Isolation of thermophilic bacteria (*Bacillus* AND *Ureibacillus*) and amplification of genes for selected enzymes

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Because catalyses often employ or produce high temperature environment, isolation of bacterial thermophiles that produce thermostable enzymes is important in industry. This study was focused on the isolation of bacteria that can grow at 55°C and higher, by using hot spring, mud spring, and solid oil sludge samples as sources of inocula added to Luria Bertini broth that were incubated at 55 to 70ºC with shaking (25 rpm). One isolate from the hot spring sample that grew at 60°C and another isolate from oil sludge sample that grew at 55ºC, were identified to belong to *Bacillus licheniformis*. An isolate from mud spring that grew at 55°C was identified to belong to *Bacillus subtilis*. Pure culture of two isolates from the oil sludge sample that could survive up to 60ºC exhibited 99% *16s rRNA* gene sequence similarity with two relatively new species, *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus*. The *apr* for alkaline protease was detected from the mud spring *B. licheniformis* while a fragment of a gene with 98% sequence similarity with the gene for Bacillopetidase F was amplified from the *B. licheniformis* isolated from oil sludge. PCR using gene-targeted primers and template DNA of the *Bacillus subtilis* isolate from mudspring, resulted in the amplification of expected size amplicons for a neutral protease, β-glycosidase, α-amylase and xylanase genes. Amplification of target gene fragments was confirmed by gene sequence analysis. Target amplicons were not generated from the *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus* isolates, suggesting the need for further studies using new or degenerate primers to amplify genes for enzymes with industrial applications. These relatively two new bacterial species that are not yet well studied, particularly at the molecular level, could be potential sources of important enzymes that allow the bacteria to survive and grow in extreme environments such as the oil sludge.

KEYWORDS

B. licheniformis, Ureibacillus, thermophile, *bprA,* Bacillopetidase

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INTRODUCTION

In several industrial processes, high temperature, pressure, and/or pH are either produced or employed in the bioconversion of raw materials to come up with the desired quality of product yield. To address this problem, thermostable enzymes that permit biocatalysis in hostile environments, including high temperatures (Haki and Rakshit 2003, Synowiecki 2010), are

sought for or developed. Some institutions ventured in protein engineering of existing mesophilic enzymes to produce thermostable ones (Clarkson et al 2001) but this approach is expensive and tedious. One approach is to isolate thermostable enzymes from thermophilic microorganisms that can survive temperatures from 55°C to 115°C (Haki and Rakshit 2003, Rakshit 2006).

In this study, thermophilic bacteria that could grow at 55 and 60°C were isolated from hot spring and mud spring of Laguna, as well as from solid oil sludge samples of an oil refinery in Manila. These environments are expected to support the presence of bacteria that can grow at high temperature. Bacteria that were isolated from these sources were used to screen for genes encoding selected enzymes that are useful in industry, such as xylanase, alkaline protease, neutral protease, α-amylase, and β-glucosidase.

The impact of xylanase lies on its capacity to hydrolyze xylan, the second most abundant polysaccharide in nature and the major hemicellulose present in a plant cell wall (Beg et al 2001, Prema 2006). Proteases hydrolyze peptide bonds in a protein molecule (Sandhya et al 2006) and its sale constitutes about 65% of the world-wide enzyme market (Haki and Rakshit 2003). Alpha-amylase, an enzyme that partially hydrolyzes starch polymers comprises the world's 30% of enzyme consumption. β-glucosidase is a cellulase and the enzyme is used in color extraction of juices, in detergents for color brightening and softening, in the pretreatment of cellulosic biomass and possibly in the production of biofuels.

Outputs from this study are expected to contribute to the goal of isolating genes for thermostable enzymes from thermophilic bacteria. Search for genes that code for thermostable enzymes was started by screening for thermophilic bacteria from different sources followed by the identification of the isolates using *16s rRNA* gene sequence analysis. DNA from bacterial isolates was then used in the PCR detection of putative genes encoding enzymes of interest, followed by sequence analysis of amplicons to confirm gene identity. The next step after this study is the isolation of the complete gene for selected industrially important enzymes. Availability of complete genes for these enzymes will pave the way for an ultimate goal of cloning and expression of genes and optimization of enzyme production in suitable bacterial hosts.

MATERIALS AND METHODS

Sample collection

Hot spring water and soil samples as well as semi solid mud spring samples were collected from a hot spring resort in Calamba, Laguna and Mt. Makiling (Laguna), respectively. Solid oil sludge samples collected from an oil refinery situated at Pandacan, Manila (Philippines), were kindly provided by Petron Philippines. The samples were stored in sterile Falcon tubes and polypropylene bags at room temperature until use.

Screening for thermophilic bacteria

Separate cultures were prepared using each of the hot spring, mud spring, and oil sludge samples. To prepare the source of inoculum, approximately 0.5 g of sample was added into 5 mL sterile distilled and deionized water. The mixture was vortexed for 2-3 minutes and allowed to stand still for 5 minutes after which 5 µL of the supernatant from each mixture was used as inoculum into sterile culture tubes containing 5 mL Luria-Bertani broth (1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v NaCl). A set of four broth cultures was prepared for each type of inoculum and one culture per set was incubated at 55, 60, 65, 68 and 70°C with shaking (225 rpm) for 18-24 hours using VS-8480SF water bath shaking incubator (Vision, Bucheon, South Korea). Negative control cultures were made by transferring 5 µL of sterile $ddH_2$to 5 mL of LB medium. The cultures were examined for growth of putative thermophiles by observing the presence of turbidity in the cultures compared to the negative control.

Isolation of pure culture

Broth cultures exhibiting turbidity were subjected to serial dilution and streak plating in LB agar plates (LB broth, 1.5% bacteriological agar). Plates were incubated at 37°C to 55°C overnight. Single bacterial colonies that grew were transferred to fresh LB agar plates and slants. Pure culture of each isolate was inoculated in LB broth to confirm the growth of each isolate at temperatures of 55°C or higher with shaking (225 rpm).

Genomic DNA extraction

Cells in the 5 mL LB broth culture were separated from the medium by centrifugation at 12000 rpm after which the cells were resuspended in 400 µL 1X TE buffer pH 8.0. The resulting bacterial suspension was processed for genomic DNA extraction using Maxwell® 16 Cell DNA Extraction Kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions.

PCR amplification of *16s rRNA gene*

The *16S rRNA* gene for each isolate was amplified using universal eubacterial forward and reverse primers (Weisberg et al 1990), fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′), respectively. Each 25µL PCR cocktail contained 1X Titanium® *Taq* buffer (Clontech, Mountain View, CA, USA), with 3.5 mM $MgCl_2$, 0.2 mM dNTP mix (Promega), 1.2 µM each of fD1 and rD1 primers, 0.2X Titanium® *Taq* polymerase (Clontech), 1.00 µL extracted

DNA template and 17.87 µL sddH_2. PCR conditions were optimized and PCR was conducted with the following conditions: 10 minutes of initial denaturation at 95°C, 30 cycles of denaturation at 95°C for 2 minutes, annealing at 42°C for 30 seconds and elongation at 72°C for 4 minutes, and a final elongation at 72°C for 20 minutes using Multigene Gradient PCR (Labnet, Edison, NJ USA).

Amplification of putative genes for selected enzymes

The primers used to amplify gene fragments of selected genes for enzymes are summarized in Table 1 and the conditions used for amplification of each gene are shown in Table 2.

The 10µL PCR cocktail contains 1X Titanium® *Taq* buffer (Clontech, Mountain View, CA, USA), with 3.5 mM $MgCl_2$, 0.2 mM dNTP mix (Promega), 0.5 µM of each forward and reverse primers, 0.25X Titanium® *Taq* polymerase (Clontech), 1.00 µL template and 6.5 µL sddH_2. PCR was carried out using Multigene Gradient PCR (Labnet, Edison, NJ USA) and the optimized PCR conditions for each enzyme (Table 2).

Sequence and BLAST analysis of amplicons

Amplicons were purified and submitted for sequencing to 1st BASE (BASE Life Sciences Holdings, Singapore). Sequences were analyzed using BLAST (BLAST; NCBI, www.ncbi.nlm.nih.gov).

Agarose gel electrophoresis and visualization of DNA

To visualize DNA after DNA extraction and PCR, the extracted genomic DNA and the reaction mixture after PCR were subjected to agarose gel electrophoresis (1% agarose) for 30 minutes at 100V. Genomic DNA or PCR amplicons were visualized under UV after ethidium bromide staining and photographed using Alpha DigiDoc Pro (Alpha Innotech, USA).

RESULTS

Isolation and identification of thermophiles

Pure culture of each of five thermophilic bacteria that exhibited growth at maximum temperatures of 55 or 60°C, were obtained from the screening protocols for thermo tolerance (Table 3). One isolate from the hot spring of Laguna grew at 60°C and was identified as *Bacillus licheniformis*. The isolate from the mud spring sample of Mt. Makiling (Laguna) was identified as *Bacillus subtilis* and grew at 55°. An isolate from the oil sludge sample that grew at 55°C belongs to *Bacillus licheniformis* while 2 other isolates from the same sample survived at 60°C and exhibited 99% *16s rRNA* gene sequence similarity with *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus.*

Amplification of gene fragments of target enzymes

PCR using DNA template from two *Bacillus licheniformis* isolates generated the expected size amplicon of about 650 and 700-bp using the alkaline protease-targeted primers (Fig.1).

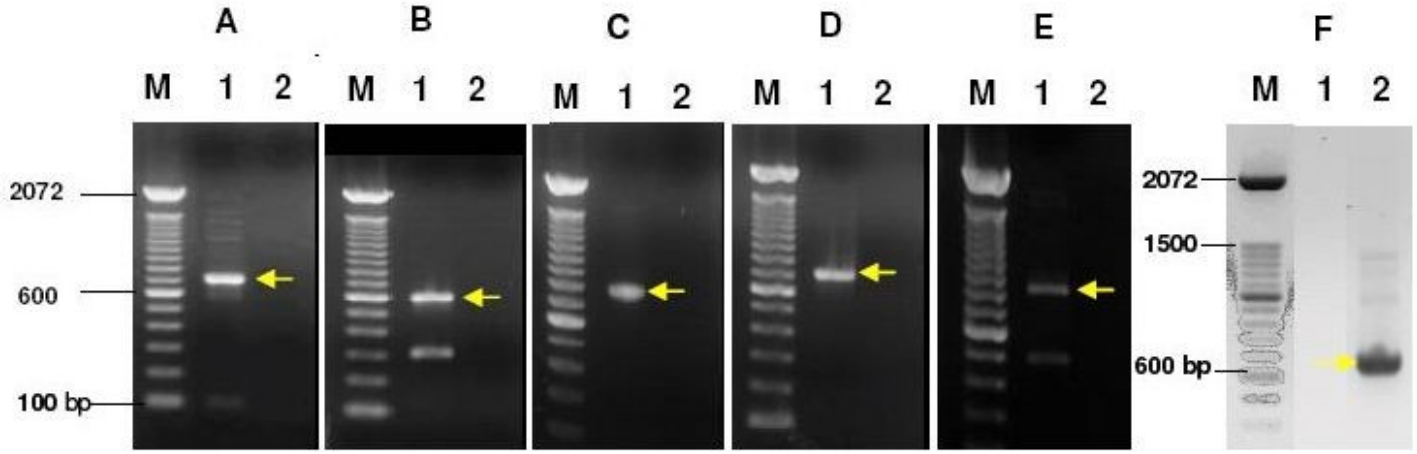

Figure 1. Amplicons (yellow arrow) of gene-targeted PCR. A (lane 1) is for PCR using DNA template from the *B. licheniformis* (from hot spring) and primers for alkaline protease gene. B-E (lane 1) is for PCR using DNA template of *B. subtilis* isolate (from mud spring) and primers for neutral protease, xylanase, -amylase, and -glycosidase genes, respectively. F (lane 2) is for PCR using DNA template of *B. licheniformis* (from the solid oil sludge) and primers for alkaline protease gene. The no template controls are in lane 2 of A-E and in lane 1 for F. M is the 100-bp Molecular Weight Marker (Invitrogen) for all gels.

Sequence analysis confirmed that the alkaline protease gene fragment was amplified in the *B. licheniformis* isolate from the hot spring. BLAST analysis of the amplicon sequence of the *B. licheniformis* isolate from the oil sludge sample, however, exhibited 98% sequence similarity with another enzyme Bacillopeptidase F (Fig.2). BLAST analysis revealed that the Bacillopeptidase F gene sequence, like the alkaline protease gene, exhibits sequence homology with the alkaline protease primers used. PCR using DNA template of the *Bacillus subtilis* isolate from mud spring of Mt. Makiling, generated expected size amplicons for four enzymes, neutral protease, xylanase, α-amylase, and β-glycosidase. The identity of the gene fragments confirmed the amplification of target gene fragments by DNA sequence analysis. PCR using all primer pairs targeting the different enzymes did not produce distinct bands for the two relatively new species that could grow in 60°C, *Ureibacillus suwonensis, Ureibacillus thermosphaericus* (data not shown).

DISCUSSION

Thermophiles are defined as microorganisms that could grow in temperatures within the range of 50°C and above (Rakshit 2006). Moderate thermophiles exist in environment with temperature range between 50°C and 80°C while hyperthermophiles can live above 80°C and are composed of microorganisms classified under the Archaea, with the exception of bacteria *Thermotoga maritima* and *Aquifex pyrophilus* (Rakshit 2006, Haki and Rakshit 2003). In general, environments considered to be conducive for thermophilic growth include geothermal site, terrestrial hot spring, continental and submarine volcanic areas and hydrothermal vents (Andrade et al 1999). In this study, samples that were used as inoculum for the isolation of moderate thermophilic bacteria were obtained from a hot spring in Laguna and mud spring in Mt. Makiling (an inactive volcano). In general, environments considered to be conducive for thermophilic growth include geothermal site, terrestrial hot spring, continental and submarine volcanic areas and hydrothermal vents (Andrade et al. 1999). Oil-contaminated soils of the desert were previously found to contain thermophilic

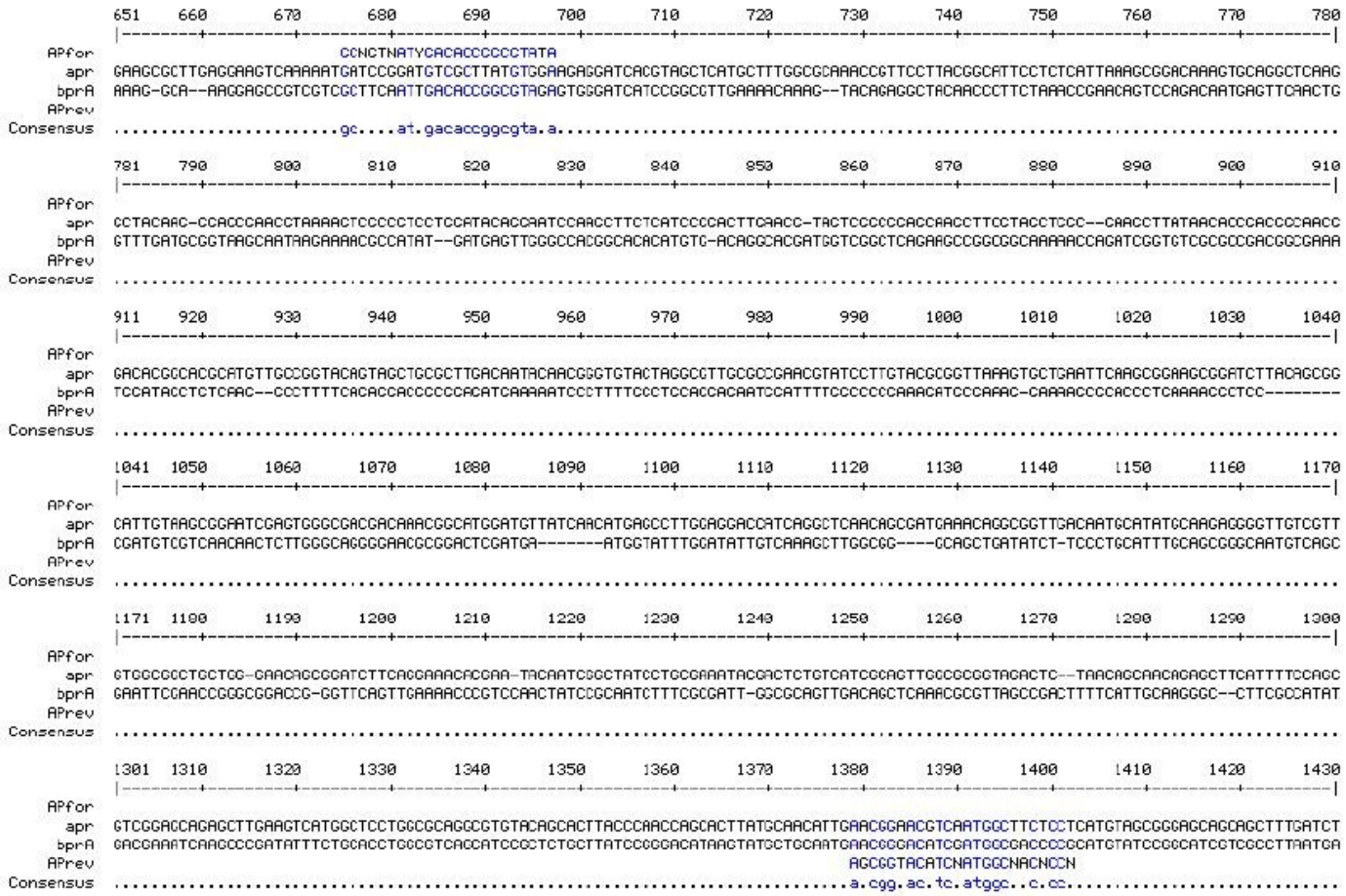

Figure 2. Sequence alignment of alkaline protease (*apr*) and bacillopeptidase F (*bprA*) genes. The attachment sites of forward (APfor) and reverse (APrev) alkaline protease primers are shown in blue.

bacteria such as *Bacillus stearothermophilus, Bacillus firmus, Bacillus sphaericus, Bacillus pallidus, Paenibacillus* sp., *Geobacillus* sp., Anoxybacillus sp., and *Saccharococcus thermophilus* (Margesin and Schinner 2001, Mohamed et al 2006). These earlier reports suggest the potentials of oil sludge sample to be used as an inoculum for the isolation of thermophilic bacteria. The presence of thermophilic bacteria in the oil sludge sample could be due to selection and survival of thermophiles when the petroleum samples were subjected to high temperature at certain points of refinery process.

Five thermophilic bacterial isolates that could grow at 55 and 60°C were obtained in this study, a *Bacillus licheniformis* from hot spring, *Bacillus subtilis* from mud spring, and three belonging to *Bacillus licheniformis*, *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus* from the solid oil sludge. The colonial morphology and cellular morphology (data not shown) of these isolates were consistent with the identification obtained using *16s rRNA* gene sequence analysis.

Three of the thermophiles isolated in this study belong to the genus *Bacillus*. One species, *Bacillus licheniformis,* is a thermophilic Gram positive rod reported with an optimum growth at 50°C but with a recorded tolerance to higher temperature (Whitaker et al 2005). The hot spring *B. licheniformis* isolate grew at 60°C while the oil sludge isolate could survive at 55°C. *Bacillus subtilis*, a well studied Gram positive rod, is a moderate thermophile. Commercial production of enzymes such as amylase, protease, pullulanase, chitinase, xylanase, lipase, among others, from *Bacillus subtilis,* represents about 60% of the commercially produced industrial enzymes (Morikawa 2000). In general, *Bacillus subtilis* is described to exhibit maximum growth at 35°C. The *B. subtilis* from mud spring in this study, however, could grow well at 55°C. The enzymes produced by this isolate are also expected to be thermostable at 55°C.

Two other bacteria isolated from oil sludge sample were found to belong to *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus* and both grew at 60°C. The bacteria exhibited colonies and cells that were Gram-negative rods (data not shown). Moreover, these have been reported to be unable to utilize sugar as carbon source (Kim et al 2006, Charbonneau et al 2010), which may explain why the bacteria were difficult to maintain in standard agar medium. Only *16s rRNA* gene and a few other gene sequences can be found in the NCBI database for both *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus*. A recent paper described the sequence analysis of the meso-diaminopimelate dehydrogenase gene from *Ureibacillus thermosphaericus* (Akita et al 2011). Recently, a draft genome sequence of a strain thermo-BF *Ureibacillus thermosphaericus,* from Ramsar hot springs in Iran (Abbasalizadeh et al 2012) was reported. These two new species of bacteria possess unknown yet numerous potentials in enzyme discovery.

Table 1. Primers used in enzyme gene-targeted PCR.

Enzyme	Primer	Primer Sequence (5'→3')	Source
Alkaline Protease	AP_F	GCNGTNATYGACACCGGCGTATA	Sacdalan DS (2007)
	AP_R	NGGNGTNGCCATNGATGTACCGCT	
Neutral Protease	NP_F	TTGTGCTTGAGACAAGCGTG	Agomaa AD (1999)
	NP_R	GCTTGTTGAAAGCACGACTG	
Alpha-amylase	Forward	5'-GGA-GGC-ATG-CAA-CGA-TGT-TTG-CAA-AAC-GAT-TC -3'	Ramsay et al (2006)
	Reverse	5'-GGG-TAC-CCG-CCG-GCA-TTT-TCT-TTC-GGT-AAG-TCC-CGTC- 3'	
β-glucosidase	F	TTTGCTGAAATGGG	Ohrmund S and Elrod S (2000)
	R	GGATCAATTTGCCANCCCC	
Xylanase	XynA/oli1	CTGAATTCGTGGTATTATACTGAAGG	Wolf et al (1995)
	XynA/oli2	CCTGATTAAGGAAGATCTGTTACC	

Table 2. Optimized PCR conditions for amplification of enzyme genes.

Enzyme		neutral protease	alkaline protease	xylanase	β-glucosidase	α-amylase
Initial denaturation		94°C (3 min)	94°C (5 min)	94°C (5 min)	94°C (10 min)	94°C (5 min)
30-35 cycles	denaturation	94°C (1.5 min)	94°C (1 min)	94°C (1.5 min)	94°C (30 sec)	94°C (1 min)
	annealing	50°C (1 min)	59°C (1 min)	58°C (1 min)	47-59°C (30 sec)	55°C (1 min)
	extension	72°C (1 min)	72°C (1 min)	72°C (2 min)	72°C (1 min)	72°C (3 min)
Final extension		72°C (3 min)	72°C (10 min)	72°C (5 min)	72°C (10 min)	72°C (10 min)

DNA from the three *Bacillus* isolates was used as template for gene-targeted PCR to detect the presence of alkaline and neutral proteases, xylanase, α-amylse, and β-glucosidase. Whereas, the expected alkaline protease gene was amplified from the hot spring *B. licheniformis*, the primer for alkaline protease amplified a gene fragment exhibiting 98% sequence similarity with Bacillopeptidase F. Sequence analysis of the amplicon revealed that the alkaline protease primers exhibit enough homology to anneal to the Bacillopeptidase F gene, resulting in the amplification of a non-target gene

fragment (Fig. 1F and 2). The Bacillopeptidase F gene that was detected, is also an industrially important enzyme that could be a subject of future work. The other target genes were not amplified in the *B. licheniformis* isolates. As expected, PCR using primers targeting the genes encoding neutral protease, xylanase, α-amylase, and β-glucosidase with DNA template from the *Bacillus subtilis* isolated from mud spring, produced the expected size amplicons (Table 3). Sequence analysis of the amplicons confirmed that fragments of the target genes for neutral protease, xylanase, α-amylase, and β-glucosidase were amplified. Additional information about these genes from the thermophiles used in this study awaits complete sequencing of genes.

Gene-targeted PCR did not produce distinct amplicons when using DNA template from the two relatively new species, *Ureibacillus suwonensis* and *Ureibacillus thermosphaericus.* This observation is consistent with the previous findings of studies that attempted to detect industrial hydrolytic exoenzymes such as esterase, amylase, xylanase, cellulase, pectinase, protease and laccase through biochemical analysis, that most isolates related to *U. suwonensis* possess nil activity for most enzymes tested (Charbonneau et al 2010, Hilden et al 2009). Furthermore, through biochemical means, the presence of an extracellular oxidase and tyrosinase and the absence of xylanase were confirmed (Vargas-Garcia et al 2006). It was also observed in earlier studies, that *U. thermosphaericus* has the capability to degrade toxic lignocellulose to degradation by-products important in the course of increased bioethanol production (Okuda et al 2008). This detoxification is achieved through the bacterium's laccase and lignin peroxidase which was biochemically determined to be produced by the bacterium (Okuda et al 2008, Vargas-Garcia et al 2006). These bacteria, therefore, could be promising and may be producing unknown yet novel enzymes important in industry. Because the bacteria are described to be unable to thrive on sugar as carbons source, these isolates could be producing enzymes that could degrade pollutants or recalcitrant chemicals as carbon source, consistent with their survival in the environment of the solid oil sludge.

CONCLUSION

Thermophilic bacteria were isolated using Luria Bertini broth and a simple selection procedure of growing the cells at elevated temperatures. The protocol may be modified in future studies to use a culture medium where sugar is not the sole carbon source in order to isolate bacteria which rely on other carbon sources for growth. Conduct of these procedures resulted in the isolation and identification of five moderate thermophiles belonging to the *Bacillus* and *Ureibacillus*. Gene-targeted PCR detected the presence of genes for proteases, xylanase, α-amylase and β-glucosidase from *Bacillus* isolates. The two relatively new species of thermophiles (*Ureibacillus suwonensis* and *Ureibacillus thermosphaericus*), isolated in this study, could be exploited further at the molecular level, as potential sources of genes for known and novel enzymes.

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NO CONFLICT OF INTEREST STATEMENT

There is no conflict of interest in the conduct of this study and the preparation of this manuscript

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Dr. Cynthia T. Hedreyda is the project leader who conceptualized the project, supervised the conduct of research and prepared the manuscript. Mr. John Jewish Dominguez and Ms. Karen Rosal performed literature search and conducted the experiments.

Table 3. Results of the tests on temperature tolerance and gene-targeted PCR of thermophilic bacteria from this study.

Source of inoculum	Maximum growth temperature	Identification using *16srRNA* gene	Gene fragment detected	Approximate size of target amplicon	Approximate Size of target gene (kb)
Hot spring	60°C	*Bacillus licheniformis*	alkaline protease	700-bp	1.329 (Mir Mohammad Sadeghi1 et al 2009)
Mud spring	55°C	*Bacillus subtilis*	neutral protease	600-bp	1.563 (Yang et al 1984)
			xylanase	700-bp	0.639 (Paice et al 1986)
			α-amylase	700-bp	1.531 (Emori and Maruo 1988)
			β-glycosidase	800-bp	1.83 (Le Coq et al 1995)
Solid oil sludge	55°C	*Bacillus licheniformis*	bacillopeptidase F	650-bp	1.509 (EMBL-EBI)
	60°C	*Ureibacillus suwonensis*	---	---	---
	60°C	*Ureibacillus thermosphaericus*	---	---	---

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