J. Trop. Agric. and Fd. Sc. 35(1)(2007): 153–157

Screening for cellulase activities in actinomycetes isolated from different locations of Peninsular Malaysia

(Penyaringan aktinomiset dari beberapa kawasan di Semenanjung Malaysia untuk aktiviti selulase)

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Key words: actinomycetes, cellulase, *Streptomyces* spp.

Abstract

A total of 282 isolates of actinomycetes were isolated from soil samples collected from few selected areas in Peninsular Malaysia (Selangor, Johor and Kuala Lumpur). These actinomycetes were observed to have different colony colours ranging from dark grey (42.6%), yellowish white (7.4%), greyish white (40.8%), white (7.8%), reddish (0.3%) and brownish (1.1%). All the isolates were screened for their cellulase activity which 37.6% (106 isolates) gave positive results. Isolate number 153, 159, 177, 184, 185, 191, 196, 200 and 262, which produced halo zone of 15.0–23.0 mm, were identified by targeting their 16S-rRNA sequence. The results obtained showed that all the isolates belong to the genus *Streptomyces* spp.

Introduction

Cellulose is the world's most abundant naturally occurring organic compounds which accumulates every year in large quantities in the form of agricultural, industrial, forest and residential waste (Mandels 1975). The potential of cellulose being a renewable source of energy was only recognized after the identification of cellulose degrading enzymes such as cellulases (Bhat and Bhat 1997).

Actinomycetes are widely distributed in soil and they play an important role in degrading lignocellulose components of plant cell walls (Lacey 1973). Most of the studies done were largely attributed to fungi, and the ability of actinomycetes in degrading the lignocellulose was neglected (Li 1997). Study done by Alam et al. (2004) showed that isolates of *Streptomyces omiyaensis* are able to produce cellulolytic enzymes. Bioconversions of cellulosic materials to desirable products involve complex processes which require a number of different enzymes (Alam et al. 2004).

In Malaysia, cellulosic materials such as agricultural and industrial residues are produced everyday by farmers and company operators. Microbiological utilization of these residues may help in the production of animal feed, food and other organic products (Alam et al. 2004). Study done at the Natick Army laboratory in the United State of America showed that cellulosic waste can be converted into glucose, soluble sugar, alcohols and single cell protein with the utilization of cellulases (Reese and Mandels 1984; Bhat and Bhat 1997). The aim of this study was to perform a prescreening and to identify cellulase producing actinomycetes.

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Materials and methods

Surveying and sample collection

Sampling of soil samples was done in 2003. Soil samples were collected 10 cm below the soil surface of the selected tree canopy. Soil samples were pre-treated by drying in open air for 1 week before isolation.

Isolation of actinomycetes from soil samples

Soil samples of 2 g each were mixed with 20 ml of sterile distilled water and shaken using an orbital shaker at 250 rpm at room temperature (±25 °C). About 200 µl of the soil suspension was then pipetted and spread onto Humic Acid (HV) agar plates (Hayakawa et al. 2004) and incubated for 7 days at ±30 °C. Grown colonies were picked up using sterile toothpick and touched onto starch casien agar (SCA) (10.0 g of soluble starch, 0.3 g of casein hydrolysate, 2.0 g of KNO_3, 2.0 g of NaCl, 2.0 g of K_2HPO_4, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$, 18.0 g of agar, 100 µg/ml of cycloheximide and 1,000 ml of distilled water) and incubated for 7 days at ±30 °C. Pure colonies of actinomycetes were obtained after several sub-cultures *(Plate 1).*

Screening of actinomycetes for cellulase activity

The isolated actinomycetes were then screened for their cellulases activity using substrate agar [1.0 g of peptone, 1.0 g of yeast extract, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of K_2HPO_4, 1.0 g of $(NH_4)_2HPO_4$, 1.0 g of Azo-CM-Cellulose (Megazyme), 15.0 g of agar; and 1,000 ml of distilled water]. Formation of halo zone after 3 days of the test indicated positive reaction *(Plate 1).*

Genomic DNA (gDNA) isolation

Isolation of gDNA was done using *BACTOZOL* KIT by Molecular Research Center, Inc. Protocol of isolation was done according to method suggested by Molecular Research Center (2005).

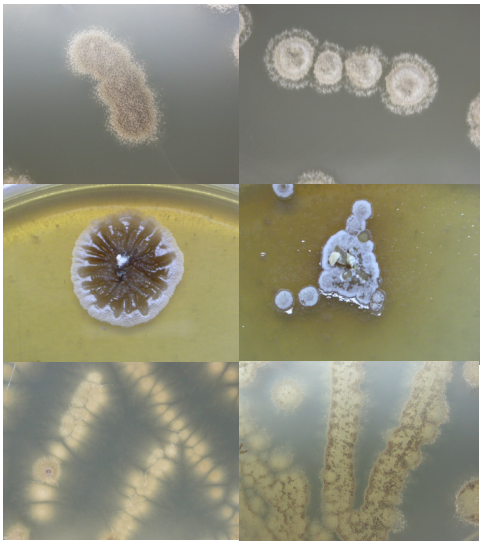

Plate 1. Some of the colony morphology of actinomycetes isolated

Polymerase chain reaction

Amplifications were performed in a 25.0 µl mixture containing 16.3 µl of sdH_2O, 2.5 µl of 10x PCR buffer (Promega), 1.5 µl of 25 mM $MgCl_2$ (Promega), 0.5 µl of 10 mM dNTP's (Promega), 0.2 µl of *Taq* polymerase (Promega), 1.0 µl for both 0.05 mM of Com1 (5'CAGCAGCCGCGGTAATAC3') and 0.05 mM of Com2 (5'CCGTCAATTCCTTTGAGTTT3') primer (Schwieger and Tebbe 1998) respectively, and 2.0 µl of genomic DNA. The reaction tube was then put into MJ Thermalcycler, which had been programmed to preheat at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s and elongation at 72 °C for 45 s before a final extension of 72 °C for 10 min. Product size estimated was 408 bp. Sterile distilled water, which substitute template DNA, was used as negative control.

Purification of PCR product

PCR product purification was done using *HiYield* Gel/PCR mini Kit supplied by Real Biotech Corporation (2005). Protocol for purification follows as stipulated by the manufacturer.

Sequencing

Purified PCR products were sent to First Base Laboratories Sdn. Bhd. which uses *ABI PRISM* 377 DNA Sequencer (Applied Biosystems) for sequencing purposes.

Interpretation of sequencing result

The obtained 16S rRNA sequences were compared to sequences in the NCBI GenBank database with the Basic Alignment Search Tool (BLAST) (Altschul et al. 1990).

Results and discussion

Isolation

From the 282 isolates obtained, the highest number of actinomycetes was isolated from Selangor soil with the total of 170 (60.3%) isolates, with only 88 (31.2%) and 24 (8.5%) isolates for Johor and Kuala Lumpur soil samples respectively. It was observed that isolation of actinomycetes from herbal area contained more population of actinomycetes rather than from banana plantation and ornamental plant area.

The variation in the number of actinomycetes isolated for each sample may be influenced by the environment where the soil samples were collected. This may be true because soil samples collected from the ornamental plants area, were from residential area.

Another reason for the less population of actinomycetes from Johor and Kuala Lumpur may be related to the agricultural practices by the owners. Herbal plants were not sprayed heavily with pesticides and fertilizers, thus the high population of microorganisms. Conversely, heavy usage of pesticides and fertilizers might be applied to the ornamental plants and banana plantations as the owner may want to protect the bananas from plant diseases such as *Fusarium oxysporum*.

About 42.6% of the total isolates produced dark grey colony, 40.8% greyish white, 7.4% yellowish white, 7.8% whitish, 1.1% brownish and 0.3% reddish colony colour *(Table 1)*. The results may have suggested that we have a diverse group of actinomycetes genera. However, according to Lo et al. (2002), actinomycetes, which produce different colony colour, may belong to the same genera if they possess the same morphological characteristics.

Cellulase screening

From the 282 isolates screened, 37.6% possessed the ability to utilize cellulose. From the total, 80.2% was from Selangor, 12.3% from Johor and 7.5% from Kuala Lumpur soil samples. The ability of actinomycetes to utilize cellulose differs from one sample source to another and this may be due to the usage of chemicals (pesticides and fertilizers) on the plants. Most of the beneficial microbes may have been killed by the dosage of pesticides used or have mutated to better adapt to the chemicals.

Nine of the most prevailing producers of cellulases were identified (isolate number 153, 159, 177, 184, 185, 191, 196, 200 and 262), with the ratio of halo zones to colony diameter between 3.00 and 4.60 *(Table 2).* The halo zones produced by actinomycetes in this study were larger than the halo zones produced by actinomycetes obtained from Amira et al. (1989). It was found that all

Table 1. Colour of actinomycetes colony as observed on SCA

Location	Colony Colour						Total isolates
	Dark grey	Yellowish white	Grey white	White	Reddish	Brownish	
Johor	37	5	38	8	0	0	88
Kuala Lumpur	15	4	0	5	0	0	24
Selangor	68	12	77	9	1	3	170
Total	120	21	115	22	1	3	282

Table 2. Ratio of size of halo zone to colony diameter of isolates at pH 7.0 at room temperature

Isolates	Ratio of halo zone (mm) to colony diameter (mm)*
153	3.00
159	4.00
177	3.80
184	3.80
185	4.60
191	3.00
196	3.40
200	4.20
262	3.20

*Diameter of colony is 5 mm

Table 3. BLAST result for best producing strains on isolates

Isolates	BLAST result
153	*Streptomyces gancidicus*
159	*Streptomyces gancidicus*
177	*Streptomyces* spp.
184	*Streptomyces malachitofuscus*
185	*Streptomyces gancidicus*
191	*Streptomyces gancidicus*
196	*Streptomyces stramineus*
200	*Streptomyces glomeratus*
262	*Streptomyces gancidicus*

these isolates belong to the same sample source. *Streptomyces gancidicus* (isolate number 159 and 185) and *Streptomycetes glomeratus* (isolate number 200) showed the biggest halo zone ratio to colony of 4.00, 4.60 and 4.20 respectively among all the actinomycetes isolated in this study. It was observed that *S. gancidicus* (isolate number 153, 159, 185, 191 and 262) were a potent producer of cellulase compared to other streptomycetes isolates in this study.

Identifying actinomycetes

All the nine isolates were identified using their partial 16S rRNA sequence. Results from BLAST of the sequences showed that all of the isolates belong to *Streptomyces* genera *(Table 3)*. This proved that the most dominant actinomycetes found in soil were *Streptomyces* and they have the ability to utilize cellulose. Study done by Amira et al. (1989) and Alam et al. (2004) supported our finding that *Streptomyces* have the ability to produce cellulases.

Conclusion

From the total of 282 isolates of actinomycetes, 106 isolates showed positive reaction in producing cellulose. Nine of the best producing isolates have been chosen to be identified using 16S rRNA methods showing that all the nine isolates belong to the genus of *Streptomyces* spp. More studies should be done using these actinomycetes to further enhance their cellulases production.

Acknowledgement

This project was supported by the Malaysian Government through RM 8th fund mechanisms.

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Abstrak

Sejumlah 282 pencilan aktinomiset telah dipencilkan dari beberapa kawasan di Semenanjung Malaysia (Selangor, Johor dan Kuala Lumpur). Kesemua aktinomiset itu memberikan warna koloni yang berbeza iaitu kelabu gelap (42.6%), putih kekuningan (7.4%), putih kekelabuan (40.8%), putih (7.8%), kemerahan (0.3%) dan keperangan (1.1%). Kesemua pencilan itu kemudiannya disaring untuk aktiviti selulase dengan 37.6% (106 pencilan) memberikan keputusan yang positif. Pencilan nombor 153, 159, 177, 184, 185, 191, 196, 200 dan 262 kemudiannya dikenal pasti dengan menyasarkan gen 16S-rRNA pencilan tersebut. Keputusan menunjukkan kesemua pencilan itu adalah daripada genus *Streptomyces* spp.

Accepted for publication on 19 October 2006