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การผลิตเอนไซม์คาร์บอกซีเมทธิลเซลลูเลสจาก *Penicillium oxalicum* HS1-3 ที่แยกได้จากดินในจังหวัดชัยภูมิ

Carboxymethyl Cellulase Production by *Penicillium oxalicum* HS1-3 Isolated from Soil in Chaiyaphum Province

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ABSTRACT

Agricultural cellulosic material is importance for bio-energy production because it is cheap, abundant and virtually inexhaustible source of renewable bioenergy. Cellulolytic enzymes are major group of enzyme that is capable degrading cellulose to glucose. This study aimed on screening of fungi producing carboxymethyl cellulase which was isolated from 48 samples of soil, wood and leaf decay in Chaiyaphum province, the northeastern part of Thailand. Only 25 fungal isolates exhibited carboxymethyl cellulase activity on plate screening assay. The selected 5 fungal isolates (HS1-3, HS1-5, HS1-7, HS1-12, HS2-5) which exhibited high carboxymethyl cellulase on the agar plate were grown and their abilities of enzyme production were determined. The 5 fungal isolates were cultured in mineral salt agar medium at pH 5.0 containing 1.0% carboxymethyl cellulose and incubated at 150 rpm, 30°C for 7 days. The results showed that fungal isolate HS1-3 exhibited the highest enzyme activity (150 U/mL). The identification of fungal isolate HS1-3 was carried out by the internal transcribed spacer region (ITS) DNA sequencing and compared with BLASTN Homology Search. The results indicated that the DNA sequence of fungal isolate HS1-3 was 100 percent (1046/1046 nucleotides) identical with *Penicillium oxalicum*, GenBank accession number HQ843504.1.

Keywords: Bioethanol, Agricultural Cellulosic Material, Carboxymethyl Cellulase, *Penicillium oxalicum*

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INTRODUCTION

At present, energy consumption is rising, while fossil fuels is depleting. Therefore, alternative energy needs to be renewable energy. Bioenergy or bioethanol is becoming a viable alternative energy, because it is clean and renewable energy source. Bioethanol can be produced through fermentation from renewable cellulosic biomass such as sugarcane bagasse, wheat straw and rice straw (Krishna et al., 2001; Lever et al., 2010; Hsu et al., 2011). Agricultural cellulosic material is cheap, abundant and increasingly important resource for future biofuel. Cellulose which is the most abundant in plant cell wall and a virtually inexhaustible source is now of great interest in commercializing technologies for bioethanol production from inexpensive biomass. The production of ethanol from cellulose involves the several steps of material pretreatment, hydrolysis (saccharification) and ethanol recovery. Hydrolysis of cellulose is the limiting step to generate the fermentable glucose. As acid hydrolysis generates hazardous waste, the enzymatic method is then an alternative efficient method in hydrolysis cellulose to fermentable sugar because this process performs under ambient condition without generation of any toxic waste.

The cellulose-degrading enzymes, cellulase, include endo-acting (endoglucanases, EGs) and exo-acting (cellobiohydrolases, CBH) enzymes, which act in a synergistic manner in biomass-degrading microbes (Dashtban *et al.*, 2009). Cellulolytic enzymes are a family of enzymes that hydrolyze the beta-1,4 linkages to glucose. These enzymes are composed of three major components including endo-(1,4)-beta-D-glucanase (endoglucanase, endocellulase, CMCase [EC3.2.1.4]), which cleaves beta-linkages at random, commonly in the amorphous parts of cellulose, exo-(1,4)-beta-D-glucanase (cellobiohydrolase, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91]), which releases cellobiose from nonreducing or reducing end, generally from the crystalline parts of cellulose and beta-glucosidase (cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cellooligosaccharides (Bhat and Bhat, 1997).
The production of cellulase enzyme is a major factor in the hydrolysis of cellulosic materials. It is important to make the process economically available for bioethanol production. Filamentous fungi have a long history of being use in the several enzymes production (Law, 2002). Cellulolytic enzymes have been mostly produced by various fungi. The production of cellulose-degrading enzymes by the genus *Penicillium* has gained interest in recent years because of their particular properties. In 2010, Ng et al. reported *Penicillium citrinum* cultured with rice bran exhibited high level production of a thermoacidophillic beta-glucosidase.

In this study, fungi producing cellulolytic enzyme were isolated from soil, wood and leaf decay in Chaiyaphum province and the capability to produce cellulolytic enzyme was investigated. The isolated and selected fungal strain which produced high activity of cellulolytic enzyme would be applied in bioethanol production process.

MATERIALS AND METHODS

Fungal isolation

Samples (soil, wood and leaf decay) were collected from tropical forest in Chaiyaphum province, the northeastern of Thailand. Fungi which produce carboxymethyl

cellulase were isolated from collected samples. One gram of sample was added into 9.0 ml of normal saline solution and spread on potato dextrose agar (PDA) plate containing streptomycin (25 µg/mL) to inhibit bacterial growth. The agar plate was incubated at 30°C for 2-7 days. The fungal hyphae was collected and purified. Pure cultures were preserved on PDA slant at 4°C.

Plate screening for fungi producing carboxymethyl cellulase

The selected fungal strains were point inoculated on mineral salt agar medium (0.1% KH_2PO_4, 0.01% $CaCl_2$, 0.02% $MgSO_4.7H_2O$, 0.05% KCl, 0.05% $(NH_4)_2SO_4$, 1.5% agar, pH 5.0) containing 1.0% carboxymethyl cellulose and incubated at 30°C for 3-7 days. Each plate was flooded with 0.2 % (w/v) Congo red solution for 15 min, and destained with 1.0 M sodium chloride (Teather and Wood, 1982). Unstained areas or clear zone indicated hydrolysis of carboxymethyl cellulose by carboxymethyl cellulase secreted from the fungi (Farkas et al., 1985).

Carboxymethyl cellulase production

The fungal strains producing large clear zone were selected to investigate carboxy methyl cellulase production in the liquid medium. Five milliliters of fungal spore suspension (approximately $1x10^8$ spores/mL) were added in 50 mL of mineral salt medium containing 1.0% carboxymethyl cellulose and incubated in an incubator shaker at 30°C for 7 days. Sample was taken to determine carboxymethyl cellulase activity and cellular growth (cell dry weight) every day for seven days.

Carboxymethyl cellulase assay

The carboxymethyl cellulase activity was determined by the colorimetric method according to Miller (1959) by using dinitrosalicylic acid reagent (DNS). The method was based on determination of the colour developed after the reaction between the reducing sugars hydrolyzed from cellulose and DNS-reagent. The cultural broth was centrifuged at 10,000 rpm for 10 min and the supernatant (0.5 mL) was added to 0.5 mL of 1% (w/v) carboxymethyl cellulose solution in 0.05 M citrate phosphate buffer, pH 5.0 and incubated at 45°C for 30 min (Sengubta et al., 2000). The enzyme reaction was stopped by adding 1.0 mL DNS-reagent and placed in a boiling water bath for 10 min. The colour of the reaction product in this mixture was measured spectrophotometrically at 540 nm. One unit of enzyme was defined as the amount of enzyme that hydrolyzed carboxymethyl cellulose to yield 1 µmol reducing sugar per min under the experimental condition.

Fugal identification

Identification of the isolate fungi was carried out by the internal transcribed spacer region (ITS) DNA sequencing using a PCR primer of ITS4 and ITS5 (White et al., 1990; Bunyard et al., 1994 and Landvik, 1996). The PCR fragment was sequenced and ITS DNA was compared with the database retrieved from GenBank.

RESULTS AND DISCUSSIONS

Isolation and identification of carboxymethyl cellulase producing fungi

Sixty five fungal isolates were selected from 48 samples of soil, wood and leaf decay in Chaiyaphum province and investigated for carboxymethyl cellulase production. Point inoculation of fungal isolates were cultured on mineral salt medium agar containing 0.1% carboxymethyl cellulose for 3-7 days and flooded with Congo red for 15 min. Congo

red has strongly interacts with polysaccharides containing beta-1,4-D-pyranosyl units (Teather and Wood, 1982). Congo red cannot interact with carboxymethyl cellulose hydrolyzed obtained from activity of carboxymethyl cellulase production from fungi resulting in clear zone around the fungal colony. Therefore, clear zone around the fungal colony approximates the amount of carboxymethyl cellulase production from fungi. The diameter of the clear zone can be measured to provide a quantitative comparison of cellulolytic activity.

Only 25 fungal isolates could hydrolyze carboxymethyl cellulose on the agar plate are shown in Table 1. The 5 fungal isolates (HS1-3, HS1-5, HS1-7, HS1-12 and HS2-5) which exhibited the different diameter between clear zone and colony zone higher than 17 mm were selected to study the production of carboxymethyl cellulase in liquid culture.

Table 1. Diameter difference between clear zone and colony zone of 25 fungal isolates on the agar plate.

Fungal isolate	Diameter difference between clear zone and colony zone (mm)	Fungal isolate	Diameter difference between clear zone and colony zone (mm)
HS1-3	**21**	HS2-13	10
HS1-4	4	HS2-15	5
HS1-5	**43**	HS2-17	1
HS1-6	10	HS2-18	10
HS1-7	**32**	HS3-8	3
HS1-11	10	HS3-9	10
HS1-12	**40**	HS3-20	5
HS1-15	6	HS3-22	1
HS1-18	2	HS3-23	10
HS2-4	2	HS3-25	2
HS2-5	**17**	HS3-26	11
HS2-9	10	HS3-28	1
HS2-12	7	-	-

Carboxymethyl cellulase production

The 5 fungal isolates (HS1-3, HS1-5, HS1-7, HS1-12, HS2-5) which exhibited large clear zone on the agar plate were selected to determine the enzyme production in batch culture. The carboxymethyl cellulase production from 5 fungal isolates is shown in Fig 1. After 1 day of cultivation, the enzyme production from fungal isolate HS1-3 was dramatically increased when compared with fungal isolates HS1-5, HS1-7, HS1-12, HS2-5. After 5 days of cultivation, fungal isolate HS1-3 produced the highest enzyme activity around 150 U/mL. Fungal isolates HS1-5, HS1-7, HS1-12 and HS2-5 exhibited enzyme activity less than 40 U/mL after incubated for 7 days.

The cellular growths of fungal strains were also determined in mineral salt agar medium containing 1.0% carboxymethyl cellulose and the results are shown in Fig 2. Isolates HS1-5, HS1-7, HS1-12 exhibited the similar growth pattern and late logarithm phase was observed about 3 days of cultivation. HS1-3 and HS2-5 exhibited growth pattern like biphasic curve the highest cell dry weight were found in 7 days. These results could be concluded that growth pattern of the 5 fungi were not associated with the ability of those fungi to produce enzyme.

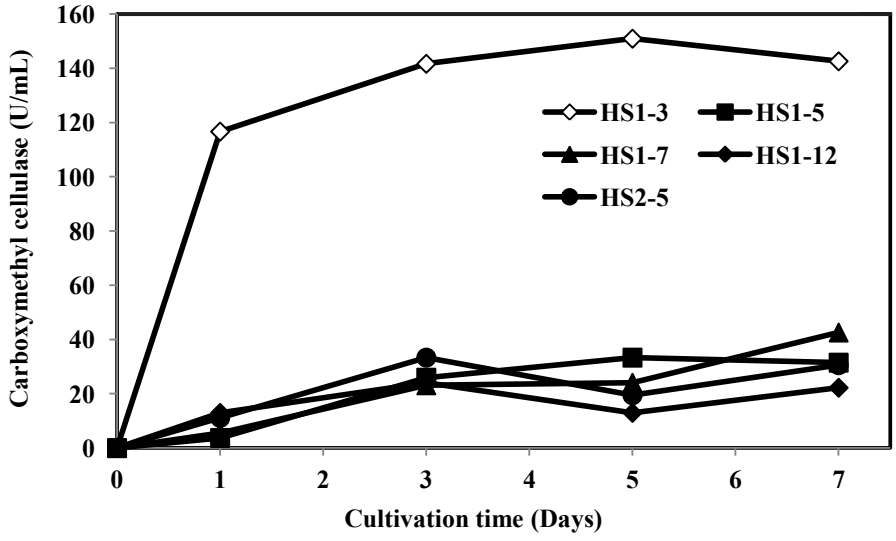

Figure 1. Carboxymethyl cellulase production from fungal isolates HS1-3 (◇), HS1-5 (■), HS1-7 (▲), HS1-12 (◆) and HS2-5 (●).

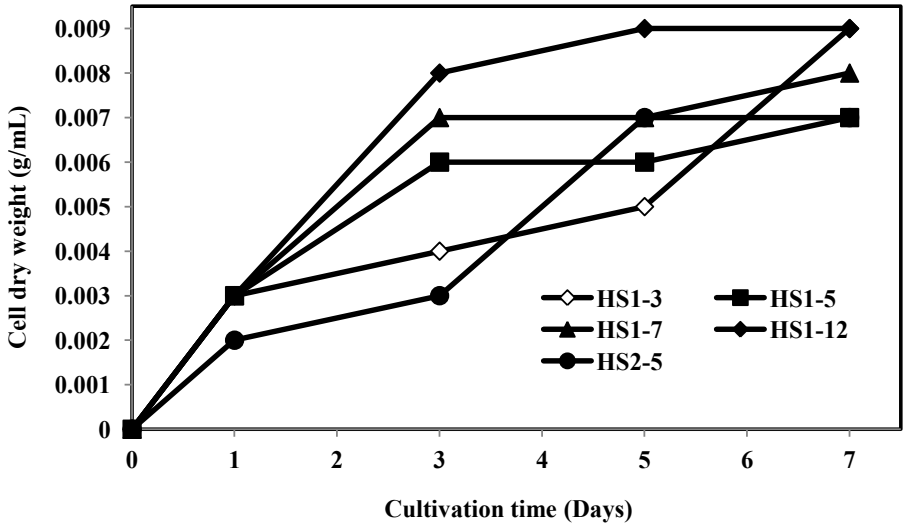

Figure 2. Cellular growth of fungal isolates HS1-3 (◇), HS1-5 (■), HS1-7 (▲), HS1-12 (◆) and HS2-5 (●)

Fungal identification

Identification of the HS1-3 isolate was carried out by the internal transcribed spacer region (ITS) DNA sequencing and compared with database in GenBank by BLASTN Homology Search. The results indicated that the sequence of fungal isolate HS1-3 was 100 percent (1046/1046 nucleotides) identical with *Penicillium oxalicum*, GenBank accession number HQ843504.1. The morphology of *P. oxalicum* HS1-3 is shown in Figure 3.

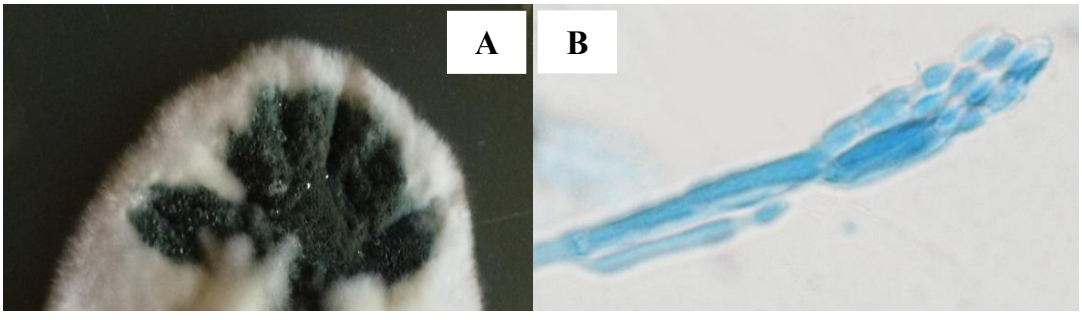

Figure 3. Morphology of *P. oxalicum* HS1-3 grown on PDA plate for 7 days (A) and examination under microscope, 1,000 x (B).

CONCLUSIONS

The productions of bioethanol from agricultural cellulosic materials are important for energy generation as biofuel. *Penicillium* spp. have been described as a large group of fungi which produce cellulolytic enzyme for many importance industrials. Several articles reported the production of cellulolytic enzyme by *P. oxalicum* (Seeley and Vandemark (1981), *P. pinophilum* (Wood et al. (1989), *P. brasilianum* (Jorgensen and Olsson, 2006) and *Penicillium* sp. (Karthikeyan et al., 2010). In our results, we isolated *P. oxalicum* HS1-3 which produced high carboxymethyl cellulase activity. Moreover, this *P. oxalicum* HS1-3 could hydrolyze filter paper and exhibited the production of beta-glucosidase and avicelase (unpublished data) at the same time. The synergistic property of cellulosic enzyme produced from *P. oxalicum* HS1-3 is quite promising as this fungus could hydrolyze cellulosic materials to glucose for ethanol production.

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