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A NOVEL LIGNOCELLULOLYTIC BACTERIUM FOR BIOCONVERSION OF RICE STRAW

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Large amounts of straw accumulate as a by-product of paddy cultivation. However, proper management of bulky straw has become a concern worldwide. We therefore explored potential bacterial isolate(s) for rice straw bioconversion. A study comprising several experiments was conducted at Composting Unit and Laboratory of Food Crops of Universiti Putra Malaysia under controlled condition. In total, 33 bacterial isolates were obtained from various sources of decomposed rice straw, such as composts or soil. The results showed that isolates UPMB7, UPMB8, UPMB10, UPMB17, and UPMB25, tested on rice-straw-powder amended media, exhibited optimum lignocellulolytic activities. UPMB7 and UPMB25 were further screened for rice straw bioconversion. Finally, the most optimal lignocellulolytic bacterial isolate, UPMB25, was identified as *Bacillus subtilis* using *16S rRNA* sequencing and tested for *in vivo* rice straw bioconversion. UPMB 25 (*B. subtilis*) decomposed rice straw at a significantly higher rate than the control, with respect to both *in-vitro* and *in-vivo* bioconversion. At day 7, UPMB 25-inoculated compost showed optimum lignin peroxidase (LiP) activity of 6.06 μmol veratraldehyde•min^{-1}•g^{-1}, endoglucanase activity of 7.37 CMC•g^{-1}, exoglucanase activity of 3.79 FPA•g^{-1}, and fluorescein diacetate (FDA) hydrolysis activity of 1428.8 µg fluorescein•min^{-30}•g^{-1} at day 14. After 4 weeks, the germination index of tomato seeds was found to be 80%, indicating the potential of UPMB25, *B. subtilis*, to be used as an efficient lignocellulolytic bacterium for large scale composting of rice straw.
Keywords: *Bacillus subtilis*, lignocellulolytic, stability, bioconversion, maturity, rice straw compost.

INTRODUCTION

Rice is a principal food crop in tropical and subtropical Asia and straw is generated as a by-product of rice cultivation. Straw occupies a large area for its low bulk density, and harbors of pests and diseases. It is not directly recycled into the field owing to its slow degradation rate. Moreover, it adds large amounts of organic carbon to the soil, which leads to the net immobilization of nitrogen. Therefore, the succeeding crops suffer from nitrogen deficiency, subsequently resulting in lower yield. Farmers usually dispose straw by open field burning, which resulted in severe problem as well as huge losses of N (up to 80%), P (25%), K (21%), and S (60%), depriving the soil of organic matter (Mandal *et al.,* 2004).

Bioconversion is a promising technique for converting rice straw into a value added end-product (Sanchez-Monedero *et al.,* 2002; Yu *et al.,* 2007). Rice straw compost is rich in silicon (Si), which serves as a potential plant nutrient source and can upgrade soil characteristics (Ma *et al.,* 2004; Khaliq *et al.,* 2006). Straw compost helps alleviate the effects of abiotic stresses, including salt and metal toxicity, drought and temperature stresses, radiation damage, and nutrient imbalances. Straw compost improves soil properties by lowering bulk density, increasing water-holding capacity, cation exchange capacity, buffering capacity, soil aeration, and infiltration rates. Straw compost containing protease, chitinase, lipase, β-1, 3 glucanase, and salicylic acid plays a vital role in plant defense systems (EL-Masry *et al.,* 2002) and is reported to increase the yield of rice by improving soil fertility, grain to straw ratio, and disease suppression (Ng *et al.,* 2012). These qualities of straw compost allow it to facilitate the organic farming of rice. In addition, foods produced from organic farming have great consumer demand as they are safe, eco-friendly, nutritious, and rich in vitamin C and iron, with more health-promoting properties than conventional foods (Vinha *et al*., 2014).

However, the degree of maturity and stability is an imperative aspect of compost application (Wu *et al.,* 2000)**.** Compost is considered mature when it becomes stable and supports microbial activity. Furthermore, the maturity of composts depends on the nature, composition, and structure of organic materials as well as the potential of microorganisms to decompose these organic substrates. Various phytotoxic compounds are produced during composting and the humification process is fed by intermediate metabolites that are generated (Perez *et al.,* 2002). Hence, the composting period and compost quality are highly dependent on the potency of microorganisms.

Bacteria have been used in the bioconversion of lignocellulosic materials due to their ability to decompose

macro-molecules (Ruberto *et al.,* 2003; Halet *et al.,* 2006; Pathak *et al.,* 2006), and can survive under extreme environmental condition during composting (Ugwuanyi, 2008**)**. In recent years, numerous methods have been developed to hasten the composting of rice straw by using lignocellulolytic fungi and actinomycetes (Khan *et al.,* 2007; Gaind and Nain, 2011; Kausar *et al.,* 2010; Kausar *et al.,* 2011; Chang *et al.,* 2012). However, thus far, few studies have investigated the composting of rice straw with lignocellulolytic bacteria. Therefore, we isolated and screened lignocellulolytic bacterial isolate(s) from ecologically related habitats of rice straw and evaluated the potential of selected bacteria for composting straw.

MATERIALS AND METHODS

***Experimental site*:** The experiments were conducted during January 2012 to August 2012 at the Composting Unit and Laboratory of Food Crops of Universiti Putra Malaysia, Serdang, Selangor, Malaysia (3° 02' N, 101° 42' E; elevation 31 m). During the experimental period, monthly average maximum and minimum temperature and RH were 34.5°C, 24.5°C and 89.5%, respectively.

***Sample collection and isolation of bacteria*:** Naturally composed rice straw, rice straw residues, and soil samples were obtained from Semerak, Pasir Puteh, Kelantan, and Universiti Putra Malaysia, Malaysia. Isolation was performed on Nutrient agar (NA, Difco^{TM}) using the plate dilution method.

In-vitro screening for lignocellulolytic activity:

***Enzymatic hydrolysis of cellulose*:** Cellulose hydrolysis was tested on Jensen's media (Jensen, 2008). Bacterial inoculum (3.0 µl) was spotted onto solidified media and incubated at room temperature for 24 h. Cellulose hydrolysis was detected by halo zone formation around the bacterial colony.

***Enzymatic hydrolysis of lignin*:** Lignin biodegradation was tested on Azure-B media following the method of Archibald (1992). Bacterial inoculum (3.0 µl) was spotted onto solidified media and incubated at room temperature for 24 h. Lignin biodegradation was assayed by colony growth on Azure-B media.

***Enzymatic hydrolysis of rice straw*:** Based on first-stage of screening, five lignocellulolytic bacterial isolates UPMB7, UPMB8, UPMB10, UPMB17 and UPMB25 were tested onto 0, 10, 20 and 30% of rice–straw–powder (RSP) amended NA media. A 3.0 µl of each bacterial inoculum was spotted onto solidified media and incubated at room temperature for 24 h. Colony growth was used to assay the lignocellulolytic activity of bacterial isolates.

***In-vitro bioconversion of rice straw*:** UPMB7 and UPMB25 showing optimum adaptation to RSP-amended media were selected for rice straw bioconversion. Two hundred fifty grams of rice straw and an equal amount of chicken manure (1:1, w/w) was mixed in a plastic bag. The mixture was inoculated with 5% (v/w) of inoculum (10^8 Cfu·mL^{-1}) (Vargas-Garcia et al., 2007) and incubated at room temperature for six weeks. Samples were collected at week 3 and 6. Total carbon and nitrogen contents were determined using the Combustion and Kjeldahl method, respectively.

***Identification of bacterial isolate*:** After three stage of screening the bacterial isolate with the best bioconversion potential, UPMB25, was identified by 16S rRNA sequencing. The isolate was grown in 5 ml of Nutrient Broth (Difco^{TM}) at room temperature for 72 h until the stationary phase was reached. Genomic DNA containing the *16S rRNA* coding region was amplified using the universal primers 27F and 1492R. PCR amplification was performed in a volume of 25 µl by mixing 1 µl of bacterial DNA, 0.15 µM of each primer, 1× PCR reaction buffer (Fermentas, USA), 0.2 mM of dNTP mix, 2.5 U of Taq polymerase (Fermentas, USA), 25 mM of $MgCl_2$ and reaming volume was top up with nuclease free water. Cycling conditions were as follows: an initial denaturation of 5 min at 94°C, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by a final extension of 5 min at 72°C. After amplification, the PCR product was purified using the QIAquick Gel Extraction Kit (QIAGEN, USA). The purified product was sent for sequencing to First Base Laboratories Sdn Bhd, Selangor, Malaysia. The obtained sequence was analyzed by BLAST and the percentage similarity was determined. A phylogenetic tree was constructed following neighbor-joining method using MEGA Version 4 (*Tamura et al.,* 2007).

***In-vivo rice straw bioconversion*:** Bioconversion was conducted using a 56×38×32 cm composter. Five kilograms of chopped rice straw (≤2.0 inches) and an equal amount of chicken manure were mixed together and placed into each composter with a hole at the bottom. The mixture contained 87.15% compostable substrates with 12.85% moisture, which was adjusted to 60% (HH2 Meter, ΔT Delta devices, Cambridge, England) by adding distilled water. The total organic carbon and initial C/N ratio were 38.32% and 30.35%, respectively. The composting substrate was placed loosely in each composter to provide aeration and inoculated with 5% UPMB25 bacterial inoculum (10^8 Cfu·mL^{-1}) (*Vargas-Garcia et al.,* 2007). Non-inoculated substrate served as a control. The mixture was turned manually every other day for the first three weeks and then once a week for aeration and homogenization of the substrate.

***Determination of enzymatic activities*:** A 25-g compost sample was placed in a 250 ml Erlenmeyer flask with 100 ml of sterile distilled water. Flasks were shaken at 150 rpm for 2 h and upper aqueous phase was taken into a 50 ml tube. The tube was centrifuged at 13000 rpm for 10 min and the supernatant was collected into 15 ml tube and stored in a deep freezer at −20°C until use. The filtrate was used for determination of lignin peroxidase (LiP), endoglucanase, and total cellulase activity. LiP activity was assayed following the method of Tien and Kirk (1984).

A 600 µl of Veratryl alcohol solution (10 mM) was mixed with 1.5 ml of distilled water, 50 µl of enzyme sample, and 600 µl of pH 2.5 tartrate buffer (0.25 M). The absorbance was measured at 310 nm 1 min after the addition of 240 µl of 5 mM H_2O_2. One unit of enzyme activity is defined as the amount of enzyme generating 1 µmol of veratraldehyde per minute under the reaction conditions. Endoglucanase and total cellulase activity were determined following the method of Ghose (1987). Carboxymethyl cellulose (CMC) and Whatman No. 1 filter paper strips (1.0 × 6.0 cm) were used as substrates to measure the endoglucanase (CMC/g of compost) and total cellulase (filter paper assay [FPA]/g of compost) activity. One CMC is the concentration of enzyme that can release 0.5 mg of reducing sugar in 30 min under the assay conditions and one FPA is the concentration of enzyme that can release 2.0 mg of reducing sugar per minute.

***Determination of fluorescein diacetate (FDA) hydrolysis*:** Fluorescein diacetate hydrolysis or total enzymatic activity of microbes was assessed using the method of Adam and Duncan (2001). Briefly, 2 g of the compost sample was taken into a 50 ml conical flask. Fifteen milliliters of 60 mM potassium phosphate buffer (pH 7.6) was added to the flask. Then, 0.2 ml of the FDA solution was added to initiate the reaction. The flasks were shaken manually, and placed in an orbital incubator at 30°C for 20 min. Fifteen milliliters of chloroform/methanol (2:1, v/v) was added to terminate the reaction. After shaking, the contents were transferred into a 50 ml centrifuge tube and centrifuged at 3000 rev·min^{-1} for 3 min. The total enzymatic activity was expressed in terms of µfluorescein/30mim /g-dry compost.

***Germination Index (GI)*:** Phytotoxicity of rice straw compost was evaluated based on germination index (GI, determined by both germination and root growth) of tomato seeds *(Zucconi et al.,* 1981).

$$\frac{Seed\,germination\,(\%)\times Radicle\,length\,of\,treatment}{Seed\,germination\,(\%)\times Radicle\,length\,in\,distilled\,water}\times 100$$

***Statistical analysis*:** All experiments were carried out using a completely randomized design (CRD) with five replications. Data were subjected to analysis of variance (ANOVA) and tested for significance by least significant difference (LSD) using the PC-SAS software 9.2. To group bacterial isolates based on their lignocellulolytic activities, data were subjected to cluster analysis using NTSYS pc 2.02.

RESULTS

***Isolation of bacteria*:** In total, 33 bacteria were isolated from 10 samples collected from different locations in Malaysia. Microorganisms were analyzed based on their cultural and morphological characteristics i.e., colony morphology, color, shape and growth patterns.

***Screening on Azure-B and carboxymethyl cellulose (CMC) amended media*:** All 33 bacterial isolates displayed diverse lignocellulolytic efficiencies to degrade lignin and cellulose on Azure-B and CMC culture media (Table 1).

Table 1. Ability of bacterial isolates to degrade cellulose and lignin on carboxymethyl cellulose (CMC) and Azure-B amended media.

Isolate No	Lignin	Cellulose
	Colony growth (mm)	Halo zone (mm)
UPMB 1	5.86 no	1.30 klm
UPMB 2	8.23 k	1.53 hij
UPMB 3	11.56 g	1.86 def
UPMB 4	8.30 k	1.70 fghi
UPMB 5	7.86 l	1.60 ghi
UPMB 6	10.86 h	1.96 cde
UPMB 7	15.30 b	2.80 a
UPMB 8	12.86 c	2.10 c
UPMB 9	4.93 st	1.13 m
UPMB 10	12.96 c	2.06 cd
UPMB 11	8.36 k	1.73 fgh
UPMB 12	5.46 pq	1.53 hij
UPMB 13	3.23 w	0.00 n
UPMB 14	4.56 u	1.36 jkl
UPMB 15	11.83 f	1.96 cde
UPMB 16	4.83 t	1.53 hij
UPMB 17	12.26 e	2.53 b
UPMB 18	12.53 d	1.80 efg
UPMB 19	10.93 h	1.73 fgh
UPMB 20	6.73 m	1.10 m
UPMB 21	5.46 pq	1.53 hij
UPMB 22	3.53 v	0.00 n
UPMB 23	8.63 j	1.73 fgh
UPMB 24	5.63 op	1.83 ef
UPMB 25	15.56 a	2.93 a
UPMB 26	5.16 sr	1.36 jkl
UPMB 27	5.93 n	1.56 hij
UPMB 28	4.83 t	1.26 lm
UPMB 29	3.70 v	0.00 n
UPMB 30	5.36 qr	1.53 hij
UPMB 31	9.53 i	1.83 ef
UPMB 32	3.23 w	0.00 n
UPMB 33	5.83 no	1.50 ijk

Means within columns with the same letters are not significantly different at 5% level of probability

Bacterial isolates grew well on media containing azure-B. Six isolates, UPMB7, UPMB8, UPMB10, UPMB17, UPMB18, and UPMB25 developed relatively larger colonies (diameter >12 mm). UPMB25 was found to develop the largest colony (15.56 mm) followed by UPMB7 (15.30 mm), UPMB10 (12.96 mm), UPMB8 (12.86 mm), and UPMB18 (12.53 mm). Twenty-nine isolates developed halo zones on CMC amended media. Five bacterial isolates, UPMB7, UPMB8, UPMB10, UPMB17, and UPMB25 showed relatively higher activity by forming larger zones (>2.0 mm); however, the other 24

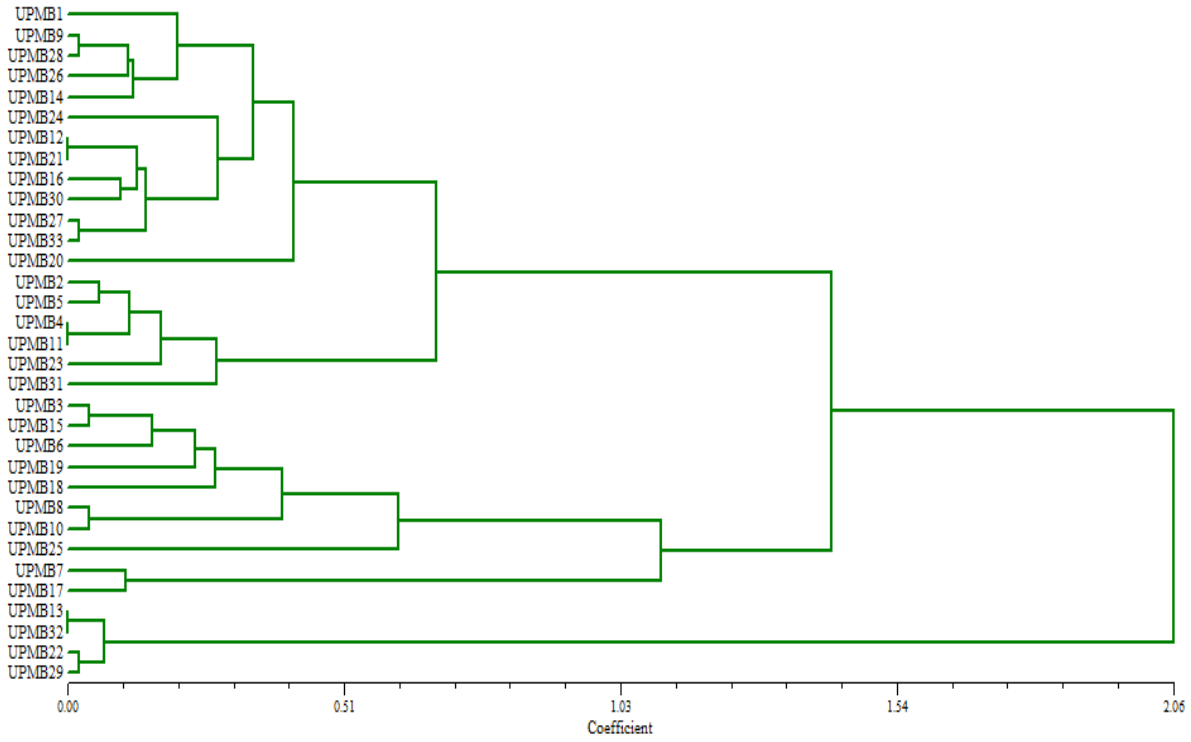

Figure 1. Clustering tree of 33-bacterial isolates based on their lignocellulolytic activity prepared using NTSYS pc 2.02 software.

isolates formed comparatively smaller zones (< 2.0 mm). UPMB 25 developed the largest halo zone (2.93 mm) followed by UPMB7 (2.80 mm), UPMB17 (2.53 mm), UPMB8 (2.10mm), and UPMB10 (2.06 mm).

Lignocellulolytic potential of the bacterial isolates was combined in a single clustering tree (Fig. 1) which generated 3 major clusters (cluster 1, 2, and 3), where the overall group dissimilarity ranged from 0.65 to 2.05. Cluster 2 comprised 10 isolates, all of which produced clear and larger halo zones and well-developed colonies on Azure-B and CMC-amended media. Cluster 1 comprised 19 isolates that showed a moderate ability to decompose lignin and cellulose. However, the 4 isolates in cluster 3 produced small colonies (≤ 3.6 mm in diameter) on Azure-B and did not produce a halo zone on CMC-amended media.

***Screening on rice straw powder (RSP) amended media*:** UPMB7, UPMB8, UPMB10, UPMB17, and UPMB25 were further screened on RSP-amended NA (Difco^{TM}) to assess their growth suitability to rice straw. Growths of bacterial isolates at different concentrations of RSP-amended media are presented in Table 2. UPMB7, UPMB8, and UPMB25 showed good adaptability up to a concentration of 30% RSP, while UPMB10 and UPMB17 maintained a higher growth rate up to a concentration of 20% and 10%, respectively. UPMB 25 developed significantly (≤0.05) larger colonies than that of other isolates at 10%, 20% and 30% RSP. However, UPMB17 showed the least adaptability to RSP-amended media.

Table 2. Effect of rice-straw-powder (RSP)-amended media on colony diameter of lignocellulolytic bacterial isolates (mm).

Isolate No	NA	NA+10% RSP	NA+20% RSP	NA+30 %RSP
UPMB7	7.45	8.90	11.42	14.50
UPMB8	6.29	8.38	10.40	11.18
UPMB10	6.43	8.38	9.00	8.68
UPMB17	8.50	8.88	8.50	8.40
UPMB25	6.88	9.46	14.66	18.20
$LSD_{0.05}$	0.42	0.39	0.78	0.65

RSP – Rice straw powder; NA− Nutrient agar.

Evaluation of UPMB7 and UPMB25 for in-vitro bioconversion of rice straw: Two bacterial isolates UPMB7 and UPMB25 that showed optimum lignocellulolytic potential and growth adaptability to RSP media were selected for *in vitro* bioconversion of rice straw.

***Carbon, nitrogen, and C/N ratio*:** The results of carbon, nitrogen, and C/N ratio during six weeks of rice straw bioconversion are shown in Table 3. Carbon content in the

Table 3. Carbon, nitrogen and C/N ratio of rice straw compost after three and six weeks of bioconversion.

Isolates	Rice straw properties					
	Three weeks			Six weeks		
	Carbon (%)	Nitrogen (%)	C/N	Carbon (%)	Nitrogen (%)	C/N
UPMB 7	39.35	1.74	22.61	34.64	1.99	17.41
UPMB25	37.83	1.96	19.30	32.09	2.12	15.14
Control	41.21	1.58	26.08	35.87	1.84	19.50
$LSD_{0.05}$	2.12	0.15	3.15	2.68	0.19	2.58

inoculated substrates was found to be lower than that of the control during the six weeks of bioconversion. After three weeks, UPMB25 showed the highest efficiency in terms of carbon bioconversion (with 37.83% remaining) followed by UPMB7 (39.35%). After six weeks, the carbon content dropped to between 32.09% and 34.64% in the inoculated substrates, while it was 35.87% in the control. UPMB25 was found to decompose carbon content (with 32.09% remaining) significantly ($p \leq 0.05$) compared to control treatment.

Total nitrogen content in inoculated substrates was significantly higher ($p \leq 0.05$) than the control during the first three weeks of bioconversion. Percent nitrogen content in the inoculated substrates ranged between 1.74% to 1.96%, while 1.58% nitrogen was recorded in the non-inoculated substrate. The highest nitrogen content was recorded in the substrate treated with UPMB 25. After week 6, a similar trend was observed for nitrogen content where inoculated treatments were recorded from 1.99% to 2.12%, while it was only 1.84% in the control treatment. At the end of bioconversion, the highest nitrogen content (2.12%) was found in substrates inoculated with UPMB25.

The C/N ratio in inoculated substrates was lower than that of the control during rice straw bioconversion. After 3 weeks, C/N ratio in the inoculated substrates ranged from 19.30 to 22.61, while it was 26.08 in non-inoculated substrate. UPMB25 significantly lowered ($p \leq 0.05$) the C/N ratio compared to UPMB7. As expected, a similar trend was observed after six weeks, where inoculated substrates were recorded from 15.14 to 17.41, while it was 19.50 in a non-inoculated substrate. The lowest C/N ratio seen in substrates inoculated with UPMB 25.

***Identification of UPMB25 using 16S rRNA sequencing*:** UPMB25 was identified by amplifying and sequencing the *16S rRNA* coding region of genomic DNA, which produced a fragment of approximately 1400 bp (Fig. 2). BLASTX sequence analysis revealed that the UPMB 25 matched *Bacillus subtilis* with 99% similarity. A phylogenetic analysis of *B. subtilis* strain UPMB 25 was performed based on the neighborhood-joining tree method with 100 bootstrap samplings (Fig. 3).

***Evaluation of UPMB25 for in-vivo bioconversion of rice straw*:** UPMB25, *B. subtilis*, was further tested for composting rice straw.

C/N ratio: The initial C/N ratio in composting substrates was 30.35, which continued to drop through the bioconversion process. As expected, the C/N ratio recorded in the inoculated treatments was significantly lower ($p \leq 0.05$) than the control at day 14 and thereafter. After day 21, C/N ratio in UPMB 25-inoculated substrate dropped to 18.33 from the initial value of 30.35, and was 24.77 in non-inoculated treatment (Fig. 4).

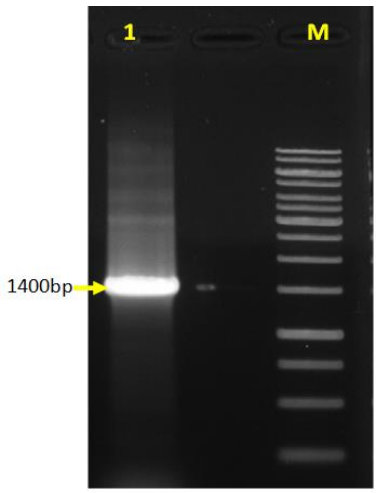

Figure 2. Agarose gel electrophoresis of the 16S rDNA PCR products of bacterialisolate UPMB25. Lane M: 1 kb DNA ladder; Lane 1: bacterial isolate UPMB25.

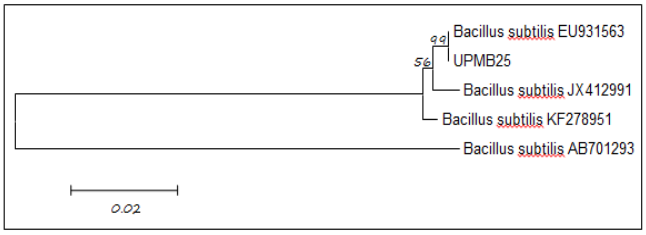

Figure 3. Neighbor-joining tree based on 16S rDNA gene sequences showing phylogenetic relationship of isolates UPMB25 to its related isolates.

***Lignin peroxidase (LiP) activity*:** LiP activity increased sharply and reached to its maximum on day 7. UPMB 25 inoculation had a significantly higher ($p \leq 0.05$) effect on LiP activity than the control for up to day 14, and dropped sharply thereafter. Highest LiP activity (6.06 μmol veratraldehyde•min^{-1}•g^{-1}) was recorded at day 7, while it was

only 2.88 μmol veratraldehyde•min^{-1}•g^{-1} dry compost in the control treatment (Fig. 5).

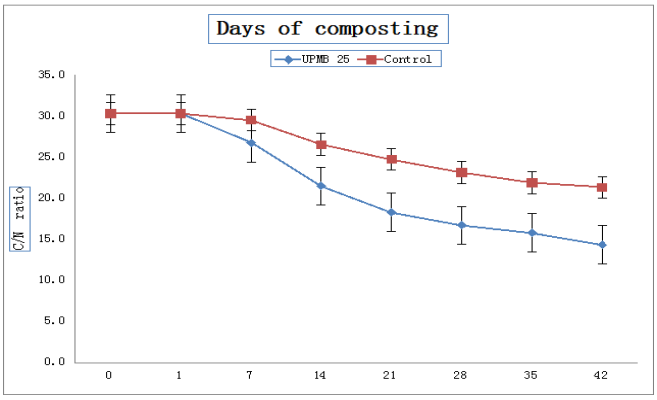

Figure 4. Effect of UPMB25 on the profile of C/N during microbial composting of rice straw.

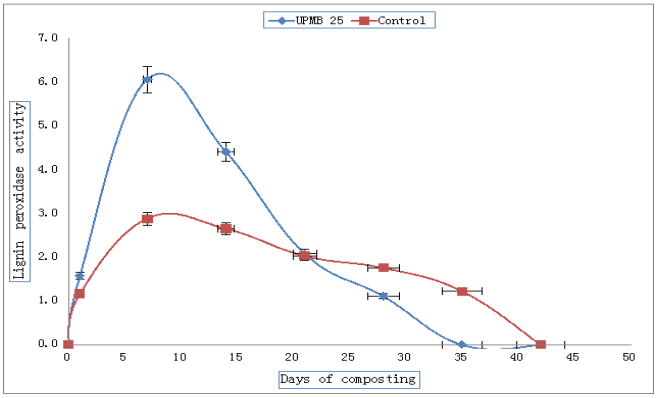

Figure 5. Effect of UPMB25 on lignin peroxidase (LiP) activity during microbial composting of rice straw.

***Endo-1, 4-β-glucanase activity*:** Endoglucanase activity of rice straw compost increased steadily and reached a maximum on day 14. UPMB 25 inoculation showed significantly higher ($p \leq 0.05$) endoglucanase activity than the control up to day 21. After 28 days, no endoglucanase activity was detected in the inoculated treatment. However, endoglucanase activity was detected till to end of the experiment in the non-inoculated treatment (Fig. 6).

***Exo-1, 4-β-glucanase activity*:** Similar to the activity of endoglucanase, exo-1, 4-β-glucanase also showed an increase up to day 14 of the experiment, followed by a gradual drop. UPMB25 inoculation had a significantly higher ($p \leq 0.05$) effect on exo-1, 4-β-glucanase activity compared to the un-inoculated condition until day 21. After day 28, exo-1, 4-β-glucanase activity also became zero in UPMB25-inoculated treatment (Fig. 7).

***Fluorescein diacetate (FDA) hydrolysis*:** Total enzymatic activity continued to increase and peaked on day 14. Throughout the composting process, bacterial inoculation had significantly higher ($p \leq 0.05$) effect than the control, except for day 42. However, at the end of the bioprocess, significantly higher ($p \leq 0.05$) total enzymatic activity was observed in the non-inoculated substrate than the inoculated substrate (Fig. 8).

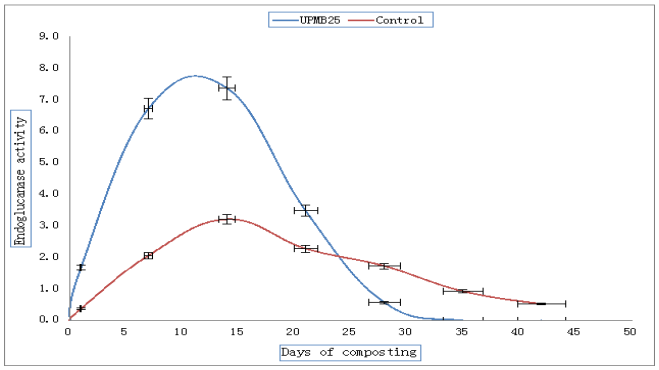

Figure 6. Effect of UPMB25 on endo-1, 4 β-glucanase activity during microbial composting of rice straw.

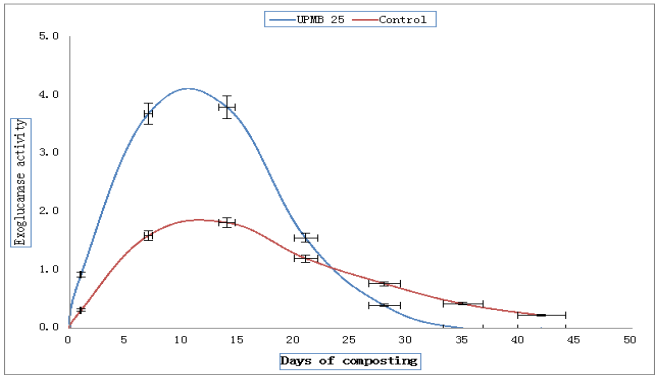

Figure 7. Effect of UPMB25 on exo-1, 4 β-glucanase activity during microbial composting of rice straw.

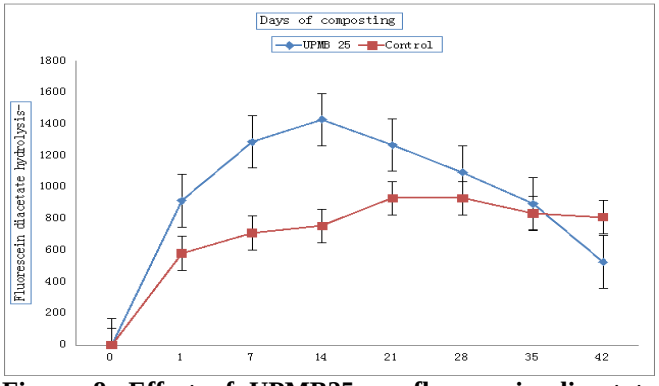

Figure 8. Effect of UPMB25 on fluorescein diacetate hydrolysis activity during microbial composting of rice straw.

Germination index (GI): The effect of bacterial inoculation on GI of tomato seeds during rice straw composting are presented in Figure 9. GI of tomato seeds dropped in the beginning, and rose afterwards as the bioprocess progressed. After day 14, GI of tomato seeds in UPMB25-inoculated treatment was significantly higher ($p \leq 0.05$) than the control. At day 28, GI of tomato seeds reached to 80% in UPMB25-inoculated treatment, while it was only 54% in the control treatment.

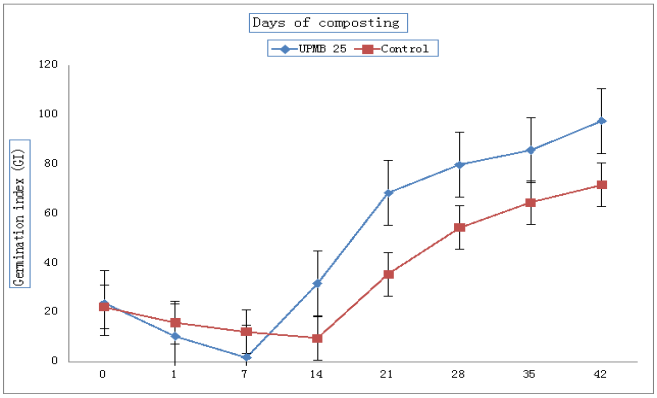

Figure 9. Effect of UPMB25 on germination index (GI) of tomato seeds during microbial composting of rice straw.

DISCUSSION

Rice straw is a heterogeneous lignocellulosic material and its use is limited due to low bulk density and high silica content. However, it can be converted to a value added end-product through microbial composting. Microbes with lignocellulolytic potential perhaps enhance the composting of rice straw through their synergistic actions with hydrolytic and oxidative enzyme systems.

Lignin in rice straw forms an irregular non-crystalline network that is highly resistant to biodegradation. Ligninolytic microorganisms can depolymerize lignin (Hofrichter, 2002). The six isolates namely UPMB7, UPMB8, UPMB10, UPMB17, UPMB18, and UPMB25, grew on Azure B media, confirming their ability to release lignin peroxidase (LiP) on such substrates. These findings were concordant with those of past studies **(**Pangallo *et al.*, 2009; *Bugg et al.*, 2011; Wang *et al.*, 2013). Bacteria cleave lignin in lignocellulosic materials through a diffusible chemical process by utilizing LiP and Phenol oxidases (Jing *et al.*, 2009). Bacteria decomposed lignocellulosic materials with hydrogen as a central intermediate to acetate and methane (Brune, 2014) and produced smaller aromatics that were imported into their cell for aromatic catabolism (Brown and Chang, 2014). Bacteria depolymerized 60.9% lignin in reeds within 15 days, under static culture conditions (Wang *et al.*, 2013). Cleavage of lignin is important in composting rice straw as lignin protects cellulose and hemicelluloses from biodegradation. Therefore, selection of bacterial isolates with ligninolytic ability was crucial in the rapid composting of rice straw.

Based on lignocellulolytic activity (Table 2) and cluster analysis (Fig. 3) five bacterial isolates UPMB7, UPMB8, UPMB10, UPMB17, and UPMB25 were selected for the second stage of screening on RSP-amended media. These five isolates were found to produce larger colonies (diameter ≥ 12.0 mm) in Azure-B and halo zone (diameter ≥ 2.0 mm) in CMC-amended media. The involvement of bacteria in the bioconversion of lignocellulosic substrates is well documented (Yang *et al.*,2002; Gilbert *et al.*, 2008). Their extracellular LiP enzyme systems catalyze the cleavage of carbon-carbon bonds in the side chains of phenolic and non-phenolic lignin substrates through oxidation and depolymerization **(**Seelenfreund *et al.*,1990). A clear halo zone, higher growth rate, and adaptability to higher concentrations of RSP confirmed that the bacterial isolates, UPMB 7 and UPMB25, efficiently utilized carbon in rice straw. These results were in line with the findings of Zainudin *et al.* (2013) who reported indigenous lignocellulolytic bacteria enhanced composting of oil palm empty fruit bunch in 40 days compared to conventional oil palm empty fruit bunch composting, which took 90 days.

Isolate UPMB 25 was selected for molecular identification where molecular phylogenetic analysis provides the basis for identification of the sequence (Singh *et al.*, 2007). *16S rRNA* sequence analysis of gene has been well-documented as a standard procedure for identification of bacteria at species, genera, and family levels (Gurtler and Mayall, 2001). The sequence analysis of UPMB25 revealed that its sequence matched *B. subtilis*, where phylogenetic analysis confirmed their relationship. *Bacillus* spp. has been well documented in the composting of lignocellulosic materials (He *et al.*, 2013). *B. subtilis* is thermo-tolerant and can maintain a high population by sporulating during composting (He *et al.*, 2013; McDonald *et al.*, 1998) and has been used successfully as bioprotectant (Mongkolthanaruk, 2012) for various crops. *B. subtilis* inhibits phytopathogens by competing for nutrients, antibiosis, and production of lytic enzymes, consequently increasing plant resistance. Therefore, composting rice straw after inoculation with *B. subtilis* could lead to the production of phytopathogen-suppressive compost.

Total organic C content of substrates inoculated with UPMB7 and UPMB25 dropped steadily compared to the control. Carbon is the building block of microbes and is utilized as energy which is lost in the form of CO_2. Therefore, carbon content in a substrate drops as the bioconversion proceeds. Maximum carbon reduction was observed in the first three weeks of bioconversion. This was owing to the presence of easily biodegradable carbon in substrates (Bernal *et al.*, 1998). Our results were in line with the findings of Devi *et al.* (2012) who found 45% carbon reduction during composting

of poultry manure and paddy straw (1:1, w/w), over a period of six months.

Nitrogen content in inoculated and control treatments was found to increase during *in vitro* bioconversion of rice straw. Compared to the control, relatively higher values were observed in substrates treated with UPMB 7 and UPMB25. Total nitrogen content increased perhaps due to the anabolism of cell structure, synthesis of enzymes, and hormones in microorganisms, and an increase of nitrogen concentration due to the volatilization of organic matter. These findings were consistent with the results of Veeken *et al.* (2001) and Raviv *et al.* (1999) who stated that total nitrogen content increased during bioconversion of sludge, lignocellulosic materials, and chicken manure.

Lignin peroxide (LiP) is involved in lignin biodegradation in rice straw. LiP activity depends on types of ligninolytic microorganisms. No LiP activity was observed after day 28 in inoculated treatment during rice straw composting. However, in the control treatment, LiP activity remained till the end of experiment indicating the availability of un-decomposed lignin substrate. Consistent low LiP activity followed by a slow gradual decline over a longer period in un-inoculated treatment indicates poor or insufficient growth of ligninolytic microorganisms, providing a clear indication of lignin biodegradation rates between inoculated and un-inoculated treatments during composting. Our results were supported by those of previous studies (Alam *et al.,* 2009; Bari *et al.,* 2009; Kausar *et al.,* 2013), where increased LiP activity was observed at an early stage during the bioconversion of lignocellulosic materials.

Endo-1,4-β-glucanases (EGs) hydrolyze the amorphic parts of cellulose microfibrils and release new terminal ends. EG activity showed an overall increase over 14 days of composting, and was followed by a gradual decline. It should be noted that LiP activity peaked at day 7, after cleavage of the lignin barrier cellulose is exposed to cellulolytic microbes (Santhi *et al.,* 2014). Therefore, the above trend in the activity of EG was expected during composting. Exoglucanases (CBHs) act on the EG-generated chain ends where CBHI interacts with reducing ends and CBHII interacts with non-reducing ends of cellulose fiber to release cellobiose molecule. Finally, β-glucosidases act on the cellobiose and release glucose (Lynd *et al.,* 2002). The highest exo-1,4-β-glucanase activity (3.79 FPA/g-dry compost) was found in the inoculated treatment on day 14; thereafter, it dropped to 0 after day 28. This trend in enzyme activity from day 14 was likely due to the reduction in cellulose content in the composting substrates. Moreover, our results were in line with the findings of Khan *et al.* (2007) who reported that microbes produced the highest level of glucanase on day 6 during the bioconversion of rice straw.

Fluorescein diacetate (FDA) hydrolysis was used to detect the total enzymatic activity in compost produced by microorganisms. The highest FDA activity was recorded in inoculated treatment on day 14 followed by a gradual drop. However, this trend was not as sharp as what was observed in case of LiP and cellulases. In the control treatment, FDA activity was higher at the end of the experiment, indicating that biodegradation occurred over a longer period. When the availability of labile-C substrates reduced microbial activity as well as the temperature also decreases. Our findings were supported by the observation of Ntougias *et al.* (2006) who found that FDA hydrolysis was related to a temperature fluctuation showing greater values at the peak temperature during composting of olive leaves. However, Ryckeboer *et al.* (2003) observed a negative correlation between temperature and FDA hydrolysis during composting of garden waste, which seems to contradict the results of our study. Nevertheless, FDA hydrolysis is a less sensitive method for detecting microbial activity, because FDA is degraded by a broad range of enzymes capable of cleaving the ester bond. Therefore, the available enzyme structure perhaps leads to overestimation of FDA values especially at the end of any composting phase (Insam, 2001; Nannipieri *et al.,* 2003).

C/N ratio is a reliable indicator for compost maturity (Inbar *et al.,* 1990). Here, the C/N ratio was found to drop both in both the *in vitro* and *in vivo* composting of rice straw, where the trend was greater in substrates treated with UPMB25, reflecting organic matter mineralization during bioconversion. During composting, carbon content dropped due to catabolism and nitrogen content increased due to anabolism in microbial cells and the volatilization effect of organic matter. These results were in line with the findings of Eiland *et al.* (2001) who stated that C/N ratio dropped during composting of *Miscanthus* straw. Jurado *et al.* (2014) also reported that *B. licheniformis* BT575, and *B. smithii* AT907 mineralized high C/N ratio and enhanced the composting of lignocellulosic materials. A C/N ratio of less than or equal to 20 is considered a mature compost if the initial value ranges from 25 to 30 (Heerden *et al.,* 2002; El Fels *et al.,* 2014). After day 21, the C/N ratio in rice straw compost inoculated with UPMB25 was 18.33, which indicated that compost treated with *B. subtilis* was mature and suitable for field application. This result was in consistent with the findings of Raut *et al.* (2009), who found that the C/N ratio was reduced to 14.7 from an initial value of 33.5 after 3 weeks of composting.

***Conclusion*:** In the present study, based on ligocellulolytic activities on Azure-B, CMC, RSP-amended media and *in vitro* bioconversion of rice straw, isolate UPMB 25 appeared as the potential lignocellulolytic bioresource for rice straw composting. After three stages of screening, the best selected bacterial isolate, UPMB 25, was identified as *B. subtilis* using *16S rRNA* sequencing, showing the highest LiP, cellulases, and FDA hydrolysis activities and germination index during *in vivo* rice straw bioconversion. Thus, showing the potential to be developed as a lignocellulolytic bioresource for rice straw composting.

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