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การคัดเลือกและการจัดจำแนกเชื้อราทนร้อนที่ผลิตเอนไซม์เซลลูเลส เพื่อการผลิตเป็นหัวเชื้อปุ๋ยหมัก

Screening and Identification of Cellulase Producing Thermotolerant Fungi as Compost Starter

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บทคัดย่อ

จุดประสงค์ของงานวิจัยคือทำการคัดเลือกและจัดจำแนกชนิดของเชื้อราที่แยกได้จากดินที่มีกิจกรรมของเอนไซม์เซลลูเลสสูงเพื่อผลิตเป็นหัวเชื้อที่จะใช้หมักปุ๋ยหมัก โดยทำการคัดแยกเชื้อราที่แยกได้จากดิน 39 สายพันธุ์ ได้ทำการคัดเลือกเบื้องต้น โดยการเลี้ยงเชื้อในสภาวะอาหารแข็งที่มีเซลลูโลสเป็นแหล่งคาร์บอนบ่มที่ 45 องศาเซลเซียส ทำการวัดอัตราส่วนของวงใสรอบโคโลนีต่อขนาดของโคโลนี จากนั้นทำการคัดเลือกอีกครั้งในสภาวะอาหารเหลวที่มีเซลลูโลสเป็นแหล่งคาร์บอนและทำการวัดค่ากิจกรรมของเอนไซม์เซลลูเลส พบว่าสายพันธุ์ FA68 ให้ค่ากิจกรรมของเอนไซม์สูงสุดและแตกต่างอย่างมีนัยสำคัญกับสายพันธุ์อื่นและรองลงมา คือสายพันธุ์ FA50 ให้ค่ากิจกรรมเท่ากับ 0.17 และ 0.13 ยูนิต/มล. ตามลำดับ จากนั้นนำเชื้อราสายพันธุ์ทั้งสองสายพันธุ์ไปทำการเลี้ยงในวัสดุเศษเหลือทางการเกษตรในสภาวะอาหารแข็ง พบว่าสายพันธุ์ FA68 เจริญได้ดีเมื่อทำการบ่มที่ 45 องศาเซลเซียส เป็นเวลา 7 วันและให้ค่ากิจกรรมของเซลลูเลสที่สูงกว่าค่ากิจกรรมของเอนไซม์ชนิดเดียวกันของสายพันธุ์ FA50 มีค่าเท่ากับ 0.23 และ 0.22 ยูนิต/กรัมสับสเทรต ตามลำดับ เมื่อทำการเลี้ยงเชื้อและศึกษาการเจริญของทั้งสองสายพันธุ์ในข้าวฟ่างเพื่อผลิตเป็นหัวเชื้อปุ๋ยหมัก พบว่าการบ่มที่ 37 องศาเซลเซียส ความชื้นเริ่มต้นร้อยละ 80 พบว่าสายพันธุ์ FA68 และ FA50 ให้อัตราการสร้างสปอร์ได้สูงสุดเท่ากับ 10.27 และ 9.46 ลอคสปอร์/กรัมเมล็ดข้าวฟ่าง หลังจากการบ่ม 6 และ 5 วัน ตามลำดับ และเมื่อทำการจำแนกชนิดของสายพันธุ์ของจุลินทรีย์ พบว่าทั้งสายพันธุ์ FA68 และ FA50 คือ จีนัส *Aspergillus*

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ABSTRACT

The purposes of this research were to screen and identify the fungi isolated from soil having high cellulase activities for producing a compost starter. Thirty-nine isolates of fungi from soil were isolated. The primary screening of the fungi was done in the solid state medium with cellulose as the carbon source and incubated at 45°C. The ratio of the clear zone and the colonial sizes were determined. The secondary screening was done in liquid state fermentation with cellulose as the carbon source and the activities of the Carboxymethylcellulase (CMCase) were measured. The CMCase activity of the isolate FA68 was the highest and followed by that of the isolate FA50 with the enzyme activity (0.17 and 0.13. IU/ml). The isolates FA68 and FA50 were then cultivated on agricultural wastes, in solid state cultivation. The isolate FA68 grew well at 45°C for 7 days and produced higher levels of CMCase activities compared to those of the isolate FA50 with activity 0.23 and 0.22 IU/g, respectively. When both the fungal isolates were cultivated and studied the growth in sorghum grains in order to produced as compost starter, it was found that at 37 °C and 80 % initial moisture content, the isolates FA68 and FA50 had the highest sporulation rates of 10.27 and 9.46 log spores/g sorghum grains after 6 and 5 days incubation, respectively. Both the isolates FA68 and FA 50 were identified to be the genus *Aspergillus*

คำสำคัญ: เชื้อราทนอุณหภูมิสูง เซลลูเลส หัวเชื้อปุ๋ยหมัก เมล็ดข้างฟ่าง การสร้างสปอร์

Keywords: Thermotolerant fungi, Cellulase, Compost starter, Sorghum grains, Sporuration

1. Introduction

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Murai et al., 1998; Hong et al., 2001) and is generally found in agaricultural wastes such as rice straw, corn cob, peanut hull and rice husk which are highly abundant in Thailand. In the past, these materials were mainly used as animal feeds and some of them were used as composts for increasing organic matter in soil. Composting is the controlled, and aerobic decomposition by microorganisms in nature. On the other hand, the period of time of the traditional composting process is very long time consuming. The use of starter in composting to speed up the nature process or to obtain a better final compost has been a controversial subject for a long time. More over, using the wastes from agricultural sources as substrates, the activity of lignocellulolytic enzymes could

be maintained and, on the other hand, the costs may be strongly reduced (Vargas-Garda, 2005). Within each of these classes, most microorganisms are able to produce several enzymes, e.g. *Tricoderma reesei*, *Aspergillus* and *Penicillium* spp. are well known as efficient producers of cellulases (Milala, et al., 2005).

The aim of this study was to screen and identify the fungi from soil having high cellulase activities for producing a compost starter in the future.

2. Materials and methods

2.1 Soil sample

Each fungus was isolated from 19 soil samples in Kanchanaburi Province. Soil samples (100 g) were collected from the top part of soil (0–15 cm deep) (Coyne, 1999) and stored at 4 ºC until being used.

2.2 Plate screening

Ten grams of each sample were added to 90 ml of saline solution. This mixture was then shaken and diluted to the final concentrations of 10^{-2}, 10^{-3} and 10^{-4}. From each dilution, 0.1 ml of it was spread in triplicate on the surface of the selective media (Applied Mandel's Medium in which cellulose was the carbon source). This medium consists of 2.0 g KH_2P0_4, 1.4 g $(NH_4)_2S0_4$, 0.3 g Urea, 0.3 g $MgS0_4$ $7H_20$, 0.3 g $CaCl_2$ H_20, 1.0 g peptone, 10.0 g α cellulose, 15.0 g agar, and 1 L distilled water. Urea was added after autoclaving, the pH was adjusted to 5.3 and the plates were incubated at 30 ºC for 3 days. Cellulolytic fungi created a clear zone around each colony on the agar were selected to grow on Potato Dextrose Agar (PDA) plates for 5-7 days at 30 ºC. Stock cultures were maintained using PDA slants at 4 ºC and were then subcultured every 6–7 weeks.

2.3 Cellulase assay

The CMCase activity was determined using cellulose agar. Mycelial disks with 5 mm diameters were cut from the edges of colonies and were used to inoculate at the centre of plates containing the above medium. The plates were incubated at 45ºC for 5 days and the cellulase activity was indicated as clear orange halos after staining with 1% congo red solution for 10-15 min following by washing for 10 min with 1 M NaCl solution. The cellulase activities were calculated by measuring the diameters of the clear zones around the colonies and the isolates having the ratio of the clear zone per colony between 1.2–2.5. (>60^{th} percentile) were selected and inoculated in cellulose broth. Flask cultivation was performed using 250 ml flasks with 50 ml culture volumes. The pH of the medium was adjusted to 5.3 after autoclaving. Each flask was inoculated with 10^6 spores/ml. Three flasks were inoculated per isolate under the conditions of 45ºC, pH

5.3 and the shaking rate of 150 rpm, for 7 days. The extracellular cellulolytic activities of the isolates were measured quantitatively by dinitrosalicylic acid (DNS) method (Mandels et al., 1976) as followed: 0.5 ml of the clear supernatant of each isolate was combined with 0.5 ml of 1% substrate (CMC) solution suspended in 50 mM citrate buffer (pH 4.8). Both solutions were incubated at 50°C for 30 min. The reaction was stopped by 3 ml of DNS reagent and boiled for 5 minutes after that the volumes of the solutions were made up to 10 ml with distilled water. The optical density of each solution was determined at 550 nm with a spectrophotometer. One unit (IU) of the enzyme activity was defined as the amount of enzyme releasing 1 μmol reducing sugar in 1 min and D-glucose was used as the standard.

2.4 The efficient digestion of agricultural wastes of the selected fungi

The isolate FA68 and FA50 were cultured in 5 agricultural wastes (coffee pulp, corn cob, dry leaves, palm fiber and rice straw) in solid state cultivation. Moisture content was adjusted by using mineral salt solution (Daniel et al., 2007) The ratio of agricultural wastes: mineral salt solution was 10 g: 10 ml and incubated at 45°C for 7 days. CMCase activities were mesured in IU/g substrate.

2.5 optimal growth conditions

The selected fungi were cultured in a solid substrate using sorghum grain as carbon source. The optimal conditions for growth of the strain was studied using the trial Factorial 4×4 with Completely Randomized Design (CRD) of 2 factors which were the temperatures (30, 37, 45 and 50°C) and the initial moisture contents (50, 60, 80 and 100%). Three replications for 5 days were done. The growth of the fungus was recorded by counting the amount of spores with a haemacytometer as log spores/g. The appropriate conditions were then selected. Once the optimal conditions for growth have been studied, the period of culture that most appropriate to the cells were collected every 2 days for 14 days and the spores were counted.

2.6 Identification of the isolates

Fungi were identified by their colony characteristics as well as their vegetative and reproductive structures as observed under the compound microscope. Some macroscopic characteristics including colour of the colony and patterns of growth of the colony were studied. Some of the microscopic characteristics as viewed under the microscope were the shape of the conidial head, the pattern of the arrangement of spores on the conidia and the shapes of the conidiophores (Bennet, 1985).

2.7 Statistic analysis

Data in 2.3 were analyzed by a one-way ANOVA and data in 2.4 analysed by two way ANOVA and comparing the differences of the averages by Duncan' New Multiple Range test at the level of 95% confidence by using the solfware package SPSS for Windows (Release 11.5; standard version).

3. Results and discussion

3.1 Screening and isolation of cellulose degrading fungi

Thirty-nine isolates of soil fungi were isolated using the ability of cellulose degradation on cellulose agar. After five days, 8 isolates gave the ratio of the clear zone per colony between 2.4-10.3 (>60th percentile). Among these isolates, FA61 showed the highest ratio of the clear zone per colony size with the value of 10.3, followed by FA06 and FA40 with the values of 8.0 and 7.7, respectively (Table 1). The result of the enzyme activity in cellulose broth was not consistent with that in agar. In broth, the results indicated that the isolate FA68 had the highest enzyme activity (0.17 IU/ml) followed by the isolate FA50 (0.13 IU/ml) (Table 2). Among fungi, *Aspergillus* and *Trichoderma* have been widely exploited for their inherent ability to produce cellulases (Zhang and lynd, 2004).

Table 1 The Ratio of clear zones per colony size incubated at 45 °C for 5 days and the cellulase activities of isolated fungi grown on CMC agar.

Isolate	Ratio of clear zone per colony size	Isolate	Ratio of clear zone per colony size
FA02	2.00 ^{fg}	FA23	1.02 ^{h}
FA04	1.21 ^{h}	FA26	1.80 ^{g}
FA05	2.60 ^{e}	FA39	2.40 ^{ef}
FA06	8.00 ^{b}	FA40	7.67 ^{b}
FA10	1.80 ^{g}	FA50	6.04 ^{c}
FA18	4.00 ^{d}	FA61	10.27 ^{a}
FA21	1.00 ^{h}	FA68	2.65 ^{e}

*The means with similar letters in a column are not significantly difference ($p < 0.05$).

Table 2 Cellulase activities of the selected fungi cultured under the conditions of 45°C, pH 5.3 with the shaking rates of 150 rpm for 2, 3, 5 and 7 days.

Isolate	Cellulase activity (U/ml)				Isolate	Cellulase activity (U/ml)			
	2 days	3 days	5 days	7 days		2 days	3 days	5 days	7 days
FA05	0.00 ^{c}	0.01 ^{d}	0.04 ^{de}	0.04 ^{d}	FA40	0.01 ^{c}	0.04 ^{b}	0.05 ^{d}	0.03 ^{e}
FA06	0.03 ^{b}	0.03 ^{c}	0.07 ^{c}	0.05 ^{c}	FA50	0.04 ^{a}	0.04 ^{b}	0.13 ^{b}	0.08 ^{b}
FA18	0.03 ^{b}	0.03 ^{c}	0.04 ^{de}	0.04 ^{d}	FA61	0.00 ^{c}	0.01 ^{d}	0.03 ^{f}	0.02 ^{f}
FA39	0.03 ^{ab}	0.03 ^{c}	0.04 ^{de}	0.04 ^{d}	FA68	0.03 ^{ab}	0.05 ^{a}	0.17 ^{a}	0.10 ^{a}

*The means with similar letters in a column are not significantly difference ($p < 0.05$)

3.2 The efficient digestion of agricultural wastes

When The isolate FA68 and FA50 were cultured in 5 agricultural wastes, it was found that FA68 gave the hightest CMCase activity, followed by the isolate FA50 in rice straw as substrate, with activity 0.23 and 0.22 IU/g substrate, respectively (Figure 1). The agricultural wastes utilized are rich in carbon content and other vital nutrients essential for fungal growth (Xianyun et al., 2008; Oberoi et al., 2010). Hence, solid state fermentation was conducted using rice straw supplemented with other nutrient rich agricultural wastes to evaluate the potential of using mono- and mixed-cultures of *Aspergillus niger and Trichoderma reesei* in production of Filter Paper Cellulase, CMCase and xylanase (DHillon et al., 2011)

3.3 Growth optimization and growth rate on solid state cultivation

From the studies of growth optimization and the period of culture in a solid culture using sorghum grains as carbon sourse in order to produce as compost starter, it was shown that the optimal conditions for growth of the two fungal isolates FA68 and FA50 were 37°C and 80% initial moisture content when cultured for 5 days. Both FA50 and FA68 gave the higest the numbers of spores when compared with other conditions and the numbers of spores were significantly different at the level of $p<0.05$ (data not shown). The numbers of spores increased to be 9.46 and 10.27 log spores/ g dry weight of sorghum grains, when cultured for 5 days for FA50 (Figure 2A) and 6 days for FA68 (Figure 2B).

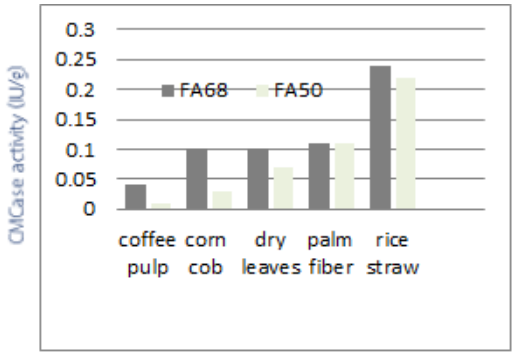


Figure 1 CMCase activities of the isolate FA68 and FA50 in 5 agricultural wastes at 45°C for 7 days

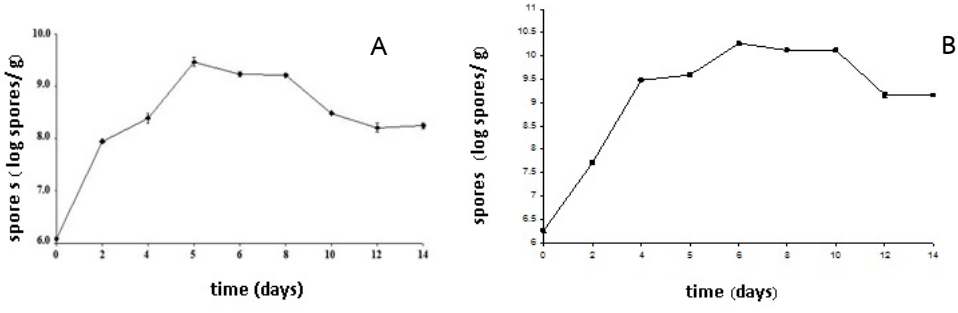
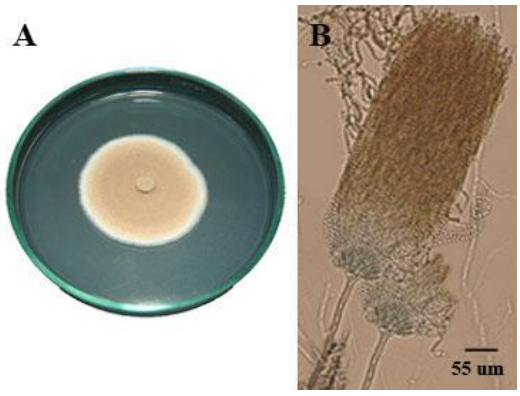
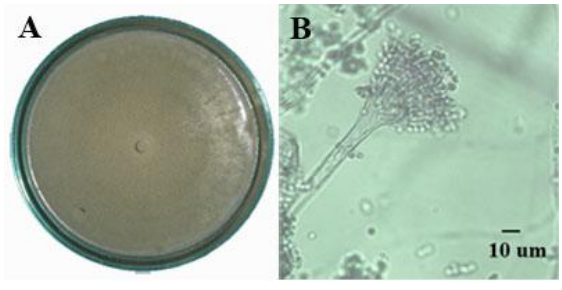


Figure 2 The growth on sorghum grain of the isolates FA50 (A) and FA68 (B)



Fiqure 3 Macroscopic and microscopic morphologies of the isolate FA50 (A) The culture of the isolate FA50 with white hyphae and brown spores grown on PDA at 30°C for 5 days. (B) Asexual state of the isolate FA50 (genus *Aspergillus*) under a microscope.



Fiqure 4 Macroscopic and microscopic: Morphologies of the isolate FA68 (A) The culture of isolate FA68 with white hyphae and green spores grown on PDA at 30°C for 5 days. (B) Asexual state of the isolate FA68 (genus *Aspergillus*) under a microscope.

3.4 Identification of the isolates

The morphological studies on the fungal isolate FA50 showed that the colonies growing on PDA at 30°C for 5 days were spherical shape with approximately 4 cm. in diameter. The hyphae were white and the spores were brown (Figure 3A). The morphological characteristics under the microscope were as follows: the hypha septate with round vesicles and the biseriate sterigmata were radially spread from the center and the spores were round (Figure 3B). The fungal isolate FA68 was able to grow quickly and created a large number of spores on PDA at the same condition and the colony diameter was about 6 cm with white hyphae and green spores (Figure 4A). The morphological characteristics of the isolate FA68 under the microscope were septate hyphae, rather round vasicles, uniseriate sterigmata and spherical spores. (Figure 4B). The above described fungal morphological characteristics showed that the two isolates were belonged to the genus *Aspergillus*. From

fungal morphological studies of the genus *Aspergillus* by the method of Raper and Funnell, 1965 which relies on colors and appearances of conidial heads of the colonies including the classification method of the fungus *Aspergillus* in the Handbook of Klich, 2002, the fungal isolates FA50 and FA68 were identified to be the genus *Aspergillus.*

4. Conclusion

In this work, we presented that the isolates FA50 and FA68 had the capability to degrade agriculture residues and to grow at high temperature (45°C). The isolate FA68 showed the highest enzyme activity (0.17 IU/ ml) followed by the isolate FA50 (0.13 IU/ ml). The optimal conditions for growth of both of the two isolates, FA50 and FA68 were at 37°C and 80 % initial moisture content and the number of spores of both of them were 9.46 and 10.27 log spores/ g dry weight of sorghum grains when cultured for 5 and 6 days, respectively. The isolates FA50 and FA68 were identified to be the genus *Aspergillus.*

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