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DGGE detection and screening of lignocellulolytic bacteria from the termite gut of *Coptotermes formosanus*

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ABSTRACT

Aims: Termites thrive in terrestrial ecosystems and play an important role in the bio-recycling of lignocellulose. The objective of this study is to isolate and detect bacteria from the termite gut of *Coptotermes formosanus* and to screen their various enzyme activities by qualitative methods. In addition, this study was aimed to isolate lignin and furfural tolerant strains for various industrial bioprocesses.
Methodology and Results: In this study, 50 worker termites of *Coptotermes formosanus* were collected from dead trees, from a forest in Taichung, Taiwan in June 2008 and the composition of the microbial flora from the termite guts was analyzed by DGGE analysis. The results proved that anaerobic and facultatively anaerobic bacteria consisting of *Acinetobacter*, *Bacteroides thetaiotaomicron*, *Escherichia coli*, and *Caulobacter* readily existed in the guts of termites. Although the majority of these gut symbionts have not yet been cultivated or identified, some related bacteria were isolated. Two isolates 1-8 and 2-2 of Genus *Bacillus*, exhibited endocellulase, protease, lipase, amylase, peroxidase and lignin peroxidase activity. Under aerobic conditions, the growth density of isolate 1-8 cultured in 1000 ppm lignin containing MSM medium was two-folds higher than cultured in MSM medium without lignin. Furthermore, the isolate 1-8 was tolerant to 20 mM furfural supplemented in the MSM medium. HPLC analysis confirmed *Bacillus* isolate 1-8 could degrade up to 15 mM furfural.
Conclusion, significance and impact of study: Hind gut bacteria from *C. formosanus* were detected by culture independent DGGE method. Also, *Bacillus* isolates 1-8 and 2-2 obtained by culture dependent methods could withstand higher concentration of furfural and as well as lignin. These isolates may be co-cultured with ethanologenic bacteria and be used as an industrial biocatalyst for biofuel production.

Keywords: lignocellulose, termite gut, *Coptotermes formosanus, Bacillus*

INTRODUCTION

Lignin is a complex chemical compound, most commonly derived from wood and cross-linked racemic macromolecule with molecular masses in excess of 10,000 units. It is relatively hydrophobic and aromatic in nature and present in plants and some algae, and is one of the most abundant organic polymers on Earth, exceeded only by cellulose (Pérez *et al*., 2002). Lignin fills the spaces in the cell wall between cellulose, hemicellulose, and pectin components for covalently linking to hemicellulose and polysaccharides, forming the lignocellulosic substrates (Chabannes *et al.,* 2001). These substrates are degraded by ligninolytic and cellulolytic enzymes which are secreted by certain fungi and bacteria. The lignin degrading enzymes present in these fungi and bacteria are manganese peroxidase, lignin peroxidase and laccase (Harazono *et al*., 2003) whereas the cellulose degrading enzymes are endo-1,4-beta-D-glucanase (endoglucanase), exo-1,4-beta-D-glucanase (exoglucanase) and beta-glucosidase (Bhat and Bhat, 1997).

In the nature, termites (Isoptera) are important for the degradation and utilization of plant matter which is constituted of cellulose, hemicellulose, lignin, and aromatic compounds. Based on their food habits, the termites are classified into three groups: wood-feeders, fungus-growers and soil-feeders respectively. As a result of their varied food habits, they have developed different pathways for the degradation of their food substrates. Termites can digest these complex organic matters through their gut system comprising of a foregut, midgut, mixed segment, first proctodeal segment, enteric valve and paunch, colon and rectum (Tokuda *et al.,* 2000; Kudo, 2009). Certain termites have a specialized hindgut flora, consisting of symbiotic bacteria and protozoa that enable them to degrade organic matter. In “higher termites”, these protozoa are absent and are known to possess prokaryotes not only in the hindgut but also in the mixed segment (Tokuda *et al.*, 2000). In “lower termites”, intestinal bacteria contribute to creating suitable conditions for symbiotic protozoa (flagellates) and metabolites from the microorganisms are considered to be absorbed across the hindgut wall in the hindgut (the

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paunch) (Harazono *et al*., 2003). The protozoa survive on a diet of carbohydrates consisting of wood particles and metabolize it to acetate and other products that are beneficial for termites (Ohkuma and Kudo, 1996). The protozoan-termite relationship is dependent on synthesizing cellulases produced by the intestinal microflora in termite (Harazono *et al*., 2003). The function of microflora in termite gut included lignocellulose digestion, acetogenesis, hydrogenesis, methanogenesis, sulfate reduction, and nitrogen fixation (Kudo, 2009). It is widely known that termites cannot survive without intestinal microorganisms and the physiological functions of symbiotic microorganisms in termites are extremely diverse. Earlier, it is proved that oxygen gradients existed in termite guts, and that oxygen is a critical co-substrate for the degradation of lignin and aromatic compounds with the help of aerobic gut microorganisms (Zimmermann, 1990; Brune *et al*., 1995).

The Formosan subterranean termite *Coptotermes formosanus* (*Isoptera: Rhinotermitidae*) are invasive species of "lower termites" from southern China. It has been transported worldwide from its native location to Japan, South Africa, Hawaii, and to the United States in the 20th century. *C. formosanus* Shiraki is currently regarded as the most destructive, invasive and economically disastrous pest in southern United States and Hawaii (Su and Tamashiro, 1987). Recently, scientists have been involved in developing second generation biofuels using lignocellulosic crops (Sukumaran *et al*., 2009). The biotechnological challenges in this process involve the breakdown of the highly stable polymers of lignocellulose to simple sugars and redirecting the carbon and electron flow in the metabolic fermentations to biofuels (such as ethanol or hydrogen). The highly efficient lignocellulose degradation ability of symbiotic microorganisms present in the termites might be regarded as a boon for the renewable and alternative fuel industry (Brune, 2007). However, on the pretreatment of lignocellulosic substrates with sulfuric acid, a toxic compound called furfural is produced, which functions as the dominant inhibitory compound for the lignocellulosic hydrolyzates (Belay *et al*., 1997 and Almeida *et al*., 2009). It is also known to act as an inhibitor in the fermentation process.

An understanding of intestinal microbes is not enough because majority of the dominant microbes in the gut, such as spirochete-shaped bacteria and protists, cannot be isolated by culture dependent methods (Kudo, 2009). Therefore, the objective of our study was to understand the composition of the microbial flora from termite guts of the termite *C. formosanus* by culture dependent and independent methods and to isolate and screen for microorganisms from the termite gut that could grow in higher concentrations of furfural and lignin. The versatility and function of *Bacillus* in *C. formosanus* will also be discussed in this paper.

MATERIALS AND METHODS

Collection of termites

Worker termites of *C. formosanus* were collected from dead trees in a forest in Taichung, Taiwan, in June, 2008. The worker termites were separated from the wood surface, surface-sterilized with 70% alcohol and used for DNA extraction.

Isolation of cultivable bacteria from the termite gut

Worker termites were surface sterilized with 70% ethanol and degutted using sterile forceps according to Long *et al*. (2010). The hind guts were homogenized, and dilution series (up to a dilution of 10^{-12}) were spread-plated on MSM medium at 37 °C (Chandra *et al*., 2007). The composition of the MSM medium per liter is as follows: 10 g Glucose, 5 g Peptone, 2.4 g Na_2HPO_4, 2.0 g K_2HPO_4, 0.10 g NH_4NO_3, 0.010 g $CaCl_2{\cdot}2H_2O$, 0.250 ml SL1 stock (240 mg H_3BO_3 / liter of distilled water), 0.050 ml of SL2 stock (160 mg $NaMoO_4{\cdot}2H_2O$ / liter of distilled water), 0.2 ml SL3 stock (200 mg $CuCl_2{\cdot}2H_2O$, 700 mg $ZnCl_2$, 200 mg $NiCl_2{\cdot}6H_2O$ / liter of distilled water), 0.08 ml SL4 stock (0.34 ml 10 N HCl, 250 mg $MnCl_2{\cdot}4H_2O$, 100 mg $CoCl_2{\cdot}6H_2O$ / liter of distilled water), 1 ml EDTA solution (500 mg / liter of distilled water) and 5 ml $FeSO_4{\cdot}7H_2O$ solution (220 mg $FeSO_4{\cdot}7H_2O$ / liter of distilled water).

DNA extraction

Fifty worker termites were degutted using fine-tipped sterile forceps and their hind guts (20 mg) were excised and suspended in Saline Tris EDTA buffer .The total microbial DNA was extracted using the Easy Tissue and Cell Genomic DNA Purification Kit, (Genemark, USA) according to the manufacturer's instructions.

Denaturing Gradient Gel Electrophoresis (DGGE)

The concentration and purity of total DNA isolated from the termite gut was measured using a NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). For the analysis of the bacterial diversity, primer pair EUB968F (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3'; GC clamp is in bold interface) and UNIV1392R (5'-ACG GGC GGT GTG TRC-3') (Nielsen *et al*., 1999) were used to partially amplify 16S rRNA gene to create a DNA fragment suitable for DGGE analysis. DGGE was performed according to the protocol described by Hayashi *et al*. (2007) using the Dcode system (BioRad Laboratories, USA). The prominent DGGE bands were excised using a sharp, sterile scalpel under UV illumination and the DNA was extracted from the gel by electroelution using an electroelutor (Genepure ELR9280). Aliquots of the supernatant (0.5 µl) were reamplified with the sample PCR mixture using touchdown conditions. The amplicons were purified using the QIAquick (Qiagen) kit, ligated into the "yT and A" cloning vector (Yeastern Biotech Co. Ltd., Taiwan) and transformed into *Escherichia coli* DH5α by the heat-shock method. The colonies were plated onto Luria-Bertani plates containing ampicillin (50 µg mL^{-1}), 7 µL of 20%

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isopropyl thio-β-D-galactoside (IPTG) and 40 µL of 2% X-gal by blue and white colony selection (Sambrook and Russell, 2001). The white colonies were selected, and colony PCR was performed using the yeast primers M13 F and M13 R (Long *et al*., 2010). The positive transformants were selected for sequencing.

Sequencing and BLAST

Sequencing of the clones was performed by the Genedragon service (Genedragon, Taiwan) using Seqman (DNAstar, USA). The 16S rDNA sequences were compared with the closest sequences deposited in the GenBank (NCBI) public database using the BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST).

Extracellular enzyme activity assay

The functional properties of the cultured isolates were analyzed for endoglucanase, xylanase, pectinase, amylase, lipase, protease, peroxidase, lignin peroxidase and laccase. The hemicellulase (xylanase) activity was determined by growing the isolates using xylan as the substrate (Chang *et al*., 2010). The endoglucanase activity was detected by using carboxymethyl cellulose (CMC) as a substrate by using the protocol of Skipper *et al.* (1985). The isolates were tested for polygalacturonase (pectinase) activity by measuring the degradation of the heteropolysaccharide pectin using a ruthenium red staining solution (0.05%) (McKay, 1988). Amylase activity was determined by the protocol of Akpan *et al*. (1999). Lipase enzyme was qualitatively analyzed using Tributyrin as substrate (Kouker and Jaeger, 1987). The proteolytic activity of the microbes was detected by the skim-milk agar method (Downes and Ito, 2001). The lignin degradation was qualitatively analyzed using syringaldazine solution (0.1% syringaldazine with 95% ethanol solution) for laccase activity (Ponting, 1999), and the drop-test method was used for lignin peroxidase activity using 0.01% guaiacol as the substrate (Okino *et al*., 2000).

Furfural tolerance assay and HPLC

In order to analyze the furfural tolerance of the bacterial isolates, different concentrations of furfural (Sigma) (0, 15, 20 and 25 mM) were added into the MSM culture medium. 1% of the test bacterium was inoculated and their growth was monitored at different time intervals (0, 12, 24 and 36 hours). The relative growth (%) was calculated as follows:

Relative growth (%) = $(OD_{furfural\text{-}MSM}/OD_{MSM})$ x 100%.

The degradation of furfural was detected quantitatively by HPLC using Agilent 1100. For this experiment, the liquid cultures were centrifuged at 12,000 rpm for 15 minutes and their supernatant collected and analyzed by the protocol of Hyman *et al*. (2008).

Growth enhancement of bacterial isolates on addition of lignin

In order to detect if the addition of lignin promoted the growth of the bacterial strains, 1% of each test bacterium was inoculated into MSM medium containing different concentrations of dealkalized lignin (TCI, Tokyo Kasei Kougyo) (0, 100, 200, 600 and 1000 ppm). The samples were collected at different time intervals (0, 12, 24, 36 hours) and their growth was measured at OD600.The relative growth (%) is calculated as follows:

Relative growth (%) = $(OD_{L\text{-}MSM}/OD_{MSM})$ x 100%.

The growth of the lignin tolerant isolates was further tested under anaerobic conditions.

RESULTS

Bacterial consortia of termite gut from *C. formosanus* Shiraki

Coptotermes formosanus Shiraki was collected from dead trees in a forest in Taichung, Taiwan. Although previous studies have employed various molecular techniques to detect the bacterial community using 16S rRNA (Husseneder *et al*., 2005; Shinzato *et al*., 2005; Kudo, 2009), the bacterial consortia of termite gut from Taiwan has not been analyzed by molecular tools like Denaturing gradient gel electrophoresis (DGGE). Earlier reports have suggested the role of intestinal bacteria, symbiotic protozoa, and endosymbiont of the protist present in the gut of the termite *C. formosanus* for lignocellulosic degradation (Hongoh *et al*., 2007). Therefore, we have initiated a study to monitor the bacterial consortia in whole hindguts of the termite by 16S ribosomal gene targeted DGGE. The DGGE profile revealed that *Acinetobacter* (band 1-1), *Bacteroides thetaiotaomicron* (band 1-3), *Escherichia coli* (band 1-2), and *Caulobacter* (band 1-4) were the dominant microbes (Figure 1).

Isolation of bacteria and lignin tolerance

Although recent studies support the direct role of the symbiotic bacteria in the hindgut of the termite in cellulose and xylan hydrolysis, only few reports have focused on the lignin-degrading bacteria. Fourteen different isolates were obtained by using culture dependent methods and were identified using 16S rRNA gene sequencing analysis (Table 1).The cultures were designated as isolate 1-1, isolate 1-2, isolate 1-3, isolate 1-5, isolate 1-8, isolate 2-1,isolate 2-2, isolate 1,isolate 13, isolate T4-2, isolate T8-2, isolate T9-3, isolate 27, and isolate T11. Out of these isolates, lignin tolerant organisms were screened using lignin and lignin related aromatic compounds as the carbon source. These bacterial strains isolated from termite gut were inoculated into MSM medium containing different concentrations of lignin. After comparing their growth based on their absorbance at 600 nm, only four bacterial strains belonging to the Genus *Bacillus*, that is;

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isolate 1-5, isolate 1-8, isolate 2-1, and isolate 2-2, were enriched by the addition of lignin. Out of the four strains, isolate 1-8 showed maximum growth (Figure 2a) when cultured at higher concentrations of lignin both aerobically and anaerobically. Under aerobic condition, the growth density of isolate 1-8 cultured with 1000 ppm lignin addition was two-folds higher than cultured in MSM medium without lignin (Figure 2b). This result indicated that isolate 1-8 possessed higher lignin utilization under aerobic condition.

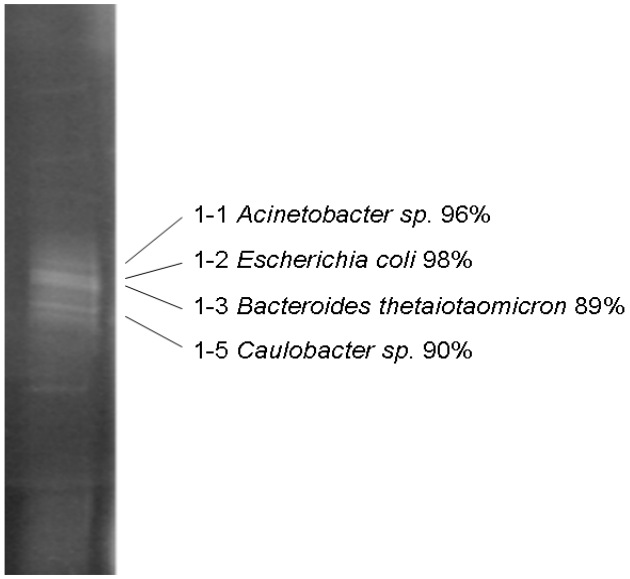
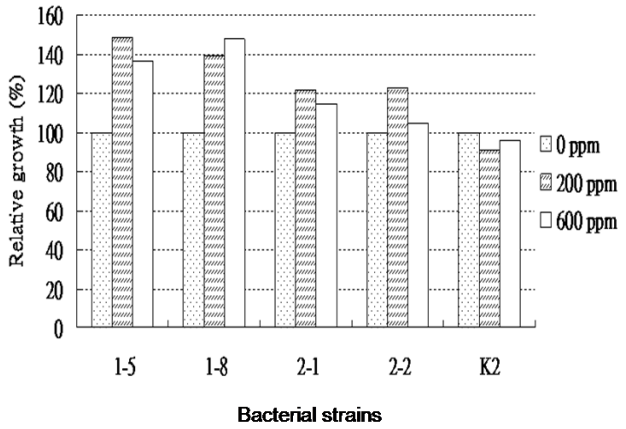


Figure 1: The 16S rRNA gene profile of gut from *C. formosanus* Shiraki by DGGE analysis.

Table 1: Bacterial isolates obtained from the hindgut of *Coptotermes formosanus* by culture dependent methods.

Isolate name	Closest neighbor	Accession number	% similarity
1-1	*Bacillus* sp	FJ528593.1	99
1-2	*Lysinibacillus fusiformis*	EU869267.1	99
1-3	*Lysinibacillus fusiformis*	FJ544400.1	99
1-5	*Bacillus* sp	EU558977.1	99
1-8	*Bacillus cereus*	FJ644692.1	99
2-1	*Bacillus thuringiensis*	EU429664.1	99
2-2, 1	*Bacillus thuringiensis*	EU429660.1	99
13	*Bacillus thuringiensis*	AM778997.1	99
T4-2	*Citrobacter farmeri*	AF025370.1	99
T8-2	*Citrobacter farmeri*	AF025370.1	100
T9-3	*Citrobacter amalonaticus*	AF025371.1	99
27	*Enterococcus avium*	EU391533.1	99
11	*Enterococcus avium*	JF947348.1	99

a)



b)

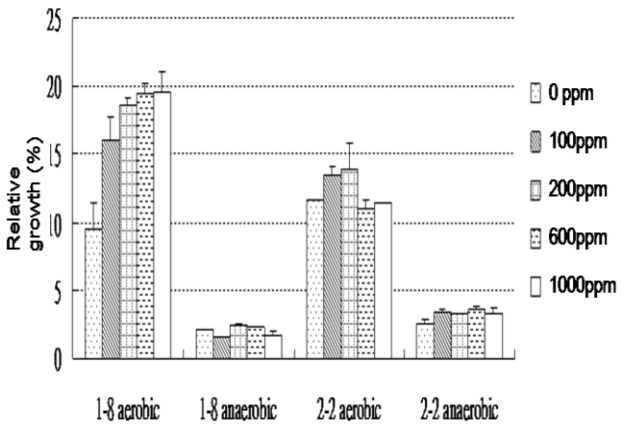


Figure 2: The relative growth analysis of bacterial isolates in MSM medium containing different concentrations of lignin. a) Relative growth (%) of isolates 1-5, 1-8, 2-1 and 2-2 in different concentrations of lignin (200ppm and 600 ppm). K2 *Bacillus thermoamylovorans* was used as the control strain. b) Relative growth (%) of isolate 1-8 and 2-2 in higher concentrations of lignin (1000 ppm) under aerobic and anaerobic conditions.

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Table 2: Qualitative enzyme profiles of the bacterial isolates obtained from the hind gut of *Coptotermes formosanus.*

Enzyme	Substrate	Condition	Strains								
			1	13	27	1-5	1-8	2-1	2-2	T8-2	K2	DH5α
Protease	Skim milk	Aerobic	+	+	-	+	+	+	+	-	+	-
		Anaerobic	+	+	+	+	+	+	+	-	+	-
Xylanase	Xylan	Aerobic	-	-	-	-	-	-	-	-	+	-
		Anaerobic	-	-	-	-	-	-	-	-	+	-
Lipase	Tributyrin	Aerobic	+	+	-	+	+	+	+	-	+	-
		Anaerobic	+	+	-	+	+	+	+	-	+	-
Endoglucanase	CMC	Aerobic	-	-	-	+	+	-	+	-	+	-
		Anaerobic	-	-	-	+	+	-	+	-	+	-
Pectinase	Pectin	Aerobic	-	-	-	-	-	-	-	-	+	-
		Anaerobic	-	-	-	-	-	-	-	-	+	-
Amylase	Starch	Aerobic	+	+	-	+	+	+	+	-	+	-
		Anaerobic	+	+	-	+	+	+	+	-	+	-

Isolate 1, 13, 27, 1-5, 1-8, 2-1, 2-2, T8-2 were the tested for their enzymatic activities. *K2- Bacillus thermoamylovorans* as the positive control strain and DH5α was used as a negative control strain. "+" indicates positive activity and "-" indicates no enzyme activity.

Table 3: Lignin degradation related peroxidase, lignin peroxidase and laccase profiles from the bacterial isolates.

Enzyme	Time (days)	*Irpex lacteus*	1	13	27	1-5	1-8	2-1	2-2	T8-2
Peroxidase	4	+	+	+	+	+	+	+	+	+
Lignin peroxidase	14	+	+	+	+	+	+	+	+	+
Laccase	15	+	-	-	-	-	-	-	-	-

Isolate 1, 13, 27, 1-5, 1-8, 2-1, 2-2, T8-2 were the tested for their enzymatic activities. *Irpex lacteus* was used as the positive control strain."+" indicates positive activity and "-" indicates no enzyme activity.

Qualitative enzyme assays

In this experiment, the extracellular enzymatic profiles of the isolates from the termite hindguts were qualitatively analyzed for protease, xylanase, lipase, endoglucanase and amylase (Table 2). The ligninolytic activity was assessed qualitatively by culturing the isolates on a petriplate containing lignin (100 ppm) and their peroxidase, lignin peroxidase and laccase were monitored (Table 3). The fungal strain *Irpex lacteus* was used as the positive control. The results indicated that all the isolates from the hindguts of *C. formosanus* exhibited protease, lipase, amylase, peroxidase and lignin peroxidase activity (Table 2 and 3). Only three lignin tolerant strains, isolate 1-5, isolate 1-8, and isolate 2-2, possessed endoglucanase activity (Table 2).

Furfural tolerance assay

In the process of lignocellulosic pretreatment, lignin-borne toxic compounds like furfural were produced and they served as a potent inhibitor of microbial growth and metabolism (Klinke *et al*., 2002; Ingram *et al*., 1999). Therefore, the following experiment was designed to analyze the ability of the bacterial isolates to grow at a higher concentration of furfural as well as degrade them to less toxic compounds. Different concentrations of furfural were inoculated with the isolates for the toxin tolerance assay and their relative growth result showed that *Bacillus* isolates 1-8 and 2-2 was resistant to 20 mM furfural with 60 % relative growth in the MSM medium at 37 °C for 24 hours. It was observed that isolate 1-8 showed higher tolerance than isolate 2-2 at 25 mM furfural concentration with 40 % relative growth (Figure 3a). The furfural degrading ability was quantitatively measured by HPLC assay at 37°C for 24 hrs .The result indicated that both the strains could degrade 98% of furfural at a lower concentration of 15 mM (Figure 3b). When the furfural concentration was raised to 20 mM, isolate 2-2 retained the higher degrading ability (97%) than isolate 1-8 (38%). Both of the strains could not degrade furfural at a higher furfural concentration of 25 mM (Figure 3b).

DISCUSSION

Termites harbored a complex microbial community in their intestines and they contributed a crucial role in the digestion of lignocellulose, as well as in the global carbon recycling. As only 1% of the gut microbes were cultivable by traditional cultivation methods, it was necessary to identify the uncultivable microbes by culture independent methods like DGGE. By DGGE-16S rDNA sequencing,

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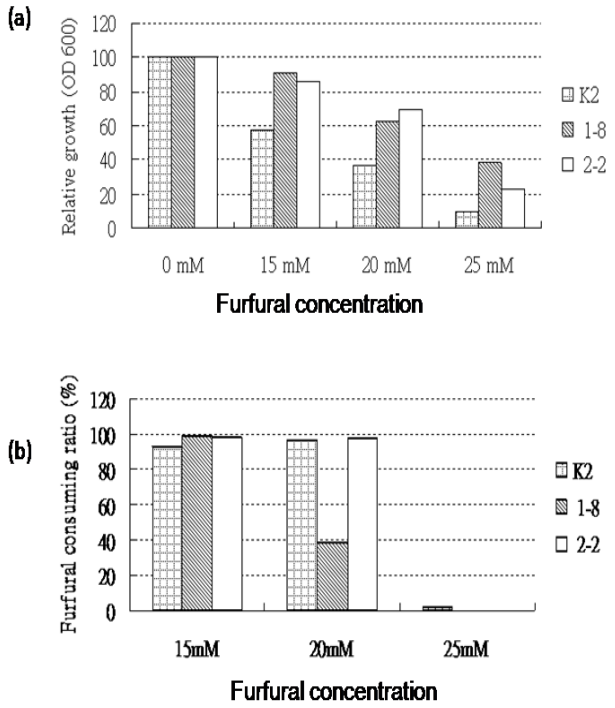

Figure 3: Furfural tolerance test and degradation. a) *Bacillus* isolates K2, 1-8 and 2-2 grown in MSM medium containing different concentrations furfural. K2 *Bacillus thermoamylovorans* was used as a control strain b) Furfural consuming assay

majority of the prokaryotes belonging to the Genus *Acinetobacter*, *Bacteriodes*, *Escherichia* and *Caulobacter* were identified; whereas *Lysinobacillus fusiformis, B. cereus, B. thuringiensis, Enterococcus avium* (Phylum *Firmicutes*) and *Citrobacter* (Phylum *Proteobacteria*) were isolated by culture dependent methods. From our study and previous studies, Phylum *Firmicutes* showed greater species diversity and were suggested to be involved in acetogenesis, sugar degradation and fermentation (Kudo, 2009; Noda *et al*., 2009). Earlier reports have suggested that most of the dominant microbes in gut of *C. formosanus* belonged to the Phylum *Bacteriodetes* and constituted 70–80% of total intestinal bacterial cells in their microbial flora (Noda *et al*., 2003; Hongoh *et al*., 2007).They were discovered as an endosymbiont of the host protist *Pseudotrichonympha grassii* in the gut of the termite *C. formosanus* by fluorescence *in situ* hybridization (FISH) analysis, transmission electron microscopy and PCR-amplified molecular analyses (Brune and Ohkuma, 2011). They were also suggested to help in the fermentation of sugars, nitrogenous compounds and cellulose digestion as well as in uric acid degradation (Husseneder, 2010). *Acinetobacter* sp. have been detected as a minor group in gut of *Coptotermes formosanus* from USA (Husseneder *et al*., 2005). Thus, it is suggested that interdependency existed between the hosts and the symbionts and that vertical transmission was a way of acquiring these symbionts (Husseneder, 2010). Co-evolution led to the continuity of these symbionts in the termite gut for several generations (Noda *et al*., 2009).

Some studies have reported that *Acinetobacter*, *Bacteroides, and Bacillus* existed in the intestine of termites and other invertebrates and were involved in the first stage of cellulose and hemicellulose hydrolysis (König *et al*., 2005; König, 2006). Moreover, the genus *Bacillus, Escherichia, Enterococcus,* and *Citrobacter* belonged to the intermediate stage of microorganisms, involved in oxidation and fermentation of cellulose and hemicellulosic substrates (König *et al*., 2005). In comparison with the rumen system that contained strict anaerobic cellulolytic bacteria such as the genera *Ruminococcus* and *Clostridium* , the termite gut harbored facultatively anaerobic or micro-aerophilic bacteria (Chang *et al*., 2010). This phenomenon might be due to the microbial proportion of the termite gut and the smaller intake of food than the rumen, which caused the larger oxygen influx in termite's gut system (Brune, 1998). A pronounced spatial differentiation in oxidation-reduction gradient that supplied with oxygen *via* the epithelium has been studied earlier (Brune and Kühl, 1996); Noirot, 1995). Thus, the microbial composition of the gut of *C. formosanus* was believed to be influenced by the above physiological factors.

After the pretreatment process of lignocellulolytic substrates with sulfuric acid, higher concentration of furfural was produced. The amount of furfural varied for different lignocellulolytic crops. After the pretreatment process, there was a washing step involved to remove excess of furfural. Only lower quantities of furfural approximately (5-10 mM) would be present on the pretreated substrates. Apparently, this concentration was found to be inhibitory to the bacteria used in fermentation process for biofuel production. Earlier reports have showed that cultures were severely inhibited at a concentration of 25 mM furfural. Some methanogens were anaerobically able to transform furfural (10 mM) to furfuryl alcohol (Belay *et al*., 1997). Therefore, 25 mM of furfural was chosen as the maximum concentration for studying the furfural tolerance. Studies have shown that furfural waste could be treated by anaerobic digestion with mixed bacterial consortia (Brune *et al*., 1982). Since *Bacillus* isolates 1-8 and 2-2 could grow aerobically as well as anaerobically, they could possibly be mixed as a co-culture with sulfate reducing bacteria that could metabolize furfuryl alcohol to acetic acid (Boopathy and Daniels, 1991) and acetate- utilizing methanogens which could convert acetate to methane and carbon dioxide. They could also probably be co-cultured with ethanologenic bacteria and encounter the limitations caused by furfural in fermentation process (Mills *et al*., 2009).

Aromatic compounds and biphenyl moieties were expected to be abundantly present in the hindgut of termites (Brune *et al*., 1995). In fact, several bacteria have been isolated and screened from *C formosanus*, and they possessed the ability to degrade chlorinated aromatic compounds, phenylalkanoic acids (breakdown products of

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lignin), phenol, and steroids (Harazono *et al*., 2003, Hayashi *et al*., 2007). In this study, we have also analyzed and isolated the microflora from *C. formosanus* guts which possess ligninolytic enzymes that could possible help in the degradation of lignin. By enrichment techniques, the lignin tolerant bacterial consortia from termite hindgut was isolated and identified as *Bacillus* isolates 1-8 and 2-2. Our studies helped in screening lignin and furfural tolerant isolates and analyzing the lignocellulolytic enzymes qualitatively. HPLC studies are yet to be done quantitatively in order to understand the lignocellulosic degradation process in detail. Earlier *Citrobacter* sp. have been isolated and characterized to be aromatic degrading microorganisms (Harazono *et al*., 2003). *Citrobacter* sp. isolate T8-2, was also detected to contain lignin degrading enzymes but they were not found to be tolerant to furfural (data not shown). These results indicated that these microbes might cumulatively play a role in the digestion of polysaccharides and aromatic compounds in the gut of *C. formosanus*.

Bacillus sp. also were discussed as the contributors in early and intermediate steps of polymer degradation under oxygen limitation in other intestinal systems of termites and lower invertebrates such as springtails, earthworms, isopods and millipedes (König, 2006). Other *Bacillus* species like *Lysinibacillus fusiformis*, *Bacillus cereus* and soil bacterium like *Bacillus thuringienesis* were also isolated in our study and probably could function in polymer degradation. Interestingly, *B. thuringiensis* is known as an effective bio-insecticidal bacterium for the control of pests such as *Lepidoptera*, *Diptera*, and *Coleoptera*, and could produce parasporal crystals with different morphology and insecticidal activity *via* its spore formation (Crickmore, 2006; Roh *et al*., 2007). Furthermore, *B. cereus* and *B. thuringiensis* strains harbored the gene cluster encoding acyl homoserine lactone (AHL) lactonases and zwittermicin A which could inhibit the growth of some gram-negative bacteria, *Oomycetes* and many plant pathogenic fungi (Handelsman *et al*., 1990; Dong *et al*., 2000). This could be the reason why the gut microflora in termite could compete with allochthonous microbes by insecticidal activity in natural ecosystems for maintaining the existing gut microflora. Some studies have reported that *Bacillus* widely enhances the germination of spores in earthworms and were also important phosphorus-solubilizing microorganisms, resulting in improved plant growth (Beneduzi *et al*., 2008). These findings widen the target range of *Bacillus* in termites besides insecticidal activity for suppressing plant pathogens and growth promotion in plants. The *C. formosanus* termites, which were invasive and difficult to control destructive pests in subtropical and tropical, might also be beneficial to kill other insects which were harmful to plants by possibly releasing the BT spores from their termite gut into the environment by defecation and *via* other termites by coprophagy and trophallaxis (Zhang *et al* ., 2003; Husseneder, 2010). Therefore from these studies and previous reports, we can prove that the termite gut of *Coptotermes formosanus* acts as a reservoir of various beneficial bacteria which could probably be useful for various industrial processes (Brune, 1998 and Hayashi *et al*., 2007).

CONCLUSION

To conclude, the *Bacillus* isolates 1-8 and 2-2 cultured from *C. formosanus* guts, possessed the peroxidase, lignin peroxidase, protease, and endoglucanase enzymes and could grow in a medium containing lignin as the sole source of carbon. The tolerance assay of furfural indicated that both the strains could tolerate higher concentrations of furfural and could also degrade furfural to lesser toxic compounds. Co-culture studies with these isolates and fermentative bacteria will be done in the future with respect to study their contribution in bio-fuel production.

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