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# ISOLATION AND IDENTIFICATION OF FUNGI ASSOCIATED WITH COMPOSTING PROCESS OF MUNICIPAL BIOSOLID WASTE

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SUMMARY

Organic waste is gradually degraded during composting process, producing carbon dioxide, water, heat, and humus, the relatively stable end product. The degradation process is carried out by living organisms, of which fungi appear to have the most important role since they break down tough debris (cellulose, lignin, and other resistant materials), enabling other microorganisms to continue the decomposition process. The objective of this study was to isolate and identify the fungi associated with large scale municipal biosolid waste composting process in Vietnam. In this study, we have isolated 10 morphologically different fungal strains from the composting materials, and classified based on morphological characteristics and 18S rDNA sequences. The results showed that these fungal strains belonged to four different genera, including *Aspergillus, Penicillium, Monascus,* and *Trichoderma*. The results would be a useful reference for further studies of diversity, and functions of fungi that involved in municipal biosolid waste composting process in Vietnam environmental conditions.

**Keywords:** *Composting, fungal biodiversity, morphological classification, 18S rDNA*

## INTRODUCTION

Composting technology has been widely used to treat biosolid waste, producing fertilizer for soil. The process is based on activities of variety of microorganisms, among those fungi play important roles due to their ability to degrade a wide range of ligno-cellulosic materials (Kumar *et al*., 2008), the major component of plant cells and the most abundant renewable organic resource. The ligno-cellulosic materials are composed of three types of polymers, namely cellulose, hemicelluloses, and lignin which are strongly engaged and persistent (Howard *et al*., 2003). In municipal biosolid wastes, ligno-cellulosic materials such as paper, carton, vegetable, and garden wastes are the major components. During composting process, fungi involve mainly at first (starting) mesophilic, and second (curing) mesophilic phase due to their low heat tolerance in comparing with bacteria (Insam, de Bertoldi, 2007). Fungi of genera *Aspergillus, Microsporium, Trichophyton, Yeast, Mucor, Penicillium, Rhizopus, Fusarium, Cladosporium,* and *Curvularia* were reported dominant in composting process of forest litter (Song *et al*., 2010), rice straw (Hefnawy *et al*., 2013), and household waste (Dehghani *et al*., 2012). However, little is known about main fungal groups existing in municipal biosolid waste composting at industrial scale in environmental conditions of Vietnam. Therefore, the objective of this study was to isolate and identify fungi which are associated with industrial scale municipal biosolid waste composting process.

## MATERIALS AND METHODS

### Sample collection

Compost samples were collected at municipal waste composting factory in Binh Duong province. At the factory, the municipal biosolid waste underwent composting process in 100 ton piles with continuous aerating. The compost samples were collected at the surface and 25-cm depth of a composting pile at the 1$^{st}$, 10$^{th}$, 20$^{th}$, 25$^{th}$, 35$^{th}$, 42$^{th}$ day during composting process, and finished

compost material (app. 90th day). The samples were quickly transported to laboratory for analyzing. Temperature of each sampling point was recorded with a thermometer.

**Isolation and morphological classification**

Three gram of composting material was suspended in 27 mL of 0.9% NaCl solution for 30 min with gently shaking at 200 rpm. After that, 100 µL of three consecutive dilutions was pipetted onto and spread evenly over petri plates containing Potato Glucose Agar (PGA) medium supplemented with chloramphenicol (100 mg/L), then incubated at 32°C, in the dark for 5 to 7 days. Single colonies were picked up and streaked on new PGA plates. After 2 days when the colonies visibly grew, but conidia had not yet been produced, 5 mm-diameter agar plugs were taken at the actively growing edge of the colony and transferred to fresh GPA plates, put in the position mycelia-side-down and at a distance of approximately 1.5 cm from the edge (or center) of the plates. Colony characteristics such as colony radius, colony appearance, time of first appearance of conidia, and type of pigmentation in the medium or conidia were recorded. Macro-morphological observation was carried out within 1 week. Micro-morphological characteristics such as vigorous growth of mycelium structure, conidiophore, phialide and conidia were recorded for 3-5 day old pure-cultures, depending on the growth rate of strains by using slide culture method and lactophenol cotton blue stain following Benson's procedure (Benson, 2002). The purified strains were classified based on their macro- and micro-morphological characteristics (Domsch *et al*., 1980).

**Genomic DNA extraction**

Genomic DNA of fungal strains was extracted using a modified method of Feng *et al*. (2010). From cultures on PGA plates, 20 mg of fungal mycelia was collected into 1.5 mL tubes containing 0.2 g glass bead (0.1 mm diameter), and 650 µL of lysis buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 1% SDS; 10 µg/mL RNase A). The mixture was vortexed for 2 min, then centrifuged at 10,000 rpm for 5 min. After centrifugation, 500 µL of supernatant was transferred into a new tube containing 100 µL of sodium acetate buffer (3.0 M, pH 5.5), and 500 µL of isopropanol, mixed and centrifuged at 10,000 rpm for 5 min to precipitate fungal DNA. DNA pellets were washed with 70% ethanol, air dried, then dissolved in 50 µL of sterile distilled water. The quality of extracted DNA samples was tested by spectrophotometer and gel electrophoresis.

**Polymerase chain reaction (PCR) to amplify 18S rDNA**

A variable region (app. 750 bp length) of 18S rDNA gene was amplified with primer pair NS1 (5' – TAGTCATATGCTTGTCTC – 3') (White *et al*., 1990) and Fung5 (5' – GTAAAAGTCCTGGTTCCCC – 3') (Smit *et al*., 1999). The PCR mixture (25 µL) contained approximately 50 ng of template DNA, 0.5 U DNA Taq-polymerase (MyTaq-Thermo scientific), 1× MyTaq PCR reaction buffer (MyTaq-Thermo scientific), and 20 pmol of each primer. A thermocycling was performed using a MyCycler Thermal cycler (Bio-Rad, UK) as follows: 94°C/5 min, followed by 30 cycles of (94°C/30 s, 47°C/40 s, 72°C/90 s), then 72°C/5 min. After that, PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained and observed under UV light.

**18S rDNA Sequencing and Phylogenetic tree building**

PCR products were purified and sequenced by ABI PRISM® 3730XL Analyzer (Macrogen sequencing service). The obtained sequences were then analyzed with Bioedit version 7.25 software and compared with 18S rDNA sequences available at NCBI database using Basic Local Alignment Search Tool (BLAST). The distance matrix for all pairwise sequence alignments was analyzed with the neighbor-joining (NJ) method of phylogenetic tree construction with 1,000 bootstrap replicates by using MEGA version 6 software.

## RESULTS

**Fungi isolation and classification**

From the samples collected at different stages of composting process, 10 fungal strains of different colony morphology were purified. Detail analyses of macro- and micro-morphological characteristics showed that these 10 fungal strains belonged to 4 genera, including *Aspergillus, Penicillium, Trichoderma, Monascus.* In more details, 7 of these strains were belonged to the genus *Aspergillus*, 1 strain to *Penicillium*, 1 strain to *Trichoderma* and 1 strain to *Monascus* (Table 1).

The results of 18S rDNA sequence analysis of these 10 strains were in agreement with

morphological classification, i.e. they belonged to four genera *Aspergillus*, *Trichoderma, Monascus,* and *Penicillium* (Table 1). A phylogenetic tree was constructed with MEGA software to overview their relationship (Figure 1), supporting that 18S rDNA sequencing is an useful tool for fungal identification (Smit *et al*., 1999).

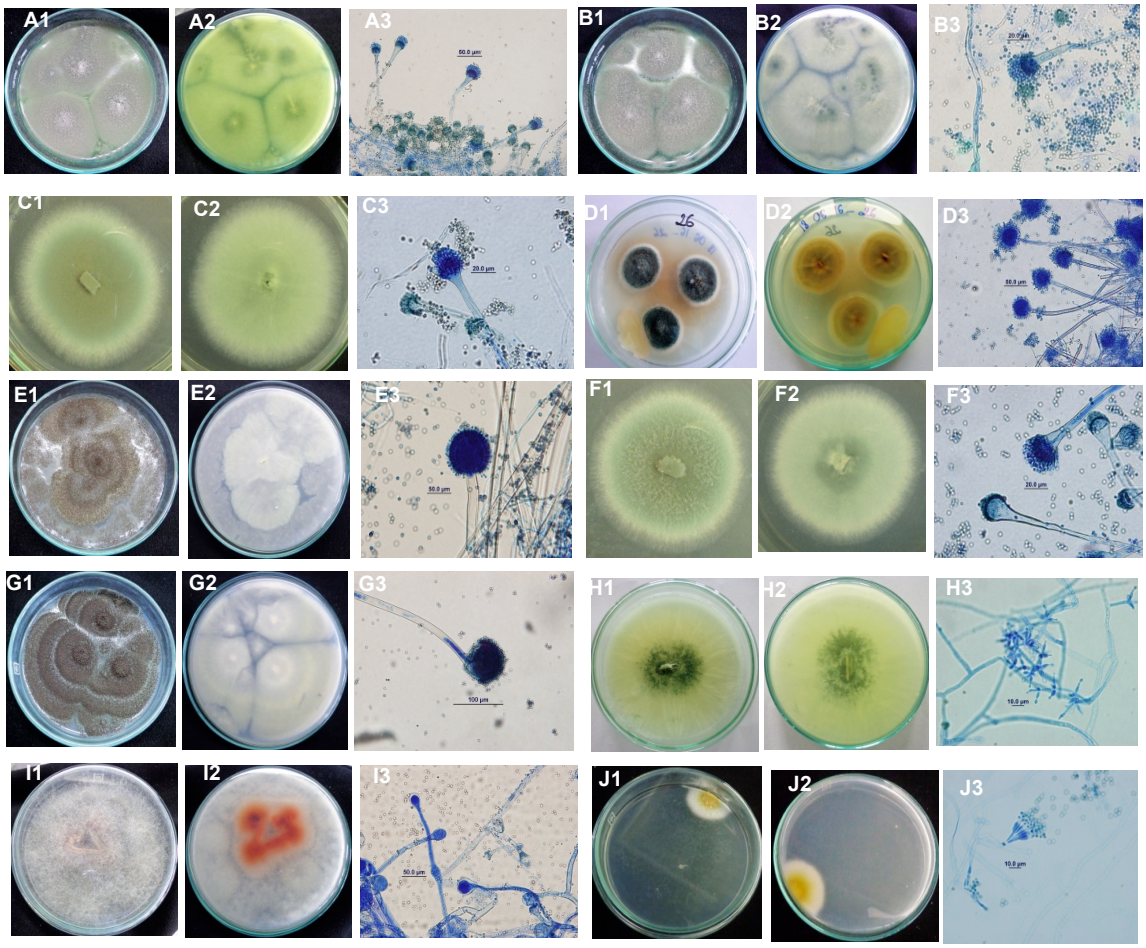


**Figure 1.** Colony morphology of fungi (front and reverse) on PGA plates at 32ºC after 5-7days and micrographs of their conidiophore. (A): Strain I; (B) Strain II; (C) Strain III; (D) Strain IV; (E) Strain V; (F) Strain VI; (G) Strain VII; (H) Strain VIII; (I) Strain IX; (J) Strain X. Scale bars: A3= D4=E3=I3=50 µM; B3= C3=F3=20 µM; G3=100 µM; H3=H4=J3=10 µM

**Table 1**. Macroscopic and microscopic characteristics of the 10 fungal strains isolated from industrial scale – municipal biosolid waste composting piles in Binh Duong, Vietnam. In the table, the fungi with similar characteristics were grouped together.

| Strain | Macroscopic characteristics | | | | | Microscopic characteristics | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Colony radius after 72 h (mm) | Color | Reverse color | Time of first observed conidia (h) | Pigmentation on medium | Conidiophores | Diameter of conidia (µm) | Shape of conidia | Identify |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| I | 57 | Powdery and black blue-green | Yellow - green | 24 | Yellow - green | | 2.5-3.1 | | |
| II | 57 | Powdery and black blue-green | Grey green | 24 | None | Conidiophores terminate in a vesicle covered with either a single palisade-like layer of phialides (uniseriate). The vesicle, phialides, and conidia form the conidial head. The phialides usually forming on the upper two-thirds of the vesicle. | 1.9 -3.0 | Globose to subglobose in chains and form compact columns (columnar). | |
| III | 59 | Powdery and black blue-green | Light green | 24 | Light green | | 2.0-3.0 | | |
| VI | 60 | Powdery and black blue-green | Light green | 24 | Light green | | 2.5-2.7 | | *Aspergillus* sp. |
| V | 50 | Black | White | 24 | None | Conidiophores are long with spherical vesicles. Conidiophore is biserate - metulae just about cover the entire surface from which the phialides extend. | 3.0-4.0 | Globular or ellipsoidal | |
| VII | 47 | Black | White | 24 | None | | 3.5-5.0 | Globular or ellipsoidal | |
| IV | 19 | Velvety green | Light orange | 48 | Light orange | Conidial heads support vesicles which are biseriate with metulae and phialides covering half to the entire vesicle. Conidial heads were radiated. | 2.0-3.5 | Round and form short chains | |
| VIII | 75 | Green | Yellow - green | 36 | Yellow - green | Conidiophores are rather short, are repeatedly branched at wide angles (approaching 90°), bearing clusters of divergent flask-shaped phialides. | 2.0-4.0 | Spherical to ellipsoidal form sticky clumps | *Trichoderma* sp. |
| IX | 77 | White | White to red | 24 | Red | Nonostiolate ascomata arising singly at the tip of stalk-like hyphae scattered on the mycelium, and an ascomatal wall composed of two distinct layers, an inner layer which results from the swelling of the tips of the stalk-like hyphae forming a vesicle-like structure and an outer layer, hyaline and ellipsoidal ascospores liberated from the cleistothecia | 5.0–15 | Globose to obovoid or obpyriform | *Monascus* sp. |
| X | 26 | White to yellow | White to yellow | 48 | Yellow | Conidiophore have a cluster of branches, each bearing a cluster of phialides (biverticillate). Phialides grouped in brush-like clusters (penicilli) at the ends of the conidiophores. | 2.5-5.0 | Globose or ellipsoidal and form long dry chains | *Penicillium* sp. |

**Table 2.** Results of 18S rDNA sequences analysis of the 10 fungal strains isolated from compost material in comparison to the available sequences on NCBI database using BLAST algorithm.

| Strain | Length of 18S rDNA sequence (bps) | Query Coverage (%) | Identity (%) | 18S rDNA identification |
|---|---|---|---|---|
| I | 631 | 100% | 100% | *Aspergillus* sp. *ISSF-T021* |
| II | 638 | 100% | 100% | *Aspergillus* sp. *ISSFT-021* |
| III | 638 | 100% | 100% | *Aspergillus* sp. *ISSFT-021* |
| IV | 659 | 99% | 99% | *Aspergillus versicolor* |
| V | 636 | 100% | 100% | *Aspergillus* sp. *PSFNRH-2* |
| VI | 638 | 100% | 100% | *Aspergillus fumigatus* |
| VII | 644 | 100% | 100% | *Aspergillus* sp. *CC-FB1* |
| VIII | 634 | 100% | 100% | *Trichoderma* sp. *S1102* |
| IX | 665 | 100% | 99% | *Monascus purpureus or Monascus ruber* |
| X | 945 | 100% | 100% | *Pennicillium* sp. *SCS-KFD08* |

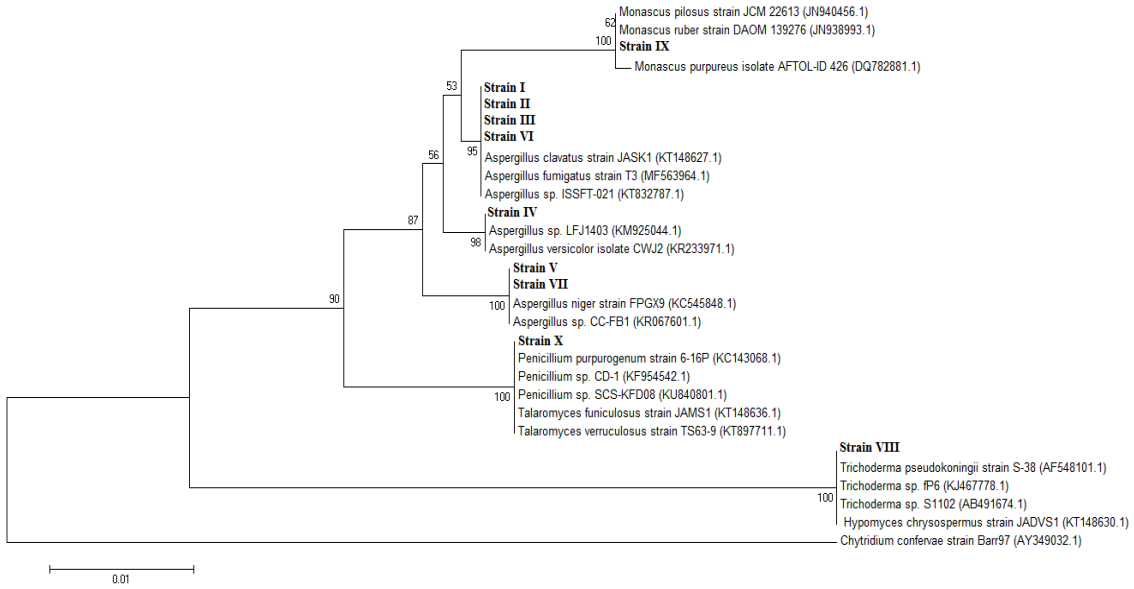


**Figure 2.** Phylogenetic tree was constructed with 18S rDNA sequence of the isolated fungi and related species using neighbor-joining method (MEGA 6 software). Numbers at branches are bootstrap values of 1,000 replications**.** The scale bar is in fixed nucleotide substitution per sequence position.

## DISCUSSION

During composting process, organic materials in municipal waste is converted into useful organic manure by microorganisms, among those fungi are important because they can decompose plant derived ligno-cellulosic materials (Kohzu *et al*., 2005). In this study, morphological and 18S rDNA classification analyses have been consistently showed that the common fungal strains in municipal biosolid waste composting process were *Aspergillus*, *Penicillium*, *Monascus*, and *Trichoderma*. Our observation was in agreement with the previous reports, indicating the abundance and important of these four fungal genera in composting process of bio-organic materials (Anastasi *et al*., 2005; Eida *et al*., 2011). The thermotolerance and capacity to degrade a wide range of organic waste of *Aspergillus*, *Penicillium* fungi may be the reasons for their dominant in bio-organic composting process (Miller, 1996).

In consistence with previous study, the data of our study showed that 7 out of the 10 fungal strains were identified as *Aspergillus*, suggesting that *Aspergillus* was the most common group in the investigated composting process (Ashraf *et al*., 2007). The fungi of *Aspergillus* genus can survive in many different environmental conditions, and possess diverse hydrolytic enzymes (amylase, protease, cellulase), therefore the fungi can degraded variety organic compounds, even complex organic compounds like lignin and cellulose, and play important roles in the composting process (Hawksworth, 2011). Besides *Aspergillus,* fungi of the other three genera have been also known for their ability to promote the speed and efficacy of composting process. Fungi of *Trichoderma,* and *Penicillium* genera have also been reported for participation in degrading a wide range of organic compounds in composting process. Recently, *Penicillium expansum* W4, a fungal strain producing ligno-cellulase, has been reported to be able to improve the quality and efficiency of composting process (Wang *et al*., 2011). In another study, *Trichoderma atroviride* has improved humic acid content in the mature compost by degradation of lignin and cellulose, xylan compounds (Maji *et al*., 2015). Fungi of *Mucor* genus have been report as the most dominant fungal genus in sawdust compost, representing 50% (9/18) of all isolates, and have high β-glucanase, mannanase, and protease activities (Hefnawy *et al*., 2013) and household waste (Dehghani *et al*., 2012).

Beside the ability to improve the efficacy and quality of composting process, fungi also play significant roles in protection of plants against pathogenic fungi. Aydi-Ben Abdallah et al. (2014) has reported that *Pythium* leak disease on potato caused by *Pythium ultimum* was controlled by culture filtrates and organic extracts from *Aspergillus* spp., originated from compost. The research of Makut and Owolewa (2011) showed that *Penicillium* sp. and *Aspergillus* sp. had the ability to inhibit the fungal pathogen *Candida albicans*. It is well known that *Trichoderma* sp. have the ability to antagonize different plant pathogens such as *Fusarium* sp., *Pythium* sp., *Rhizoctonia* sp., and has been widely used in agriculture. Moreover, *Trichoderma* can enhance the plant growth and development, as well as support plants to respond to stress conditions such as drought and soil salinity.

## CONCLUSION

In conclusion, the results of this study have revealed that fungi of four genera *Aspergillus, Penicillium, Monascus*, and *Trichoderma* were associated with municipal biosolid waste composting process at industrial scale in Vietnam. It is call for further study for better understanding of their roles in degradation of organic compounds in composting process, and maturation of composting materials. The understanding of composting-associated fungi is necessary for carrying out monitor and their utilization to improve the performance of industrial biosolid composting process.

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# PHÂN LẬP VÀ ĐỊNH DANH CÁC VI NẤM THAM GIA VÀO QUÁ TRÌNH Ủ COMPOST CHẤT THẢI RẮN SINH HOẠT ĐÔ THỊ

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TÓM TẮT

Trong quá trình ủ compost, các chất thải rắn có nguồn gốc sinh học sẽ bị phân hủy bởi các vi sinh vật, tạo ra carbon dioxide, nước, nhiệt và các chất mùn (compost). Trong các vi sinh vật, nhóm vi nấm có vai trò quan trọng trong việc phân giải các hợp chất bền vững như cellulose, lignin và các vật liệu khác. Mục tiêu của nghiên cứu này là phân lập và xác định các nhóm vi nấm tham gia vào quá trình ủ compost chất thải rắn sinh học đô thị ở quy mô công nghiệp tại Việt Nam. Chúng tôi đã quan sát thấy có 10 chủng vi nấm có sự khác biệt

về hình thái hiện diện trong quá trình ủ compost, các chủng vi nấm này sau đó được định danh dựa trên các phân tích chi tiết về hình thái và trình tự 18S rDNA của chúng. Kết quả thí nghiệm cho thấy các chủng vi nấm chiếm ưu thế thuộc về bốn chi khác nhau bao gồm *Aspergillus, Penicillium, Monascus* và *Trichoderma*. Các kết quả này sẽ là dữ liệu tham khảo hữu ích cho các phân tích sâu hơn về sự đa dạng và chức năng của các vi nấm trong quá trình phân hủy chất thải rắn sinh học đô thị ở Việt Nam.

***Từ khóa*****:** *Chất thải rắn hữu cơ, phân compost, đa dạng sinh học vi nấm, quy mô công nghiệp, phân loại hình thái, 18S rDNA.*