the heterogeneity of the composting process and the diversity of active microbial populations.

Temperature within the compost mass not only influences microbial growth and metabolism but also plays a role in eliminating harmful organisms like helminths, bacteria, and protozoa (Waszkielis et al. 2013). The result of present study observed thermophilic phase temperatures consistent with those reported by Cao et al. (2020), reaching a peak of 62.00°C within five days in treatment T1. Similar self-heating patterns during the mesophilic and thermophilic stages have been documented, with temperatures remaining above 55°C for 30 days before gradually declining during cooling and maturation (Meng et al. 2019). The thermophilic phase is crucial for pathogen elimination and waste stabilization, with metabolic heat gradually dissipating through compost biodegradation. Notably, the addition of *Trichoderma* sp. in present study expedited temperature increase, reaching 62.00℃ by day 5, suggesting its potential as a compost activator.

pH is another critical parameter for evaluating compost maturity. Azim et al. (2018) observed the production of organic acids during early decomposition stages, favoring fungal growth and cellulose/lignin breakdown. Mature compost typically exhibits a near-neutral pH of 7-8 due to organic acid neutralization. Our study's pH progression mirrored that of (Hashim et al. 2022), reaching near-neutral levels after 98 days. Zhang and Sun (2016) identified a pH range of 5.5-8.0 as optimal for microbial activity, cautioning that acidic conditions can impede degradation rates.

Moisture content, as highlighted by Pezzolla et al. (2021), serves as a medium for nutrient transfer, influencing porosity, temperature, oxygen uptake, and microbial activity. Our moisture levels ranged from 35.60% (T1) to 47.20% (T5), aligning with Jain et al. (2019), who reported a 57% moisture content in finished compost. A decrease in moisture content over time is indicative of decomposition and compost maturation.

Microbial community dynamics, including those of *Trichoderma* sp., offer insights into the degradative capacity of the compost mix (Ling et al. 2014). Results demonstrated that *Trichoderma* sp. densities exceeding 1×10^6 CFU/g in all treatments after 30 days, underscoring its role in the composting process. This fungus releases hydrolytic enzymes to break down complex molecules, generating readily available compounds that enhance agricultural potential and ecological balance (Awasthi et al. 2015; Rastogi et al. 2020).

Nitrogen, phosphorus, and potassium content are key indicators of organic fertilizer quality. Our results revealed variations in total nitrogen content depending on the source of sludge, with shrimp pond sludge yielding higher levels compared to snakehead fish pond sludge. Overall, our organic fertilizer boasted higher potassium, phosphorus, and nitrogen levels compared to those reported by Thanh et al. (2023).

The C/N ratio is fundamental for establishing a balanced compost mix. Temporal changes in the C/N ratio reflect organic matter degradation rates, with carbon conversion to CO2 being a key driver. A finished C/N ratio below 20 typically indicates stabilization and maturity (Damaceno et al. 2021), although this should not be considered an absolute indicator (Vico et al. 2018). Marhuenda-Egea et al. (2007) suggested a C/N range of 15-25 around day 20 of composting. Our study observed similar C/N ratios ranging from 14.76±0.01% (T6) to 23.05±0.27% (T4), showcasing compost-specific variations. This contrasts with Chang et al. (2023), who reported C/N ratios below 20, but aligns with the 12-26 range observed in composted fresh grass/wheat straw in salmon trout fish pond sediment (Dróżdż et al. 2020b).

Given the current challenges facing agriculture, organic fertilizer presents a promising avenue for sustainable production. The results of this study demonstrates the potential of isolated *T. asperelloides* S25 as a component of microbial organic fertilizer, opening doors for future research and sustainable agricultural practices. The overall findings confirm the suitability of our treatments for producing high-quality granular bio-compost, offering farmers a valuable resource derived from agricultural byproducts.

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