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Isolation and Screening of Cellulolytic Fungi from Spent Mushroom Substrates

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Abstract Isolation of fungi from spent mushroom substrate of *Pleurotus* sp., *Ganoderma lucidum*, *Auricularia polytricha*, *Coprinus fimetarius* and *Schizophyllum commune* form mushroom farms in southern Thailand. Isolation was by dilution pour plate technique on glucose ammonium nitrate agar (GANA). All fungal were tested for their ability to produce the hydrolytic enzyme cellulase on 1% carboxymethyl cellulose (CMC). A total of 24 fungal species were detected. Twenty-three fungal species were anamorphic fungi and one was a species from basidiomyces. Twenty-one species obtained from spent of *Pleurotus* sp., 14 species form spent mushroom substrate of *Auricularia polytricha*, 12 species from spent mushroom substrate of *Ganoderma lucidum*, nine species from spent mushroom substrate of *Schizophyllum commune* and *Coprinus fimetarius*. The frequency of fungi was high in genus *Trichoderma*, *Aspergillus* and *Penicillium*. Two species, *Trichoderma harzianum* and *Aspergillus flavus* were dominant species and found all spent mushroom substrates. Whereas two species, *Aspergillus niger* and *Aspergillus oxalicum* showed strong cellulolytic activity on agar plates.

Keywords: Cellulase, Diversity, Enzyme, Fungi, Spent mushroom substrate

Introduction

Fungi play a very important role in the biodegradation and conversion processes during composting. The biomass ratio of fungi to prokaryotes in compost is about 2:1 (Wiegant, 1992). Moreover, fungi can survive in extreme conditions. They mainly are responsible for compost maturation (Miller, 1996) such as spent mushroom substrate.

Spent mushroom substrate is the left-over substrate after completion of harvesting the mushroom, harboring both bacteria and fungi (Manikandan *et al*., 2012). Mushroom substrate preparation is the result of microbial community succession. Microbial community change results in changes in

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substrate in such a way that the growth of another microbial community is favored. The biological degradation of cellulose has great importance in the activity of many living systems. A cellulolytic enzyme system consists of three major components: endo-ß-glucanase, exo- ß -glucanase and ß -glucosidase (Agriculture and Consumer Protection, 2011). Cellulolytic organisms can convert cellulose into various economically important products such as monomeric sugars, single cell, protein, antibiotics, and compost of everyday use for man (Gautam *et al*., 2011). Use of cellulosic material has been thought in recent years to contribute to the production of food, industry and energy. Fungal enzymes are importance in agriculture, biotechnology, industry and medicine, as they are often more stable (at high temperature) than the enzymes derived from plants and animals (Maria *et al*., 2005; Tang *et al*., 2008).

Many researchers studied about extracellular enzyme by microorganisms from many sources (Choi *et al*., 2005; Jahangeer *et al*., 2005; Maria *et al*., 2005; Taeochapoempol *et al*., 2010; Gautam *et al*., 2011; Wongpisal *et al*., 2016). Thermophilic filamentous fungi and actinomycetes are widely used for industrial production of specific, stable and active enzyme. Gessner (1980) studied degradative enzyme production by salt-marsh fungi and the results showed 20 fungi to be capable of degrading cellulose, cellobiose, lipids, pectin, starch, tannic acid and xylan. Kumaresan and Suryanarayanan (2002) studied the extracellular enzymes form foliar endophytic fungi and reported that *Rhizophora apiculata* had involvement in litter degradation after senescence. Moreover, Jahangeer *et al.* (2005) screened the cellulolytic ability of fungi from native environments and the results showed that 67.83% of fungi produced cellulolytic enzymes such as *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp., *Alternaria* sp., *Penicillium* sp. and *Rhizopus* sp. In other research, Wongpisal *et al*. (2016) studied fungi associated with oil palm leaf litter and reported that four fungal species produced strong cellulolytic activity on carboxyl methyl cellulose agar (CMC) belonging to *Aspergillus flavus*, *A. niger*, *Penicillium funiculosum* and *P. janthinellum*, those are more effective for compost production.

On the other hand, some have reported of cellulolytic fungi studies on spent mushroom substrate, such as on *Pleurotus cystidiosus* (Kitpaisarn, 1985), *Pleurotus* spp. (Manikandan *et al*., 2012) *Pleurotus sajor-caju* (Singh *et al*., 2012) *Agaricus bisporus* (Kitpaisarn, 1985; Ivors *et al*., 2002; Singh *et al*., 2012; Chandel *et al*., 2013), *Volvariella volvacea* (Singh *et al*., 2012) and *Lentinula edodes* (Chandel *et al*., 2013). In agriculture, cellulolytic fungi in most studies of biodegradation processes have emphasized their role because of their capability of producing and secreting high amounts of enzymes (Maki *et al*., 2009). Therefor the objective of this present study is to investigate the

diversity of fungi associated with five different spent mushroom substrates via *Pleurotus* sp., *Auricularia polytricha*, *Ganoderma lucidum*, *Coprinus fimetarius* and *Schizophyllum commune* and select some of those with high capability of producing of cellulolytic enzyme for future use.

Materials and methods

Isolation and identification of fungi

The spent mushroom of *Pleurotus* sp., *A. polytricha*, *G. lucidum*, *C. fimetarius* and *S. commune* were collected from 80 mushroom farms in Nakhon Si Thammarat, Patthalung, Songkhla, Suratthani and Trang Province, all in southern Thailand. Isolation of fungi was done with dilutions pour plate technique. One ml of 1×10^{-5} serial dilution was pipetted into each of four replicates of glucose ammonium nitrate agar (GANA) with streptomycin sulfate (300 µg/ml), which was cooled to 45 °C, and poured into Petri dishes. The dishes were incubated at room temperature for 3 days and then examined for fungal growth. All colonies were collected with pure culture by hypha tip method and maintained at 4 °C for further use. The culture was identified based on the microscopic and macroscopic characteristics.

Percentage occurrence of taxon A = (number of spent mushroom samples on which each fungus was detected/ total number of spent mushroom samples examined) × 100%. Fungal taxa with a percentage occurrence equal to or higher than 10% were regarded as dominant species.

Screening for cellulase enzyme

All fungi were tested for their ability to produce the hydrolytic enzyme cellulase in a plate assay method using 1% carboxymethyl cellulose (CMC) in a basal salt medium (cellulolysis basal medium; CBM) (Gautam *et al.*, 2011). A 5 mm diameter mycelial plug on MEA was put onto the CMC agar, thereafter incubated at 28-32 ^{o}C in darkness. When the colony diameter was approximately 30 mm, the plates were flooded with 0.25% w/v aqueous iodine (I2 and KI) and left for 15 minutes, then poured off the staining material and washed the agar surface with distilled water. Then, the plates were flooded with 1M NaCl to distain for 5 minutes and then the distaining fluid was poured off. The zone of cellulose hydrolysis appeared as a clear zone around the colony (Wongpisal *et al.*, 2016).

Extracellular cellulolytic activity was measured by the method described by Choi *et al.* (2005); enzymatic reaction ratio = the ratio of clear zone diameter to that of colony diameter. The test results were classified into 4

categories as follows (Taeochapoempol *et al*., 2010; Xu and Yang, 2010). Strong reaction (+++): the extracellular enzyme ratio was higher than or equal to 2. Medium reaction (++): the extracellular enzyme ratio was less than 2 but more than 1. Weak reaction (+): the extracellular enzyme ratio was equal to or less than 1 but more than zero. No reaction (-): there is no reaction at all (Pointing, 1999).

Results

Diversity of fungi on spent mushroom compost

A total of 24 fungal species collected from five different samples of spent mushroom substrate. Twenty-one species obtained from spent mushroom substrate of *Pleurotus* sp., 14 species from spent mushroom substrate of *A. polytricha*, 12 species from spent mushroom substrate of *G. lucidum* and nine species obtained from spent mushroom substrate of *S. commune* and *C. fimetarius* (Table 1, 2).

Fungal taxonomic and dominant species

A total of 24 species were detected by dilution plate technique, comprised of 23 anamorphic fungi and one basidiomyces (Table 1). The frequencies of fungi were high in genus *Trichoderma*, *Aspergillus* and *Penicillum*, and were found six, five and three species, respectively. Two species (8.33%), *Aspergillus flavus* and *Trichoderma harzianum* were found overlapping in all spent mushrooms substrates. Five species (20.83%), *Alternaria* sp., *Aspergillus niger*, *Neurospora* sp., *Penicillium janthinellum* and *P. oxalicum* were found in four spent mushroom substrate. Five species (20.83%), *Lasiodiplodia theobromae*, *Curvularia lunata*, *Fusarium oxysporum*, *Penicillium* sp. and *Trichoderma* sp. were found overlapping in three spent mushroom substrates. Six species (25.00%), *Aspergillus* sp., *A. fumigatus*, *T. atroviride*, *T. koningii*, *T. viride* and *Rhizoctonia solani* were found overlapping in two spent mushroom substrates. While, *Alternaria alternata* and *Cunninghamella elegan*s were found on *G. lucidim* and *A. polytricha* spent mushroom substrate only.

Fungal diversity and species richness

The dominant fungi on the spent mushroom substrate, with over 10% occurrence in each mushroom are shown in Table 1. Anamorphic fungi (23

species) were the dominant group. On the other hand, only one species was found in basidiomycetes. Twenty-one dominant fungal species were found in spent of *Pleurotus* sp. comprising *Alternaria* sp., *A. alternata, Aspergillus* sp., *A. niger, A. flavus, A. fumigatus, A. tubingensis, Lasiodiplodia theobromae, Chaetomium* sp., *Cunninghamella elegans, C. lunata, Fusarium* sp., *F. solani, Neurospora* sp., *Penicillium* sp., *P. janthinellum, P. oxalicum, R. solani, Trichoderma* sp., *T. atroviride, T. harzianum, T. inhamatum, T. koningii* and *T. viride.* Six species, *Aspergillus* sp., *A. niger, A. flavus, Neurospora* sp., *P. janthinellum* and *R. solani* were dominant species found in spent mushroom substrate of *A. polytricha*.

Six species, *F. oxysporum, Neurospora* sp., *P. oxalicum, Trichoderma* sp., *T. harzianum* and *T. inhamatum* were dominant species found in spent of *G. lucidum.* Nine species, *Alternaria* sp., *A. flavus, L. theobromae, Chaetomium* sp., *C. lunata, F. oxysporum, P. oxalicum, T. atroviride* and *T. harzianum* were dominant fungi found in spent mushroom substrate of *C. fimetarius*. Three species, *A. niger, A. flavus* and *Neurospora* sp. were dominant species found in spent mushroom substrate of *S. commune*.

Cellulolytic fungi

Twenty–four fungal species form spent mushroom substrates were tested for their ability to produce cellulase enzymes. Twenty species (83.33%) showed some degree of clearance of the cellulose-containing medium during incubation periods of 2–4 days. Two of the fungal species (8.33%), *A. niger* and *P. oxalicum* showed strong cellulolytic activity (+++) on agar plates. Ten species (41.67%) belonging to *A. flavus, A. tubingenesis, L. theobromae, F. oxysporum, F. solani, Neurospora* sp., *Penicillium* sp., *P. janthinellum, R. solani* and *T. harzianum* showed medium cellulolytic activity (++) on agar plates. Eight fungal species (3.33%) including *Alternaria* sp., *A. alternata, Aspergillus* sp., *A. fumigatus, Chaetomium* sp., *Curvularia lunata, Trichoderma koningii* and *T. viride* showed weak reaction (+) on agar plates. Four fungal species, *Cunninghamella elegans, Trichoderma* sp., *T. atroviride* and *T. inhamatum* did not show any clearance (–) of the cellulose-containing medium (Table 3).

Table 1. Type of fungi species were isolated from spent mushroom substrates

Taxa	Spent mushroom substrate				
	***Pleurotus* sp.**	***A. polytricha***	***G. lucidum***	***C. fimetarius***	***S. commune***
Basidiomycetes					
Rhizoctonia solani	23.81	14.29			
Deuteromycetes					
Alternaria sp.	14.28	7.14		22.22	9.09
A. alternata			7.69		
Aspergillus sp.	19.05	14.29			
A. niger	71.43	28.57	7.69		27.27
A. flavus	61.90	14.29	7.69	33.33	36.36
A. fumigatus	23.81	7.14			
A. tubingenesis	19.05				
Chaetomium sp.				11.11	
Cunninghamella elegans		7.14			
Curvularia lunata	33.33		7.69	22.22	
Fusarium oxysporum	23.81		15.38	33.33	
F. solani	28.57				
Lasiodiplodia theobromae	47.62	7.14		22.22	
Neurospora sp.	33.33	14.29	15.38		18.18
Penicillium sp.	33.33		7.69		9.09
P. janthinellum	23.81	14.29	7.69		9.09
P. oxalicum	23.81	7.14	15.38	44.44	
Trichoderma sp.	23.81	7.14	15.38		
T. atroviride	23.81			11.11	
T. harzianum	19.05	7.14	30.77	11.11	9.09
T. inhamatum	19.05	7.14	15.38		
T. koningii	14.29				9.09
T. viride	19.05				9.09
Total	21	14	12	9	9

Table 2. Diversity indices of fungi on spent mushroom substrates

Spent mushroom substrates	No. of species	Index *D*	Index *H*
A. polytricha	14	0.9423	2.5239
C.fimetarius	9	0.9123	2.0868
G. lucidum	12	0.9476	2.4504
Pleurotus sp.	21	0.9477	2.9374
S. commune	9	0.9150	2.1972

Table 3. Type of fungal species produced cellulase enzyme on carboxymethyl cellulose agar

Fungal species	Hydrolysis capacity[1/]	Number of day (size 30 mm)
Alternaria sp.	+	2
A. alternata	+	2
Aspergillus sp.	+	2
A. niger	+++	2
A. flavus	++	2
A. fumigatus	+	2
A. tubingenesis	++	2
Chaetomium sp.	+	3
Cunninghamella elegans	–	2
Curvularia lunata	+	3
Fusarium oxysporum	++	3
F. solani	++	3
Lasiodiplodia theobromae	++	3
Neurospora sp.	++	3
Penicillium sp.	++	4
P. janthinellum	++	4
P. oxalicum	+++	3
Rhizoctonia solani	++	3
Trichoderma sp.	–	2
T. atroviride	–	2
T. harzianum	++	2
T. inhamatum	–	2
T. koningii	+	2
T. viride	+	2

[1]/Hydrolysis capacity (cm.) + = < 1.00 cm; ++ = 1.01–2.00 cm, +++ = 2.01–3.00 cm, ++++ = > 3.00 cm.

Discussion

Diversity of fungal on spent mushroom substrate

Spent mushroom substrate is a by–product of mushroom production. It is rich in diverse microorganism. The present study provides comprehensive data about the relative composition of microorganisms in composts based on different spent mushroom substrates simples. In the results, twenty-four fungal species were isolated from five different of spent mushroom substrates which were varied in their fungal population. The versatility of microorganisms present in spent mushroom substrate depends on the type of formula used for mushroom cultivation and kind of mushroom cultivated (Singh *et al*., 2012). The spent mushroom substrate of *Pleurotus* was the highest in fungal diversity obtained 21 species, all of them being dominant fungi such as *Aspergillus*

niger, A. flavus, L. theobromae, Penicillium sp., *Neurospora* sp. and *C. lunata* etc. The results according to previous researchers who studied the spent mushroom substrate of *Pleurotus* showed that it was the one with the highest population of fungi and bacterial (Ahlawat and Singh, 2011; Goyal and Soni, 2011; Chandel *et al*., 2013; Adedeji and Aduramigba Modupe, 2016). Ahlawat and Singh (2011) reported that *A. fumigatus, S. commune* and *Pezizomycotina* sp. were the dominant fungi found on the spent mushroom substrate from *Pleurotus sajor-caju*. While, Adedeji and Aduramigba Modupe (2016) reported that the spent mushroom substrate of the oyster mushroom (*P. sajor-caju*) contained diverse microorganisms including fluorescent *Pseudomanas* spp., *T. viride, Bacillus* spp., *Penicillium* spp. and *Aspergillus terrus*. Moreover, Lee *et al.* (2011b) reported that spent mushroom compost of white button mushroom contained diverse microorganisms including fluorescent *Pseudomonas* sp., *Bacillus* sp., *Trichoderma* sp. and *Actinomyces*.

Normally, the fungi were isolated form spent mushroom substrate such as *T. harzianum, T. polysporum, T. viride, P. janthinellum, Mycogene periciosa, Aspergillus bisporus, A. niger, Rhizopus* spp. and *Penicillium* spp. (Lee *et al*., 2011a; Manikandan *et al*., 2012; Singh *et al*., 2012; Chandel *et al*., 2013; Remya and Beena, 2015). Ashraf *et al.* (2007) studied fungi, bacteria and actinomycetes form 11 different composts showed 119 genera such as *Aspergillus, Trichoderma, Mucor, Penicillium, Alternaria, Cladosporium, Monilia, Helminthosporium, Coccidioides* and *Scedosporium*. Of those isolated, those of the genus *Aspergillus* were the most prevalent followed by *Bacillus* (20%). Eman and El- Zaben (2010) reported that optimum temperature requirements of different types of microorganisms found in spent mushroom substrate of different mushrooms varied between the types. With the intention of elucidating the physical structural attributes of spent mushroom substrate the ability of the microbes to survive there can also be fully used. Moreover, environment, including temperature, moisture and pH were effected for microorganisms in the spent mushroom substrate.

Cellulolytic fungi on spent mushroom substrate

Out of twenty-four fungal species isolated form spent mushroom substrates that were tested for their ability to produce cellulase enzymes. Two fungal species showed the highest production of cellulase (+++) namely *A. niger* and *P. oxalicum*. There are some reports concerning the biomass of microorganisms including bacteria or fungi and the activity of cellulolytic enzymes on spent mushroom substrate (SMS) (Goyal and Soni, 2011; Chandel *et al*., 2013). Especially, fungi are very essential for producing the diverse

enzymes that can degrade natural polymeric compounds such as cellulose, pectin and starch. Kitpaisarn (1985) studied cellulolytic fungi form spent mushroom substrate of *Pleurotus csytidiosus* and *A. bisporus* and reported that 31 isolates were recorded by *Humicola* sp. was the highest enzyme activity. While, Chandel *et al.* (2013) studied cellulolytic fungi from spent mushroom of *A. bispous* and *Lentinula edodes* and reported that 45 isolates were detected. Of which 23 isolates showed the sought-for cellulase activity. The isolates were identified as *Trichoderma, Aspergillus, Rhizopus* and *Penicillium*. According to this experiment, the genus *Aspergillus*, *Trichoderma* and *Penicillium* were the dominant fungi obtained from all spent mushroom substrate, and can produce high extracellular enzymes on CMC agar. Agriculture and Consumer Protection (2011); Gautam *et al.* (2011) and Wongpisal *et al.* (2016) reported that fungi of the genera *Trichoderma* and *Aspergillus* are thought to be cellulase producers, and crude enzymes produced by these fungi are commercially available for agricultural use. Moreover, Dubey and Maheshwan (2010) reported that the cellulolytic fungi such as *Aspergillus, Trichoderma, Penicillium* and *Trichurus* accelerate composting for efficient recycling of dry crop wastes with high C:N ratio and reduce the composting period by about month.

Moreover, Choi *et al*. (2007) reported that microorganisms such as fungi and bacteria that were isolated from spent mushroom substrate are of potential benefit to controlling plant disease. Especially, if fungi are provided with the right conditions, they can be antagonistic towards a number of important soil-borne microbial pathogens (Larkin and Fravel, 1998; Lee *et al*., 2011b; Adedeji and Aduramigba Modupe, 2016). Potentially antagonistic microflora from spent mushroom substrate against soil borne pathogens were reported on ginger such as *T. koningii*, *T. viride* and *T. harzianum* (Whipps, 2001; Remya and Beena, 2015). *Trichoderma* spp. was usually contaminated in spent mushroom substrate. It can be useful for reducing pathogen amounts in soil.

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