Indigenous actinomycetes from empty fruit bunch compost of oil palm: Evaluation on enzymatic and antagonistic properties

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Three actinomycetes isolates were isolated from empty fruit bunch (EFB) compost of oil palm. They were identified as Nocardiopsis sp. (ac9), Streptomyces violaceorubidus (6ca11) and Streptomyces sp. (ac19). The enzymatic potential of the three isolates were established from assays on hydrolytic and lignolytic activities. The production of amylases, cellulases and xylanases were higher in isolates Nocardiopsis sp. (ac9) and S. violaceorubidus (6ca11), at both mesophilic (40 ± 2°C) and thermophilic (60 ± 2°C) temperatures. The contrary, lignolytic activities were higher at mesophilic temperature. Streptomyces sp. (ac19) showed higher lignolytic activities at both temperatures compared to the other two isolates. These three isolates were also antagonistic towards the pathogenic Ganoderma boninense. Nocardiopsis sp. (ac9) was superior with 91.4% inhibition. From this study, it is established that indigenous actinomycetes isolates have both enzymatic and antagonistic potential for use in composting and Ganoderma control, respectively. We recommend the application of actinomycetes into the compost heap during mesophilic stages to allow progressive composting and for compost to be enriched with actinomycetes.

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1. Introduction

Empty fruit bunches (EFB) of oil palm is a major waste problem in oil palm plantations. In the recent years, this waste is managed in a more environmental-friendly approach by converting them into compost. Composting presents an attractive option in EFB management as composting can successfully reduce the initial volume and weight of the EFB by 85% and 50%, respectively (Saletes et al., 2004). Composted EFB also has increased C:N ratio and higher nutrient values which benefits crops when the compost is returned to the soil (Lim and Chan, 1987).

The composting process is primarily a microbiological process, regulated by the indigenous microbes found in the compost heap. During composting, organic material is converted to carbon dioxide, humus and heat, by compost microorganisms (Tuomela et al., 2000). As composting takes place, a gradual succession of microbial communities occurs as a response to the different stages of the composting process, namely the mesophilic, thermophilic and curing stage. Various groups of microbes are involved in the composting process, with bacteria more often dominating the mesophilic and thermophilic stages, while fungi and actinobacteria in the curing stage (Tang et al., 2004). Mesophiles predominantly hydrolyse simple sugars and starch, while thermophiles are involved in the breakdown of hemicellulose, cellulose, lignocellulose and lipids. However, complex organic compounds like lignin are mainly degraded by thermophilic fungi and actinomycetes at optimum temperature of 40–50°C (Tuomela et al., 2000). The expression of the enzymes by the respective groups of microbes is therefore temperature-dependent, and would subsequently impact the rate of composting of the EFB. Efficient composting is highly desirable as it ultimately results in the degradation of 44%, 34% and 20% of cellulose, hemicellulose and lignin residues in EFB, respectively (Saletes et al., 2004).

Indigenous actinomycetes from EFB compost are good candidates for exploration. Actinomycetes are natural antibiotic-producers, able to inhibit various soil-borne pathogens such as Rhizoctonia solani (Tuitert et al., 1998; Postma et al., 2003; Patil et al., 2010), Fusarium oxysporum f.sp. cubense (Getha and Vikineswary, 2002), and Verticillium dahlia (Aouar et al., 2012; Cuesta et al., 2012). The role of actinomycetes in composting is favoured by their natural hyphal-branching growth, which allows for the penetration and degradation of insoluble substrates such as lignocellulose (McCarthy, 1987). Therefore, aside from forming an integral part of the microflora responsible for nutrient recycling, actinomycetes contribute immensely in the degradation of lignocellulose substrates in the compost. Another advantage in using actinomycetes for composting is that as prokaryotes, actinomycetes are more amenable to and have higher success rate in strain improvement strategies by genetic manipulation and large-scale
culturization (McCarthy, 1987). Hence, biomass generation of actinomycetes for compost application is achievable.

In this study, indigenous species of actinomycetes were isolated from EFB compost (curing-stage) obtained from an oil palm mill in Lahad Datu, Sabah, Malaysia. Briefly, isolation was performed using the spread-plate technique, from which pure cultures were subsequently established using the streak-plate technique on Actinomycetes Isolation Agar (AIA) (Difco). The cultures were incubated at 40 ± 2 °C until use.

2.2. Enzymatic analysis

For enzymatic analysis, the cultures were first established by inoculating a loopful of the pure cultures in 30 ml Actinomycyes Broth (Difco) and incubated at 40 ± 2 °C and 60 ± 2 °C for 5 days. The cultures were then centrifuged (4600 rpm, 20 min) (Hettich Universal 320R) and the supernatant obtained was filtered through a 0.22 μm membrane pore filter (Sartorius). The cell-free filtrates containing the crude enzymes were then used to assess for hydrolytic (amylase, cellulase, xylanase) and lignolytic activities, using the Dinitrosalicylic acid (DNS) (Wood et al., 2012) and Remazol Brilliant Blue R (RBBR) (Machado et al., 2005) assays, respectively. Triplicates were prepared for each parameter analysed.

Briefly, the DNS assay was conducted using 1.0 ml of crude enzyme extract and 1.0 ml of corresponding substrate in 0.05 M citrate buffer solution (pH 4.5) (R&M Chemicals). For amylase and enzyme extract and 1.0 ml of corresponding substrate in 0.05 M hydrolytic (amylase, cellulase, xylanase) and lignolytic activities, the reaction mixture was incubated 1 h at 40 ± 2 °C and 60 ± 2 °C. The absorbance was then read at 585 nm. RBBR decolourization was determined by the difference between the absorbance values of the test sample and control, and expressed as percentage of decolourization (%) of RBBR.

2.3. in vitro screening for antagonistic activity

in vitro screening for antagonistic activities of the actinomycetes against G. boninense (provided by Prof. Dr Sariah Meon, Universiti Putra Malaysia) was carried out by dual culture assay. Actinomycetes isolates were first streaked vertically on Nutrient Agar (NA), 3 cm and 6 cm away from the closest and opposite periphery of the petri dish. The plate was incubated at 37 ± 2 °C for 2 days, followed by the inoculation of a mycelial plug of G. boninense (5 mm diameter) at 3 cm away from the actinomycetes streak. This now gives a good placement of 3 cm equal distance of the streak and mycelial plug and from both the periphery of the plate. The inoculated plates were incubated at 37 ± 2 °C for 7 days. Procedure was repeated to prepare the control plates, substituting actinomycetes streak with sterile distilled water. Triplicates were prepared. The inhibitory effect of the actinomycetes towards G. boninense is measured by calculating the percentage inhibitory of radial growth (PIRG) as follows:

PIRG(%) = (R1 – R2)/R1 × 100%

where R1: radial growth of G. boninense in control plate and R2: radial growth of G. boninense co-inoculated with actinomycetes.

2.4. Identification of isolates

Identification of the three actinomycetes isolates was performed based on the sequencing of their 16S rRNA gene sequence. The genomic DNA of each isolate was first extracted using the GF-1 commercial kit (Vivantis). Polymerase chain reactions (PCR) were then performed using the actinobacteria-specific primers; S-C-Act-0235-a-S-20: 5’-GGGCTATCACTTGT TGC-3’ and S-C-Act-0878-a-A-19: 5’-CCGACTCCACGCGCG GG-3’ (Zucchi et al., 2011). The amplified 16S rRNA amplicon was sequenced by First BASE Laboratories Pte. Ltd., and the sequences analysed using BLAST software to determine their homologs.

2.5. Statistical analysis

All statistical analysis was analysed using ANOVA performed with the R software (Knezevic et al., 2007). Means were compared with Tukey’s tests (HSD(0.05)) or a T-test (α=0.05) where appropriate.

3. Results

3.1. Hydrolytic and lignolytic activities

Of the three isolates tested, ac9 and 6ca11 produced relatively higher levels of amylase, cellulase and xylanase, compared to ac19 (Fig. 1A and B). Ac9 and 6ca11 have comparable cellulase and xylanase activities at both incubation temperatures; while amylase activity was significantly higher in ac9 with 477.734 μg min⁻¹ ml⁻¹ against 345.970 μg min⁻¹ ml⁻¹ by 6ca11 at 40 ± 2 °C (Fig. 1A). Hydrolytic activities of ac19 were inferior to both ac9 and 6ca11 at both temperatures.

Higher enzymatic activities were also observed when isolates were incubated at a higher temperature (60 ± 2 °C) (Fig. 1B),...
suggesting possible thermotolerant nature of isolates and the thermosensitivity of the enzymes. Isolate 6ca11 was observed to benefit the most in high temperature condition, showing significant increases in amylase, cellulase and xylanase activities with 496.383, 592.418 and 283.218 %, respectively at 60 ± 2 °C, compared to 345.970, 380.970 and 188.812 %, respectively at 40 ± 2 °C (Fig. 2A). Contrary, isolates ac9 and ac19 produced significantly different levels of enzyme activities in response to the higher temperature. Although cellulase and xylanase activities were significantly higher for isolate ac9 at 60 ± 2 °C, amylase activities were not significantly increased (Fig. 2B). For isolate ac19, only xylanase activity was significantly higher at 60 ± 2 °C (Fig. 2C).

Lignolytic activities by ac9, ac19 and 6ca11 were discovered to be temperature sensitive. Lignolytic activities were detected at 40 ± 2 °C for all isolates, and were generally higher compared to activities at 60 ± 2 °C (Fig. 3). At 60 ± 2 °C, ac9, ac19 and 6ca11 recorded only 3.42, 9.92 and 1.33 % of RBBR decolorization, respectively (Fig. 3). These values were a significant contrast to the % of decolorization by the isolates at 40 ± 2 °C, which recorded 15.22, 15.78 and 3.67 %, respectively (Fig. 3).

3.2. Antagonistic activities

All three isolates showed good antagonistic activities towards G. boninense in the dual culture plate assay. Isolates ac9 and 6ca11 were superior compared to isolate ac19, with PIRG values of 91.4 % and 86.4 %, respectively against 69.1 % by ac19 (Fig. 4). Inhibition was primarily due to the inhibitory compounds produced as growth was clearly inhibited at the clearing zone (inhibition zone).

Competitive exclusion via overgrowth and mycoparasitism was not observed in all three isolates (data not shown).

3.3. Identification of isolates

Isolate ac9 was identified as Nocardiopsis sp. with 99 % similarity (602 bp/606 bp) to Nocardiopsis sp. (JN942137.1) based on their 602 bp sequence after BLAST analysis. For isolate 6ca11, it was identified as similar to Streptomyces violaceorubidus (99 % similarity, 527bp/528 bp). The final isolate (ac19) was identified as Streptomyces sp. (100 % similarity, 619 bp/619 bp).

4. Discussion

This study generated some novel findings especially on the discovery of indigenous actinomycetes from EFB with antagonistic properties towards G. boninense, and the preliminary profiling of their enzymatic capacity to enhance the composting process. Two of the actinomycetes isolates were Streptomyces spp. (6ca11 and ac19), which are typical species of the actinomycetes community (Miyashita et al., 1982; Cuesta et al., 2012). The third isolate, a Nocardiopsis, is a non-Streptomyces actinomycetes with distinct lineage within the order of Actinomycetales (Rainey et al., 1996). The discovery of Nocardiopsis sp.(ac9), S. violaceorubidus (6ca11) and Streptomyces sp. (ac19), and their antagonistic potential towards G. boninense (particularly Nocardiopsis sp. (ac9) (91.4 % PIRG)), presents an interesting option to the biocontrol strategies for G. boninense. To date, reports on the bioactivity of Nocardiopsis sp. (ac9) and Streptomyces spp. (6ca11, ac19) towards G. boninense is lacking. Instead, Nocardiopsis sp. is more established for their antibacterial activities towards a host of human pathogens such as Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus faecalis and Staphylococcus aureus (Vinhal et al., 2008). Antagonism of Streptomyces spp. is more common than Nocardiopsis sp. (Aouar et al., 2012), with known broad-spectrum antibacterial and antifungal activities towards Micrococcus luteus, Candida albicans, Salmonella, S. aureus (Bensultana et al., 2010), and Sclerotium rolfsii (Boukaew et al., 2011). Therefore, this study may be the first few to document that Nocardiopsis sp. (ac9), S. violaceorubidus (6ca11) and Streptomyces sp. (ac19) from EFB compost, have potential as biocontrol agents towards G. boninense.

The three isolates from this study can be considered as possible thermophiles based on the definition purposed by Taha et al. (2007). In their study, thermophilic nature is proven when isolates are able to tolerate incubation period at 44 °C for 7 days. In this study, cultures grew very well at 40 ± 2 °C although incubation at 60 ± 2 °C did not produce colony growth on AIA plates (data not shown). As such, we consider our isolates as tolerant to mesophilic and initial thermophilic temperatures, able to withstand the composting process as they were successfully isolated from the curing-staged composts. The thermo-tolerant nature of all three isolates is further strengthened with earlier reports of similar thermophilic characteristics in species of Streptomyces (Strom, 1985) and Nocardiopsis (Stamford et al., 2001; Yan et al., 2011).

It is apparent that the thermostable enzymes produced by Nocardiopsis sp.(ac9), S. violaceorubidus (6ca11) and Streptomyces sp. (ac19) observed in this study, were most probably attributed to the inherent thermophilic nature of the isolates. Although the actinomycetes isolates were not extreme thermophiles, their extracellular enzymes were thermostable. This was evident when hydrolases have higher activities at 60 ± 2 °C, despite the inability of the isolates to grow at the same temperature on AIA plates. The extracellular enzymes produced by these isolates are significant as they can accelerate substrate degradation through a process that is
independent of cells. This cell-independent process was also observed in our previous study using the thermophilic bacterium Ureibacillus terrenus (Ting et al., 2013). We also observed a distinctive variant in the thermostability of enzymes within species of Streptomyces as S. violaceorubidus (6ca11) produced more thermostable amylases, cellulases and xylanases, compared to Streptomyces sp. (ac19). Our results for Nocardiopsis sp. (ac9) also differed from other reports where optimum temperatures for hydrolytic activities varied. We postulate this may be due to the origin of the Nocardiopsis sp. and their subsequent response to temperature. In this study, higher cellulase and xylanase activities of Nocardiopsis sp. (from compost) were reported at 60 ± 2 °C while amylase activities were not significantly different at both temperatures. This did not resemble observations by Stamford et al. (2001) who identified the optimum amylolytic activity for the endophytic Nocardiopsis strain from yam bean was at 70 °C, and at 40 °C for cellulytic and xylanase activity for a Nocardiopsis strain from soil (Saratale and Oh, 2011).

Contrary to observations for hydrolytic activities, all three actinomycetes isolates were found to have better ligninase activities at 40 ± 2 °C than at higher temperature. This suggested the possibility that ligninase-activity is a cell-dependent process, in which when cell growth is affected by the high temperature; the ligninase activities were also affected. Comparisons with ligninase activities of thermophilic U. terrenus further reiterate this as thermophilic bacterial growth at 60 ± 2 °C leads to significantly
higher ligninase activities (Ting et al., 2013). Among the three isolates tested, the ligninase activities of Streptomyces sp. (ac19) were the highest at both temperatures. This was rather unexpected as hydrolytic enzyme levels for ac19 were inferior to the other two isolates. Therefore, it appears that for Streptomyces sp. (ac19), ligninases and not cellulases could be primarily involved in the breakdown of lignocellulose compounds. Mason et al. (1988) postulated this as well when the molecular weight of a single enzyme by Streptomyces cyaneus postulated this as well when the molecular weight of a single enzyme by Streptomyces cyaneus was 190,000 daltons and not 140,000 daltons as reported by Hobbs et al. (1977). The observation allows the further investigations and development of EFB compost, and documented their gratitude to Monash University Malaysia for the facilities to complete the project.

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References


Sakatsu, S., Saletes, S., Siregar, A.F., Caliman, J.P., Liwang, T., 2004. Ligno-cellulose composting: a variety of soil-borne pathogens such as Fusarium oxysporum f.sp. radicis-lycopersici, Pythium ultimum and R. solani (deBrito et al., 1995). As such, we expect the incorporation of Nocardiosis sp.(ac9), S. violaceorubidus (6ca11) and Streptomyces sp. (ac19) into EFB compost to render similar beneficial effects against G. boninense.

To conclude, this study has successfully identified three indigenous actinomycetes isolates from EFB compost, and documented their enzymatic and antagonistic potential. This preliminary observation allows the further investigations and development into harnessing the biomass or the enzyme derivatives of Nocardiosis sp.(ac9), S. violaceorubidus (6ca11) and Streptomyces sp. (ac19), to accelerate the composting process and for control of Ganobera.


