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Characterization and Utilization of Pulp and Paper Mill Sludge Digesting Thermophilic Bacteria in Composting Process

(Pencirian dan Penggunaan Enapcemar Kisar Pulpa dan Kertas Menghadamkan Bakteria Termofili dalam Proses Pengkomposan)

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ABSTRACT

Pulp and paper mill sludge (PPMS) was found to be poorly colonised with thermophilic microorganisms. However, evidence to support the need for inoculation to facilitate PPMS composting has only been demonstrated in one instance. In this study, we aimed to: screen and identify PPMS digesting thermophilic bacterial strains; investigate effects of the mixture of selected thermophilic bacterial strains on PPMS digestion; and utilize this mixture as start inoculum in PPMS composting and assess the quality of compost product. The results showed that eleven thermophilic bacterial strains were isolated from Bai Bang PPMS by the enrichment culture method. Among these, three strains which reflected high growth rates on the plates of Minimal Media Agar supplemented with Bai Bang PPMS and showed hydrolytic and ligninolytic activities on the agar plates containing appropriate inductive substrates were selected. Based on the morphological, biochemical characteristics and 16S rRNA gene sequencing, they were identified as Bacillus subtilis. *The inoculation with the mixture of selected strains enhanced remarkably Bai Bang PPMS digestion. The dry weight decrease, volatile suspended solids removal, dehydrogenase and protease activities in the inoculated sludge were 2.1-, 1.5-, 1.3- and 1.2-fold higher, respectively, compared to the non-inoculated sludge. The assessment of compost quality based on stability using the alkaline trap method and maturity using the germination and root elongation test showed that the inoculated compost was stable and mature while the non-inoculated compost was unstable and immature. These thermophilic bacterial strains therefore have great potential for Bai Bang PPMS composting.*

Keywords: Bacillus subtilis*; composting; pulp and paper mill sludge; thermophilic bacteria*

ABSTRAK

Enapcemar kisar pulpa dan kertas (PPMS) didapati kurang dipengaruhi oleh mikroorganisma termofili. Walau bagaimanapun, bukti untuk menyokong keperluan inokulasi bagi memudahkan pengambilan PPMS hanya ditunjukkan dalam satu keadaan. Objektif kajian ini adalah untuk: menilai dan mengenal pasti strain bakteria termofili mencerna PPMS; mengkaji kesan campuran strain bakteria termofili yang dipilih pada pencernaan PPMS; dan menggunakan campuran ini sebagai inokulum permulaan dalam pengkomposan PPMS dan menilai kualiti produk kompos. Keputusan kajian menunjukkan bahawa sebelas strain bakteria termofili telah diasingkan daripada Bai Bang PPMS dengan kaedah budaya pengayaan. Antaranya, tiga strain yang mencerminkan kadar pertumbuhan tinggi pada plat Minimal Media Agar ditambah dengan Bai Bang PPMS dan menunjukkan aktiviti hidrolitik dan ligninolitik pada plat agar yang mengandungi substrat yang sesuai bagi induktif dipilih. Berdasarkan ciri morfologi, biokimia dan urutan gen rRNA 16S, ia dikenali sebagai Bacillus subtilis. *Inokulasi dengan campuran strain terpilih telah meningkatkan penguraian Bai Bang PPMS. Penurunan berat badan kering, aktiviti penyingkiran pepejal terampai, dehidrogenase dan protease dalam enapcemar yang disuntikkan masing-masing adalah 2.1-, 1.5-, 1.3- dan 1.2 kali lebih tinggi berbanding dengan bukan berasaskan enapcemar. Penilaian kualiti kompos berdasarkan kestabilan menggunakan kaedah perangkap alkali dan kematangan menggunakan percambahan dan pemanjangan akar ujian menunjukkan bahawa kompos yang diinokulasi adalah stabil dan matang manakala kompos yang tidak disuntik adalah tidak stabil dan tidak matang. Oleh itu strain bakteria termofili mempunyai potensi besar untuk pengomposan Bai Bang PPMS.*

Kata kunci: Bacillus subtilis*; bakteria termofili; enapcemar kisar pulpa dan kertas; pengkomposan*

INTRODUCTION

Pulp and paper mill sludge (PPMS) is the main organic residual material generated from the wastewater treatment of pulp and paper industry. The properties of PPMS depend on the type of raw material, paper and pulp making process and wastewater treatment (Pervaiz & Sain 2015). In Vietnam, most of PPMS generated from the wastewater treatment is currently incinerated and landfilled, posing a serious threat to the environment and also wasting the valuable recyclable resource. Composting of PPMS could help reduce its volume, degrade potentially toxic compounds, eliminate harmful pathogens, recover energy

and nutrients therein and produce compost suitable for agricultural uses (Campbell et al. 1995; Gregory & Daniels 1999). Therefore, composting is one of the economically viable and environmentally acceptable methods to recycle PPMS. However, PPMS was not easily digested by microorganisms (Jackson & Line 1997), resulting in rather long and uneconomical retention periods in the conventional bioreactors. Meanwhile, several previous studies reported that thermophilic microorganisms are capable of speeding up sludge digestion process due to their unique thermostable extracellular enzyme systems (Hasegawa et al. 2000; Sakai et al. 2000; Shiota et al. 2002; Roman et al. 2006; Yan et al. 2008). However, evidence to support the need for inoculation of effective microbial decomposers to accelerate PPMS composting has only been demonstrated in one instance (Davey 1953). Therefore, the objectives of this study were to: screen and identify PPMS digesting thermophilic bacterial strains; investigate effects of the mixture of selected thermophilic bacterial strains on PPMS digestion; and utilize this mixture as start inoculum in three-month composting process of PPMS supplemented with rice straw as bulking agent and assess the quality of compost product.

Materials and Methods

Sample collection and determination of PPMS properties

The PPMS was sampled from the wastewater treatment system of Bai Bang paper mill located in Phong Chau, Phu Tho, Vietnam (hereafter referred to as "Bai Bang PPMS"). Water content were determined by the gravimetric method (TCVN 6648:2000- ISO 11465:1993). The pH in KCl was measured with a glass electrode in a 1:5 suspension of sludge in 1 M KCl (TCVN 5979:2007- ISO 10390:2005). Total C after dry combustion was determined according to TCVN 6642-2000 (ISO 10694:1995). Total N was determined by the modified Kjeldahl method (TCVN 6498:1999- ISO 11261:1995). Total P was determined by the colorimetry method (TCVN 8940:2011). Total K was determined by flame photometry (TCVN 8660:2011). The total number of thermophilic microorganisms was estimated by the dilution agar plate method on Plate Count Agar (Merck 1.05463) for 3 days at 60°C.

Isolation, selection and identification of thermophilic bacterial strains

The enrichment medium was composed of 10% (w/v) Bai Bang PPMS, 1% (w/v) carboxymethyl cellulose (CMC), 0.5% (w/v) yeast extract, 0.1% (w/v) peptone and 0.1% (w/v) K_2HPO_4 in deionized water (pH7.5). This medium was autoclaved at 121°C for 30 min prior to use. The enrichment culture was prepared by adding 0.1% (w/v) of Bai Bang PPMS into 1 L of the medium in a 3 L-Erlenmeyer flask and incubated on a shaker (New Brunswick, Innova 44R, Eppendorf) at 150 rpm, 60°C for 7 days. From the culture suspension, to isolate as many strains possible, 100 µL of 10-fold diluted enrichment culture was spread on different nutrient media such as Nutrient Agar (Merck 1.05450), Tryptic Soy Agar (Merck 1.05458), and Luria-Bertani Agar (Merck 1.10283). The plates were incubated at 60°C for 2-3 days. Subsequently, isolates were subcultured on Minimal Media Agar (0.05% (w/v) yeast extract, 0.01% (w/v) peptone, 0.01% (w/v) K_2HPO_4, 1.5% (w/v) agar in deionized water) supplemented with 1% (w/v) of Bai Bang PPMS (pH7.5). Isolates forming large colonies (>2 mm in diameter) which reflected high growth rates on the plates of Minimal Media Agar supplemented with Bai Bang PPMS were continuously screened for extracellular enzyme activities. Protease, amylase, cellulase, ligninolytic activities were tested by the diffusion method on the agar plates supplemented with 1% (w/v) casein, soluble starch, CMC and lignin, respectively, at pH7.5. Isolates which showed hydrolytic and ligninolytic activities were selected for further experiments. The colony morphology and hemolytic property were visually detected by naked eyes on Blood Agar (Merck 1.10886) after incubating at 30°C for 48 h. The colony was stained by Gram staining method and cell morphology was observed with a light microscope (BX50F4, Olympus, Japan). The biochemical characteristics were tested using the API 50 CHB kit consisting of 49 carbohydrates associated with the API 20 E strip (bioMerieux). Genomic DNA was isolated following protocol of Sambrook & Russell (2001). Amplifications of 16S rRNA gene were performed using the universal primers 341F (5'-CCT ACG GGA GGC AGC AG-3') with a GC clamp and 907R (5'- CCG TCA ATT CCT TTR AGT TT-3') (Kuang et al. 2009). Purified PCR products were sequenced with an Applied Biosystems 3130xl Genetic Analyzer. Analysis of 16S rRNA sequences was performed using Applied Biosystems Sequencing Analysis Software v.5.3. The 16S rRNA gene sequences were compared against the nucleotide collection at GenBank database using BLAST (Altschul et al. 1997) and submitted to DDBJ/EMBL/GenBank to get the respective accession numbers. The multiple alignments of sequences, calculation of nucleotide substitution rates and construction of a Neighbor-Joining phylogenetic tree were carried out in accordance with the method of Kumar et al. (2016).

Effects of the mixture of thermophilic bacterial strains on PPMS digestion

The mixture of three selected thermophilic bacterial strains was used as an inoculum for the investigation of Bai Bang PPMS digestion. A loopful of each strain preserved on the slant was inoculated into the 500 mL Erlenmeyer flask containing 200 mL of LB-broth (Miller 110285) and incubated on a shaker (New Brunswick, Innova 44R, Eppendorf) at 150 rpm and 35°C for 12 h, followed by 60°C for 36 h. Next, the culture suspension was centrifuged at 6,000 rpm for 15 min and then the pellet was diluted with distilled water to achieve an OD_{600} of 0.8. The mixture was pooled by mixing 100 mL of diluted culture from each strain. The inoculated sludge

was prepared by adding the mixture into 2 L of unsterilized medium containing 20% (w/v) Bai Bang PPMS, 0.5% (w/v) yeast extract and 0.1% (w/v) K_2HPO_4 at an initial pH of 7.5. The non-inoculated sludge was prepared by the same procedure but received no inoculation. Both sludges were incubated on the shaker at 150 rpm, 60°C for 10 days. Sampling was carried out on a daily basis for 10 days to determine the dry weight, volatile suspended solids (VSS), protease and dehydrogenase activity. The dry weight was determined after drying the solids at 105°C for 24 h. The VSS content of supernatant filtered through a 0.45 µm-MF Millipore mixed cellulose ester membranes was determined in accordance with Standard Methods (Rice et al. 2012). The protease activity was assayed by the method of Okamura et al. (2007). Briefly, a volume of 0.5 mL of the filtrate and 2% casein solution (2.0 g in 100 mL of 0.4 M of Tris-HCl buffer, pH8.5) was incubated for 15 min at 60°C. One mL of 0.44 M trichloroacetic acid was added to the mixture and kept it for 30 min. The formed precipitate was filtered with filter paper. Next, 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin reagent were added to 0.5 mL of this filtrate and absorbance was detected at 660 nm. One unit of absorbance is expressed as 1 UmL^{-1} protease activity. Dehydrogenase activity was evaluated with the method proposed by Lopez et al. (1986). This method is based on the measurement of the colour produced on reduction of the original substrate, INT 2-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride to INT-formazan by the oxidative effect of the dehydrogenase enzymes. The samples were diluted to a final concentration of 1 g VSS L^{-1}. At a final volume of 5 mL, 0.5 mL of INT reagent was added at 3.95 mM and was then incubated for 30 min in darkness. The reaction was stopped by the addition of 1 mL of 37% (v/v) buffered formaldehyde. Extraction was effected by placing 2 mL of the INT mixture/sample in a test tube, which was then centrifuged at 1500 g for 15 min. The supernatant was separated and in order to extract the formazan, 5 mL of 95% (v/v) ethanol was added to the pellet and ultrasound at 40 KHz frequency, 30°C for 10 min in a digital ultrasonic bath (Nahita, Model 626/6, Alea Equipment) was employed to encourage dilution. Finally, the extract was centrifuged once more and the absorbance of the supernatant was measured at 480 nm wavelength. INT-dehydrogenase activity was calculated in equivalent oxygen units using the following formula:

$$\text{DHA} = \frac{1024 \times D480 \times V}{v \times C \times t \times F}$$

where DHA, INT-dehydrogenase activity ($mgO_2\ gVS^{-1}d^{-1}$); 1024, the conversion factor (Lopez et al. 1986); D_{480}, absorbance at 480 nm wavelength; *V*, final volume of dissolvent used to extract formazan (mL) volume of reagent used and sample treated (mL); *C*, concentration of volatile solids (gL^{-1}); *t*, incubation time (min); *F*, dilution factor.

COMPOSTING OF BAI BANG PPMS AND ASSESSMENT OF COMPOST QUALITY

The composting materials consisted of Bai Bang PPMS and rice straws collected from a rice field at Ha Dong, Ha Noi, Vietnam. The PPMS was passed through a 2 mm mesh sieve and the rice straws were cut into particle size of 2-3 cm prior to use. The materials were prepared by mixing the PPMS with rice straws in the proportions (volume basis) of PPMS (25%) and rice straws (75%) to obtain an appropriate C:N ratio (30:1). The composting process was carried out in a two-can bioreactor with an 80 L can containing materials placed inside a 120 L can. Placing the can that holds materials inside another container helps alleviate any odor and the outside container can also be used to collect leachate. For the experimental compost pile, the mixture of three selected thermophilic bacterial strains prepared as mentioned above was inoculated into the 135 dm^3 of materials at the start-up stage of composting process and designated inoculated compost. Meanwhile, for the control compost pile, the same materials were composted without the inoculation and designated non-inoculated compost. The attention was paid to initial moisture content (60%). The piles were turned periodically to maintain aerobic condition and homogenize the mixture. After three months, each compost sample consisted of three sub-samples collected from different points throughout each pile and homogeneously mixed together to ensure representative sampling. The compost stability was assessed by CO_2 evolution via alkaline trap method described in TMECC method 05.08-B (Thompson 2002). Prior to conducting the test, the moisture content of the samples was adjusted to 50% and the samples were pre-incubated at 37°C for 36 h in unsealed bags to acclimate the microbial community. Subsequently, 25 g of sample were placed in a 100 mL beaker and 250 mL of NaOH (1M) was placed in another 100 mL beaker. Both beakers were placed in the 4 L jars. The jar lid was screwed down tightly to prevent any gas exchange. To take into account the initial CO_2 content of the 4 L jar, a control jar contained an empty beaker as well as 20 mL NaOH in second beaker was also prepared. These sealed jars were placed in the incubator for 72 h at 37°C. The CO_2 evolved during respiration was absorbed by NaOH trap. After the incubation period, NaOH solutions were collected for titration by adding a volume of 1 M HCl until a preset pH was measured by an indicator electrode (pH7.0). CO_2-C resulting from the titration method was calculated according to the following equation:

$$CO_2\text{-C} = \frac{(B-V) \times N \times E \times (\frac{Tr}{A})}{\left(\frac{t}{24}\right) \times w \times \left(\frac{100-mc}{100}\right) \times VS}$$

where CO_2-C, evolved CO_2-C (mg CO_2-C gVS^{-1} d^{-1}); *B*, volume of HCl needed to titrate the blank to the end point (mL); *V*, volume of HCl needed to titrate the sample to the end point (mL); *N*, normality of acid used for titration; *E*, equivalent weight to convert to mg (C = 6); *Tr*, trapped volume (mL); *A*, aliquot titrated (mL); *t*, incubation time

(h); *w*, compost weight (g); *mc*, compost moisture content (%); *VS*, volatile solids content in the sample (dry basis).

The compost maturity was evaluated using *in vitro* germination and root elongation test described in TMECC method 05.05-B (Thompson 2002) with garden cress (*Lepidium sativum* L.). Fresh compost was mixed with distilled water at 1:2 (w/v) using a shaker for 30 min and then filtered using a filter paper. Ten mL of the filtered mixture would be extracted and added to filter paper in a sterilized Petri dish, after that eight seeds were evenly distributed on the filter paper. The set up would be inocubated in dark condition at 25°C for 48 h. After 48 h of incubation in the dark, the seed germination and root length of the eight plants in the different strengths of extracts (10×dilution, 3×dilution and full strength) were determined. The seed germination percentage and root elongation of the plants in deionized water were also measured and used as the control. A 5 mm primary root was used as the operational definition of seed germination (USEPA 1982). The relative seed germination, relative root growth and germination index (GI, the product of relative seed germination and relative root elongation) were calculated as follows:

$$\text{Relative seed germination (\%)} = \frac{\text{Number of seeds germinated in extract}}{\text{Number of seeds germinated in control}} \times 100$$

$$\text{Relative root growth (\%)} = \frac{\text{Mean root length in extract}}{\text{Mean root length in control}} \times 100$$

$$\text{Germination index (GI)} = \frac{(\text{\% Realtive seed germination}) \times (\text{\% Relative root growth})}{100}$$

STATISTICAL ANALYSIS

For the sludge digestion assay, results were reported as means ± standard error of three replicates. Student t-test was used to calculate the significance value between the experiment and control samples. A P value of <0.05 was defined as statistical significant. All statistical analysis was carried out using Microsoft Excel 2010.

RESULT AND DISCUSSION

PROPERTIES OF BAI BANG PPMS

Bai Bang PPMS was a mixed sludge formed by the combination of primary and secondary sludges and dewatered partly by a mechanical process. It had pH value of 8.04. The alkalinity of sludge typically arised from sulphite method used in the pulping and paper production process. Although many microorganisms are capable of growing and liberating the appropriate enzymes for the hydrolysis of materials over a wide pH range, in most cases, degradation occurs most rapidly at neutral pH, indicating the pH of the sludge may need to be decreased prior to composting. The sluge possessed rather low gravimetric water content (48.9%). A high press dewatering efficiency may be responsible for this case. The N content of the sludge (1.85%) was in accordance with the report that combined paper mill biosolids usually contain 1.0-2.5% N by dry weight. However, the C:N ratio of the sludge (14.5:1) was lower compared to the report that it ranged from 100:1

TABLE 1. Morphological, physiological and biochemical characteristics of bacterial strains

Tests		Strain		
		C1	C5	V18
Colony	Form		round	
	Diameter (mm)	2-3	3-4	2-3
	Color		creamy	
	Surface	wrinkled	flat	rough
	Margin		undulate	
Cell form, size (μm)		rod (1.0×2.0)	rod (1.0×3.0)	rod (0.8×2.0)
Gram stain			+	
Optimal temperature			45-60°C	
Catalase			+	
Oxidase			-	
Hemolysis			-	
API 20E				
ONPG	ß-galactosidase		+	
ADH	Arginine dihydrolase		-	
LDC	Lysine decarboxylase		-	
ODC	Ornithine decarboxylase		-	
CIT	Citrate utilization		+	
H_2S	Hydrogen sulfide production		-	
URE	Urea hydrolysis		-	
TDA	Deaminase		-	
IND	Indole production		-	
VP	Acetoin production		+	
GEL	Gelatinase		+	
NIT	Nitrate Reduction		+	

(Continued)

(*Continue*) TABLE 1.

Tests		Strain		
		C1	C5	V18
API 50CHB				
GLY	Glycerol		+	
ERY	Erythritol		-	
DARA	D-Arabinose		-	
LARA	L-Arabinose		+	
RIB	D-Ribose		+	
DXYL	D-Xylose		+	
LXYL	L-Xylose		-	
ADO	D-Adonitol		-	
MDX	Methyl-ßD-xylopyranoside		-	
GAL	D-Galactose		-	
GLU	D-Glucose		+	
FRU	D-Fructose		+	
MNE	D-Mannose		+	
SBE	L-Sorbose		-	
RHA	L-rhamnose		-	
DUL	Dulcitol		-	
INO	Inositol		+	
MAN	D-Rannitol		+	
SOR	D-sorbitol		+	
MDM	Methyl-αD-mannopyranoside		-	
MDG	Methyl-αD-glucopyranoside		+	
NAG	N-acetylglucosamine		-	
AMY	Amygdalin		-	
ARB	Arbutin		+	
ESC	Esculin Ferric citrate		+	
SAL	Salicin		+	
CEL	D-Cellobiose		+	
MAL	D-Maltose		+	
LAC	D-Lactose		-	
MEL	D-Melibiose		-	
SAC	D-Saccharose (Sucrose)		-	
TRE	D-Trehalose		-	
INU	Inulin		+	
MLZ	D-Melezitose		-	
RAF	D-Raffinose		+	
AMD	Amidon (starch)		+	
GLYG	Glycogen		+	
XLT	Xylitol		-	
GEN	Gentiobiose		+	
TUR	D-Turanose		+	
LYX	D-Lyxose		-	
TAG	D-Tagatose		-	
DFUC	D-Fucose		-	
LFUC	L-Fucose		-	
DARL	D-Arabitol		-	
LARL	L-Arabitol		-	
GNT	Potassium Gluconate		-	
2KG	Potassium 2-ketogluconate		-	
5KG	Potassium 5-ketogluconate		-	
XLT	Glycerol		-	
GEN	Erythritol		-	
TUR	D-Arabinose		-	
LYX	L-Arabinose		-	

+: positive; - : negative

to 20:1 in combined paper mill biosolids (Curnoe 1998). Since the rate of decomposition of organic materials is proportional to the concentration of N, evidence suggested that addition of N to achieve a C:N ratio of 35:1 or below would be required to stimulate microbial degradation of the materials. The sludge also contained a low concentration of P (0.65%) and K (0.12%). Although P and K are usually not limiting in most materials for biodegradation to occur, supplementation of the sludge with such nutrients may be required to increase the likelihood of microbial growth and activity. It was also found to be poorly colonised with thermophilic microorganisms (1.5×10^2 CFU g^{-1}), suggesting that the inoculation of sludge may benefit the composting process.

ISOLATION, SELECTION AND IDENTIFICATION OF THERMOPHILIC BACTERIA

Eleven thermophilic bacterial strains were isolated from Bai Bang PPMS. Among these, three strains namely C1, C5 and V18 which were able to form large colonies (>2 mm in diameter) reflecting high growth rates on the plates of Minimal Media Agar supplemented with 1% Bai Bang PPMS and showed protease, amylase, cellulase and ligninolytic activities on the plates containing 1% casein, soluble starch, CMC and lignin were selected for further experiments. Their morphological and biochemical characteristics (Table 1, Figure 1) were in agreement with those of genus *Bacillus* (Krieg & Holt 1984; Logan & Berkeley 1984).

The 16S rRNA sequences of strains C1 and C5 had 100% similarity with those of *Bacillus subtilis* BSn5 (CP002468.1) and *B. subtilis subsp. spizizenii* TU-B10 (CP002905.1). On the other hand, the 16S rRNA sequence of strain V18 showed a perfect match with that of *B. subtilis* IAM 12118 (NR 112116.1) and *B. subtilis* NCDO 1769 (NR 118972.1). The 16S rRNA sequences of strains C1, C5 and V18 have been deposited in the DDBJ/EMBL/GenBank database with accession numbers KX397360, KX397361 and KX397362, respectively. These strains were considered to be *B. subtilis* and identified as *B. subtilis* C1 (KX397360), *B. subtilis* C5 (KX397361) and *B. subtilis* V18 (KX397362). The phylogenetic tree constructed by the Neighbor-Joining method is shown in Figure 2.

EFFECTS OF THE MIXTURE OF THERMOPHILIC BACTERIAL STRAINS ON PPMS DIGESTION

The inoculated sludge achieved a significantly higher digestion efficiency than that found in the non-inoculated

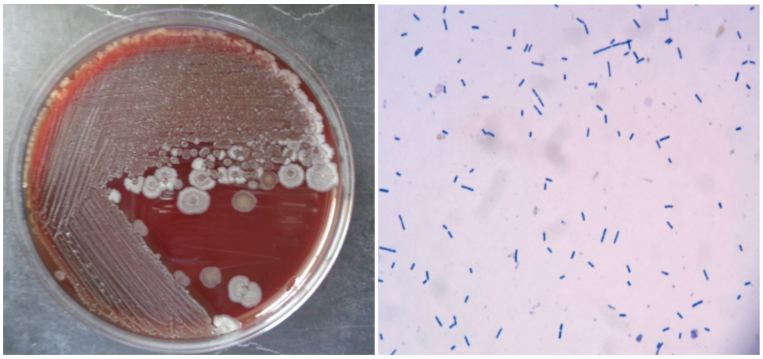

FIGURE 1. Colony morphology and Gram staining of bacterial strain C5

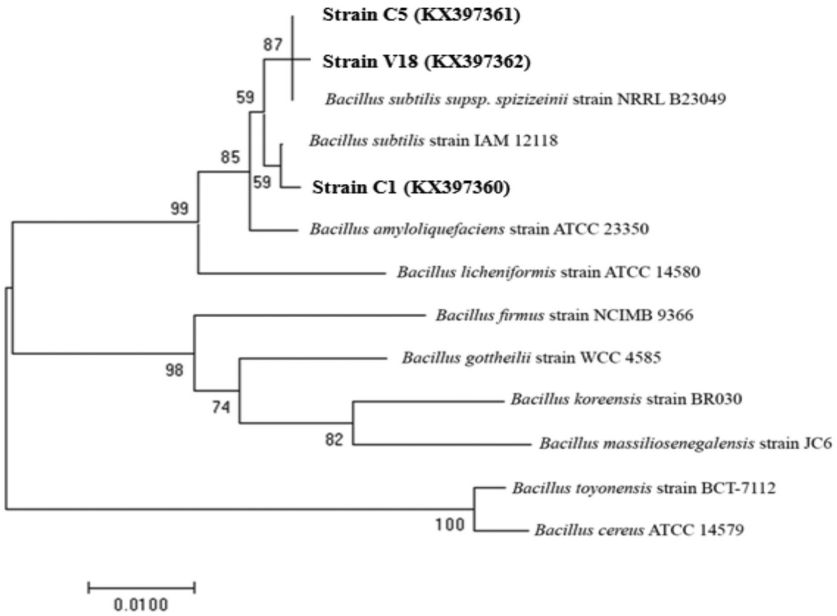

FIGURE 2. The phylogenetic tree was constructed by the Neighbor-Joining method. The optimal tree with the sum of branch length 0.20637887 was shown. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (2000 replicates) was shown next to the branches. The bootstrap consensus tree was taken to represent the evolutionary history of the sequences analyzed. The evolutionary distances were computed using the p-distance method and were in the units of the number of base substitutions per site. The scale bar represents approximately 0.01 changes per average nucleotide position

sludge (p<0.05) (Figure 3). It may be due to the unique thermostable enzyme system produced by thermophilic *B. subtilis* strains in the extreme habitat (Silvania et al. 2015). In this experiment, hydrolytic activities of the mixture of selected thermophilic bacterial strains cultivated at 60°C are shown in Figure 4. Through digestion under thermophilic conditions, the insoluble sludge biomass was transformed into soluble molecules which were subsequently metabolized to CO_2 and water, resulting in a decrease in its dry weight. The dry weight decrease increased linearly up to day 8 of incubation and then the curve gradually leveled off to day 10. The solid fraction of the inoculated sludge decreased approximately 52.5%, whereas, for the non-inoculated sludge, only 25.5% decrease was observed after 10 days of incubation (Figure 3(a)). Contemporaneously, Bai Bang PPMS mineralization occurred during the digestion process in which a removal of VSS took place. The rapid removal of VSS was probably related to more vigorous metabolism in the initial period due to abundant organic substrates and high temperature (60°C) which facilitated rapid growth of thermophiles. The highest VSS removal rate reached about 45% at day 8 in the inoculated sludge and did not change significantly over the following two days of the experiment, while only 30% change was observed in the non-inoculated sludge (Figure 3(b)). In addition, protease is considered to be the main enzymatic reaction in the digestion sludge (Kim et al. 2002). Therefore, protease activity should be an important factor for the sludge reduction efficiency. Protease activity of the inoculated sludge increased rapidly and reached at the highest value (0.85 U/mL) after 7 days, then maintained at this level until day 8 and gradually declined thereafter. On the contrary, protease activity of the non-inoculated sludge displayed a gradual increasing trend for the first 6 days of digestion and then fluctuated within a narrow range (0.62-0.67 U/mL) until the end of digestion trial (Figure 3(c)). Besides, dehydrogenase activity associated with the presence of living bacterial cells was also considered as a good indicator of microbial activity in the stability of sludge (Sánchez et al. 2006). In this experiment, there was an initial decrease in dehydrogenase activity, then a slight increase in dehydrogenase activity was observed and continued to day 8 after which there was a gradual decrease in this activity. This trend of dehydrogenase activity was found in both of inoculated and non-inoculated sludges (Figure 3(d)). The existence of positive correlation between the thermophilic bacterial population density and dehydrogenase activity was also stated when, for instance in the inoculated-sludge, the initial number of thermophilic bacteria (1.5×10^6) was declined at day 2 (0.8×10^5 CFU mL^{-1}), then increased till day 8 (1.4×10^6 CFU mL^{-1}) and decreased gradually towards day 10 (0.9×10^6 CFU mL^{-1}). The initial decrease in dehydrogenase activity may be due to the adaptation of the bacterial strains to new culture conditions. However, once acclimatization had been achieved, an increase in dehydrogenase activity was observed. The decrease in thermophilic bacterial population density that resulted in decrease in dehydrogenase activity at the latter period of incubation was most likely due to the exhaustion of available nutrients and the lysis of bacterial cells (Snow 1996).

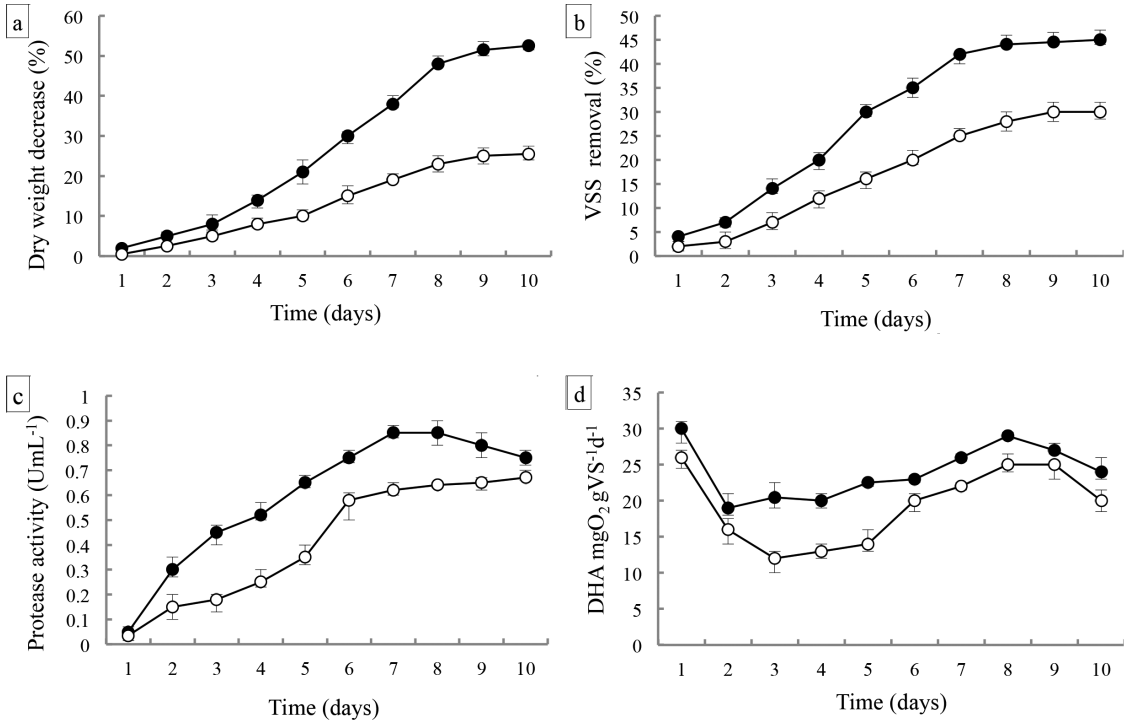

FIGURE 3. Dry weight decrease (a), VSS removal (b), protease activity (c) and dehydrogenase activity (d) of inoculated sludge (black circle) and non-inoculated sludge (white circle) during digestion process at 60°C for 10 days

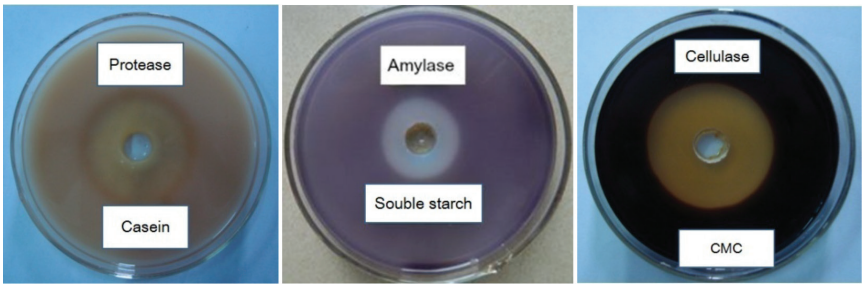

FIGURE 4. Hydrolytic activities of the mixture of selected thermophilic bacterial strains

STABILITY AND MATURITY OF COMPOST

In three-day incubation test of alkaline trap method, the non-inoculated compost sample produced 8.5 mg CO_2-C g VS^{-1} d^{-1}. By contrast, the inoculated compost sample yielded a CO_2 production rate of 3.2 mg CO_2-C g VS^{-1} d^{-1}. The compost stability is associated with the state of organic matter and the resultant microbial activity therein. Evolution of CO_2-C was considered as result of the respiration of compost microorganisms. These microbial respiration values were in the range suggested for stable compost (2-4 mg CO_2-C g VS^{-1} d^{-1}) for the inoculated compost and unstable compost (8-9 mg CO_2-C g VS^{-1} d^{-1}) for the non-inoculated compost (Thompson 2002). Moreover, the inoculated compost had a dark brown color and produced no foul smell in moist condition. It means that the inoculated compost was indeed stable. Use of stable compost will prevent nutrient tie up and maintain or enhance oxygen availability in soil (Buchanan et al. 2001). On the other hand, in *in vitro* germination and root elongation test with garden cress (Figure 5), the relative seed germination and root growth increased with the dilution of the non-inoculated compost extract. It can be explained that the non-inoculated compost may contain amounts of free ammonia, certain organic acids or other water-soluble compounds which can limit seed germination and root development (Buchanan et al. 2001). However, the relative seed germination was constant and root growth decreased with the dilution of the inoculated compost extract. For the full strength of the extract, the lower values of relative seed germination (85.5%) and root growth (87.9%), indicating a lower GI value (75.2%), was found for the non-inoculated compost extract. Meanwhile, the significantly higher values of relative seed germination (94.6%) and root growth (137.5%), indicating a higher GI value (130.1%) was found for the inoculated compost extract. The inoculated compost GI value was in the range suggested for compost with the absence of phytotoxicity (> 80%) and the non-inoculated compost GI value was in the range suggested for compost with moderate phytotoxicity (50-80%) (Thompson 2002). In other words, the inoculated compost was a mature product free of the potentially phytotoxic components and even considered a phyto-nutrient or phyto-stimulant (GI> 100%). The accelerated stability and maturity of the inoculated compost could be due to the inoculation of thermophilic *B. subtilis* strains that shortened appreciably the start-up period of sludge digestion process (Fang et al. 2001) and enhanced the sludge digestion by their thermophilic enzymes (Sakai et al. 2000). Since immature and poorly stabilized composts may pose a number of problems during storage, marketing and use (Buchanan et al. 2001), utilization of PPMS thermophilic bacteria as start inoculum in composting process is very essential to produce the acceptable quality composts.

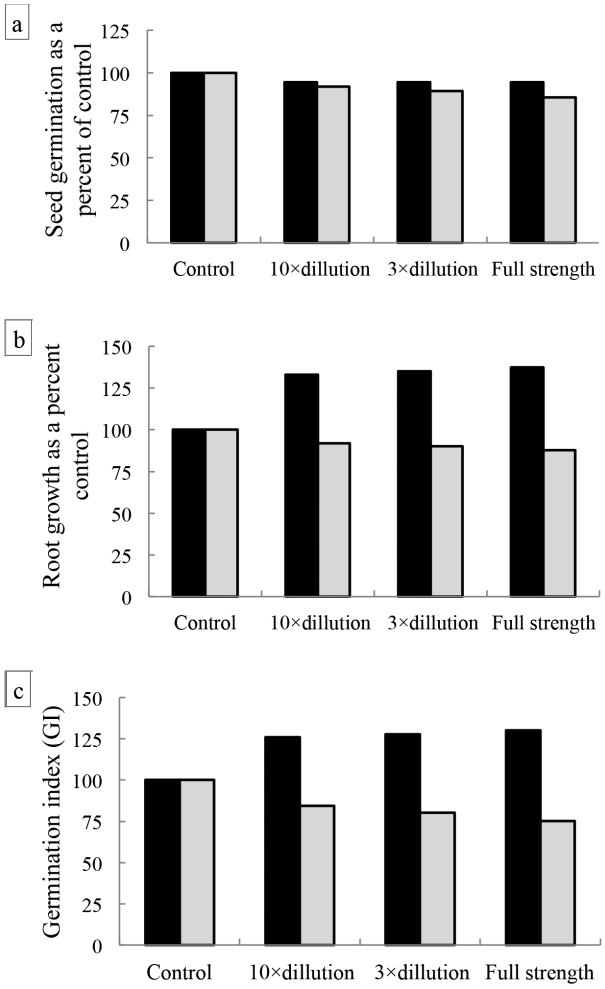

FIGURE 5. Relative seed germination (a), relative root growth (b) and germination index (c) of garden cress (*Lepidium sativum* L.) at control and different strengths of the extracts from inoculated compost (dark bar) and non-inoculated compost (light bar)

CONCLUSION

The inoculation with the mixture of three thermophilic bacterial strains including *B. subtilis* C1 (KX397360), *B. subtilis* C5 (KX397361) and *B. subtilis* V18 (KX397362) enhanced remarkably the Bai Bang PPMS digestion. Moreover, the utilization of the mixture as start inoculum in composting process of Bai Bang PPMS supplemented with rice straw as bulking agent improved considerably the quality of compost product. This result provided a persuadable evidence to support the need for inoculation to facilitate composting process of Bai Bang PPMS in particular and PPMS in general.

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