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Tropical Soil Fungi Producing Cellulase and Related Enzymes in Biodegradation

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Abstract: The objective of this study was to screen, identify and characterize of cellulolytic fungi from various soil management fields; organic, young organic, semi-chemical and chemical soil from Surin rice fields, Thailand. Fungi from various type of soils were isolated by dilution plating technique on Reese minimal medium supplemented with cellulose powder and rice straw then incubated at 25°C for 3-7 days. The isolated fungi were screened and identified using slide culture technique. The enzymatic activities were assessed by qualitative method for cellulase, xylanase, peroxidase and laccase activities. Two-hundred and fifty-eight fungi isolates found in Surin rice fields belonging to the genus *Penicillium* (5 species), *Paecilomyces* (4 species), *Aspergillus* (3 species), *Acremonium* (2 species), *Chaetomium* (2 species), *Alternaria* (1 species), *Bipolaris* (1 species), *Curvularia* (1 species), *Fusarium* (1 species), *Humicola* (1 species), *Mucor* (1 species), *Nigrospora* (1 species), *Phoma* (1 species), *Pyrenochaeta* (1 species), *Pythium* (1 species), *Rhizopus* (1 species), *Sporotrichum* (1 species) and *Trichoderma* (1 species). Out of 29 fungal species clearly showed different in enzymatic activities. Most tropical soil fungi had ability to produce cellulase, xylanase, laccase and peroxidase, respectively. The highest capacity was found only in cellulase. *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum* and *Trichoderma* sp. showed the highest potential to produce cellulase. Eleven species of soil fungi showed high capacity in xylanase activity. For laccase and peroxidase activity, there were found in 2 species. The results also revealed that only ten showed highest carboxymethyl cellulase, xylanase, peroxidase and laccase activities by qualitative screening method for enzymatic assay. They were identified as *Aspergillus niger*, *Acremonium* sp., *Aspergillus* sp., *Chaetomium murorum*, *Humicola grisea*, *Mucor* sp., *Paecilomyces victoriae*, *Penicillium janthinellum*, *Penicillium lanosum* and *Trichoderma* sp. These tropical soil fungi will be beneficial to use for biodegradation and decomposition of agricultural residues, especially rice straw.

Key words: Screening, identification, soil fungi, microbial enzyme, carboxymethyl cellulase, xylanase, laccase, peroxidase, qualitative test, hydrolysis capacity

INTRODUCTION

The expansion of rice cultivation has led to produce large amount of rice straw (Gadde *et al*., 2009b). About 50% of the dry weight of the rice plant makes up straw waste (Abdulla and El-Shatoury, 2007; Kausar *et al*., 2010) and each year the straw from rice farming has increased and accumulated every year. Because major components of rice straw are cellulose and hemicellulose encrusted by lignin and only small amounts of protein, it is resistant to microbial decomposition compared to straw from other protein-rich grains such as wheat and barely (Parr *et al*., 1992). In addition, rice straw is considered as disease infestation, unstable nutrients and slow rate in degradation. Therefore, the post-harvest rice residue normally is eliminated by open-field burning in several countries such as Philippines, Thailand and India (Gadde *et al*., 2009a, b; Kausar *et al*., 2010). Currently, effects of paddy straw burning in the fields are coming serious concern all over the world because this process emits many pollutants. For example, carbon dioxide, carbon monoxide, methane, nitrous oxide, sulphur dioxide, polychlorinated dibenzofurans (PCDFs), polychlorinated dibenzo-p-dioxins (PCDs) etc. (Gadde *et al*., 2009a; Kausar *et al*., 2010). These pollutants represent a threat to public health and pose an environmental pollution problem. Especially, polycyclic aromatic hydrocarbons (PAHs), PCDFs and PCDs, several scientific researches have been reported that these pollutants are carcinogenic substances which can be cause severe impacts on human health. Emissions from field burning activities also release many respiratory particles of <10 μm size (PM10, particulate matter less than 10 μm in diameter) which deteriorate local air quality and cause high personal exposure (Belal and El-Mahrouk, 2010; Oanh *et al*., 2011).

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Thailand about 48% of rice residues is burned in the field (Gadde *et al*., 2009b; Kausar *et al*., 2010; Oanh *et al*., 2011). Thailand produced massive amounts of rice straw more than other countries because availability in rice cultivation over 3-4 times a year. Also together with many patterns of rice cultivations by direct dry seeding, wet seeding and indirect seeding under organic, young organic, semi-chemical and chemical management practices and there are in wet and dry seasons. Therefore, rice straw residues are varieties in moisture and temperature conditions. It continuously accumulated year round. Although, rice straw composes of three major components, the chemical compositions of tissue part as total ash, insoluble ash (silicon), cellulose, hemicellulose and lignin contents varied greatly among the different area (Jin and Chen, 2007; Rodriguez *et al*., 2009). The biodegradation of rice straw are related with soil microorganisms (Kuhad *et al*., 1997; Yu *et al*., 2007) as Actinomycetes (Abdulla and El-Shatoury, 2007; Minamiyama *et al*., 2003; Xu and Yang, 2010) bacteria (Xu *et al*., 2005) and fungi (Chandra *et al*., 2007; Hart *et al*., 2002; Kumar *et al*., 2008; Stepanova *et al*., 2003). Many researches have been reported that fungi showed the most efficiency for biodegradation processes (De Castro *et al*., 2010; Liang *et al*., 2010). However, this process takes longer time to apply and need optimal environments. The soil fungi isolated from the agricultural soil is able to produce cellulolytic enzymes for degradation process and make rice straw degradation more feasibility. The soil fungi from direct agricultural area can reduce the problems of time consuming and environmental factors for biodegradation process and also reducing the cost for soil improvement. In addition, it also reduced air and soil pollutions that affect human health from open air burning. The screening of soil fungi producing cellulases and related enzymes in biodegradation is important for Thailand. The objective of this research is to isolate and screen tropical soil fungi for lignocellulose degrading enzyme productions from various paddy soils. It is hoped that this will be a step towards providing a method of accelerated decomposition of rice straw in paddy fields.

MATERIALS AND METHODS

Isolation and identification of soil fungi: About 1 g of soils from various Surin rice fields in Thailand, as organic, young organic, semi-chemical and chemical management practices were collected between August 2010-December 2011. The soil samples were suspended into sterile distilled water and aliquots of the resulting suspension were inoculated onto Reese minimal medium by dilution plating technique (Kumar *et al*., 2008; Yang *et al*., 2003). After 7-14 days of incubation at 30°C, several different colonies were purified and then characterized by morphological characteristics. Based on the methods of colony observation with a stereoscope and microscopic features of fungal strains with a light microscope; squash mounts stained with Lactophenol and Cotton blue, used to identify fungal cultures to species level (Alves-Prado *et al*., 2010; Barnett and Hunter, 1999; Watanabe, 2002).

Inoculation procedure: Pure cultures were cultivated on basal medium (LMB) containing: KH_2PO_4 (1 g L^{-1}), ammonium tartrate (0.5 g L^{-1}), $MgSO_4.7H_2O$ (0.01 g L^{-1}), $CaCl_2.2H_2O$ (0.01 g L^{-1}), yeast extract (0.001 g L^{-1}), $CuSO_4.5H_2O$ (0.001 g L^{-1}), $Fe(SO_4)_3$ (0.001 g L^{-1}) and $MnSO_4$ (0.001 g L^{-1}). LBM medium was supplemented 0.4% w/v glucose and solidified with 1.60% w/v agar (Difco). All enzymatic assays were inoculated with agar disc (6 mm in diameter) of active mycelia from 5 day-old cultures on LBM medium cultivated in Petri dishes and incubated under the dark at 25°C (Tortella *et al*., 2008).

Qualitative enzymatic assay: The soil fungi were screened qualitatively for the production of degrading enzymes; cellulase, xylanase, laccase and lignin peroxidase. The media used for detecting the enzyme were prepared as follows (Minamiyama *et al*., 2003; Pointing, 1999; Tortella *et al*., 2008; Xu and Yang, 2010).

Cellulase activity: The Cellulolysis Basal Medium (CBM) was prepared containing: KH_2PO_4 (0.5 g L^{-1}), ammonium tartrate (0.5 g L^{-1}), $MgSO_4.7H_2O$ (0.1 g L^{-1}), $CaCl_2.2H_2O$ (0.01 g L^{-1}), yeast extract (0.001 g L^{-1}), $CuSO_4.5H_2O$ (0.001 g L^{-1}), $Fe(SO_4)_3$ (0.001 g L^{-1}) and $MnSO_4$ (0.001 g L^{-1}). LBM medium was supplemented with carboxymethyl cellulose 2% w/v (Sigma) and 1.6% of agar (Difco) and was autoclaved at 121°C for 20 min the medium was aseptically transferred to petri dishes and inoculated with a 6 mm agar disc cut from a 5 day old fungal culture of each strain separately and incubated at 25°C in darkness and, when the colony diameter was approximately 30 mm, were flooded with aqueous Congo red (2% w/v) for 15 min. Then, the agar surface was washed with distilled water and plates were flooded with NaCl (1 M) for 1.5 min. Production of cellulase was observed by the formation of a yellow-opaque area around the colonies.

Xylanase activity: The Xylanolysis Basal Medium (XBM) was prepared containing: ammonium tartrate (5 g L^{-1}), KH_2PO_4 (1 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), yeast extract (0.1 g L^{-1}), $CaCl_2.2H_2O$ (0.001 g L^{-1}). The XBM medium was supplemented with xylan, 4% w/v (Sigma) and 1.6% w/v of agar (Difco) and autoclaved at 121°C for 20 min. The medium was aseptically transferred to petri dishes and inoculated with an 6 mm agar disc of isolated

soil fungi. The petri dishes were incubated in the dark at 25°C and, when the colony diameter was approximately 30 mm, were flooded with iodine dye (0.25% w/v aqueous I_2 and KI) for 5 min. Then, the agar surface was rinsed with distilled water. Production of xylanase enzyme was observed by the formation of a yellow-opaque area around the colonies compared to a blue/reddish purple color standard for non-degraded xylan.

Laccase activity: Laccase activity was determined in all isolated strains via the reaction with ABTS in basal medium (LBM) as the previous mentioned media. The basal medium was supplemented with 1 g L^{-1} of 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 16 g L^{-1} of agar (Difco). The medium was autoclaved at 121°C for 20 min. One milliliter of a separately sterilized 20% w/v aqueous glucose solution was added aseptically to each 100 mL of growth medium. The medium was subsequently aseptically transferred to petri dishes and inoculated with an 6 mm agar disc of fungal culture. Production of laccase was observed by the formation of a green color in the growth medium.

Peroxidase activity: Azure B agar was used for the production of lignin peroxidase. The LMB medium supplemented with 0.01% w/v of Azure B and 1.6% w/v agar (Difco). The medium was autoclaved at 121°C for 20 min. One milliliter of a separately sterilized 20% w/v aqueous glucose solution was added aseptically to each 100 mL of growth medium. The medium was subsequently aseptically transferred to petri dishes and inoculated with an 6 mm agar disc of fungal culture. The clearance of blue cloured medium is positive reaction of lignin peroxidase.

Hydrolysis capacity (HC value): HC was used to evaluate the capacity of degrading enzymes of soil fungi by diameter of the clearing zone/diameter of the colony (Xu and Yang, 2010).

RESULTS

Two-hundred and fifty-eight fungi isolates found in Surin rice fields belonging to the genus *Penicillium* (5 species), *Paecilomyces* (4 species), *Aspergillus* (3 species), *Acremonium* (2 species), *Chaetomium* (2 species), *Alternaria* (1 species), *Bipolaris* (1 species), *Curvularia* (1 species), *Fusarium* (1 species), *Humicola* (1 species), *Mucor* (1 species), *Nigrospora* (1 species), *Phoma* (1 species), *Pyrenochaeta* (1 species), *Pythium* (1 species), *Rhizopus* (1 species), *Sporotrichum* (1 species) and *Trichoderma* (1 species). Qualitative screening of the fungal cultures indicated that 29 strains showed various levels in cellulase, xylanase, peroxidase and laccase (Table 1). The most of strains presented in

Table 1: Qualitative enzyme production of soil fungi on solid plates of cellulase, xylanase, laccase, and peroxidase

		HC value			
Isolate No.	Soil fungi	Cellulose Congo red agar	Xylan agar	ABTS agar	Azure-B agar
1	*Acremonium alternatum*	***	*	*	*
2	*Acremonium* sp.	***	***	**	**
3	*Alternaria padwickii*	**	*	*	*
4	*Aspergillus brevipes*	***	**	**	**
5	*Aspergillus niger*	****	***	*	*
6	*Aspergillus* sp.	****	***	*	*
7	*Bipolaris oryzae*	**	*	*	*
8	*Chaetomium brasiliense*	**	*	*	*
9	*Chaetomium murorum*	****	***	*	*
10	*Curvularia lunata*	**	**	**	**
11	*Fusarium* sp.	*	*	*	*
12	*Humicola grisea*	***	***	***	*
13	*Mucor* sp.	***	**	***	*
14	*Nigrospora* sp.	***	**	**	*
15	*Paecilomyces inflatus*	**	***	**	**
16	*Paecilomyces puntoni*	**	**	**	*
17	*Paecilomyces roseolus*	**	***	**	**
18	*Paecilomyces victoriae*	***	***	*	***
19	*Penicillium janthinellum*	***	***	*	*
20	*Penicillium corylophilum*	*	*	*	*
21	*Penicillium lanosum*	***	***	**	**
22	*Penicillium nigricans*	***	**	**	**
23	*Penicillium* sp.	***	**	**	*
24	*Phoma* sp.	**	**	*	*
25	*Pyrenochaeta terrestris*	*	*	*	*
26	*Pythium ultimum*	*	*	*	*
27	*Rhizopus* sp.	***	*	*	*
28	*Sporotrichum* sp.	*	*	**	*
29	*Trichoderma* sp.	****	***	**	***

HC value: Hydrolysis capacity, diameter of the clearing zone/diameter of the colony divided in 4 levels as *<1.00,**1.01-2.00, ***2.01-3.00 and ****>3.00

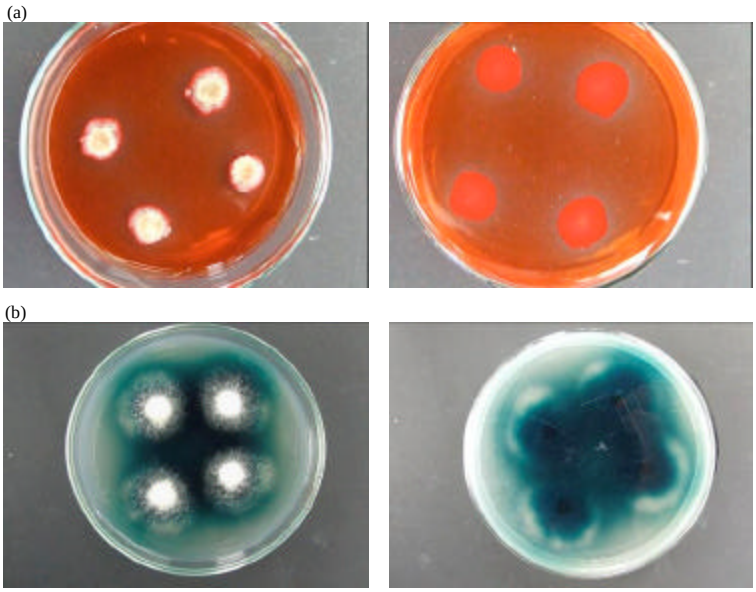

Fig. 1(a-b): Qualitative assays of cellulase and laccase activity, (a) CMC agar and (b) ABTS agar

cellulase (CMCase) activity followed xylanase, laccase and peroxidase, respectively. Four species of soil fungi that showed highest CMCase activity (>3.00 HC) were identified as *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum* and *Trichoderma* sp. Whereas 12 species at high level (2.01-3.00 HC) were *Acremonium alternatum*, *Acremonium* sp., *Aspergillus brevipes*, *Humicola grisea*, *Mucor* sp., *Nigrospora* sp., *Paecilomyces victoriae*, *Penicillium janthinellum*, *Penicillium lanosum*, *Penicillium nigricans*, *Penicillium* sp. and *Rhizopus* sp. While 11 soil fungi showed high level of xylanase activity (2.01-3.00 HC) were *Acremonium* sp., *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum*, *Humicola grisea*, *Paecilomyces inflatus*, *Paecilomyces roseolus*, *Paecilomyces victoriae*, *Penicillium janthinellum*, *Penicillium lanosum* and *Trichoderma* sp. The high level of laccase and peroxidase activities was obtained only two species. *Humicola grisea* and *Paecilomyces inflates* were laccase producers. *Paecilomyces victoriae* and *Trichoderma* sp. were peroxidase (Table 1, Fig. 1).

In addition, the results also clearly found that out of 29 soil fungal species, the ten most efficient capacity for CMCase, xylanase, laccase and peroxidase activity (HC >2.00 at least 2 activities) was identified as *Acremonium* sp., *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum*, *Humicola grisea*, *Mucor* sp., *Paecilomyces victoriae*, *Penicillium janthinellum*, *Penicillium lanosum* and *Trichoderma* sp. according to Fig. 2.

DISCUSSION

This research indicated that many fungi strains isolated from agricultural soil in Surin province showed high efficiency of enzyme activity for biodegradation, especially, the genera of *Trichoderma* and *Aspergillus*. They also showed higher in enzymatic activities than other areas compared with other reported studies. *Trichoderma* from agriculture soil was HC value of cellulase and laccase >3.00 while *Trichoderma* from other areas was ranged between 2.00-2.50 (Gochev and Krastanov, 2007), 2.00-2.50 for cellulase and 1.61-2.22 for laccase (Toyama and Toyama, 2001). In addition, Xu *et al.* (2006) pointed out that the genera *Aspergillus* showed the diameter of the clearing zone/diameter of the colony between 1.30-1.90 for laccase activity. Dhouib *et al.* (2005) reported that *Trichoderma* species were only 1.00-1.25 of the diameter of the clearing zone/diameter of the colony for laccase activity. These variations may be the affects of agricultural management practices on paddy soils such as fertility amendment, water content, cropping system, plant cover, residue composition, straw burning and environmental factors (Bastias *et al.*, 2009; Braun *et al.*, 2010; Grishkan *et al.*, 2008; Meriles *et al.*, 2009; Wang *et al.*, 2010; Whitelaw-Weckert *et al.*, 2007). Bulluck *et al.* (2002) found that organic and synthetic fertility amendments significantly influenced soil microorganisms on organic and conventional farms. Especially, beneficial soil fungi in the genus *Trichoderma*, the numbers of *Trichoderma* species were

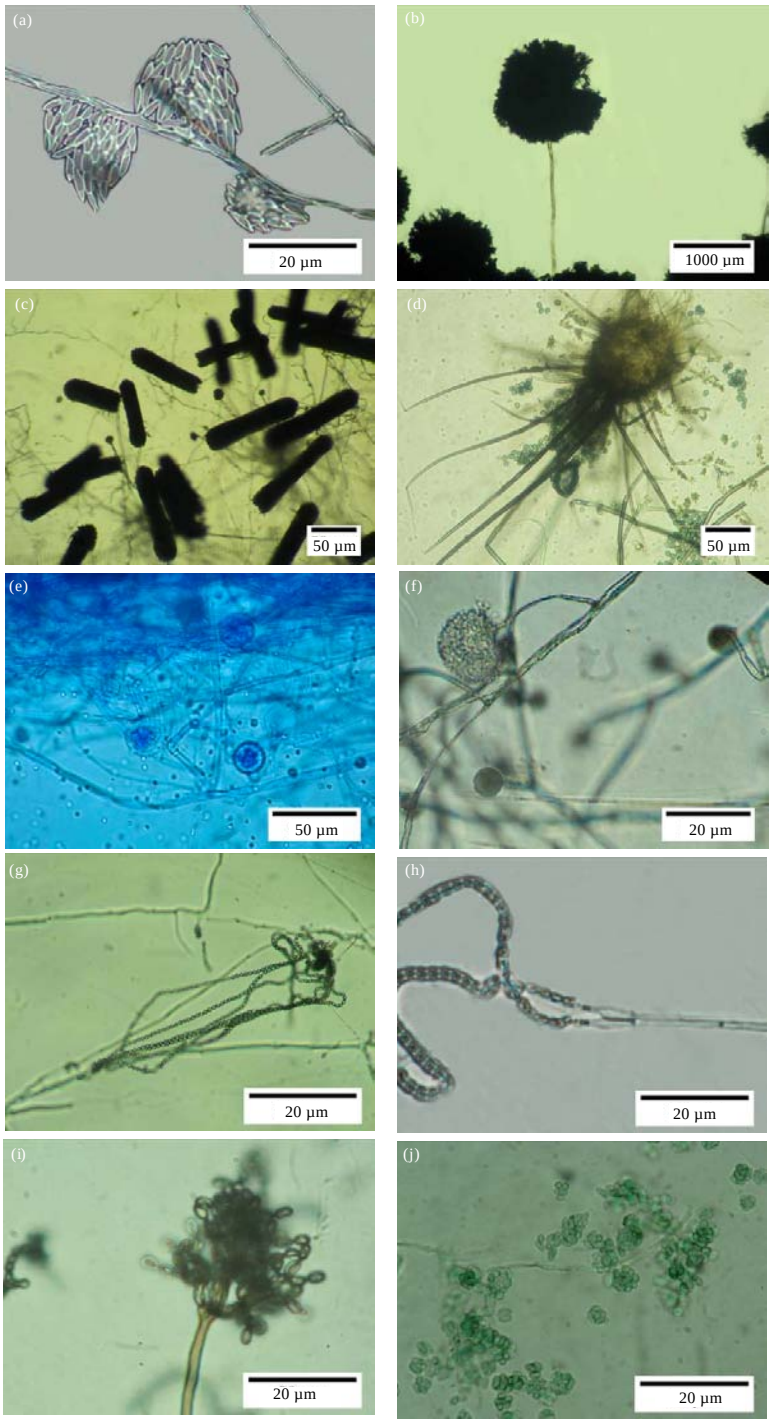

Fig. 2(a-j): Morphology of soil fungi isolated from various paddy soils having lignocellulose degrading enzymes, (a) *Acremonium* sp., (b) *Aspergillus niger*, (c) *Aspergillus* sp., (d) *Chaetomium murorum*, (e) *Humicola grisea*, (f) *Mucor* sp., (g) *Paecilomyces victoriae* (h) *Penicillium janthinellum*, (i) *Penicillium lanosum* and (j) *Trichoderma* sp.

higher in soils from fields with a history of organic than conventional production. The densities were also increased over time in fields with a conventional history but were remained lower over time in soils from organic compared to conventional fields. Soils with alternative amendments had also higher population densities of *Trichoderma* sp. than soils amended with synthetic fertilizers in vegetable. The occurrence and distribution of culturable soil fungi were significant differences in species distribution patterns with respect to soil pH, moisture, carbon, chlorophyll a, salinity, elevation and solar inputs (Connell *et al*., 2006). Arenz and Blanchette (2011) found that most soil fungi positively correlated with percent carbon and nitrogen while soil pH and conductivity were negatively correlated. In addition, Surin rice field located in northeast of Thailand. It has a tropical climate, with the annual temperature at 32°C and humidity averaged at 65% which is optimal condition for the growth of microorganisms (Reanprayoon and Yoonaiwong, 2012). These directly result to soil fungi population densities and biodegradation of rice straw. This research also found that the genera *Trichoderma* and *Aspergillus* are dominant genera in tropical soils in agreement with many studies. Qiao *et al*. (2008) indicated that fungi belong to the important components in soil microbial biomass of forests. *Aspergillus* and *Acremonium* were the most dominant of fungi isolated from forest soils (Cabello and Arambarri, 2002; Qiao *et al*., 2008). For agricultural soils, *Aspergillus* and *Trichoderma* were the prevalent genera (Donner *et al*., 2009; Jaime-Garcia and Cotty, 2010; Kausar *et al*., 2010; Liu *et al*., 2008; Porras *et al*., 2007; Shukla *et al*., 2012). For biodegradability of soil fungi, many reports also indicated that *Penicillium*, *Paecilomyces and Aspergillus* were effective in degrading plastics (Bansal *et al*., 2012; Kausar *et al*., 2010; Kim *et al*., 2000). These isolated fungi could be used for biodegradations of natural and synthetic materials in soil effectively.

CONCLUSION

Twenty nine strains of soil fungi were isolated from various agricultural soils as organic, young organic, semi-chemical and chemical soil from Surin rice fields, Thailand. Only ten strains found high activity in lignocellulolytic enzyme productions. Most tropical soil fungi showed ability to produce cellulase, xylanase, laccase and peroxidase, respectively. *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum* and *Trichoderma* sp. showed the highest potential to produce cellulase. *Acremonium* sp., *Aspergillus niger*, *Aspergillus* sp., *Chaetomium murorum*, *Humicola grisea*, *Paecilomyces inflatus*, *Paecilomyces roseolus*, *Paecilomyces victoriae*, *Penicillium janthinellum*, *Penicillium lanosum* and *Trichoderma* sp. found in xylanase activity. For peroxidase, *Paecilomyces victoriae* and *Trichoderma* sp. were peroxidase producers while *Humicola grisea* and *Paecilomyces inflates* were laccase producers. The result also revealed that ten species showed highest carboxymethyl cellulase, xylanase, peroxidase and laccase activities by qualitative screening method for enzymatic assay. They were identified as *Aspergillus niger*, *Acremonium* sp., *Aspergillus* sp., *Chaetomium murorum*, *Humicola grisea*, *Mucor* sp., *Paecilomyces victoriae*, *Penicillium janthinellum*, *Penicillium lanosum*, *Trichoderma* sp. These tropical soil fungi will be a step towards providing a method of accelerated decomposition of agricultural residues and for biodegradation process.

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