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Full Length Research Paper

Development of a potential lignocellulolytic resource for rapid bioconversion of rice straw

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This study was conducted at the Laboratory of Food Crops and Floriculture, Institute of Tropical Agriculture, Universiti Putra Malaysia to characterize the potential lignocellulolytic bacteria for rapid bio-bioconversion of rice straw. Fifty isolates of bacteria were isolated from several *in-situ* and *in-vitro* sources. Isolates B3, B13, B21, B37 and B46 showed the highest enzymatic activities to starch, carboxymethyl cellulose (CMC) and Azure B amended media. They were further screened on rice straw powder (RSP) amended media for their adaptability on rice straw (RST). The isolate B37 obtained from thermophilic phase of *in vitro* composting of rice straw and showing the optimum lignocellulolytic activity and adaptation to RSP amended media was identified as *Bacillus pumilis.*

Key words: Rice straw, lignocellulose, microbial degradation, *Bacillus pumilis*.

INTRODUCTION

Rice (*Oryza sativa*) is the most important food crop in the world after wheat, with more than 90% currently grown in Asia. It is grown on an area of 154.493 million hectares (ha), yielding 636.493 million tons of paddy (USDA, 2008) that generates huge amount of rice straw as by-product. It is not recycled in the soil due to its bulk volume, slow degradation rate, harboring of diseases and weed problems (Devevre and Horwath, 2000), as well as poor yield caused by short-term negative effect of nitrogen immobilization (Buresh and Sayre, 2007). In addition, it is not suitable for animal feed as forage or even in engineering applications due to its poor quality (Jenkins et al., 1997). Hence, the farmers usually dispose the huge amount of straw by *in-situ* burning. This results in on one hand a waste of renewable organic source and on the o ther hand leads to emission of green house gases (Badrinath et al., 2006). It also contributes to emission of harmful air pollutants, including polycyclic aromatic hydrocarbons (PAHs) (Korenaga et al., 2001), poly-chlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), referred to as dioxins (Gullett and Touati, 2003; Lin et al., 2007) which have significant toxicological properties and notably potential carcinogens that can cause severe impacts on human health.

Keeping in mind the harmful effects of open field-burning of rice straw, as well as the convenience of farmers, an economical, environment friendly and low labor intensive strategy should be adapted for effective utilization of rice straw. Rice straw is a potential food source for microorganisms and application of rice straw compost into soil has long-term impact in nutrients recycling and maintaining soil fertility (Gaind et al., 2006), increasing microbial number, microbial biomass, and

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enzyme activity (Hayano et al., 1995). Inoculation with lignocellulolytic microbes might be an effective alternative to *in-situ* burning (Kumar et al., 2008) to make the rice straw composting process economically viable in a short period of time. The compost serves as an excellent source of nutrients in organic farming to mitigate the ill-effects of using chemical fertilizer.

Bacteria are well known for their ability to decompose complex molecules, particularly lignocellulosic components, which make them important agents in decomposition process (Pathak et al., 2006; Halet et al., 2006; Ruberto et al., 2003). They hasten the bioconversion of lignocellulosic components by producing lignocellulolytic enzymes (Lee et al., 2008), releasing soluble sugar from rice straw (Lei and VanderGheynst, 2000) for surviving in extreme environmental conditions (Ugwuanyi, 2008). Thus, the aim of this study was to isolate, select and characterize the potential bacteria from ecologically related habitats for the rapid bio-degradation of rice straw.

MATERIALS AND METHODS

Isolation of lignocellulolytic bacteria

Several *in situ* samples were collected from decomposed rice straw, rice straw residues and soil collected from dairy and goat farms, and rice fields of Universiti Putra Malaysia and Kuala Selangor, Malaysia. Five cores of samples were randomly taken from 5 cm depth and pooled in a clean plastic bag and stored at 4°C until use. On the other hand, *in-vitro* samples were obtained from different phases of a composting process at regular intervals conducted at the Composting Unit, Universiti Putra Malaysia. Isolation was done by using nutrient agar (Difco^{TM}) and incubated at 28 ± 2°C for 24 to 48 h. The plates were examined regularly. The single colony of bacteria was transferred aseptically onto nutrient agar to obtain the pure culture. Pure cultures were kept on nutrient agar as stock at 4°C until required for further studies.

In vitro screening for lignocellulolytic activity

Enzymatic degradation of starch

The ability of the isolates of bacteria to degrade starch was tested by using 10% starch amended nutrient agar. The solidified media was inoculated with 3 µL of bacteria suspension (10^8 CFU ml^{-1}) and incubated at 28 ± 2°C for 24 h. The colony growing on the media was flooded with iodine solution (10%) and allowed to be in contact for 30 s. Un-hydrolyzed starch reacts with iodine and turn black and hydrolyzed starch creates a 'halo zone' around the colony.

Enzymatic degradation of cellulose

Cellulose degradation was tested on Jensen's media [20.0g Sucrose, 1.0 g K_2HPO_4, 0.1g $FeSO_4$, 0.5 g $MgSO_4.7H_2O$, 0.5 g NaCl, 0.005 g $NaMoO_4$, 2.0 g $CaCO_3$, 15.0 g agar and 1.0 L distilled water] amended with 2.0 g carboxymethyl cellulose (CMC) (Jensen, 1940). The media were autoclaved at 121°C for 45 min and 20 ml of media was poured into Petri dish. The solidified medium was inoculated with 3 µL of bacteria suspension (10^8 CFUmL^{-1}) and incubated at 28 ± 2°C for 24 h. Then, the media was flooded with an aqueous solution of Congo red (1.0 mg/ml media) for 15 min. Subsequently, the Congo red solution was poured off and the plate was flooded with 1 M NaCl for 15 min. Degradation of cellulose was visualized as a halo zone around the colony. The diameter of the halo zone was used to assay for the degree of cellulose degradation (Teather and Wood, 1982).

Enzymatic degradation of lignin

Lignin degradation was tested using the media proposed by Archibald (1992) with some modifications. The media contained (g/L) $(NH_4)_2HPO_4$ 1.0, KCl 0.2, $MgSO_4.7H_2O$ 0.2, yeast extract 2.0, glucose 2.0, Azure B (0.01% w/v) 0.1, agar 15.0 and 1.0 L distilled water. All reagents were dissolved in distilled water and autoclaved at 121°C for 20 min and 20 ml was poured into each Petri plate. The solidified medium was inoculated with 3 µL of bacteria suspension (10^8 CFU ml^{-1}) and incubated at 28 ± 2°C for 24 h. Lignin degradation was indicated by the growth of colony on the media and growth diameter was recorded.

Lignocellulolytic activity of rice straw powder (RSP) amended cultural media

Five isolates (B3, B13, B21, B37 and B46) of bacteria based on previous *in vitro* test were further screened at different percentages (0, 10, 20 and 25%) of RSP amended nutrient agar. The culture media were autoclaved at 121°C for 45 min and 20 ml of media was poured into Petri dish. The solidified media were inoculated with 3 µL of bacteria suspension (10^8 CFU mL^{-1}) and incubated at 28 ± 2°C for 24 h. Radial growth was recorded.

Identification

The best isolate of bacteria (B37) in term of lignocellulolytic activities and growth in RSP amended media was identified using the BIOLOG identification system (Biolog Inc., 3938, Trust Way, Hayward, CA, USA) with the software Microstation system release version 4.20.

Experimental design and data analysis

All the experiments were conducted using completely randomized design (CRD) with five replications. The data were subjected to analysis of variance (ANOVA) and tested for significance using Least Significant Difference (LSD) by PC-SAS software (SAS Institute, Cary, NC, 2001). For the microbial isolates based on their biochemical activities, data were subjected to cluster analysis for grouping and a clustering tree was constructed using S-PLUS (Struyf et al., 1997).

RESULTS AND DISCUSSION

A total of 50-isolates of bacteria, 30 from *in situ* sources and 20 from *in vitro* sources of composted rice straw are presented in Tables 1 and 2, respectively.

In vitro lignocellulolytic activity

All the 50-isolates of bacteria assessed on media containing starch, cellulose and Azure-B for their lignocellulolytic potentials in terms of starch, cellulose and lignin degradation are presented in Tables 1 and 2, respectively. When all the isolates of bacteria were tested to Starch amended nutrient agar (Difco ^{TM}) media, 30

Table 1. The ability of bacteria to degrade starch, cellulose and lignin tested on media containing starch, carboxymethyl cellulose and Azure B (*in situ* sources).

Isolate number	Source of isolate	Starch		Cellulose halo zone (mm)	Lignin colony growth (mm)
		Positive (+)	Negative (-)		
B1	Dairy farm		–	0.00^{n}	3.20^{o}
B2	Dairy farm	+		2.44^{fg}	3.60^{no}
B3	Dairy farm	+		3.40^{bc}	14.40^{bc}
B4	Dairy farm	+		2.18^{ghi}	3.40^{no}
B5	Dairy farm	+		2.20^{ghi}	6.40^{fg}
B6	Dairy farm		–	0.00^{n}	3.60^{no}
B7	Dairy farm		–	0.00^{n}	3.40^{no}
B8	Dairy farm		–	1.66^{klm}	3.80^{mno}
B9	Goat farm	+		0.00^{n}	7.00^{f}
B10	Goat farm		–	1.76^{klm}	3.60^{no}
B11	Goat farm		–	1.46^{m}	4.20^{ko}
B12	Goat farm		–	0.00^{n}	4.20^{ko}
B13	Goat farm	+		3.30^{bc}	14.00^{c}
B14	Goat farm	+		2.32^{gh}	3.60^{no}
B15	Rice field	+		2.10^{hij}	4.40^{jn}
B16	Rice field		–	0.00^{n}	3.60^{no}
B17	Rice field	+		0.00^{n}	4.20^{ko}
B18	Rice field		–	1.72^{kl}	4.36^{jn}
B19	Rice field		–	1.78^{kl}	4.40^{jn}
B20	Rice field	+		1.84^{jkl}	3.20^{o}
B21	Rice field	+		3.60^{ab}	15.20^{ab}
B22	Rice field	+		1.78^{kl}	5.20^{hk}
B23	Rice field	+		1.74^{kl}	3.60^{no}
B24	Rice field	+		0.00^{n}	3.20^{o}
B25	Rice field	+		1.90^{ijk}	9.20^{e}
B26	Rice field		–	0.00^{n}	3.40^{no}
B27	Rice field	+		1.74^{klm}	4.20^{ko}
B28	Rice field		–	1.68^{klm}	5.60^{ghi}
B29	Rice field	+		0.00^{n}	4.80^{im}
B30	Rice field		–	1.54^{lm}	6.00^{fgh}
$LSD_{0.05}$				0.31	1.00

Values having the same letter(s) in a column do not differ significantly at 5% level of probability.

isolates were found to produce halo zone after staining with iodine solution (Figure 1). The ability of the isolates to grow well and form halo zone proves that they had excreted starch degrading enzymes amylases on starch amended media and used the substrate as energy source. Amylases enzymes are divided into endo-amylases and exoamylases. Initially, endoamylases randomly catalyze hydrolysis of starch molecule, causing the formation of linear and branched oligosaccharides; subsequently exoamylases hydrolyze the intermediate substrate to glucose molecules (Gupta et al., 2003). Therefore, bacterial isolates producing halo zone on starch amended media truly represent their capability to produce endoamylases and exoamylases enzymes on such environment. These observations were in line with the past research experiences (Reddy et al., 2003; Agrawal et al., 2005) where bacteria convert starch molecules to glucose by producing amylases enzymes.

Twenty-nine isolates of bacteria were found to develop halo zone on carboxymethyl cellulose (CMC) media after staining with Congo red solution (Figure 1). Seven isolates (B3, B13, B21, B37, B39, B41 and B46) showed relatively higher activity by forming larger zone (>3.00 mm), whereas other 22 isolates formed comparatively smaller zone (< 3.00 mm) than the seven isolates. Isolates B37 (3.80 mm) was found to form the largest halo zone followed by B46 (3.76 mm), B21 (3.42 mm), B3 (3.40 mm), B13 (3.30 mm), B41 (3.12 mm) and B39 (3.08 mm), respectively. Cellulose is a polysaccharide of glucose units linked by glycosidic bonds. Synergistic action of cellulases enzyme systems (hydrolytic endoglucanases, exoglucanases and glucosidases) is

Table 2. The ability of bacteria to degrade starch, cellulose and lignin tested on media containing starch, carboxymethyl cellulose and Azure B (*in vitro* sources).

Isolate number	Source of isolate	Starch		Cellulose halo zone (mm)	Lignin colony growth (mm)
		Positive (+)	Negative (-)		
B31	Mesophilic stage	+		0.00^{n}	3.60^{no}
B32	Mesophilic stage	+		1.72^{klm}	5.00^{hl}
B33	Mesophilic stage		–	2.66^{ef}	3.40^{no}
B34	Mesophilic stage	+		0.00^{n}	3.60^{no}
B35	Mesophilic stage		–	0.00^{n}	3.40^{no}
B36	Thermophilic stage		–	0.00^{n}	4.00^{mno}
B37	Thermophilic stage	+		3.80^{a}	15.60^{a}
B38	Thermophilic stage	+		3.12^{cd}	11.80^{d}
B39	Thermophilic stage	+		3.54^{ab}	7.00^{f}
B40	Thermophilic stage		–	0.00^{n}	5.00^{hl}
B41	Thermophilic stage	+		2.90^{de}	11.00^{d}
B42	Thermophilic stage		–	1.80^{jkl}	6.00^{fgh}
B43	Thermophilic stage	+		0.00^{n}	6.80^{f}
B44	Thermophilic stage		–	1.82^{jkl}	5.60^{ghi}
B45	Maturation stage	+		0.00^{n}	4.40^{jn}
B46	Maturation stage	+		3.76^{a}	15.20^{ab}
B47	Maturation stage	+		0.00^{n}	5.40^{gj}
B48	Maturation stage	+		0.00^{n}	3.40^{no}
B49	Maturation stage	+		0.00^{n}	3.20^{o}
B50	Maturation stage		–	1.62^{klm}	3.40^{no}
$LSD_{0.05}$					1.00

Values having the same letter(s) in a column do not differ significantly at 5% level of probability.

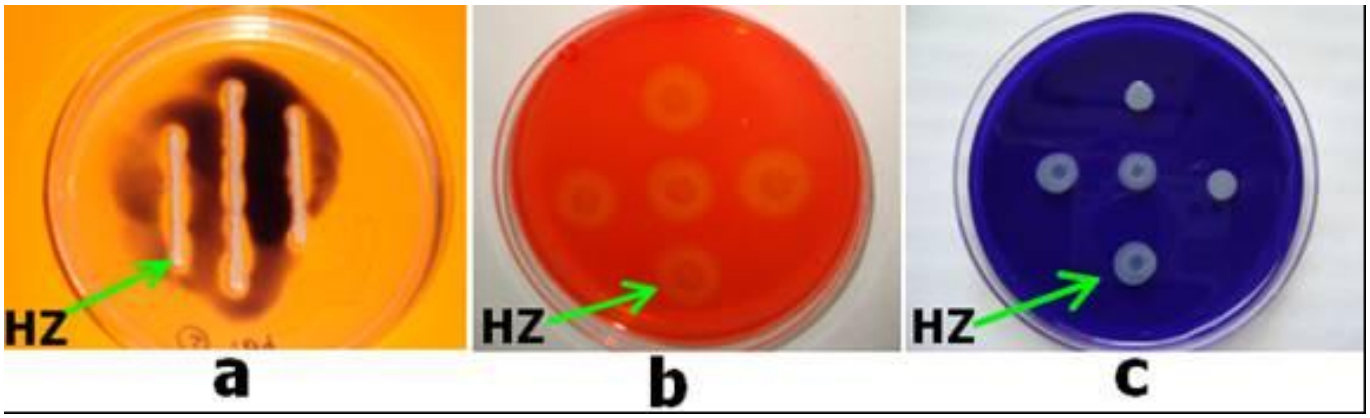

Figure 1. Ability of bacteria to degrade (a) starch, (b) cellulose and (c) lignin as indicated by the formation of halo zone (CZ) and colony diameter (CG) on plate containing starch, carboxymethyl cellulose and Azure-B.

essential in the total bioconversion of cellulose to glucose. CMC is used for the detection of endo-glucanases activity of microorganisms (Hankin and Anagnostakts, 1977) which catalyze the initial bioconversion of cellulose polymer. Endoglucanases cleave glycosidic bonds and expose cleft-shaped open active sites for subsequent degradation. Therefore, the isolates of bacteria forming halo zone on CMC media proved that they might have the ability to produce extracellular hydrolytic endoglucanases enzymes.

Previous investigations also showed that *Bacillus* spp. decompose lignocellulosic materials into glucose by producing extracellular endoglucanases, exoglucanases and ß-glucosidase enzymes (Niranjane et al., 2007; Maki et al., 2009). Cellulose is the key component and com-prises approximately 43 to 49% of the dry weight of rice

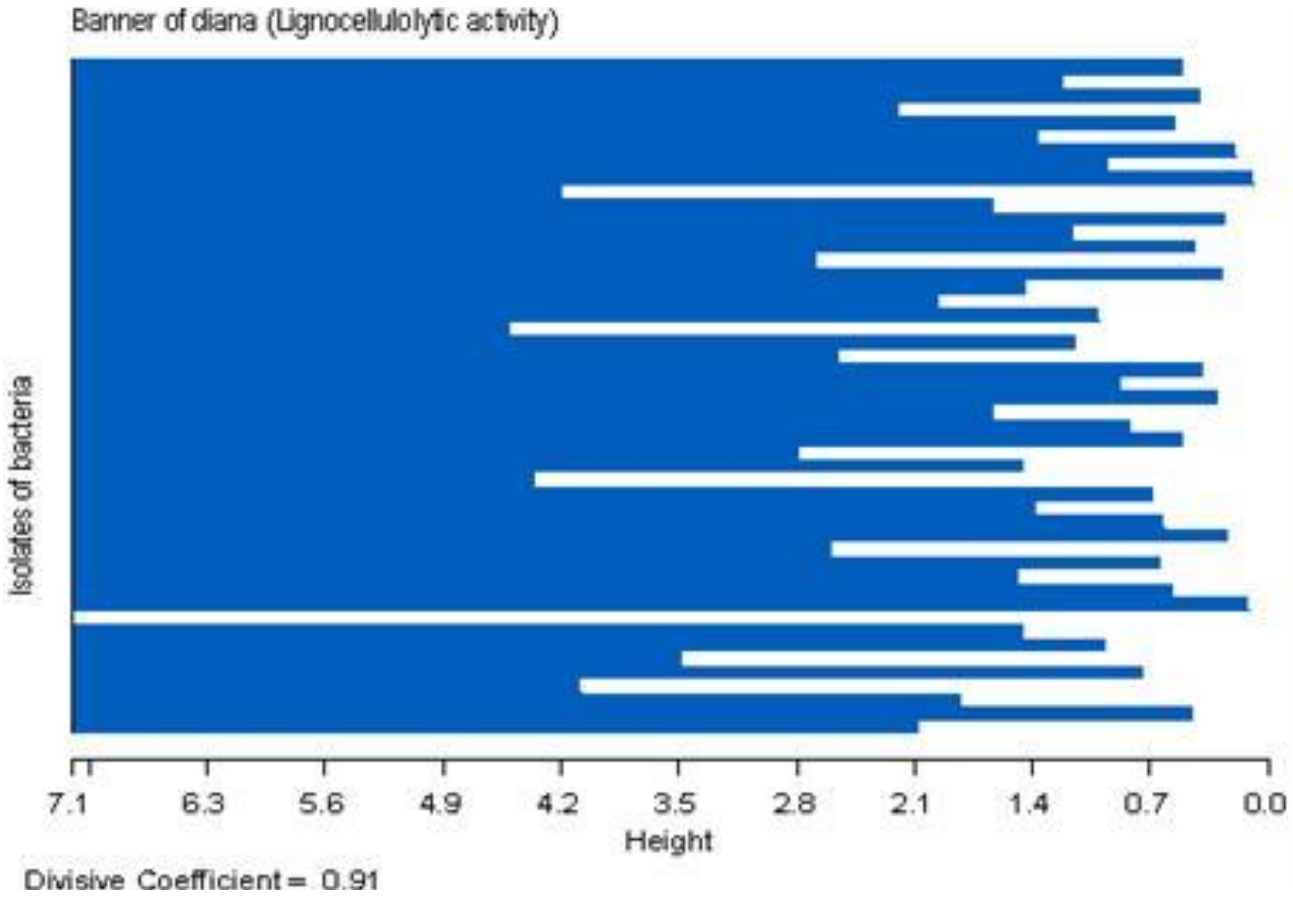

Figure 2. Lignocellulolytic activities of 50-bacterial isolates represented in a banner by using Diana divisive hierarchical method.

plant (Lin et al., 2001). Therefore, screening of the isolates of bacteria having the cellulolytic capability is a crucial step in the rapid bioconversion of rice straw as synergistic relationships between ligninolytic and cellulolytic microorganisms might lead to a total degradation of lignocellulosic rice straw to glucose with the release of CO_2 and water.

All the 50 isolates of bacteria were also found to grow well on the media containing Azure-B (Figure 1). Among them, 5 isolates (B3, B13, B21, B37 and B46) were found to produce relatively larger colony (>14.00 mm). The isolate B37 (15.60 mm) produced the largest colony over the isolates of B21, B46, B3 and B13 with the colony diameter of 15.20, 15.16, 14.40 and 14.00 mm, respectively. Lignin forms an irregular non-crystalline network in the plant cell wall, which is very resistant to microbial degradation. Only microbes having the ligninolytic enzyme systems (oxidoreductase enzymes) are able to mineralize lignin to carbon dioxide (Martin, 2002). Isolates of bacteria developing colony on Azure B media depict that they might be able to produce oxidoreductases enzymes on such environment for lignin bioconversion. This observation was found to be consistent with the report of Vicuna (1988) who stated that several bacteria could degrade, modify or solubilize the lignin polymer more efficiently even their fungal counterpart because of their more specific enzymatic systems. Lignin depolymerization is the key step in the bioconversion of lignocellulosic materials as lignin encrusts the cellulose and hemicelluloses and protects them from microbial decomposition. Therefore, screening of the isolates of bacteria having the ligninolytic potentiality was quite logical for the rapid decomposition of rice straw.

The lignocellulolytic potentials of 50 isolates of bacteria to the enzymatic degradation of starch, cellulose and lignin were combined in a single clustering tree that was derived from divisive hierarchical method by using S-PLUS software. The divisive coefficient was high (0.91) which indicates a good clustered structure. Both the banner (Figure 2) and the clustering tree (Figure 3) have been plotted, to facilitate comparisons between them. The clustering tree gave two major clusters (clusters 1 and 2), where overall group dissimilarity was above 6.0. Cluster 1 included 9 isolates both from *in situ* and *in vitro* sources and this cluster was further split into 2 sub cluster (1A, 1B). Sub cluster 1A contained 5 isolates (B3, B13, B21, B37 and B46) which were able to produce

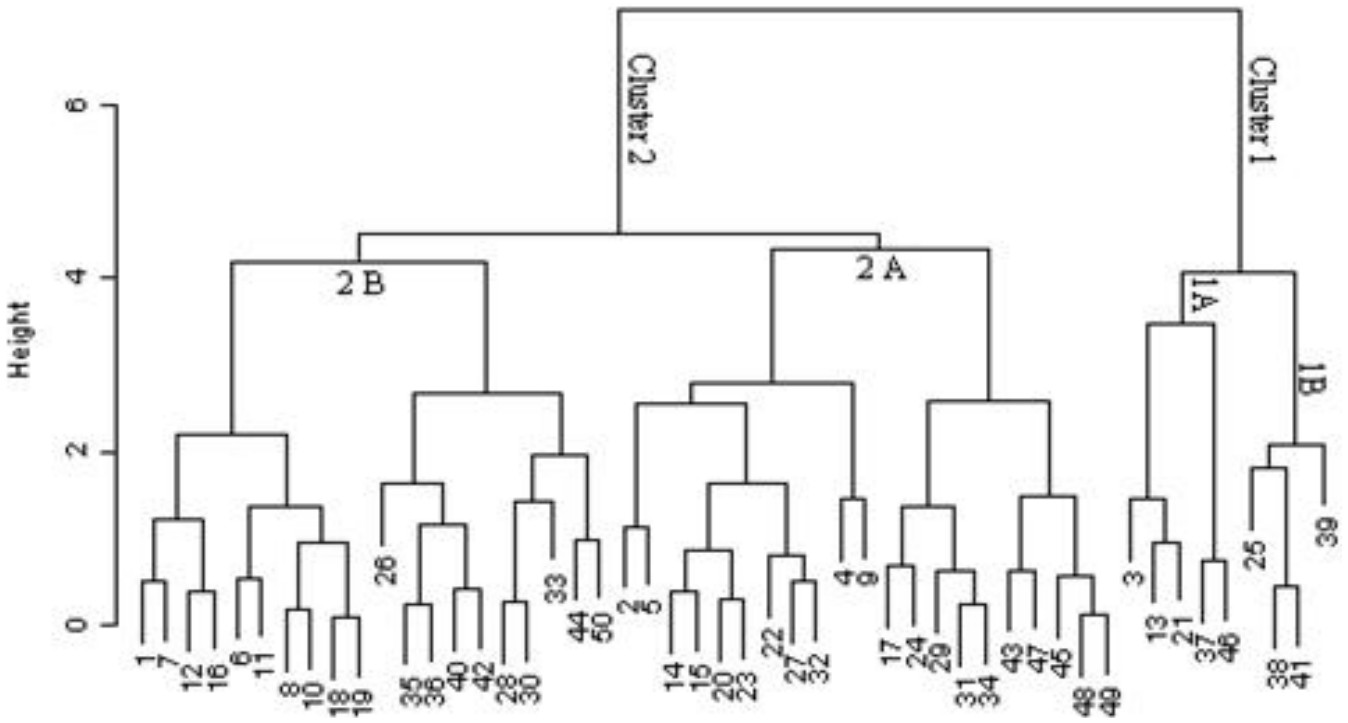

Figure 3. Clustering tree of 50 bacterial isolates by using Diana divisive hierarchical method.

Table 3. Growth (mm) of five selected lignocellulolytic bacteria on rice straw amended NA cultural media.

Isolate code	NA	NA+10%	NA+20%	NA+25%
B3	5.82^{d}	7.70^{b}	8.00^{d}	7.90^{d}
B13	8.10^{a}	9.00^{a}	10.30^{b}	9.90^{b}
B21	6.40^{c}	8.20^{b}	9.10^{c}	8.90^{c}
B37	6.90^{b}	9.50^{a}	13.60^{a}	17.10^{a}
B46	6.40^{c}	8.20^{b}	8.20^{d}	8.70^{c}
$LSD_{0.05}$	0.31	0.65	0.72	0.41

Values having the same letter(s) in a column do not differ significantly at 5% level of probability.

prominent larger halo zone and well developed colony on media containing starch, CMC and Azure-B. On the other hand, sub cluster 2B included 20 isolates showing the lowest lignocellulolytic potential, where 7 isolates (B1, B6, B7, B16, B26, B35, B36) were not able to produce halo zone on starch and CMC media although a little colony (≥ 3.40 mm) on Azure-B media was observed. Based on the analysis, five best isolates of bacteria from sub cluster 1A were selected for lignocellulolytic activities to rice straw powder (RSP) amended media.

Lignocellulolytic activity of selected bacteria isolates tested on rice straw powder (RSP) amended media

The growth of five lignocellulolytic isolates (B3, B13, B21, B37, and B46) of bacteria on different concentration (0 to 25%) of rice straw powder (RSP) amended nutrient agar (Difco^{TM}) are presented in Table 3. Three isolates (B13, B21, and B37) were found to grow well until 20% level of RSP amendment media, while only one isolate (B37) showed well adaptability to 25% level of RSP amendment media. Isolate B37 developed significantly ($p \leq 0.05$) higher colony having prominent halo zone at 25% level of RSP amended media compared to all other isolates with the colony diameter of 17.10 mm. On the other hand, the isolate (B3) showed the lowest adaptability to RSP media, where it maintained an increasing trend only up to 10% of RSP amended media, thereafter decline with further increases of RSP concentration in the cultural media (Figure 4).

Rice straw is a complex lignocellulosic material, thus, synergistic actions of oxidative and hydrolytic enzymes are necessary for the bioconversion of rice straw. The

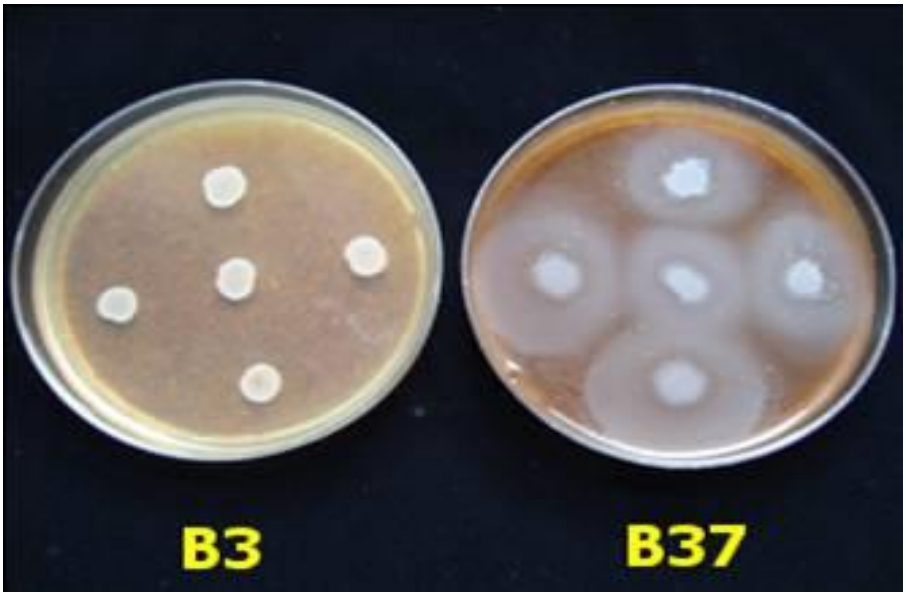

Figure 4. Growth of bacterial isolates (B3 and B37) at 25% level of RSP amended NA cultural media.

higher growth rate, halo zone and well adaptability to higher concentration of RSP amended media revealed that isolate (B37) might efficiently utilized the RSP as carbon source by producing lignocellulolytic enzymes on RSP amended media. Bacteria were well documented in the rapid composting of lignocellulosic materials (Yang et al., 2002; Gilbert, et al., 2008). The highest treatment concentration (25% RSP media) for this study was reasonable because the majority of the bacteria isolate (B3, B13, B21, and B46) that were not able to cross 20%, except for isolate B37. Molla et al. (2002) also reported 25% sludge powder (SP) media as the highest treatment concentration to screen potential lignocellulolytic microbes for the bioconversion of domestic wastewater sludge. Considering the bio-chemical characteristics and adaptability to rice straw amended cultural media, isolate B37 that was isolated during thermophilic stage (Table 2) of *in vitro* composting of rice straw and was identified as *Bacillus pumilis* based on Biolog identification system of version 4.20.

Conclusion

In this study, fifty isolates of bacteria were isolated from several natural and induced rice straw compost sources. Five isolates (B3, B13, B21, B37 and B46) were selected based on their enzymatic degradation of starch, cellulose and lignin.

They were further screened against RSP amended media. After a two-stage screening, the isolate B37 showing the optimum lignocellulolytic potential identified as *Bacillus pumilis* perhaps could play important role in the composting of rice straw.

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