International Food Research Journal 17: 45-53 (2010)

Highly thermostable extracellular lipase-producing *Bacillus* strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing

Akanbi, T. O., Kamaruzaman, A. L., Abu Bakar, F., Sheikh Abdul Hamid, N., Radu, S., Abdul Manap, M. Y. and *Saari, N.

Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract: The activities of lipase produced by five lipases-producing thermophilic bacteria strains (SY1, SY5, SY6, SY7 and SY9) isolated from Selayang Hot Spring in the western part of Peninsular Malaysia were analyzed and compared. SY7 and SY9 had considerably higher lipolytic activities than those of SY1, SY5 and SY6. Thermostabilities of lipase produced by all strains were determined after heating at 80°C for 30 minutes. Strain SY7 retained the highest lipolytic activity of 77%, while others had infinitesimally low thermostability (retaining less than 34% of their original activity) at the same temperature and time. SY7 was chosen for further characterization because it showed exceptionally high lipase activity and thermostability. It was identified as belonging to Bacillus species by the conventional Gram-staining technique, Biochemical tests and Biolog Microstation system. By using 16S rRNA gene sequencing, strain SY7 generated the same expected PCR product with molecular weight of 1500 base pair. It displayed 98% sequence similarity to Bacillus cereus strain J-1 16S rRNA gene partial sequence with accession number: AY305275 and has been deposited in the database of Genbank.

Keywords: Thermostable, lipase, 16S rRNA, *Bacillus* strain

Introduction

Lipases can be naturally and readily discovered from the earth's flora and fauna. However, most commercial lipases are produced from microbes (Jaeger *et al*., 1994). Schmidt *et al*. (1994), Luisa *et al.* (1997) and Limpon *et al.* (2007) reported on several *Bacillus* spp. as the main sources of lipolytic enzymes. Lipase-producing bacteria have been found in diverse habitats such as soil contaminated with oil, dairies, industrial wastes, oilseeds and decaying food (Sztajer *et al*., 1988), compost heaps, coal tips and hot springs (Wang *et al*., 1995).

Lelie *et al.* (2005) reported that microbial lipases have been immensely used for biotechnological applications in dairy, detergents and textile industries, production of surfactants and oil processing industries. It has also been widely used in pharmaceutical industries in the production of enantiometrically pure pharmaceuticals, since they have a number of unique characteristics coupled with distinct substrate specificity namely regio-specificity and chiral selectivity (Kim et. al., 1998).

A large number of beneficial thermophiles which produced lipases with good thermal stabilities have been found in diverse habitats (Wang *et al*., 1995). Their thermal stabilities, particularly in the absence of appreciable amounts of water, increases their attractiveness industrially (Nirupama *et al.,* 1998). Thermostable lipases have a lot of applications in the processing of food, domestic, industrial wastes, cosmetic, detergents and pharmaceutical industries (Ghosh *et al.* 1996; Saxena *et al*. 1999).

In this study, we report selection of *Bacillus cereus* isolated from a Malaysian hotspring, among others as a thermostable lipase-producing bacteria and its identification based on more reliable identification systems of 16S rRNA analysis in order to proceed for further confirmation of its identity.

*Corresponding author.
Email: *nazamid@putra.upm.edu.my*
Tel.: +603-89468371; Fax: +603-89463552

Materials and Methods

Isolation and screening of lipase-producing bacteria

Hot water samples with temperature between 50 and 60°C and pH ranging from 7.68 to 8.05 were collected from Selayang Hot Spring, Malaysia. In the laboratory, water samples were diluted with 1% sterilized peptone water and then plated onto Rhodamine B-olive oil agar plates (Kouker and Jaeger, 1986). The medium contained the following per litre: nutrient agar, 28 g; sodium chloride, 4 g; Rhodamine B, 10 mg; and 31 ml olive oil. pH of the medium was adjusted to pH 7.0 by addition of sodium hydroxide before it was autoclaved. Inoculated plates were incubated for 48 h at a moderately high temperature of 45°C capable of facilitating colonial growth of bacteria present in the hot water samples. Positive lipase-producers can be distinguished by the presence of orange fluorescent halos surrounding the colonies present on the plates observed under UV rays (Major Science, UV Transilluminator, Taiwan).

Culture conditions for thermophilic lipase production

The positive isolates were incubated in quadruplicate plates of tryptone soy broth for 48 h at 45°C. The culture aliquots were then chilled rapidly at 4°C and centrifuged at 6000 rpm for 15 min at 4°C to rupture the intact cell. The supernatant obtained were filtered through a Whatman No.1 filter paper. The crude lipase solution was obtained by filtering through a Millipore 0.22 µm filter membrane and concentrated to 10-fold by ultrafiltration using a Millipore PLGC UF membrane with a nominal exclusion limit of 10 kD at 4°C under 2.0 bar of nitrogen pressure. The extract was then assayed for lipase activity.

Determination of lipase activity

Lipase activity was measured by titrating free fatty acids released by hydrolysis of olive oil using the titration method (Lee and Rhee, 1993). Olive oil substrate emulsion contained 10% (w/v) olive oil, 10% (w/v) Arabic gum, 0.5 M sodium chloride and 20 mM calcium chloride was blended for 2 min at the maximum speed in a waring blender. 20 ml substrate was mixed with 2 ml of the lipase enzyme and incubated in a reciprocal shaker water bath at 125 rpm for 30 min at 30°C. The lipase solution for the positive control was boiled in a water bath for 10 min before addition of the reaction mixture. The reaction was terminated by adding 10 ml ethanol: acetone (1:1) and titrated with 0.02 N sodium hydroxide until the end point was reached with phenolphthalein (0.1%) as an indicator. One unit of lipase activity (U) was defined as the release of 1 µmol of fatty acid per min under the conditions above. The amount of fatty acid liberated and lipase activity were calculated.

Comparison of thermostability of lipase

Thermostability of lipase was determined by incubating each lipase for 30 minutes at 40, 50, 60, 70 and 80°C. The lipases were chilled rapidly at 4°C and the remaining activity was measured as described above.

Morphological characterization

The five isolates (SY1, SY5, SY6, SY7 and SY9) were categorized on the basis of their morphological characteristics. Colony characteristic was observed after cultivation on nutrient plate agar for 48 hours. The cellular morphology was determined by bright field microscopy of a Gram-stained preparation (Johnson and Case, 1986).

Biolog Microstation system

Each isolate was tested in the BIOLOG® system. These test panels provide a standardized micro method utilizing 95 different carbon sources for the identification of a broad range of Gram-negative and Gram-positive bacteria, respectively. The only preliminary test required to differentiate between Gram positive-rod spore forming bacteria (GP-ROD SB) from Gram positive-coccus (GP-COCCUS) or Gram positive-rod (GP-ROD) bacteria was Gram-staining. To differentiate between Gram negative-non-enteric (GN-NENT) bacteria from Gram negative-enteric (GN-ENT), preliminary oxidase and triple sugar iron agar tests were carried out. The standard BIOLOG® procedure was used, and the pattern of carbon substrate utilization was observed at 4 to 6 hours and 24 hours. The MicroPlates were read either visually or with the Biolog MicroStation or OmniLog System and compared to the database. Any reading from the top left well (A-1) was subtracted from values on the other 95 wells, because it did not contain a substrate.

Conventional biochemical test methods

The taxonomical characteristics of the bacteria were determined by conventional biochemical test methods (Sneath *et. al.,* 1986; Holt *et. al.,* 1994; Osterhout *et. al.,* 1998; MacFaddin 1980, 2000)

DNA preparation and PCR amplification

DNA was extracted from SY7 cultures by a modification of the procedure described by El-Helow (2001). After the physiological test of the isolates' growth through various range of temperatures and

pH, 2 ml of an overnight culture was harvested by centrifugation and resuspended in 180 µl of 20 mM Tris-Cl (pH 8.0), 2 mM EDTA and 1.2% Triton X-100. A total of 20 mg ml^{-1} lysozyme was added and the samples incubated at 37°C for 30 min and continued in accordance to the manufacturer's instructions with DNeasy Tissue Kit (Qiagen, UK). The resulting supernatant containing the DNA extract was transferred to a sterile microcentrifuge tube, quantified by spectrophotometer readings at A_{260} and A_{280} and stored at -20°C.

Each genome of DNA used as a template was amplified by PCR with the aid of the primer sets of F95 and R15 for the 16S rRNA gene. All the PCR products were confirmed by a second PCR reaction using the internal primers F684, F114 and R704 that were designed in the DNA sequences of the first PCR product. The PCR mixture contained 100 ng template DNA, 2.5 U *Taq* polymerase (BIOTOOLS Labs), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM of dNTP mixture, 1.5 mM $MgCl_2$, 0.5 µM (each) of the primer, and made up to 100 µl with sterile distilled water. The PCR protocol consisted of denaturation at 94°C for 1 min and subsequent 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 73°C for 30 s. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in a 1.2% agarose gel with 1X TBE buffer and 1 kb DNA ladder (BIORON) as the molecular marker, followed by ethidium bromide staining. The remaining PCR products for sequencing were purified with a QIAquick PCR Purification kit (Qiagen, UK) as recommended by manufacturer.

16S rRNA sequencing and data analysis

Sequence analysis was performed on a 1500-bp PCR product using five different primers (F95, R15, F684, F114 and R704) for sequencing reactions. The primers were synthesized and supplied by Invitrogen Life Technologies (Carlsbad, California). The sequences analysis was performed using the ABI PRISM 3100 DNA Sequencer and ABI PRISM BigDye Terminator Cycle Sequencing (Perkin Elmer, Massachusetts, USA). The 16S rRNA sequences determined in this study were aligned and compared with other 16S rRNA genes in the GenBank by using the NCBI Basic Local Alignment Search Tools, nucleotide (BLASTn) program (http://www. ncbi. nlm.nih.gov/BLAST/). The 16S rRNA gene sequence described in this paper has been deposited into the Genbank Data Library and has been assigned the accession number AY305275.

Results and Discussion

Screening of lipase producing bacteria

Five bacterial strains were found to be positive as lipase-producers, as indicated by the presence of orange fluorescent halos around the colonies, when the Rhodamine B agar plates were observed under UV rays. The fluorescence observed was due to the hydrolyzed substrate reacting with Rhodamine B. Hou and Johnston (1992) suggested that the formation of fluorescent was due to Rhodamine B dimers complexed with mono- or diglycerides and fatty acid.

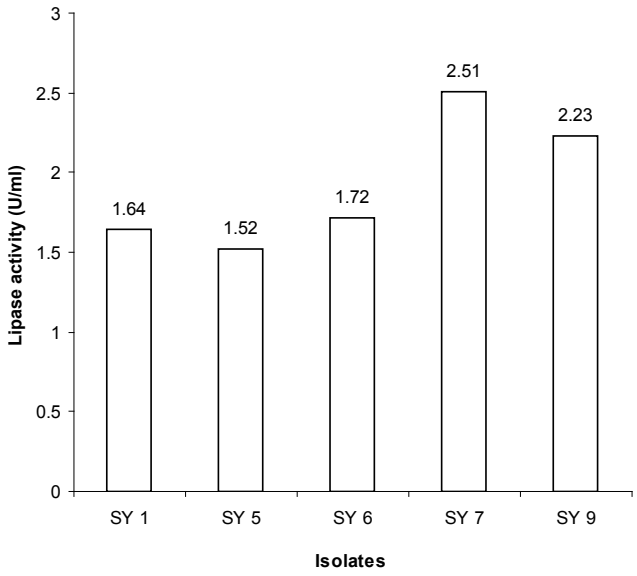

Figure 1. Lipolytic activities of positive lipase-producing thermophilic bacteria isolated from Selayang Hot spring, Malaysia

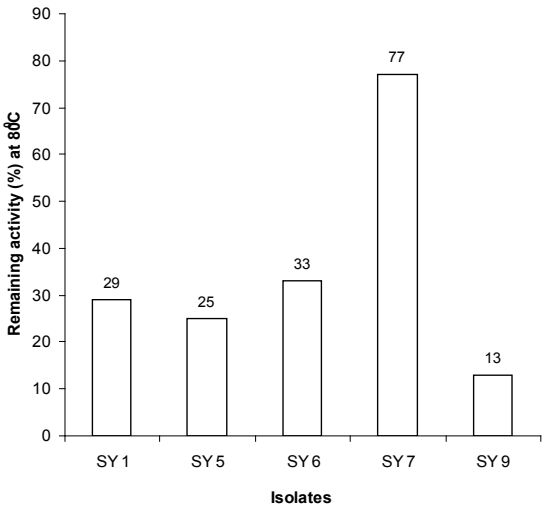

Figure 2: Thermostability of Lipases from lipase-roducing thermophilic bacteria isolated from Selayang Hot spring

Comparative lipolytic activity of positive bacterial lipase producer

Figure 1 shows that isolate SY7 exhibited the highest lipolytic activity of 2.51 U/ml followed by isolate SY9 with an activity of 2.23 U/mL. Isolates SY1, SY5 and SY6 showed activities less than 2.0 U/mL. Labuschagne *et al.* (1997) observed that the *Flavobacterium odoratum* lipase exhibited maximum activity of 6.5 U/ml at higher temperatures in the range of 50 to 65°C. However, the reported maximum lipase activity of *Pseudomonas putida* 3SK was found to be between 35 and 40°C, and this activity sharply decreased after incubation at 45°C (Lee and Rhee, 1993).

Thermostability

Extracellular lipases extracted from selected isolates were incubated at 80°C for 30 minutes for the thermostability test. Figure 2 shows the results of the thermostability test for all the isolates. Isolate SY7 produced the most thermostable lipase with 77% of its original activity retained at 80°C, while others retained less than 34% of their original activities. The thermal stability of lipase produced by SY7 at elevated temperature is similar to those reported elsewhere (Janssen *et al.,* 1994; Wang *et al.,* 1995; El-shafei and Reskallah, 1997; Kim *et al.,* 1998; Lee *et al.,* 1999).

However, the poor thermal stability of lipases from other isolates is similar to that reported by Nishio et al. (1987). He confirmed some bacterial lipases' ability to retain about 25% of their activities after incubation at above 70°C. Thus, SY7 was chosen for further characterization, because it showed exceptionally high lipase activity and thermostability.

Colony morphology, Gram- reaction and microscopy

Cellular morphology of SY7 was studied using bright field microscopy of the Gram-stained preparation. The strain was tested and observed to be Gram-positive with short rods, raised umbonate, beige colour and have ellipsoidal endospores at the terminal end. An array of Gram- positive lipase producing bacteria have been screened and identified El-Helow *et al.,* 2001; Nazimah *et al.,* 2003; Bora *et al.,* 2007). An isolated lipase-producing rod-shaped bacterium with ellipsoidal endospores at the terminus of the swollen cells was reported by Sneath *et al.* (1986). Dong-Woo *et al.* (1999) also isolated a rod-shaped lipase-producing thermophilic bacterium from an Indonesia hotspring.

Physiological tests

From Table 1, SY7 was able to grow well throughout the incubation temperature regime. Nazimah *et al.* (2003) reported many lipase producing bacteria isolated from hotspring having the abilities to grow in a broad range of temperature. However, strain SY7 had no growth at pH 5, indicating that lower pH do not favour its growth. It had normal growth up to pH 9 and weaker growth at pH 10. Reports have shown that many lipase producing bacteria grow optimally at pH values less than 10, while few can grow at pH values below 5 (Brado *et al.,* 1999; Kulkarni *et al.,* 2002; Bayoumi *et al.,* 2007).

Biochemical test

Results of biochemical tests carried out at 35, 37 or 42°C and 45°C are presented in Table 2. These temperatures were chosen because microbial growths are common at those temperatures. With reference to the Bergey's Manual of Determinative Bacteriology (Holt *et. al.,* 1994) and Biochemical Test for Identification of Medical Bacteria, the biochemical characteristics of strains SY7 were compared to biochemical characteristics of *Bacillus halodurans* (Horikoshi, 1991). *Bacillus halodurans*, is able to grow in alkaline medium up to pH 10 and at high temperature such as 55°C. These two characteristics of *Bacillus halodurans* were similar to strain SY7. However, from the results obtained, only 63% of the biochemical characteristics of SY7 were similar to *Bacillus halodurans*. According to MacFaddin (2000), microorganisms often give conflicting results because of mutation or of the type of media used for isolation, cultivation, identification and maintenance. Therefore, further biochemical tests were carried out to confirm the identity of the bacteria.

Table 1. Effect of temperature and pH on the growth behavior of SY7

Parameters	Growth response
Temperature (°C)	
25	+
30	+
35	+
40	+
45	+
50	+
55	+
60	+
pH	
5.0	-
6.0	+
7.0	+
8.0	+
9.0	+
10.0	$+^{w}$

Note: (+) positive results; (-) negative results; and (^{w}) weak

Table 2. Results of biochemical tests for the Gram-positive rods bacteria at 35, 37 or 42°C and 45°C

Biochemical tests	Results at	
	35, 37 and 42°C	**45°C**
Hemolysis 5% blood agar		
Carbohydrate fermentation:		
Arabinose	+ve	+ve
Glucose	+ve	+ve
Lactose	+ve	+ve
Mannitol	+ve	+ve
Xylose	-ve	-ve
Adonitol	+ve	+ve
Galactose	+ve	+ve
Inositol	+ve	+ve
Sorbitol	+ve	+ve
Cellobiose	+ve	+ve
Glycerol	-ve	-ve
Citrate (Simmons)	-ve	-ve
Esculin	-ve	-ve
Gelatin liquefaction	-ve	-ve
Indole	-ve	-ve
Motility	+ve	-ve
Nitrate reduction	+ve	+ve
Oxidation-fermentation glucose	Inert	Inert
Phenylalanine deamination	-ve	-ve
Growth in:		
5.0% Sodium chloride	-ve	-ve
7.5% Sodium chloride	+ve	+ve
Starch hydrolysis	-ve	-ve
Urease production	+ve	+ve
Voges-Proskauer	-ve	-ve
Catalase	+ve	+ve
Methyl Red	-ve	-ve
Denitrification	-ve	-ve

Table 3. Identification and Confirmation of Bacillus genus lipase-producing thermophilic bacteria strain SY 7

Techniques	Identification	
	SY 7	Similarity Index
Phenotypic		
Biochemical tests	*Bacillus halodurans*	63%
Biolog Microstation	*Bacillus halodurans*	0.540
Genotypic		
16SrRNA	*Bacillus cereus*	98%

Biolog Microstation system

Isolate SY7 was identified using the Biolog Microlog GP2 microplate. Biolog identified SY7 as *Bacillus halodurans* with a similarity index of 0.540 (Table 3). It has been reported in the Biolog's technical literature that *Bacillus* species are difficult to identify because they can give 'false-positive' reactions (Tang *et al.,* 1998). A number of possible causes have been proposed, which include sporulation, utilization of lysed cell material, and utilization of stored endogenous substrate or extracellular polysaccharides. The problem of 'false-positives' requires further study. Therefore, precise confirmation of the strain was done using 16s rRNA gene sequencing technique.

16S rRNA sequence analysis of lipase-producing thermophilic bacteria isolates

Genomic DNA of SY7 was purified, and the 16S rRNA gene was amplified using F95 and R15 primers. The fragment obtained was sequenced and the results were aligned against the 16S rRNA sequences using the NCBI's BLASTn program. The partial sequence analysis for the 1411 bases of 16S rRNA gene of the isolate SY7 was determined and compared with those of reference *Bacillus* sp. The nucleotide sequence of the 16S rRNA gene of SY7 strain was determined to confirm the species designation and the taxonomic positioning. Figure 3 shows the sequence comparison of strain SY7 and *Bacillus cereus*. The 16S rRNA gene sequence homology between strain SY7 and *Bacillus cereus* strain J-1 was high (98%). In addition, other closely related species belonging to cereus group as recognized by Gene Bank blast search have similarity indexes in the range of 96 – 98%. Therefore, strain SY7 belonged to Bacillus cereus (Table 3)

Conclusion

It is significant in this study that a full taxonomic study of strain SY7 helped to establish its position relative to the genera Bacillus. In this study, the 16S rRNA gene sequence was found to provide frequent phylogenetically useful information. Direct sequence determination of 16S rRNA gene fragments represents a highly accurate and versatile method for identification of bacteria to the species level. Therefore, the identification of lipase-producing bacteria should not only rely on phenotypic methods, but should be confirmed by the beneficial genotypic techniques such as 16S rRNA sequence analysis.

Acknowledgements

We appreciate the Malaysian Ministry of Science, Technology and Innovation for the financial support awarded to Professor Dr. Nazamid Saari under IRPA Project No. 01-02-04-0514.

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SY 7        CCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGG  60
SY 7        ACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    ACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGG  120
SY 7        GCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    GCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGT  180
SY 7        CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCA  240
SY 7        ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGA  300
SY 7        CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC  360
SY 7        GCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    GCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTG  420
SY 7        CTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG  480
SY 7        CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCG  540
SY 7        CGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT  600
SY 7        GGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTACCGATGAAAT  660
            |||||||||||||||||||||||||||||||||||||||||||||||||| || |||||||
AY305275    GGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAAT  660
SY 7        GCGTACAGATATGGAGGAACACCCGTGGCGAAGGCGACCTTCTGGTCTGTAACTGACACT  720
            ||||| ||||||||||||||| ||||||||||||||| ||||||||||||||||||||||
AY305275    GCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACT  720
SY 7        GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC  780
SY 7        GATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCA  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    GATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCA  840
SY 7        CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCAC  900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCAC  900
SY 7        AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA  960
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA  960
SY 7        TCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCAT  1020
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    TCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCAT  1020
SY 7        GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT  1080
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT  1080
SY 7        GATCTTATTTGCCATCATTCACTTGGGCACTCTAAAGTGACTGGCGCTTACAAACCGGAG  1140
            ||||||| |||||||||||| | |||||||||||||| ||||||| || | ||||||||||||
AY305275    GATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG  1140
SY 7        GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGTTCC  1200
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| | |
AY305275    GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC  1200
SY 7        AATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATTTCATAAAACCGTTTT  1260
            |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||| |
AY305275    AATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCT  1260
SY 7        CAGTTCGGATTGTAGGCTGCAACTCGCCTTCATGAAGCTGGAATCGTTAGTAATCGCGGA  1320
            |||||||||||||||||||||||||||||| |||||||||||||||| ||||||||||||||
AY305275    CAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGA  1320
SY 7        TCACCATGCCGCGGTGAATACGTTCCCGGCCCTTGTACACACCGCCCGTCACACCACGAG  1380
            ||| |||||||||||||||||||||||| |||||||||||||||||||||||||||||||
AY305275    TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAG  1380
SY 7        AGTTTGTACCACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGA  1440
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
AY305275    AGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGA  1440
SY 7        CAGTGGATTGGGGCGAAGTCGTAACAAGGTAGCCCGAAT  1479
            |||  |||||||| ||||||||||||||||||||||||||
AY305275    CAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCCGAAT  1479
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Figure 3. 16S ribosomal RNA gene sequence alignment comparison of *Bacillus cereus* (SY 7) as subject compared to the published sequence of *Bacillus* cereus strain J-1 16S rRNA gene partial sequence (AY305275) with 1479 nucleotide

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