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Novel multifunctional plant growth–promoting bacteria in co-compost of palm oil industry waste

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Previously, a unique co-compost produced by composting empty fruit bunch with anaerobic sludge from palm oil mill effluent, which contributed to establishing a zero-emission industry in Malaysia. Little was known about the bacterial functions during the composting process and fertilization capacity of this co-compost. We isolated 100 strains from the co-compost on 7 types of enumeration media and screened 25 strains using *in vitro* tests for 12 traits, grouping them according to three functions: plant growth promoting (fixation of nitrogen; solubilization of phosphorus, potassium, and silicate; production of 3-indoleacetic acid, ammonia, and siderophore), biocontrolling (production of chitinase and anti-*Ganoderma* activity), and composting (degradation of lignin, xylan, and cellulose). Using 16S rRNA gene sequence analysis, 25 strains with strong or multi-functional traits were found belong to the genera *Bacillus*, *Paenibacillus*, *Citrobacter*, *Enterobacter*, and *Kosakonia*. Furthermore, several strains of *Citrobacter sedlakii* exhibited a plant growth-stimulation *in vivo* komatsuna plant cultivation test. In addition, we isolated several multifunctional strains; *Bacillus tequilensis* CE4 (biocontrolling and composting), *Enterobacter cloacae* subsp. *dissolvens* B3 (plant growth promoting and biocontrolling), and *C. sedlakii* CESi7 (plant growth promoting and composting). Some bacteria in the co-compost play significant roles during the composting process and plant cultivation after fertilization, and some multifunctional strains have potential for use in accelerating the biodegradation of lignocellulosic biomass, protecting against *Ganoderma boninense* infection, and increasing the yield of palm oil.

[**Key words:** Co-compost; *Citrobacter sedlakii*; *Enterobacter cloacae* subsp. *dissolvens*; *Bacillus tequilensis*; Plant growth promoting]

Oil palm (*Elaeis guineensis* Jacq) is one of the most prolific oil–producing crops worldwide. Its oil–saturated fruits can be used to produce two valuable vegetable oils (palm oil and palm kernel oil) and numerous industrial value-added sub-products. The increase in area of monoculture plantations for producing fresh fruit bunches (FFB), however, has also increased the amount of industrial processing waste and risk of outbreak of endemic disease, the severe basal stem rot disease caused by fungi of *Ganoderma boninense* (1). In the field, the two main waste biomasses are oil palm frond (OPF) generated from daily pruning and FFB harvesting activities and oil palm trunk (OPT) generated during replanting cycles. These are left on the ground to dry naturally, and their decaying time depends on the environmental conditions. On the other hand, palm oil mills generate both solid and liquid wastes during oil extraction activities. Two major waste types are the lignocellulosic-rich empty fruit bunch (EFB) and palm oil mill effluent (POME), which has high biochemical (BOD) and chemical (COD) oxygen demand values (2), potentially contributing to several environmental issues such as river pollution and greenhouse gases emission (3).

Co-composting POME with EFB is one technique for treating these waste products. This technique has advantages over the single EFB composting method as a shorter composting period, higher mineral content, and lower C/N ratio in the produced co-compost. Recently, a new co-composting process was developed in a zero-discharge pilot-plant (4). The POME is first subjected to methane fermentation for biogas production, and then, a mixture of the thickened POME anaerobic sludge and shredded EFB is treated to yield co-compost. This system can potentially eliminate the need for an open ponding system for POME treatment while simultaneously reducing greenhouse gas emissions and its water-polluting potency. The advantages of this new system over the conventional co-composting method have been discussed (5) and include the production of value–added biogases, a co-compost

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with better physicochemical properties, and no requirement for additional exogenous composting microorganisms.

The function of the bacteria in the co-compost, in particular lignocellulosic degradation activity, was demonstrated in our recent study (4). Apart from cellulose- and xylan-degrading traits, it was not known if the bacteria would be beneficial for plant growth and inhibit the growth of *G. boninense* harbored in the co-compost, which is scattered on the plantations as a soil amendment material around the palm trees. In soil, insoluble organic matter and minerals are usually held in soil colloids; otherwise, they would be flushed out through runoff. One way to access the insoluble mineral nutrients contained in soil is through mineralization activities. The solubilization of soil minerals by plant–associated bacteria has been shown to enhance mineral uptake and promote plant growth (6). In some cases, these plant growth promoting (PGP) bacteria also exhibit biological control traits and are capable of suppressing plant pathogens via direct growth inhibition, competition for essential minerals such as iron (7), production of antibiotics, and induction of plant systemic resistance response (8). Therefore, the co-compost produced from the new zero-discharge pilot plant may contain multifunctional bacteria that may be able to promote seedling growth or prevent infection by *G. boninense* when used as a planting medium, as well as accelerate the degradation of OPF and OPT when scattered back on the plantation. The aim of this study was to isolate, characterize, and identify the multifunctional bacteria harbored in co-compost, particularly focusing on three functions: plant growth promotion, *G. boninense* growth inhibition, and lignocellulosic biomass degradation under *in vivo* and *in vitro* conditions.

MATERIALS AND METHODS

Isolation of bacteria The co-compost sample used for bacterial isolation was randomly collected from a co-compost pile produced at the co-composting pilot plant located at Universiti Putra Malaysia (UPM), Malaysia, in January 2015. Ten grams of this co-compost was suspended in 90 ml of sterile saline water (0.85% NaCl) and incubated overnight at 37°C with shaking (120 rpm). The mixture was filtered by passing the co-compost extract through cheesecloth, followed by serial dilution to 1×10^{-4}; 100 µl of the diluted filtrate was then plated on isolation medium prepared in triplicate using the spread plate technique. The isolation media used in this study included trypticase soy agar (TSA), TSA amended with 0.25% magnesium trisilicate (TSASi) (9), nitrogen (N)–free and bacillus (B) agar (10), Pikovskaya's (P) agar (11), co-compost extract (CE) agar, and CE amended with 25% magnesium trisilicate (CESi) agar. CE agar was modified from soil extract agar using 50 g co-compost instead of soil. The CE extract was first prepared by autoclaving 50 g co-compost in 950 ml distilled water at 121°C for 15 min and filtering through Whatman qualitative filter paper (grade 4). CE agar was prepared by adding 2 g/l glucose, 1 g/l yeast extract, 0.5 g/l K_2HPO_4, and 15 g/l agar to the resulting dark CE filtrate and bringing up the final volume to 1 l with distilled water before autoclaving. Incubation was carried out at 37°C, and a single colony was picked up from each isolation media and subcultured as needed until a pure culture was obtained for further characterization.

Cultivation of *G. boninense* A pure culture of *G. boninense* strain ATCC 204072 originally isolated from oil palm trees in Malaysia was purchased from the American Type Culture Collection (ATCC). The fungus was first recovered from frozen ampoules according to the ATCC's recommendations except that potato dextrose broth (PDB) was used instead of potato dextrose agar (PDA). Because of the low survival rate of sterile mycelium on agar plates during long-term storage and because *G. boninense* only produces basidiocarps and spores naturally on wood, we developed a cost-efficient method for the continuous production of *G. boninense* seed cultures in liquid broth and for the preparation of glycerol stock for ultralow temperature storage. In detail, a mycelium plug of *G. boninense* obtained from a 5-d-old culture on PDA was sub-cultured in a 500 ml conical flask with 300 ml of modified glucose-asparagine (GA) medium (12,13) and incubated at 25°C with shaking at 120 rpm. This slightly modified medium contained 15 g/l glucose, 1 g/l yeast extract (Difco, Becton Dickinson, Franklin Lakes, NJ, USA), 3 g/l soy peptone (Nacalai Tesque, Kyoto, Japan), 4.8 g/l L-asparagine, and 1.25 g/l K_2HPO_4 with the initial pH was adjusted to pH 4.5 using 1 N HCl. Numerous circular mycelial masses were continuously produced throughout the incubation period, and the culture remained sterile and active after 3 months without the need for sub-culturing. The *G. boninense* glycerol stock was prepared in a similar way by transferring three circular mycelial masses from a 7 d-culture to a sterile cryotube containing fresh GA medium in 40% glycerol. The fungus remained active after 2 years of storage at −80°C.

Preparation of inoculum and incubation conditions for each isolated strain Purified isolated strains were grown on TSA at 37°C for 24 h. A bacterial suspension of each isolated strain was prepared by transferring fresh colonies to sterile saline water in a 10 ml test tube and adjusted to a 2.0 McFarland standard (approximately 0.5 optical density at a wavelength of 610 nm). All *in vitro* screenings of PGP, biocontrolling and composting traits in Petri dishes were carried out by inoculating each test medium with 10 µl of bacterial suspension and incubating at 37°C in an inverted position for 5 d before results were recorded, unless otherwise stated.

Characterization of plant growth promoting traits Plant growth promotion was assigned as the first functional group (group I) in this study and consisted of multiple traits as described below. The ability of isolated strains to fix nitrogen was determined on nitrogen–free medium. Visible colony growth on the agar indicated positive nitrogen fixation, whereas bacteria that could not fix nitrogen were unable to grow on this medium. The abilities of isolated strains to solubilize inorganic phosphorus, potassium, and silicate were screened on Pikovskaya's medium containing 0.5% insoluble tricalcium phosphate (11), Aleksandrov medium containing 0.5% mica (potassium aluminium silicate) (14), and glucose agar medium containing 0.25% magnesium trisilicate (9), respectively, as the key indicators. Phosphate–solubilizing, potassium–solubilizing, and silicate–solubilizing bacteria dissolved these insoluble minerals, resulting in the formation of a clear halo zone around the colony. The diameter of the colony (D2) and the halo around each colony (D1) in millimeters (mm) were measured. The solubilizing index (SI) was calculated and expressed using the following equation (15):

$$SI = [(D2 - D1)/D1] \times 100 \quad (1)$$

The production of 3-indoleacetic acid (IAA) was evaluated on trypticase soy broth (TSB) supplemented with 5 mM tryptophan (16). In brief, after incubation at 37°C with shaking for 48 h, cultures were centrifuged, and 0.5 ml of the supernatant was reacted with 1 ml Salkowski reagent (prepared by mixing 2 ml 0.5 M $FeCl_3$, 49 ml distilled water and 49 ml 70% perchloric acid) at room temperature for 25 min. The absorbance of the pink color at 530 nm was measured and calculated to obtain the IAA concentration (µg/ml) using a standard curve (10 points; $r^2 = 0.99$) of 3-indoleacetic acid (Sigma-Aldrich, MO, USA). The production of ammonia (NH_3) was determined on TSB after incubation at 37°C with shaking for 72 h. The concentration of ammonia in culture supernatants (µg/ml) was quantified using the indophenol blue method by measuring the absorbance at 625 nm after a 30 min incubation at room temperature (17). Ammonia was quantified using a standard curve (10 points; $r^2 = 0.99$). The detection of siderophore production by isolates was determined using the universal chrome azurol S (CAS) assay (18). Yellow precipitation around a colony indicated a positive result for siderophore production.

Characterization of biocontrolling traits Biocontrol was assigned as the second functional group (group II) in this study and consisted of antagonistic and chitinase traits. Growth inhibition of *G. boninense* ATCC 204072 was screened using a dual culture assay (19). For inoculation, a 3-d-old circular mycelial mass was first placed 2 cm from the center of a Petri dish containing TSA medium. All *Ganoderma*-inoculated plates were incubated overnight at 30°C before the same Petri dish was inoculated the next day with 10 µl of bacterial suspension 2 cm from the center against the fungus. Anti-*Ganoderma* (Anti-G) activity was considered positive if a growth inhibition zone formed between the *G. boninense* and tested strain after incubation for 7 d at 30°C in the dark.

The production of chitinase was tested on basal salt medium containing 1% (w/v) colloidal chitin, and 0.7 g/l K_2HPO_4, 0.3 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.01 g/l $FeSO_4$, 0.001 g/l $ZnSO_4$, 0.001 g/l $MnSO_4$, 0.25 g/l $(NH_4)_2SO_4$, and 1.0 g/l yeast extract (Becton Dickinson) (20). Ten grams of commercial chitin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in 100 ml of 85% phosphoric acid and stirred magnetically at 180 rpm inside a 4°C cold room. The thick dissolved chitin solution was carefully poured into a 2 l beaker containing 1.5 l of cold water. The colloidal chitin formed was separated by filtration using Whatman filter paper (grade 4) with the assistance of a vacuum pump. The retained cake was flushed with tap water through vacuum filtration until the filtrate had a pH of 7.0 as indicated by the pH indicator phenol red, which had been added to the filtrate. The semi-vacuum-dried colloidal chitin was used for preparation of colloidal chitin agar as described by the author. A clearing zone around a bacterial colony indicated the ability of the isolate to degrade chitin in the chitin agar.

Characterization of composting traits Composting was assigned as the third functional group (group III) in this study. A degradation study of the plant lignocellulosic biomasses lignin, xylan (hemicellulose), and cellulose was carried out using commercial kraft lignin (KL, Nacalai Tesque, Kyoto, Japan), xylan from beechwood (Xy, Sigma-Aldrich) and carboxymethyl cellulose (CMC, Katayama Hagaku, Osaka, Japan). The ability of a strain to utilize lignin was checked on minimal salt medium kraft lignin (MSM-KL) agar supplemented with a low amount of glucose and yeast extract. This medium contained 15 g/l agar, 4.55 g/l K_2HPO_4, 0.53 g/l KH_2PO_4, 0.5 g/l $CaCl_2$, 0.5 g/l $MgSO_4$, 5.0 g/l NH_4NO_3, 0.25 g/l methyl blue, 0.5 g/l KL, 2.0 g/l glucose and 1.0 g/l yeast extract (Becton Dickinson) (21). A white

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