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# Species Identification of Thermo-tolerant *Bacillus* Isolates Using 16S rDNA, gyraseB Gene (*gyrB*) and Enzyme Gene Sequence Analysis

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**Twenty four thermo-tolerant *Bacillus* isolates that tested positive in preliminary enzyme plate assays were subjected to 16S rDNA sequence analysis, which revealed that identification results were not consistent with conventional biochemical identification in eighteen isolates. Identification inconsistencies were resolved in sixteen isolates by *gyrB* sequence analysis that gave single species identification, consistent with 16S rDNA sequence analysis. One isolate was identified as *B. subtilis* based on similar results from the conventional approach and 16S rDNA analysis. Ambiguous identification was observed in seven isolates with16S rDNA and *gyrB* sequences exhibiting 96-100% sequence identity with two or more closely related *Bacillus* species. Four isolates with ambiguous identification exhibited significant 16S rDNA and *gyrB* sequence identity with a group of *Bacillus* that includes *B. cereus, B. thuringiensis,* and *B. anthracis*. Each of three remaining isolates with ambiguous identification exhibited significant rDNA and/or *gyrB* sequence identity with a different group, a group of bacteria that includes *B. vietnamensis* and *B. aquimaris*, a group with *B. safensis* and *B. pumilus* and another with *B. methylotrophicus* and *B. amyloliquefaciens*. Enzyme gene-targeted polymerase chain reaction (PCR) amplified partial gene sequences of at least one of the enzymes protease, cellulase, amylase, and phytase in each of fourteen isolates. The enzyme genes exhibited 98-99% sequence identity with genes reported in the database for *Bacillus* species that matched the identification results. Additional phenotypic and molecular markers that could distinguish closely related *Bacillus* species are necessary to resolve ambiguous identification.**

Keywords: amylase, Bacillus, gyrB, protease, 16S rDNA, thermo-tolerant

## INTRODUCTION

Thermo-tolerance is an important characteristic that is often desired for bacterial strains used in industry. This is because during the bioprocessing of materials that makes use of bacteria (Haki and Rakshit 2003), increased temperature is either produced or used in the bioconversion of raw materials to come up with the desired quality and quantity of the products. High temperature during bioprocessing is one of the problems that need to be addressed when using microbial enzymes in industry. It is either the bacteria could not survive the increased temperature or the bacterial enzymes undergo protein denaturation with increased temperature (Pandey and Ramachandran 2006). To address this problem, it is necessary to isolate thermo-tolerant

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bacteria that could produce thermostable enzymes at temperatures 50° C and above (Haki and Rakshit 2003, Rakshit 2006). There is significant demand and high cost of microbial-based enzyme importation for local industrial processes. Use of bacteria that produce thermostable enzymes or isolation of thermostable enzymes from thermo-tolerant *Bacillus* has not been extensively explored in the Philippines.

This research made use of twenty four thermo-tolerant bacterial isolates that were previously isolated from different sources and are currently maintained in the Philippine National Collection of Microorganisms (Biotech UP Los Baños, College, Laguna). The bacteria were initially identified using conventional biochemical tests to belong to *Bacillus,* a group reported to include thermophiles (Kristajansson 1989). Preliminary evaluation revealed that these thermo-tolerant isolates produced one or more enzymes like protease, cellulase, amylase, and pectinase, suggesting that these bacteria could be potential sources of industrially important enzymes.

This study was conducted to confirm species identification of twenty four thermo-tolerant *Bacillus* isolates using 16S rDNA sequence analysis. Sequence analysis of *gyrB,* another universal and conserved gene, was also performed to address identification inconsistencies obtained from the conventional biochemical approach and the 16S rDNA sequence analysis. Enzyme gene-targeted PCR was performed using primers that target genes for industrially important enzymes (including cellulase, protease, amylase, and phytase) and DNA templates from the *Bacillus* isolates. BLAST analysis of putative enzyme gene amplicons provided the information on identity of enzyme gene. Moreover, BLAST analysis gave the bacterial species that possess the same enzyme gene with significant (97-100%) and highest sequence identity. The study identified enzyme producing thermo-tolerant *Bacillus* that may be used directly in industrial processes. Some of these enzyme producing bacterial isolates could be potential subjects of strain improvement research for increased enzyme activity or enzyme stability using new approaches such as directed evolution and protein engineering.

## MATERIALS AND METHODS

The bacterial isolates − derived from soil samples (planted with pili, ramie, and fig), "bagoong" (shrimp paste) from different areas in the Philippines and "tapuy" (rice wine) from Ifugao − were provided by the Philippine National Collection of Microorganisms (PNCM, Biotech, UP Los Baños).

### Isolation, growth, and maintenance of bacterial isolates

Pure culture of each isolate was grown in 5 mL Luria-Bertani (1% w/v tryptone, 0.5% w/v yeast extract, and 1% w/v NaCl) broth incubated at 37° C with shaking (225 rpm) using Vision shaking incubator (Vision, Bucheon, South Korea) or in LB agar slants (LB broth, 1.5% w/v agar).

### Screening for thermo-tolerant bacteria

Inoculum (20 µL) from overnight LB broth cultures (grown at 35° C) of each isolate was added to three replicates of LB broth in 40 mL Erlenmeyer flasks. The flask cultures were incubated at 50, 55, 60, and 65° C in a shaker water bath (Vision Scientific Co. Ltd., Kyoungi-Do, South Korea) for 24 h and 48 h. Presence of growth was determined by measuring turbidity using NanoDrop™ (Thermo Scientific, Wilmington, DE, USA) set at 600 nm following manufacturer's protocol.

### Protease, cellulase, amylase, and pectinase agar plate assay

Selected isolates were screened for protease, cellulase, amylase, and pectinase plate assay activity. Agar assay plates contain 1.0% gelatin and 1.0% casein, 0.5% carboxymethyl cellulose, 0.4% soluble starch and mineral salt agar with 0.2% citrus pectin, to test for protease, cellulase, amylase, and pectinase enzyme activity, respectively (Siddalingeshwara et al. 2010, Meddeb-Mouelhi et al. 2014, Deb et al.

2013, Rehman et al. 2015). Protease activity was evaluated by observing clearing zones surrounding the bacterial growth. Cellulase activity was determined by observing clearing zones surrounding the colonies after adding Congo red solution (1% w/v) for 15 min, followed by destaining with 1 M NaCl for the same duration of time. Amylase activity was visualized by observing clearing zones after adding Gram iodine. Pectinase activity was detected by observing clearing zones in plate growth culture after adding potassium iodide solution.

### Genomic DNA template preparation

Bacterial cells were obtained from 1.5 mL of overnight LB broth culture of each isolate. Cells were harvested by centrifugation (7,000 x g for 1 min) and re-suspended in 200 µL dH2O. The resulting bacterial suspension was subjected to genomic DNA extraction using ZR Fungal/ Bacterial DNA Miniprep™ (Zymo Research, Irvin, CA, USA) following the manufacturer's instructions. The genomic DNA was subjected to 1% agarose gel electrophoresis with in-gel 0.5 µg/ mL ethidium bromide stain using Labnet electrophoresis tank at 100V for 30 min. DNA was visualized under UV and genomic DNA concentration and purity were assessed using NanoDrop™ (Thermo Scientific, Wilmington, DE,

USA). Extracted genomic DNA was stored at -20° C until use in polymerase chain reaction (PCR).

**Identification of bacterial isolates**

Isolates were identified using morphological and physiological tests VITEK II ID System and VITEK MS (BioMérieux, Inc. Hazelwood. MO, USA) following manufacturer's protocols. Bacterial identification was also performed using 16S rDNA and *gyrB* sequence analysis. PCR was performed using extracted genomic DNA as template and universal primers for 16S rDNA and *gyrB* (Table 1). The following PCR profiles were used for 16S rDNA amplification: 5 min at 94º C; 30 cycles of 30 s at 94ºC, 30 s at 50º C, and 45 s at 72º C; and 7 min at 72º C. The following profiles were used for *gyrB* amplification: 5 min at 94º C; 30 cycles of 30 s at 94º C, 45 s at 59º C, and 45 s at 72º C; and 7 min at 72º C.

**Detection of genes for amylase, protease, cellulose, and phytase**

PCR conditions for gene-targeted primer pairs were optimized using the extracted genomic DNA as template and following optimized PCR profiles for each gene to be amplified. The following profiles were used for amplifying amylase gene: 5 min at 94º C; 30 cycles of 30 s at 94º C, 30 s at 58º C and 50º C for *amyE, amyS,* and *sAP* respectively, 30 s at 72º C; and 7 min at 72º C. Another primer set targeting the cellulase gene (*cel)* was used with the following profiles: 5 min at 94º C; 30 cycles of 30 s at 94º C, 30 s at 59º C, 30 s at 72º C; and 7 min at 72º C. The primer set to amplify the gene for phytase was used with 5 min at 95º C; 35 cycles of 1 min at 95º C; 1 min at 52º C, 1 min at 72º C; and 10 min at 72º C.

All amplicons (16S rDNA, *gyrB*, and enzyme genes) were sent to 1st BASE (BASE Life Sciences Holdings, Singapore) for sequencing. The sequence identity was obtained using Basic Local Alignment Search Tool (BLAST, Altschul et al. 1997). Sequences were aligned with matches from the database using Multalin (http://multalin.toulouse. inra.fr/multalin/).

## RESULTS

**Bacterial isolates**

The list of twenty four isolates used in this study, source of bacteria, results of preliminary enzyme plate assays, and the highest temperature that resulted to bacterial growth in LB broth (measured in terms of turbidity change using Nanodrop cell culture measurement), are summarized in Table 2. The isolates exhibited growth within the range of 50-65° C and produced one or more of the following enzymes: protease, cellulase, amylase, and pectinase in agar plate assays.

**Identification of thermo-tolerant *Bacillus***

All twenty four isolates used in this study were classified under the genus *Bacillus* using the conventional biochemical bacterial identification protocols. In eighteen of twenty four isolates studied, results of conventional

**Table 1**. PCR primers used in the study.

| Gene | | Primer sequence (5' to 3') | Source | Amplicon size (bp) |
|---|---|---|---|---|
| 16S rDNA | F | AGAGTTTGATCMTGGCTCAG | Marchesi et al. 1998 | 1,450 |
| | R | GGTTACCTTGTTACGACTT | | |
| gyraseB gene (*gyrB)* | F | GAAGTCATCATGACGTTCTGCA | Yamamoto & Harayama 1995 | 1,200 |
| | R | AGCAGGTACGGATGTGCAGCC | | |
| *amylase* gene (*amyE*) | F | AAAGAGAGTGGCGAGATGAGG | This study | 880 |
| | R | CAAACGTACCGCGTCGTAAA | | |
| *amylase* gene (*amyS*) | F | TGTGCAGCCGCTGAAGAATA | This study | 1,600 |
| | R | TCAATGGGGAAGAGAACCGC | | |
| *serine alkaline protease* gene (*sAP*) | F | AAYATGGAYGTNATHAAYATG | This study | 450 |
| | R | ARNCCYTTNCCRTARTARAA | | |
| *cellulase* gene (*Cel)* | F | AGAGCCAAAATGATGCGAAGG | This study | 1,500 |
| | R | TTCATCCACAACGCAAACCTC | | |
| *phytase* gene (*Phy*) | F | CTGTCTGATCCTTATCATTT | Bawane et al. 2011 | 1,059 |
| | R | TCCGCTTCTGTCGGTCA | | |
| neutral protease gene (*NP*) | F | TTGTGCTTGAGACAAGCGTG | This study | 660 |
| | R | GCTTGTTGAAAGCACGACTG | | |

**Table 2**. Results of preliminary enzyme plate assays and test for temperature tolerance on Bacillus isolates from different sources.

| Isolate code | Source of isolate | Enzyme detected by enzyme plate assay | Maximum temp tolerated (°C) |
|---|---|---|---|
| 1262 | *Ficus ulmifolia* roots from Taal volcano island | protease pectinase | 65 |
| 1268 | Fish sauce (bagoong) Capiz, Philippines | protease, amylase | 60 |
| 1269 | Fish sauce (bagoong) Lingayen, Philippines | protease | 65 |
| 1271 | Fish sauce (bagoong) Camarines Norte, Philippines | protease | 55 |
| 1272 | Fish sauce (bagoong) Lingayen, Philippines | amylase | 65 |
| 1277 | Rice wine (tapuy) from Ifugao, Philippines | protease, cellulase, amylase | 55 |
| 1279 | Rice wine (tapuy) from Ifugao, Philippines | cellulase, amylase protease | 65 |
| 1280 | Rice wine (tapuy) from Ifugao, Philippines | protease, cellulase | 65 |
| 1567 | Soil planted with ramie | pectinase | 60 |
| 1569 | Soil | cellulase | 65 |
| 1572 | Soil | amylase | 55 |
| 1573 | Soil | cellulase, amylase. protease | 55 |
| 1574 | Soil | cellulase | 55 |
| 1575 | Soil | amylase | 55 |
| 1576 | Soil | protease | 55 |
| 1056 | Soil | cellulase, protease, | 55 |
| 109*2* | Soil | cellulase | 55 |
| 10100 | Culture Collection | amylase | 55 |
| 10101 | Culture Collection | cellulase | 55 |
| 10102 | Hotspring in Laguna, Philippines | protease pectinase | 55 |
| 10103 | Hotspring in Laguna, Philippines | protease, amylase, cellulase | 55 |
| 10104 | Hotspring, in Laguna, Philippines | protease pectinase | 55 |
| 10105 | Pili kernel, Philippines | protease, amylase, cellulase | 65 |
| 1320 | Culture collection | amylase, protease | 55 |

bacterial identification at the species level were not consistent with the identification using the 16S rDNA sequence analysis (Table 3). Several isolates exhibited high 16S rDNA sequence identity with more than one closely related *Bacillus* species. The *gyrB* sequence analysis was used as an additional approach for species identification to help resolve identification inconsistencies, resulting in species identification consistent with identification results of 16S rDNA sequence analysis for sixteen isolates (Table 4). One isolate (1056) was identified as *Bacillus subtilis* based mainly on the conventional approach and 16S rDNA sequence and was not subjected to *gyrB* sequence analysis.

The BLAST results of 16S rDNA and *gyrB* from four (1268, 1272, 1279, and 1275) of seven isolates with ambiguous identification, exhibited highest sequence identity with same genes from *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. toyonensis.* One isolates with ambiguous identification (1576) exhibited highest 16S rDNA and *gyrB* sequence identity with a group that includes *B. safensis* and *B. pumilus.* The 16S rDNA from isolate 1269 matched genes from *B. vietnamensis* and *B. aquimaris* but its *gyrB* did not give significant match with the same species. Isolate 1573 gave 100% sequence identity of 16S rDNA and *gyrB* with genes from both *B. methylotrophicus* and *B. amyloliquefaciens*.

Additional data from enzyme gene-targeted PCR contributed confirmation of species identification in thirteen isolates that were identified based on consistent BLAST analysis results of 16S rDNA and *gyrB* gene sequences (Table 4). BLAST analysis results on enzyme gene sequences for each of 13 isolates revealed highest sequence identity with genes from *Bacillus* species consistent with species identification. Isolate 1056 that was identified as *B. subtilis* also has a phytase gene that exhibited highest (99%) sequence identity with phytase gene from *B. subtilis*. BLAST results of 16S rDNA and *gyrB* identified isolate 1573 to belong to *B. methylotrophicus* or *B. amyloloquefaciens* and genes for serine alkaline protease and cellulase from this isolate also exhibited highest sequence identity with these genes in these two species. Three of six isolates with ambiguous species identification (1268, 1272, and 1279) possess an alpha amylase gene that exhibit 100% sequence identity with amylase gene from *B. cereus*, one of the possible species identification suggested by 16S rDNA and *gyrB* sequence analysis.

The BLAST results on *gyrB* and enzyme gene sequences were crucial in resolving species identification ambiguities from 16S rDNA analysis in some isolates (Tables 3 and 4). In isolate 1262, top hits (with 100% sequence identity) based on 16S rDNA sequence are *B. stratosphericus* and *B. pumilus* but BLAST result of *gyrB* showed highest sequence identity (99%) with *gyrB* of *B. pumilus.* Serine alkaline protease gene from isolate 1262 also exhibits 98% sequence identity with genes from *B. pumilus.* Significant sequence identity of *gyrB* and protease gene of *B. pumilus* with genes from isolates 10102 and 10104 was also the major basis for classifying both isolates under *B. pumilus*. The 16S rDNA in isolate 1277

**Table 3**. Species identification of Bacillus isolates based on conventional approach and BLAST analysis of 16S and gyrB sequences.

| Isolate code | **Conventional Biochemical Approach** | **BLAST result of 16S rDN*A* sequence analysis (% sequence identity)** | **BLAST result of *gyrB* sequence analysis (% sequence identity)** |
|---|---|---|---|
| 1262 | *Paenibacillus polymyxa* | *B. stratosphericus* LSR9.1 (100%)<br>*B. pumilus* KU-BF1(100%)<br>*B. pumilus* NS-31 (100%) | *B. pumilus* TUAT (99%)<br>*B. pumilus* GR-8 (99%)<br>*B. pumilus* GLB197(99%) |
| 1268 | *Brevibacillus laterosporous* | *B. cereus* ASDS9 (99%)<br>*Bacillus sp.*CMJ37RA(99%)<br>*B. anthracis* (99%) | *B. cereus* FRI-35 (99%)<br>*B. cereus* Q1 (99%)<br>*B. thuringiensis* (99%) |
| 1269 | *Bacillus licheniformis* | *Bacillus sp* VCM5 (99%)<br>*B. vietnamensis* KJ-WC ( 99%)<br>*B. aquimaris* H063 (99%) | *B. amyloliquefaciens* (73%)<br>*B. subtilis* (73%)<br>*Bacillus sp* SDL1 (73%) |
| 1271 | *Bacillus licheniformis* | *B. pumilus* CSR12 (99%)<br>*B. pumilus* CSE 32 (99%) | *B. pumilus* (99%) |
| 1272 | *Bacillus sphaericus* | *B. anthracis* Ames(100%)<br>*B. cereus* ATCC 14579(99%)<br>*B. cereus* JCM 2152 (99%) | *B. cereus* F837/76 (100%)<br>*B. cereus* 03BB108 (99%)<br>*B. thuringiensis* HD571(99%) |
| 1277 | *Bacillus licheniformis* | *B. subtilis* ASC4 (97%)<br>*B. amyloliquefaciens* (97%)<br>*B. tequilensis* (97%) | *B. subtilis* Y98b (98%)<br>*B. vallismoris* (90%)<br>*B. axarquiensis* (90%) |
| 1279 | *Bacillus pumilus* | *B. thuringiensis* ATCC 10792 (99%)<br>*B. thuringiensis* IAM 12077 (99%)<br>*B. toyonensis* BCT-7112 (99%) | *B. thuringiensis* serovar *tohokuensis* (98%)<br>*B. cereus* (98%)<br>*B. toyonensis* (97%) |
| 1280 | *Bacillus pumilus* | *B. subtilis subsp. spizizenii* ATCC 6633 (100%)<br>*B. subtilis* JCM 1465(100%)<br>*B. subtilis* BRC 13719 (100%) | *B. subtilis* Y98b (100%)<br>*B. subtilis* subsp. *subtilis* RO-NN-1 (99%)<br>*B. subtilis* subsp. *subtilis* CU1050 (99%) |
| 1567 | *Bacillus sphaericus* | *B. sphaericus* (97%) | *B. sphaericus* (86%) |
| 1569 | *Bacillus licheniformis* | *B. clausii* DTM1 (99%)<br>*B. clausii* IHBB (99%)<br>*B. clausii* GB-B15 (99%) | *B. clausii* KSM-K16 (96%)<br>*B. clausii* ENTPRo (96%) |
| 1572 | *Bacillus pumilus* | *B. cereus* BRL-J (100%)<br>*B. cereus* IARI- SOB 15 (100%)<br>*Bacillus sp* IRAI-CFB 20 (100%) | *B. cereus* NC7401 (99%)<br>*Brevibacillus brevis* CQUBb (99%)<br>*B. cereus* CICC 10041 (99%) |
| 1573 | *Bacillus subtilis subsp. subtilis* | *B. amyloliquefaciens* ZYTC101 (100%)<br>*B. methylotrophicus* B25 (100%)<br>*B. amyloliquefaciens* BA100 (100%) | *B. methylotrophicus* B25 (100%)<br>*B. amyloliquefaciens* MBE1283 (100%)<br>*B. amyloliquefac*iens D15 (100%) |
| 1574 | *Bacillus subtilis subsp. subtilis* | *B. amyloliquefaciens* YW2 (99%)<br>*B. methylotrophicus* MER TA (99%) | *B. amyloliquefaciens* BGP20 (97%)<br>*B. subtilis* B-1 (97%) |
| 1575 | *Bacillus licheniformis* | *B. cereus* L12 (99%)<br>*B. anthracis* J4 (99%) | *B. cereus* H6 (98%)<br>*B. cereus* D17 (98%)<br>*B. thuringiensis* AlHakam (98%)<br>*B. anthracis* A16R (98%) |
| 1576 | *Bacillus sp* | *B. safensis* SL-40 (99%)<br>*B. safensis* KNUSC1009 (99%)<br>*B. safensis* 1-Si-2-5-7-T (99%)<br>*B. pumilus* ISE 24 (99%) | *B. safensis* VKM B-711 (99%)<br>*B. pumilus* FO-038 (99%) |
| 1056 | *Bacillus subtilis subsp. subtilis* | *B. subtilis* VT03 KC512905.1 (96%)<br>*B. subtilis* H10-5-5 (96%)<br>*B. subtilis* W1 (96%) | Not done |
| 1092 | *Bacillus thuringiensis* | *B. thuringiensis* R112-100 (99%)<br>*B. thuringiensis* R112-97 (99%) | *B. thuringiensis* serovar *israelensis* AM65-52 (99%)<br>*B. thuringiensis* HD1002 (99%) |
| 10100 | *Bacillus stearothermophilus* | *B. cereus* RSA21 (100%)<br>*B. anthracis* Larissa(100%)<br>*B. thuringiensis* MS8 (100%) | *B. cereus* F837/7 (98%)<br>*B. cereus* 03BB108 (98%)<br>*B. cereus* 03BB108 (98%) |
| 10101 | *Bacillus sp.* | *B. subtilis* HB-1 (99%)<br>*B. subtilis* B18 (99%)<br>*B. tequilensis* CGX5-1 (99%) | *B. subtilis* BY-2 (99%)<br>*B. subtilis BSn5* (99%)<br>*Bacillus sp. YP1* (99%) |

Table 3. continued next page . . . .

| | | | |
|---|---|---|---|
| 10102 | *Bacillus subtilis subsp subtilis* | *Bacillus sp. MT7* (99%)<br>*B. pumilus* NJ-V (99%)<br>*Bacillus sp.* BAB-*5881* (99%) | *Bacillus sp. R31 2014* (99%)<br>*B. pumilus* Klu7 (99%) |
| 10103 | *Bacillus subtilis subsp subtilis* | *B.subtilis* VITM 6 (97%)<br>*B.subtilis* H10-5-5 (97%)<br>*B.subtilis* 16-5G (97%) | *B.subtilis* E2 (98%) |
| 10104 | *Bacillus pumilus* | *B. pumilus* NJ-V (99%)<br>*Bacillus sp.* 7-3 (99%)<br>*B. altitudinis* W10 (99%) | *B. pumilus* GLB197 (99%) |
| 10105 | *Bacillus subtilis* | *B. methylotrophicus* HQB246 (100%)<br>*B. subtilis* RSS-1 (100%)<br>*B. amyloliquefaciens* HXD-5 (100%) | *B. amyloliquefaciens* h47 (99%)<br>*B. amyloliquefaciens plantarum* G4b (99%)<br>*B. amyloliquefaciens* NMSX4 (99%) |
| 1320 | *Bacillus licheniformis* | *Bacillus licheniformis* (99%) | *B. licheniformis BCRC 11702* (99%)<br>*B. licheniformis BCRC 14353* (99%)<br>*B. licheniformis K106c* (99%) |

**Table 4**. Suggested species identification based on BLAST analysis results on 16S rDNA, gyrB and enzyme genes.

| Isolate code | Identification based on 16 rDNA & gyrB gene sequence analysis (% sequence identity) | BLAST result of enzyme (% sequence identity) |
|---|---|---|
| 1262 | *Bacillus pumilus* 16S rDNA (99%) *gyrB* (99%) | *B. pumilus* alkaline serine protease (98%) |
| 1268 | *Bacillus cereus*<br>*Bacillus anthracis* 16S rDNA (99%)<br><br>*Bacillus cereus*<br>*Bacillus thuringiensis gyrB* (99%) | *B. cereus* alpha amylase (100%)<br>*B. thuringiensis* (98%) |
| 1269 | *Bacillus vietnamensis* or *Bacillus aquimaris*<br>16s rDNA (99%) *gyrB* /no conclusive data | No data for gene detection |
| 1271 | *Bacillus pumilus 16s rRNA* (94%) *gyrB* (99%) | *B. pumilus* serine alkaline protease (99%) |
| 1272 | *Bacillus cereus* 16S rDNA (99%) *gyrB* (100%)<br>*Bacillus anthracis*_16S rDNA (100%) *gyrB* none<br>*Bacillus thuringienis* 16S rDNA none *gyrB* (99%) | *B. cereus* alpha amylase (100%) |
| 1277 | *Bacillus subtilis* 16S rDNA (97%) *gyrB* (98%) | *B. subtilis* serine alkaline protease (97%)<br>*B. subtilis* cellulase (98%)<br>*B. subtilis* neutral protease (99%) |
| 1279 | *Bacillus thuringiensis* 16S rDNA (98%) *gyrB (*98%)<br>or *Bacillus cereus* 16S rDNA (97%) *gyrB (*98%) | *B. cereus* alpha amylase (100%) |
| 1280 | *Bacillus subtilis* 16S rDNA (99%) *gyrB* (98%) | *B. subtilis* neutral protease (100%)<br>*B. subtilis* endo-1,4-beta-glucanase (99%) |
| 1567 | *Bacillus sphaericus* 16S rDNA (97%) *gyrB* (86%) | gene detection, no data |
| 1569 | *Bacillus clausii* 16S rDNA (99%) gyrB (96%) | gene detection, no data |
| 1572 | *Bacillus cereus* 16S rDNA (100%) *gyrB* (99%) | *B. cereus* alpha-amylase (100%)<br>*B. cereus* neutral protease (99%) |
| 1573 | *Bacillus methylotrophicus*<br>*Bacillus amyloliquefaciens* 16S rDNA (100%) *gyrB* (100%) | *B. methylotrophicus* endoglucanase (99%)<br>*B. methylotrophicus* serine alkaline protease (100%)<br>*B. amyloliquefaciens* serine protease (98%)<br>*B. amyloliquefaciens* cellulase (98%) |
| 1574 | *Bacillus amyloliquefaciens* 16S rDNA (99%) *gyrB* (97%) | gene detection, no data |
| 1575 | *Bacillus cereus*<br>*Bacillus anthracis* 16S rDNA (99%) *gyrB* (98%) | gene detection, no data |
| 1576 | Bacillus safensis 16S rDNA (99%) *gyrB* (99%)<br><br>*Bacillus pumilus* 16S rDNA (99%) *gyrB* (99%) | gene detection, no data |
| 1056 | *Bacillus subtilis* VT03 16S rDNA (99%) *gyrB* / no data | *B. subtilis* phytase gene (99%) |
| 1092 | *Bacillus thuringiensis* 16S rDNA (99%) *gyrB* (98%) | gene detection, no data |

Table 4. continued next page . . . .

| | | |
|---|---|---|
| 10100 | *Bacillus cereus* 16S rDNA (100%) *gyrB* (98%) | *B. cereus* alpha amylase (100%) |
| 10101 | Bacillus subtilis 16S rDNA (99%) *gyrB* (99%) | *B. subtilis* protease (99%)<br>*B. subtilis* endo-beta-1,4-glucanase (95%) |
| 10102 | *Bacillus pumilus* 16S rDNA (99%) *gyrB (*99%) | *B. pumilus* serine alkaline protease (99%) |
| 10103 | *Bacillus subtilis* 16S rDNA (97%) *gyrB* (98%) | *B. subtilis* cellulase gene (99%) |
| 10104 | *Bacillus pumilus* 16S rDNA (99%) *gyrB* (99%) | *B. pumilus* serine alkaline protease (98%) |
| 10105 | *Bacillus amyloliquefaciens* 16S rDNA (100%) *gyrB (*99%) | cellulase, protease, amylase assay (+)<br>*B. amyloliquefaciens* endoglucanase gene (98%)<br>*B. amyloliquefaciens* alkaline serine protease (100%) |
| 1320 | *Bacillus licheniformis* 16S rDNA (100%) *gyrB (*99%) | *B. licheniformis strain* CICIM B102 alkaline protease (99%) |

matched two other species in addition to *B. subtilis*, but BLAST analysis of *gyrB* and genes for enzymes serine alkaline protease, neutral protease and cellulase exhibited significant sequence identity with genes from *B. subtilis*. The 16S rDNA of isolate 10100 matched three different species but *gyrB* and alpha amylase gene matched *B. cereus* with 98 and 100% sequence identity, respectively. Isolate10105 exhibited ambiguous identification based on 16SrDNA but gave single species *B. amyloliquifaciens* identification using *gyrB.* Sequences of endoglucanase and serine alkaline protease genes of isolate 10105 also exhibited highest sequence identity with the same genes from *B. amyloliquifaciens*.

## DISCUSSION

Several studies have reported the isolation of thermotolerant bacteria that belong to the genus *Bacillus* from thermophilic and mesophilic environments (de Souza et al. 2001, Beffa et al. 1996, Huang et al. 1998). In this study, bacteria that were isolated from different sources (Table 2) and initially identified to belong to the genus *Bacillus* using conventional approach, were provided by the Philippine National Collection of Microorganisms (PNCM, Biotech UP Los Baños). Use of these *Bacillus* isolates was advantageous since pure cultures were available, thus eliminating the tedious procedures of sample collection, isolation and preliminary identification.

All isolates used in this study exhibited growth at 50° C or higher, consistent with the reports that many species under the genus *Bacillus* are thermo-tolerant (Raincy et al. 1994, Marteinsson et al. 1996, Aanniz et al. 2015). Genes for protease, cellulase, amylase, and pectinase were amplified from several isolates and it is expected that these thermo-tolerant bacteria also posses temperature stable enzymes for possible industrial applications. When the preliminary identification of the isolates based on conventional approach was verified using 16S rDNA sequence analysis, eighteen of twenty four isolates were observed to exhibit species identity different from the results of conventional approach (Table 3). One major objective of this study, therefore, is to address inconsistencies in species identification of the *Bacillus* isolates. Accurate identification would pave the way to gaining relevant prior knowledge available in the literature about each isolate, including the industrially significant enzymes the species can produce.

It is common to experience conflicting species identification or ambiguous results between conventional biochemical tests and the molecular approach using 16S rDNA sequence analysis especially with genus containing very closely related species like *Bacillus* (Berthier and Ehrlich 1998, Almeida and Araujo 2013). The *gyrB*, a gene for gyrase B enzyme (subunit B protein of DNA gyrase or topoisomerase type II), is another molecular marker used in bacterial identification because like the 16S rDNA, *gyrB* is universal and highly conserved. In certain cases, because *gyrB* molecular evolution rate is higher than that of 16S rDN*A* (Yamamoto and Haryama 1995), *gyrB* sequence analysis could be more discriminatory for some closely related species. In this study, *gyrB* sequence analysis was performed and BLAST results of *gyrB* sequences contributed to the resolution of identification inconsistencies for sixteen isolates.

Ambiguity in bacterial species identification is encountered if the bacteria belong to closely related species that possess 16S rDNA and *gyrB* sequences exhibiting significant sequence identity. Ambiguity was observed in seven isolates. For isolate 1573, the molecular approaches were not able to provide a single species identification because 100% sequence identity in both 16S rDNA and *gyrB* was observed with genes from *B. mehylotrophicus* and *B. amyloliquefaciens*. Even genes for serine alkaline protease and endoglucanase in isolate 1573 exhibited significant sequence identity with the same genes in both species. This observation is expected and is consistent with previous reports that genome comparison could not distinguish type strain *B. amyloliquefaciens* subsp. *plantaraum* from type strain *B. methylotrophicus* (Dunlap

et al. 2015). Ambiguity in identification could only be addressed by additional phenotypic and molecular markers that will be useful in distinguishing closely related bacterial species.

The results of enzyme gene-targeted PCR were able to show which isolates have genes for industrially important enzymes protease, cellulase, amylase, and phytase (Table 4). Enzyme gene sequence analysis is not a routine procedure for species identification in bacteria but BLAST analysis of enzyme gene amplicons did not only identify the enzyme genes, but also provided the database information on what bacterial species possess genes with significant sequence identity. It is expected that the genes for the same enzyme produced by different bacterial species exhibit a few to several nucleotide sequence variations that may or may not result in corresponding amino acid variation of the protein. Genes that have no obvious evolutionary relatedness might have short segments that are similar to one another but with overall low percentage of similar nucleotide sequences (Brown 2002), while genes from the same species are expected to exhibit high sequence identity. BLAST results in this study revealed that out of several genes reported in the database on a specific enzyme gene, the amplified genes from the isolates exhibited highest sequence identity with genes from the species consistent with identification results in 14 isolates. Additional enzyme gene sequence detection and analysis from the remaining ten isolates may also provide results consistent with species identification. In three isolates with ambiguous species identification, an alpha amylase gene that exhibit 100% sequence identity with amylase gene from *B. cereus*, was present. *B. cereus* is one of the suggested species identification based on 16S rDNA and *gyrB* sequence analysis. Comparison of alpha amylase gene sequences from other close relatives of *B. cereus* may be valuable to determine if alpha amylase gene sequence variation could help resolve identification ambiguity.

This study shows that a combination of conventional biochemical tests and molecular approaches (e.g., 16S rDNA*, gyrB)* and enzyme gene sequence analyses, are valuable in bacterial species identification. Inconsistencies of identification results from conventional biochemical tests and molecular approaches are expected in very closely related species and ambiguity in bacterial species identification may also be encountered if the bacteria that belong to closely related species that possess 16S rDNA and *gyrB* sequences exhibiting significant sequence identity with two or more species. Resolution of identification ambiguities awaits discovery of additional phenotypic and/or molecular markers that could help distinguish closely related species. Members of closely related species may be distinguished from one another in terms of presence or absence of an enzyme gene. If the different species posses the same enzyme genes, presence of significant nucleotide variation in specific enzyme genes among different species may contribute in distinguishing closely related bacterial species. Enzyme gene sequence analysis data in 14 isolates evaluated in this study pointed out to the same species obtained from the standard identification protocols using 16S rDNA and *gyrB* sequence analysis. Use of enzyme gene sequence analysis − as an additional procedure to verify identification results − merits further evaluation.

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