Comparison of Conventional Plate Assays with DNA-based Screening Protocols for Protease and Cellulase Production from Putative *Bacillus* Isolates

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ABSTRACT

Putative *Bacillus* isolates obtained from mud and soil samples of Mt. Makiling, Los Baños, Laguna were screened for the production of either cellulase or alkaline protease using cellulose and casein plate assays, respectively. Five out of eight isolates assayed for cellulase activity tested positive. DNA from these eight samples were extracted using the modified ROSE method for slot blot hybridization with cellulase gene probe. Out of the five samples positive for the cellulose plate assay, only three exhibited hybridization results. DNA from the eight isolates were used as templates for PCR amplification using primers specific for *B. subtilis* cellulase gene. Out of the eight isolates, two produced the expected 350-bp cellulase amplicon. Another set of ten putative *Bacillus* isolates were screened for the production of alkaline protease using casein plate assay. Four isolates exhibited protease activity. Genomic DNA extracted from ten isolates were subjected to slot blot hybridization using a fragment of alkaline protease gene from *B. pumilus*. Five isolates, of which four previously tested positive for protease activity, showed positive hybridization signals. PCR analysis using primers designed based on alkaline protease gene of *B. pumilus* showed that only one isolate produced the expected 320-bp PCR amplicon. These data suggest that biochemical plate assays may be advantageous for isolating bacteria that produce different types of cellulase and alkaline protease, while DNA-based methods are useful in detecting specific target genes but may therefore miss out on novel or variant enzymes.

Keywords: Bacillus, alkaline protease, cellulase, slot blot hybridization, Polymerase Chain Reaction (PCR)

INTRODUCTION

Various *Bacillus* species have been found to excrete extracellular enzymes, which are very important for the degradation and hydrolysis of several biomass polymers (Rao et al., 1998; Ferrari et al., 1993; Pero & Sloma, 1993). The two major enzymes that were included in this study are alkaline protease and cellulase; both of which are essential components of a wide range of products in the detergent, food, and chemical industry (Ferrari et al., 1993).

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Identification of potential enzyme-producing bacteria is an important step in searching for useful and novel enzymes. Traditional biochemical methods of screening for enzymes involve growing the microorganisms on an agar plate containing the appropriate enzyme substrate. On the other hand, DNA-based methods, such as DNA hybridization and polymerase chain reaction (PCR), are based on the detection of the particular gene coding for the target enzyme.

This study aimed to compare the conventional plate assay with PCR and DNA hybridization as screening methods for protease and cellulose producing bacteria. Specifically, putative *Bacillus* isolates were screened for the production of alkaline protease and cellulase using plate assays. The genomic DNA of these bacterial isolates was then tested for the presence of genes homologous to *Bacillus* alkaline protease and cellulase genes using polymerase chain reaction (PCR) and slot blot hybridization.

MATERIALS AND METHODS

Bacterial samples

Eighteen putative *Bacillus* isolates were obtained from soil and mud samples of Mt. Makiling, Los Baños, Laguna. Those obtained from soil were designated as 1SA-23, 3SA-9, 1SM-9, 1SM-18, 3SA-13, 1SM-8, 4SM-8, and 5SA-17, while those from mud were 3MA-11, 6MA-6, 6MM-4, 7MM-30, 4MA-13, 7MM-12, 3MA-13, 4MM-33, 5MA-14, and 7MM-17. The isolates were routinely grown in nutrient agar slants at 37°C overnight and were stored at 4°C for further use.

Traditional biochemical assay

Bacillus isolates were screened for their ability to excrete either alkaline protease and cellulase using traditional plate assay techniques. The casein plate assay was used as test for the presence of alkaline protease. The isolates were line-inoculated onto a Luria-Bertani (LB)–agar medium containing 1% casein and incubated overnight at 37°C.

The cellulase assay used the modified procedure of Yeoh et al. (1985) for screening cellulolytic fungi. Isolates were streaked on plates containing 0.1% (w/v) $NaNO_3$, 0.5% (w/v) NaH_2PO_4, 0.5% (w/v) KCl, 0.1% (w/v) $MgSO_4$, 0.14% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4, 0.3% (w/v) carboxymethyl cellulose and 2% (w/v) agar. After incubation at 37°C overnight, the plates were flooded with deep blood-red cellulose stain (0.8% I_2 crystals, 0.8% (w/v) KI, 8 mM KCl) for 5 to 10 minutes or until clearing zones appeared around the periphery of bacterial colony that excreted cellulase.

DNA extraction

Total DNA was extracted from overnight nutrient-broth cultures of the isolates following a procedure based on the ROSE protocol of Steiner et al. (1995). Bacterial cells were pelleted at 20,800 RCF for one minute (Eppendorf centrifuge 5415 C), washed with 1 ml 0.85% NaCl, and treated with 300 µL modified ROSE buffer [10 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 1% (w/v) sarkosyl, and 1% (w/v) polyvinylpolypyrrolidone (PVPP)]. Samples were incubated at 90°C for 20 minutes in a water bath. Tubes were then placed on ice or stored at 4°C. The supernatant containing the genomic DNA, was collected after centrifugation at 10,600 RCF for 5 minutes.

PCR amplification

The genomic DNA of all bacterial isolates were used as template for PCR amplification using primers specific for alkaline protease and cellulase genes, respectively.

ROSE genomic DNA extracts were diluted 1:54 with sterile water prior to PCR amplification. PCR was performed using the Perkin-Elmer GeneAmp PCR System 2400 thermocycler (Perkin Elmer Corp., Singapore) with a reaction volume of 20 µL containing 1x PCR buffer, 1.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each of the forward and reverse primers, 0.025 U/µL Taq DNA polymerase, and 1 µL (50-100 ng) diluted DNA extract.

PCR conditions for alkaline protease gene amplification include an initial denaturation at 94°C for 5 minutes; 40 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 40 seconds, extension at 72°C for 45 seconds; and a final extension at 72°C for 7 minutes. The same conditions were used for the amplification of cellulase gene, except for the annealing temperature, which was set at 51°C for 40 seconds.

DNA hybridization

Template DNA preparation

DNA extracted from the selected bacterial isolates were boiled for 10 minutes, and immediately placed on ice for 1-2 minutes. A volume of 50 µL of the DNA template was applied onto a nylon membrane pre-soaked in 1x Tris-EDTA (TE) buffer using a PR 648 Slot Blot Filtration Manifold (Amersham Pharmacia Biotech; California, USA). The membrane blotted with DNA was soaked in a denaturation Solution 1 (0.5M NaOH, 1.5 M NaCl) for 10 minutes and then transferred into Solution 2 (0.1M Tris pH 7.5, 1.5M NACl) for another 10 minutes. The blotted membrane was air-dried and subjected to UV cross-linking using GS Gene Linker (Bio-Rad Laboratories, USA) before pre-hybridization with a solution containing 5x SSC, 1% blocking reagent, 0.1% sarkosyl, 0.02% SDS) at 68°C for one hour.

Hybridization with labeled probe

PCR amplification products containing fragments of either *B. pumilus* alkaline protease gene or *B. subtilis* cellulase gene, were labeled with digoxigenin, following the protocol described by Roche Molecular Biochemicals (Mannheim, Germany) to produce the probe. The labeled gene probes were allowed to hybridize with the DNA templates on the membrane at 68°C overnight.

Detection of hybridization

After hybridization and prior to detection, the membrane was washed twice in a solution containing 2x SSC and 0.1% SDS at room temperature for 5 minutes, with shaking. The membrane was further washed twice in a solution containing 0.1x SSC and 0.1% SDS at 65°C for 5 minutes. Hybridization signals were detected using an enzyme-linked immunoassay with anti-Dig-AP (digoxigenin-alkaline phosphatase) Fab fragments and NBT-BCIP (nitroblue tetrazolium chloride-5-bromo-4-chloro indoyl phosphate toluidine salt) substrate following the manufacturer's procedure (Roche Molecular Biochemicals; Mannheim, Germany).

RESULTS AND DISCUSSION

Biochemical assay

Screening for either cellulase or alkaline protease production of the 18 putative *Bacillus* isolates was done by cellulose and casein plate assay, respectively. Bacterial isolates with positive cellulase activity are detected by the formation of a clearing zone around the bacterial colony after the addition of a cellulose stain onto the plate. These isolates are said to utilize cellulose as carbon source, and convert the substrate into cellobiose and glucose sub-units. On the other hand, casein assay is a general method used to screen for microorganisms that excrete alkaline proteases in the surrounding medium. Incorporating casein as the sole carbon source into an agar medium will detect alkaline protease-secreting organisms by the presence of a clear halo of hydrolyzed casein around the growing colony.

Among the eight putative *Bacillus* isolates used for cellulose plate assay, four were obtained from soil (1SA-23, 1SM-9, 1SM-18, and 3SA-9) and the other four were taken from mud (7MM-30, 3MA-11, 6MA-6, and 6MM-4). Out of these eight samples, two isolates from soil and three isolates from mud were shown to have cellulase activity (Table 1). For casein plate assay, only four out of 10 bacterial samples were positive for alkaline protease activity. Two out of the four putative *Bacillus* isolates from soil exhibited alkaline protease activity, while only two of the six screened soil isolates were found to secrete alkaline protease.

Table 1. Summary of protein and DNA-based screening for cellulase performed on eight initial bacterial isolates.

Sample	Cellulose assay	Slot blot hybridization	PCR
7MM-30	+	+	+
1SA-23	+	+	-
6MM-4	+	+	+
3MA-11	-	+	-
1SM-18	-	+	-
1SM-9	+	-	-
6MA-6	+	-	-
3SA-9	-	-	Not tested

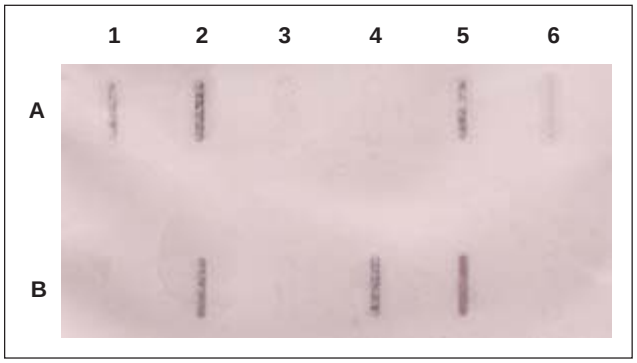

Fig. 1. *Slot blot hybridization of DNA templates prepared using modified ROSE method with a probe prepared from a fragment of B. subtilis cellulase gene*. Lanes A1 to A5 and B2 to B5 contain DNA extracted from unknown bacterial isolates: 7MM-30, 1SA-23, 3SA-9, 1SM-9, 6MM-4, 3MA-11, 6MA-6, and 1SM-18, respectively. Lanes A6 and B6 contain the positive (*B. subtilis*) and negative (*B. thuringiensis*) genomic DNA controls, respectively. Lane B5 contains cellulase gene fragment from *B. subtilis*, while lane B1 contains only TE buffer.

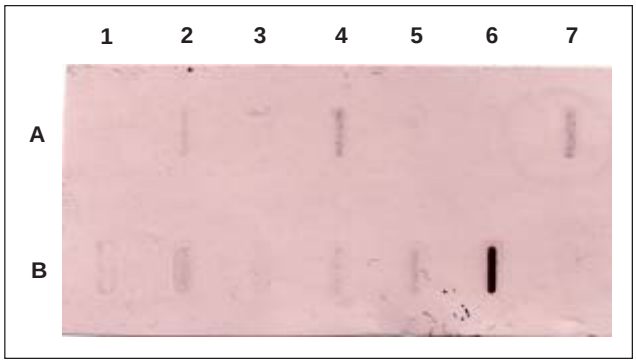

Fig. 2. *Slot blot hybridization of DNA templates prepared using modified ROSE method with a probe prepared from a fragment of B. pumilus alkaline protease gene*. Lanes A1 to A5 and B1 to B5 contain DNA extracted from unknown bacterial isolates: 7MM-12, 4SM-8, 4MM-13, 1SM-8; 7MM-12, 3SA-13, 5SA-17, 3MA-13, 4MM-33, 5MA-14, respectively. Lanes A7 and B7 contain the positive (*B. pumilus*) and negative (*B. thuringiensis*) genomic DNA controls, respectively. Lane B6 contains alkaline protease gene fragment from *B. pumilus*, and lane A6 contains only TE buffer.

DNA hybridization

All hybridization assays require careful probe selection, optimization of hybridization conditions, and strict attention to quality control to achieve the sensitivity and specificity required for routine screening. Slot blot hybridization assays permit qualitative detection of the presence of a cellulase and alkaline protease gene in a DNA template using specific probes prepared from *Bacillus* species. Optimized hybridization parameters that gave a signal for the positive control (DNA template from *Bacillus subtilis*) and no hybridization for the negative control (DNA template from *Bacillus thurigiensis*) was used in the screening for target genes from unknown isolates. Out of the five samples positive for the cellulose plate assay, only three exhibited hybridization with the cellulase gene probe prepared from *B. subtilis* (Fig. 1). However, two other samples that were negative for the plate assay gave positive hybridization results. The possible explanations for this are: (1) cellulase gene is present but the expressed protein is not secreted; and (2) a gene with a different function is homologous to *B. subtilis* cellulase gene.

On the other hand, all isolates positive for the casein plate assay also showed positive results in slot blot hybridization using a fragment of an alkaline protease gene from *B. pumilus* as probe (Fig. 2). This suggests that the isolates possess an alkaline protease gene homologue. However, another isolate negative in the casein plate assay also demonstrated positive hybridization signal, suggesting that enzyme may be produced but not excreted to the medium.

Polymerase chain reaction

PCR involves multiple cycles of template denaturation, primer annealing, and primer elongation to amplify a specific gene that is present in the template DNA.

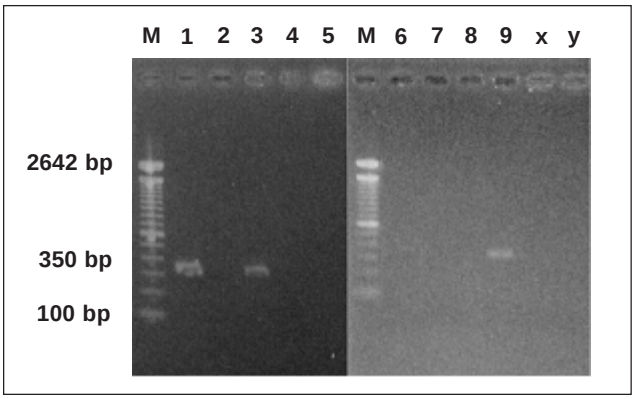

Fig. 3. *Amplified fragments of B. subtilis cellulase gene generated from DNA templates extracted using modified ROSE*. Lanes 1-8: 7MM-30, 1SA-23, 6MM-4, 3MA-11, 1SM-18, 1SM-9, 6MA-6, and 3SA-9, respectively. Lane 9 contains amplified cellulase gene fragment of *B. subtilis* (M4-11). Lane M contains the 100-bp DNA ladder. Lanes x and y contain the negative controls *B. thuringiensis* and PCR mix, respectively.

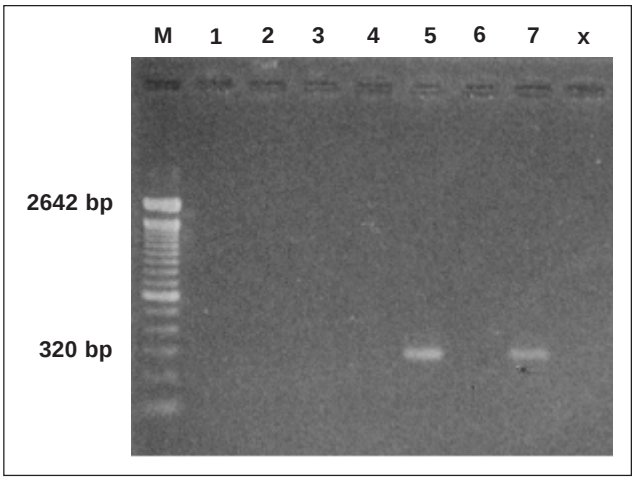

Fig. 4. *Amplified fragments of B. pumilus alkaline protease gene generated from DNA templates extracted using modified ROSE*. Lanes 1-6: 4MM-33, 5MA-14, 4SM-8, 5SA-17, 1SM-8, and 7MM-17, respectively. Lane 7 contains amplified alkaline protease gene fragment of *B. pumilus* (M3-8). Lane M contains the 100-bp DNA ladder (Invitrogen Life Technologies). Lane x contains the negative control (PCR mix).

Optimized parameters that produced the expected amplicon using a positive control (DNA template of *Bacillus subtilis* for cellulase and DNA from *Bacillus pumillus* for protesase) that carries the target gene, were used in PCR screening for target genes from unknown test isolates. Using DNA templates prepared by modified ROSE method, two isolates produced the expected 350 bp cellulase amplicon (Fig. 3). On the other hand, only one out of four isolates positive for casein plate assay produced the expected 320 bp alkaline protease gene fragment (Fig. 4).

Comparison of the different screening methods

Tables 1 and 2 show the summarized results for all screening methods for cellulase and alkaline protease, respectively. The number of samples that were positive for amplification of target gene was found to be less than those positive for DNA hybridization. This suggests that PCR is a more specific method in detecting target genes. PCR is largely dependent on the annealing of primers designed from *Bacillus* species. Under stringent conditions and in the absence of a specific DNA template sequence that is complementary to the primers used, no amplification of target genes will occur. However, it is possible that the samples may have homologous alkaline protease genes, and that the PCR primers were not able to anneal to their target sites due to some sequence variation.

Table 2. Summary of protein and DNA-based screening for alkaline protease performed on ten additional bacterial isolates.

Sample	Cellulose assay	Slot blot hybridization	PCR
3SA-13	-	-	Not tested
4MA-13	-	-	Not tested
7MM-12	-	-	Not tested
3MA-13	-	-	Not tested
1SM-8	+	+	+
4MM-33	+	+	-
5MA-14	+	+	-
4SM-8	-	+	-
5SA-17	+	+	-
7MM-17	-	-	-

The considerably less number of bacterial isolates that were positive for both DNA-based screening methods, as compared to the original number of samples positive for the plate assays, suggests that some isolates may possess cellulase and alkaline protease genes with significant sequence variation from those of the gene probes or primers which were based on *Bacillus* enzymes.

Table 3. Comparison of PCR and slot blot hybridization in terms of cost effectivity and labor intensiveness.

Screening procedure	Cost per sample (inclusive of a positive and a negative control)	Total time of screening (hour)	Number of samples analyzed per batch
PCR (using Perkin-Elmer GeneAmp PCR System 2400 thermocycler)	PhP 230.00	5	24
Slot blot hybridization	PhP 90.00	48	48

An ideal detection or screening system should be applicable to a vast majority of microbial strains, show good reproducibility over a long period of time, and yield consistent results. Moreover, the technique should be simple, inexpensive, and require less labor. Among the three screening procedures used, the traditional biochemical assay is the cheapest and easiest method to perform. It requires cheap and easily accessible reagents, and only basic microbiological and aseptic techniques are needed to culture microbial strains. However, biochemical assays do not specifically detect certain target enzymes.

DNA-based screening protocols may be more expensive and labor-intensive to perform as compared to biochemical assays, but they are more specific in detecting particular genes. Slot blot hybridization can be used to screen large numbers of DNA samples. A drawback for this method is the fact that several steps have to be performed before a result is obtained. Moreover, it is difficult to distinguish cross-hybridization of the probe to other genes since complementarity between the probe and the DNA template need not be 100% homologous for a signal to be produced. PCR, on the other hand, is a very sensitive method. However, the presence of even minute amount of contaminating DNA and inhibiting factors could affect PCR results. Moreover, PCR relies on an optimized PCR parameters and a properly designed pair of primers. Designing of primers specific for a particular gene requires prior knowledge of the gene's sequence.

As reflected on Table 3, slot blot hybridization is more labor intensive, but it is relatively cheaper than PCR and can analyze a large number of samples in one batch, thereby making it more favorable for use in routine screening.

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