CHARACTERIZATION AND IDENTIFICATION OF HIGH CELLULASE-PRODUCING BACTERIAL STRAINS FROM PHILIPPINE MANGROVES[1]

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ABSTRACT

Five promising cellulase-producing bacterial strains from soils collected from various mangrove sites in the country were characterized phenotypically and identified using conventional approach and, alternatively, by rapid identification through the Analytical Profile Index (API) system. They were identified as follows: BBCS-11 as *Bacillus cereus*; BBCS-14 as *Bacillus licheniformis*; BOrMGS-2 and BOrMGS-3 as *Bacillus pumilus*; and BBoB2L2-2 as *Bacillus* sp. The results generated from this study provided data regarding species of *Bacillus* producing cellulase enzyme and impart additional knowledge regarding the bacterial diversity of mangrove forests in the Philippines.

KEYWORDS: Bio-prospecting, Conservation, Bacterial Diversity, Mangrove Forests

INTRODUCTION

There is very little information, if any, on the microbial diversity of the mangrove ecosystem in the Philippines. Being a natural sink, microbial interaction in the mangroves is very dynamic, converting plant litter to nutritious food sources for marine organisms. Mangrove lignocellulose has been reported to support a vast range of micro-organisms. About a thousand species from 585 genera have been cited elsewhere for fungi alone (Pointing et al., 1998, Jones and Alias, 1997). However, the microbial flora of our mangrove ecosystem is still practically undocumented and unexplored.

Previous studies have revealed that mangrove microorganisms possess important degradative enzymes of industrial importance (Monsalud et. al. 2003; Monsalud et. al, 2006). This study aimed to characterize and identify high cellulase-producing bacterial strains that have been screened and optimized in an earlier study (Tabao and Monsalud, 2010).

This study was conducted at the Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños from April 2007 to March 2009.

METHODOLOGY

Sources of Bacterial Strains

All test organisms were provided by the Philippine National Collection of Microorganisms (PNCM), National Institute of Molecular Biology and Biotechnology (BIOTECH). The mangrove test cultures were previously isolated from decaying leaves and twigs, water, and soil at various mangrove sites in Batangas, Bicol, Bohol, Cebu, and Occidental and Oriental Mindoro, Philippines (Monsalud et al, 2003; Monsalud et al, 2006).

Characterization and Identification of the Bacterial Strains

Morphological, cultural, biochemical and taxonomic characteristics were examined in accordance with the established methods of Sneath et al. (1986) and available keys were also used for identification (Sneath et al. 1986, Reva et al. 2001). Alternatively, rapid identification was done through the Analytical Profile Index (API) identification system employing API 50 CHB strips which were used to biochemically characterize 24-hr to 48-hr old cultures. Identification procedures were performed following the manufacturer's instruction.

RESULTS AND DISCUSSION

Characterization and Identification of the High-Cellulase Producers

Morphological, cultural and physiological characterizations of the high-cellulase producing mangrove bacterial strains are summarized in Table 1.

Strain BBCS-11. Using the simplified key for identifying aerobic spore-forming bacteria by Reva et al. (2001), this strain was found to belong to the *Bacillus cereus* and related species. Using the key to *Bacillus* species determination by Sneath et al. (1986), on the other hand, this strain was found to be any among *Bacillus anthracis*, *Bacillus mycoides*, *and Bacillus cereus*. To further elucidate the identity of the strain, an additional method was, thus, used. In mannitol-egg-yolk-polymyxin agar (MYPA), BBCS-11 was found to be a non-mannitol fermenter, and was lecithinase-positive. Comparing these characteristics gathered to those in the Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986), strain BBCS-11 was finally identified as *Bacillus cereus*.

Table 1. Morphological, cultural, and physiological characterization of high-cellulase producing strains.

CHARACTERISTICS	BBCS-11	BBCS-14	BBoB2L2-2	BOrMGS-2	BOrMGS-3
Morphological Characteristics Gram reaction, shape arrangement, and size (μm)	Gram-positive rods in singles, pairs, and chains; 3x1	Gram-positive rods in singles, pairs, and chains; 1.5-2x0.7-0.8	Gram-positive rods in singles, pairs, and chains; 3x1	Gram-positive rods in singles, pairs, and short chains; 1.5x0.5	Gram-positive rods in singles, pairs, and chains; 1.5x0.5
Endospore shape, cell distension and motility	central, ellipsoidal; non-distended; motile	central to sub-terminal,ellipsoidal; non-distended; motile	central,ellipsoidal; non-distended; motile	central to sub-terminal, ellipsoidal; non-distended; motile	central to sub-terminal, ellipsoidal; non-distended; motile
Cultural Characteristics Colonial morphology on Marine Agar at 30ºC for 48 hours	1-2 mm circular to irregular, white to off white, opaque, smooth, dull, flat, erose margin	1-2mm circular to rhizoid, white, opaque, smooth, shiny, flat, erose to filamentous margin	2-4 mm irregular to spindle, white, opaque, dull, flat, erose margin	1-2 mm circular to spindle, cream to white, opaque, smooth, shiny, flat, entire to erose margin	1-3 mm circular to rhizoid, white to off-white, translucent to slightly opaque, smooth, dull, flat, entire to erose margin
Range of %NaCl, pH, and T° for growth	0-1.5% NaCl, pH 4-9, 25-45°C	0-10% NaCl, pH 5-10, 25-50°C	0-10% NaCl, pH 5-10, 25-45°C	0-5.0% NaCl, pH 5-10, 25-45°C	1.5-7.5% NaCl, pH 5-10, 25-45°C

Table 1. Continued.

CHARACTERISTICS	BBCS-11	BBCS-14	BBoB2L2-2	BOrMGS-2	BOrMGS-3
Physiological Characteristics[1]					
O_2 requiremen[2]	FA	FA	FA	A	A
Oxidase reaction	+	+	+	+	+
Catalase reaction	+	+	+	+	+
Urea hydrolysis	-	-	-	-	-
Indole production	-	-	-	-	-
H_2S production	-	-	-	-	-
Citrate utilization	+	+	+	+	+
Mixed acid production	-	-	-	-	-
Acetoin production	+	+	+	+	+
Nitrate reduction	+ (to NO or N_2)	+ (to NO or N_2)	+ (to NO or N_2)	+ (to NH_3)	+ (to NH_3)
Lysine decarboxylase	-	-	-	-	-
Argine decarboxylase	-	-	-	-	-
Gel liquefaction	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Lipolysis	+	-	-	+	+
Starch hydrolysis	+	+	+	-	-
Tyrosine production	wk	-	-	-	-
Melanin production	-	-	-	-	-
Sugars utilized[3]:	glu, suc, fru, mal,	glu, suc, fru, lac, gal, mal, ara, raf, mne, mnl	glu, fru, lac, mal, mnl	glu, suc, fru, lac, ara, mne, mnl	glu, suc, fru, mne, mnl

[1]Test reactions: (+) – positive reaction, (-) – negative reaction, (wk) – weak positive reaction

[2]O_2 requirement: A – aerobic, FA – facultative anaerobic

[3]Sugar utilization: glu – glucose, suc – sucrose, fru – fructose, lac – lactose, gal – galactose, mal – maltose, ara – arabinose, raf – raffinose, mne- mannose, mnl – mannitol.

Strain BBCS-14. Using the keys to the identification of aerobic spore-forming bacteria (Reva et al. 2001) and to the determination of the *Bacillus* species (Sneath et al., 1986), BBCS-14 was identified as *Bacillus licheniformis*. This was also in accordance with the phenotypic data described in the Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986).

Strain BBoB2L2-2. Using Reva et al. (2001) key, strain BBoB2L2-2 was shown to belong to *B. cereus* and related species group, and was said to be any among *B. anthracis, B. mycoides,* and *B. cereus* when the key of Sneath et al. (1986) was used. API 50 CHB was then used alternatively for identification, and it was found that the profile produced by the strain was a very good identification to the genus *Bacillus*. The closest significant taxon was *B. cereus* 1; however, the identification strength was only 50%. Considering the very low ID strength for *B. cereus* from the API 50 identification which primarily makes use of sugar utilization, lecithinase test was conducted since lecithinase is typically produced by *B. cereus*. However, MYPA result showed that Strain BBoB2L2-2 did not exhibit lecithinase activity. Further tests, therefore still needs to be done to elucidate the identity of this strain.

Strain BOrMGS-2. To identify this strain, the key to the determination of species of *Bacillus* (Sneath et al. 1986) was used and the strain turned out to be *Bacillus pumilus*. Using the Reva et al. (2001) key, on the other hand, showed inconclusive result due to the strain not being able to grow at 50ºC. It was confirmed using API 50 CHB, however, that BOrMGS-2 is indeed *Bacillus pumilus*, where it noted an excellent identification with 99.9% certainty. The phenotypic characteristics of the Strain gathered were also in accordance with the Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986).

Strain BOrMGS-3. BOrMGS-3 was identified using the keys described by Sneath et al. (1986) and it was also identified as *Bacillus pumilus* using the former way, while an inconclusive result was obtained with the latter due to the Strain not being able to grow at 50ºC. As a confirmatory step, the API 50 CHB was used. The result showed a 99.9% certainty that BOrMGS-3 is indeed *Bacillus pumilus*, noted as an excellent identification. The data gathered about the Strain was compared and the identification, consequently, was affirmed using the Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986).

Implication and Relevance of the Identities of Bacteria from Several Philippine Mangrove Areas

This study characterized and identified five high cellulase-producing strains. All of these five were found to be endospores-formers belonging to the *Bacillus* sp. (Table 2) and all of them were isolated from mangrove forest soil. Soil is generally accepted as the primary habitat of *Bacillus* species (Sneath et al. 1986).

Bacillus species are known to produce cellulases, some of which are exploited in the industries. Ariffin et al. (2006) claimed that bacterial cellulases are more often than not effective catalysts. The effectiveness of the cellulases produced by *Bacillus* spp. probably differs from species to species. Three species of *Bacillus* identified in this study are *B. cereus* (BBCS-11), *B. licheniformis* (BBCS-14), and *B. pumilus* (BOrMGS-2 and BOrMGS-3).

Table 2. The sources, identities, and enzyme activities of the high-cellulase producing mangrove bacterial strains.

STRAIN	MANGROVE SOURCE AND SITE	CELLULASE ACTIVITIES (U ml^{-1})	IDENTITY
BBCS-11	Soil, Bicol	56.60	*Bacillus cereus*
BBCS-14	Soil, Bicol	66.50	*Bacillus licheniformis*
BBoB2L2-2	Leaf, Bohol	50.33	*Bacillus* sp.
BOrMGS-2	Soil, Oriental Mindoro	51.04	*Bacillus pumilus*
BOrMGS-3	Soil, Oriental Mindoro	48.70	*Bacillus pumilus*
Control Strain:			
BIOTECH 1240	NA	54.80	*Cellulomonas* sp.

It has been observed as early as 1987 by Kawai et al. (1988) that a neutrophilic strain of *Bacillus pumilus* produces alkaliphilic CMCase (a type of cellulase). Ariffin et al. (2006) also reported a strain of *Bacillus pumilus* producing cellulase at pH range of 5 to 9. The highest activity produced by the strain reported by Ariffin et al. (2006) was 0.079 U ml^{-1} which is very much lower compared to 51.04 U ml^{-1} by the strain *Bacillus pumilus* (BOrMGS-2) in this study. Due to the probable alkaline nature of the cellulases of BBCS-11, BBCS-14, and BOrMGS-2 they have great potential for the detergent industry, but not discounting the fact that they may have several uses in the pulp, textile and paper industries. They may also have some agricultural uses as cellulose is the most abundant waste material due to plants having fibers with mostly cellulosic material.

B. licheniformis is said to be an important producer of several exoenzymes. Veith et al. (2004), who worked on mapping the genome of the species, found prominent genes that coded for these exoenzymes and one of them coded for cellulase proving that *B. licheniformis* is indeed a cellulase producer. It is on the basis of *B. licheniformis* having genes coding for different exoenzymes that makes it a great industrial potential. In fact *B.*

licheniformis was never reported to be pathogenic and is in actual use for large-scale industrial production of exoenzymes as it can secrete large quantities (up to 20–25 g L^{-1}). Strain BBCS-14, having quite a high cellulase activity (66.50 U ml^{-1}), could possibly be used for enzyme production in the industrial scale. Not only are they important to industries, they have some environmental uses as well. Rojas et al. (2001) have reported *B. licheniformis* as one of plant growth-promoting bacteria and their potential use as reforestation agents in mangroves forests.

Bacillus cereus strains are also known for their cellulase however they are better known for their being causative agents for food spoilage (Sneath et al. 1986) and their risk potential or pathogenicity to humans and animals is high (Stackebrandt 2006). *B. cereus* is commonly found in the soil and is believed to be part of the transient permanent cellulolytic population of the intestinal flora of termites due probably to being ingested with the food of termites and other insects (Brune 2006). Although its pathogenicity and being able to colonize human intestinal tracts remains to be elucidated, it is recommended that BBCS-11 and BBoB2L2-2 (which is very similar to BBCS-11), even with its high cellulase activity, should be used with caution for any industrial application.

CONCLUSION

The data gathered in this study provides us a glimpse of some of the dynamics of the production of cellulase and cellulase-substrate interactions of several strains belonging to the genus *Bacillus* which could probably help taxonomists, enzymologists, and even some industrialists in their own researches. This study gave us a glimpse as well on the wealth of the mangrove forests in the country. Therefore, conservation efforts must be done to preserve them as they may harbor potential agents for possible exploitation not only for the industries but also for agriculture and the environment, like the ones presented in this study thus far.

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LITERATURE CITED

Ariffin H, Abdullah N, Umi Kalsom MS, Shirai Y, Hassan MA. 2006. Production and characterization of cellulase by *Bacillus pumilus* EB3. *Int. J. Eng. Tech*. 3(1):47-53.

Brune A. 2006. Symbiotic associations between termites and prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E. *The Prokaryotes*. New York, USA: Springer Science. pp. 43-46.

Jones, EBG and Alias, SA. 1997. Biodiversity of Mangrove Fungi. In: *Biodiversity of Tropical Microfungi*. Hyde, K.D. (ed.). Hongkong University Press, Hongkong. Pp.71-92.

Kawai S, Okoshi H, Ozaki K, Shikata S, Ara K, Ito S. 1988. Neutrophilic *Bacillus* strain, KSM-522, that produces an alkaline carboxymethyl cellulase. *Agric. Biol. Chem*. 52(6):1425-1431.

Monsalud, R.G. Papa, IA, Zulaybar, TO, Clavillas, JDL. 2006. Isolation, Preservation and Identification of Microorganisms from the Mangrove Ecosystem in Mindoro with Degradative and Antimicrobial Potential. Commission on Higher Education – UP Los Baños-Zonal Research Council (CHED-UPLB ZRC) Project Terminal Report. 178 pp.

Monsalud, RG, Zulaybar, TO, Alip, CG. 2003. Isolation, Preservation **a**nd Identification of Microorganisms from Mangrove Ecosystem with Degradative and Bio-Control Potential. University of the Philippines – Center for Integrative and Developmental Studies (UP-CIDS). Project Terminal Report.

Pointing, SB., Vrijmoed, L.P., Jones, EBG. 1998. *Botanica Marina.* 41:293-298.

Reva ON, Sorokulova IB, Smirnov VV. 2001. Simplified technique for identification of the aerobic spore-forming bacteria by phenotype. *Int. J. Sys. Evol. Microbiol*. 51:1361-1371.

Sneath PHA, Mair NS, Sharpe ME, Holt JG. 1986. *Bergey's Manual of Systematic Bacteriology*. Vol. 2. USA: The Williams and Wilkins Co.

Stackebrandt E. 2006. Defining taxonomic ranks. In: *The Prokaryotes*, edited by Dworkin, M., S. Falkow, E. Rosenberg, K.H. Schleifer, and E. Stackebrandt. New York, USA: Springer Science. pp. 29-57.

Tabao, NS and Monsalud RG. 2010. Screening and Optimization of Cellulase Production of *Bacillus* Strains from Philippine Mangroves. Manuscript submitted for publication at *Phil. J. of Syst. Biol.*

Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, Baumer S, Henne A, Liesegang H, Merkl R, Ehrenreich A, Gottschalk G. 2004. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *J. Mol. Microbiol. Biotechnol*. 7:204-211.