SCREENING AND OPTIMIZATION OF CELLULASE PRODUCTION OF *BACILLUS* STRAINS ISOLATED FROM PHILIPPINE MANGROVES

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ABSTRACT

Two-hundred and twenty-five mangrove bacterial strains previously isolated from several mangrove areas in the Philippines were qualitatively screened for cellulase production and 154 were found to be capable of cellulase production. Among them, ten strains showed very strong positive cellulase activities, which were then quantified. Five with the statistically highest activities, specifically, BBCS-11, BBCS-14, BBoB2L2-2, BOrMGS-2, and BOrMGS-3, including *Cellulomonas* sp. BIOTECH 1240 (control), were further subjected to partial optimization studies. The effects of substrate, pH, incubation temperature and incubation time on cellulase productoin were determined. The cellulase activities (U mL^{-1}) of the strains under optimized conditions were: 54.80 (Control); 56.60 (BBCS-11); 66.50 (BBCS-14); 50.33 (BBoB2L2-2); 51.04 (BOrMGS-2); and 48.70 (BOrMGS-3). The promising cellulase producers were previously identified: BBCS-11 as *Bacillus cereus*; BBCS-14 as *Bacillus licheniformis*; BOrMGS-2 and BOrMGS-3 as *Bacillus pumilus*; and BBoB2L2-2 as *Bacillus* sp.

KEY WORDS: Bio-prospecting, Conservation, Bacterial Diversity, Bacterial Enzymes, Mangrove Forests.

INTRODUCTION

Microbes generating cellulases play an active role in the degradation of mangrove forest detritus, which is mainly composed of cellulosic plant material. Detritus is very important in the mangrove forests as they can be sources of nutrients to different life forms in the area when fully (or maybe even partially) degraded (Holguin et al. 2001). Cellulase and its producers can then be considered important in the continuing life and chemical cycles in mangrove forests. They are vital as well in industries which manufacture products that are important in our daily lives.

This study aimed to screen for bacterial strains obtained from different mangrove areas in the Philippines that are cellulase producers, quantify and optimize the cellulolytic activities of strong-cellulase producing strains and identify them. 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 2006 to March 2008.

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. The positive control strain, *Cellulomonas* sp. BIOTECH 1240 is a known cellulose-enzyme producer held in the PNCM collection.

Screening for Cellulase Producers

Preliminary qualitative enzymatic activity determination was done following the method described by Laurent et al. (2000). A total of 225 mangrove bacterial strains were inoculated on plates of carboxymethyl cellulose solid medium CMCSM containing (g L^{-1}): Sodium Carboxymethyl Cellulose (Na-CMC) 1.2, Yeast Extract 0.5, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, $NaNO_3$ 0.3, Agar 15.0, and supplemented with commercially available 1.5% marine salts. The plates were incubated at 30°C for 5-10 days. Cellulase activity was determined by staining the plates with Congo red dye (0.1 g L^{-1}) for 15 minutes. The plates were then washed with a 1 M NaCl solution to reveal cellulase activity, which was indicated by an orange halo surrounding the colony against a red media background. The diameters of the haloes around the colonies were measured and those with diameters of more than 10 mm were considered.

Cellulomonas sp. BIOTECH 1240, identified to be a strong cellulase producer, was used as the positive control. Being a non-mangrove strain, it was inoculated in CMCSM without supplementary marine salts.

Quantitative Measurement of Cellulase Activity

Cellulase activity was assayed using the protocol described by the Food and Agriculture Organization of the United Nations (FAO) with some modifications (FAO Food and Nutrition Paper 52: Compendium of Food Additive Specifications Addendum 8 (JECFA) [Updated 2000]). Ten high cellulase-producing strains, namely, BOrMBS1-3, BOrMFS-9, BOrMFS-10, BOrMFS-11, BOrMGS-2, BOrMGS-3, BOrMGT-9, BBCS-11, BBCS-14, and BBoB2L2-2 were inoculated in carboxymethyl cellulose liquid medium (CMCLM) supplemented with 1.5% marine salts and incubated on a gyratory shaker for 24 to 48 hrs at 30°C. *Cellulomonas* sp. BIOTECH 1240, used earlier as the positive control for the qualitative cellulase plate assay was also

inoculated in CMCLM but without the supplementary salts. Cell-free culture supernatants were obtained by centrifugation at 10,000 rpm for 5 minutes and were used as working sample solutions.

A mixture containing 1 mL of CMC substrate solution and an equal volume of a sample both pre-warmed to 40.0 ± 0.1°C for 5 mins was dispensed in a test tube and incubated in a water-bath maintained at 40.0 ± 0.1°C for 10 minutes. To stop the enzymatic reaction, 4 ml of 3,5-dinitrosalicylic acid (DNS)-lactose solution was added and mixed. Subsequently, the tubes were covered and placed in a water bath at 100°C for 15 minutes and then cooled to room temperature with a cooling water bath. Insoluble substances were removed by centrifugation (10,000 rpm, 5 min). The absorbance of the samples, and a reagent blank that has undergone the same process as mentioned above like the rest of the samples, was determined against a water blank using a spectrophotometer set at 540 nm. One cellulase unit (U) is defined as the amount of enzyme that liberates reducing sugar at a rate of 1μmol min^{-1}.

Partial Optimization of Conditions for Cellulase Production and Enzyme Activity

Partial optimization of conditions for cellulase production and enzyme activity of the top five strains (in terms of enzyme activity) was done by following the quantitative photometric assay steps with some modifications.

The effect of substrate type on cellulase production during growth in media was done by substituting CMC with crystalline cellulose (Avicel). Incubation, was extended up to eight days to determine the effect of the age of the cultures on the production of cellulase assaying after one, three, six and eight day/s. The incubation temperature (temperature during reaction) was lowered to 30°C and pH, were varied to pH 5, 7, and 9 to account for slightly acidic, neutral, and basic conditions by adding either 1N HCl or 1N NaOH.

Experimental Design and Statistical Analysis

All quantitative data in this study were statistically analyzed using Analysis of Variance (ANOVA) under a completely randomized design (CRD). All experiments were performed in triplicates. Means were compared using the Least Significant Difference Test (LSDT).

RESULTS AND DISCUSSION

Bacterial Strains Producing Cellulase

Of the 225 bacterial strains screened for cellulase production, 154 (69%) tested positive. Of these, 10 strains namely BOrMBS1-3, BOrMFS-9, BOrMFS-10, BOrMFS-11, BOrMGS-2, BOrMGS-3, BOrMGT-9, BBCS-11, BBCS-14, and BBoB2L2-2 showed very strong positive reactions indicated by

a clearing zone of more than 10 mm in diameter. Out of these 10 strains, eight were isolated from soil, one was isolated from twigs, and also only one from leaves. Usually, one would equate cellulase producers with those in association with plant leaves or twigs. However, it can be noted that detritus in mangrove forest floors contain a significant amount of mangrove plant material (leaf litter) and that cellulase producers are one of the major players for the conversion/transformation of this materials such that it is not uncommon to find a lot of strong cellulase producers in the mangrove forest soils (Zhuang and Lin 1993).

Quantification of Cellulase Activity

The assay for quantifying cellulase activity described in this study is based on the ability of the cellulase enzyme produced by the strains to hydrolyze CMC to reducing sugars. The resulting increase in reducing groups were measured using 3,5-dinitrosalicylic acid (DNS). The aldehyde functional group present in glucose, for example, is oxidized and the DNS is simultaneously reduced to 3-amino, 5-nitrosalicylic acid. This oxidation-reduction process eliminates the remaining glucose, which may be the reason for the termination of enzyme reaction process (Miller 1959). Table 1 shows how the activities of the ten strains, screened as high-cellulase producers in the preliminary screening, compare to that of the control strain, which is a known high-cellulase producer. It can be noted that strain BBoB2L2-2 exhibited the highest cellulase activity (48.87 U mL^{-1}) which was obtained at the 24^{th} hour of incubation and was followed by BOrMGS-2 with 41.13 U mL^{-1}. The difference between the activities of the two strains is statistically significant. The cellulase activities of these two strains are 53% and 44% higher than that of the control strain. The ten strains including *Cellulomonas* sp. BIOTECH 1240 (control) had their greater cellulase activities on the 48^{th} hour of incubation.

At 48 hours of incubation, BBCS-14 exhibited the highest cellulase activity (38.40 U mL^{-1}), which is significantly different from the rest of the strains. The next highest cellulase activities were exhibited by BBoB2L2-2 and BOrMGS-3 with 32.53 and 33.70 U mL^{-1}, respectively, which are not significantly different. These three mangrove strains exhibited higher cellulase activity (36%, 27%, and 24%, respectively) than that produced by the positive control *Cellulomonas* sp. BIOTECH 1240. A drastic drop on the values of the enzymatic activities of BOrMGS-2 and BBoB2L2-2, from the 24^{th} hour to the 48^{th} hour of incubation, was shown. This drastic drop may be accounted for by the aggressive depolymerization of the substrates by the enzymes of these strains, which could also be responsible for the rapid depletion of substrates, hence lower activities at extended incubation.

The strains with the highest activities in the initial quantification were selected for partial optimization of their cellulase production and enzyme activities.

Table 1. Cellulase activity (U mL^{-1}) of the promising bacterial mangrove strains obtained at 24- and 48-hour incubation periods.

STRAIN	CELLULASE ACTIVITY (U mL^{-1})*	
	24 HOURS	48 HOURS
Cellulomonas sp. BIOTECH 1240 (control)	23.17^{d}	24.73^{cd}
BBCS-11	22.33^{de}	25.63^{c}
BBCS-14	33.27^{c}	**38.40^{a}**
BOrMBS1-3	17.27^{fg}	21.63^{def}
BOrMFS-9	18.67^{defg}	21.80^{def}
BOrMFS-10	15.40^{g}	19.00^{f}
BOrMFS-11	18.03^{efg}	20.90^{ef}
BOrMGS-2	41.13^{b}	23.70^{cde}
BOrMGS-3	30.23^{c}	32.53^{b}
BOrMGT-3	20.23^{def}	22.77^{cde}
BBoB2L2-2	**48.87^{a}**	33.70^{b}

*means with same letter designations are not significantly different at α = 0.050 by Least Significant Difference Test (LSDT).

Effect of substrate type on cellulase production

To determine the effect of substrate type on cellulase production the substrates used in the quantitative assay were varied. The original substrate carboymethyl cellulose (Sigma) or CMC, and alternative substrate cellulose (Avicel) were used. The peak cellulase activities (U mL^{-1}) of the strains using CMC were higher than those obtained using avicel as shown in Table 2. This trend was probably due to the crystalline nature of avicel. According even to Koukielo et al. (2005), degrading crystalline cellulose to glucose needs at least the cooperation of three enzymes: endoglucanase, exoglucanase, and β-glucosidase. As for carboxymethyl cellulose, only endoglucanases are needed to degrade it (Robson and Chambliss 1989).

Table 2. Peak cellulase activities of the selected strains with carboxymethyl cellulose and avicel as substrates.

STRAIN	PEAK CELLULASE ACTIVITIES (U mL^{-1})	
	CMC	AVICEL
Cellulomonas sp. BIOTECH 1240 (control)	39.41	19.50
BBCS-11	35.61	24.70
BBCS-14	47.40	23.04
BBoB2L2-2	47.80	48.87
BOrMGS-2	38.40	34.89
BOrMGS-3	41.80	18.50

Effect of incubation temperature on cellulase activity

Those that produced significantly greater cellulase activities (U mL^{-1}) at the original assay temperature, 40ºC, include BBCS-11 (39.40), BBCS-14 (47.70), and BBoB2L2-2 (47.80) 6+. Those with greater activities (U mL^{-1}) at 30ºC incubation temperature for growth were the control (52.70), BOrMGS-2 (46.30), and BOrMGS-3 (48.30). There is still a need to further test these strains using different temperatures to determine which would produce the highest enzyme activity.

Effect of pH on cellulase activity

Majority of the strains registered peak cellulase activities (U mL^{-1}) at a basic pH (pH 9), of which included the control (54.80), BBCS-11 (56.60), BBCS-14 (66.50), and BOrMGS-2 (51.04). These values decreased as pH approaches neutral or more acidic. Only BBoB2L2-2 registered the peak cellulase activity at neutral pH (pH 7) with 50.33 U mL^{-1}, while BOrMGS-3 registered its peak cellulase activity at an acidic pH or pH 5 with 48.70 U mL^{-1}.

Lee et al. (1985) suggest that the difference or the change in values of a strain's activities may be due to either the influence of pH on the synthesis of the enzyme protein or its effect on the stability of the enzyme molecule itself.

Effect of incubation period or age of culture on cellulase production

In 2000, Laurent et al. (2000) claimed that the cellulase activity of *Chryseomonas luteola*, a cellulase and pectate lyase producer, extended to the end of exponential phase and could as well be assayed during early stationary phase. This information could very well be exploited by industries as the resilience of cultures is important for cost effective measures.

All of the strains had their peaks on the 6^{th} day except for BBCS-11, which had its peak on the first day. It was observed that peak cellulase production of all strains can still hold out until the 8^{th} day but in decreasing trend which can be due to the depletion of substrate.

Identification of the High-Cellulase Producers

Characterization and identification of the promising strains were carried out and reported in detail in another paper (Tabao and Monsalud, 2010). The identities of the selected promising strains were BBCS-11 as *Bacillus cereus*; BBCS-14 as *Bacillus licheniformis*; BOrMGS-2 and BOrMGS-3 as *Bacillus pumilus*; and BBoB2L2-2 as *Bacillus* sp.

Implication and Relevance of the Cellulase Activities of the Mangrove *Bacillus* Strains

The optimized conditions of the cellulases of the strains are summarized in Table 3.

Table 3. Values of the optimized parameters for the cellulase production and cellulase activities of selected mangrove bacteria.

STRAIN	OPTIMIZED PARAMETERS				CELLULASE ACTIVITY (U mL^{-1})
	During Cellulase Production (Growth in Media)		During Enzyme Reaction with Substrate		
	Substrate*	Incubation Time (days)	Temperature (˚C)	pH	
Cellulomonas sp. BIOTECH 1240	CMC	6	30	9	54.80
B. cereus BBCS-11	CMC	1	40	9	56.60
B. licheniformis BBCS-14	CMC	6	40	9	66.50
Bacillus sp. BBoB2L2-2	CMC	6	40	7	50.33
B. pumilus BOrMGS-2	CMC	6	30	9	51.04
B. pumilus BOrMGS-3	CMC	6	30	5	48.70

*CMC – Carboxymethyl cellulose

Peak cellulase activities by most of the mentioned strains were observed to be greater at pH 9 (alkaline pH) except for *B. pumilus* BOrMGS-3 which had greater activity at pH 5 (slightly acidic) and BBoB2L2-2 which had greater activity at pH 7 (neutral). It is not unusual for these neutrophilic strains to have produced cellulases with high activities outside neutral pH. The highest activity produced by the *B. pumilus* strain EB3 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 *B. pumilus* BOrMGS-2 in this study. The conditions for production in this study and that of Ariffin et al. (2006) were not exactly the same. In both studies CMC was used as subtrate and the same production medium was also used. However, instead of acetate buffer, sodium citrate buffer was used in this study. Furthermore, Ariffin et al (2006) used 575 nm wavelength to read their samples while in this study the samples were read at 540 nm following the method described in the FAO Food and Nutrition Paper #52 (2000). These dissimilarities may account for differences in the enzyme activities of the strains being compared, but it is still notable since it could mean one of two

things: (1) The method used in this study may be more sensitive than the one used by Ariffin et al. (2006), or (2) Strain BOrMGS-2 may indeed be a better cellulase-producer.

Due to the probable alkaline nature of the cellulases of *B. cereus* BBCS-11, *B. licheniformis* BBCS-14, and *B. pumilus* 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* 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.

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 mangrove *Bacillus* strains. This study gives us a hint as well on the microbial wealth of mangrove forests in the Philippines which can be harnessed for biotechnological processes.

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