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# ISOLATION AND MOLECULAR IDENTIFICATION OF OBLIGATE THERMOPHILES FROM HOT SPRINGS IN BA RIA – VUNG TAU AND KHANH HOA PROVINCES, VIETNAM

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SUMMARY

Environments with temperatures from 50°C to 80°C are rare in nature and are almost exclusively associated with geothermal regions including hot springs, solar-heated soils and volcanic areas. Thermophilic bacteria already exist and prefer in such habitats. Since innate tolerance to thermal environment and potential chassis for extracellular enzymes such as lipase, protease and amylase, which are utilized widely in the industrial fermentation, thermophilic bacteria have been becoming one of the objects for microbiologists worldwide, recently. This study aimed to isolate and identify thermophilic bacteria from hot springs in several provinces in Vietnam such as Ba Ria - Vung Tau and Khanh Hoa. In the results, six moderate thermophilic bacterial strains (namely BM7, BS5, NS1, NS3, NS4, and NW6) that could grow at 55ºC were purified from the hot spring ecosystems. All micro morphology of isolates were recorded as rod-shaped, Gram positive, and endospore forming. The results of 16S rDNA sequencing and phylogenetic analysis showed that these isolate belonged to group I of *Bacillus* genus (the thermophilic group). The isolated strains NS1, NS3, NS4, BS5, NW6 and BM7 were identified to belong to the *Bacillus* genus, species as *Bacillus* sp. Resulting strains are potential candidates for industrial applications due to its stable fitness in a hash environment, particularly at high temperature. In addition, this study provides a useful insight into the diverse community of thermophilic bacteria (*Bacillus* spp.) in several hot springs of Vietnam, that can be applied as bacterial cell factories to produce biomaterials, biofuels, or valuable compounds in the future.

**Keywords:**16S rDNA, *Bacillus* sp., hot spring, thermophilic bacteria, phylogenetic

## INTRODUCTION

Thermophilic bacteria are able to survive and grow at high temperature (45ºC - 80ºC). Geothermal ecologies including hot springs, solar-heated soils and volcanic areas contain extremely poor nutrition. However, such thermophilic habitats are rich concentration of trace elements and natural gases ($H_2S$, $H_2$, $CH_4$ and $CO_2$) (Anna-Louise *et al*., 2001). The diversity of microbes in hot environments is composed of heterophilic bacteria, cyanobacteria, methanogen, and chemolithoautotrophic bacteria (Satyanarayana *et al*., 2013).

Previous studies demonstrated that hot springs are reservoirs of thermophilic bacteria (Stöhr *et al*., 2002; PinzónMartínez *et al*., 2010). These strains are aerobic, rod shape, Gram-positive, forming spore bacteria and linked closely to *Geobacillus* sp., *Anoxybacillus* sp. and *Aeribacillus* sp., *Brevibacillus thermoruber*, *Paenibacillus* sp. and *Bacillus licheniformis* (Stohr *et al*., 2001; Martinez *et al*., 2010; Verma *et al*., 2014). Besides high temperature,

thermophilic bacteria are able to grow in the highly acidic environments (pH = 0 – 3) or the alkaline environments (pH = 10 – 12) (Anna-Louise *et al*., 2001) and produce extracellular thermophilic enzymes such as lipase, protease and amylase (Sharma *et al*., 2002). Other enzymes including cellulase, xylanase, chitinase and keratinase also were extracted from these strains to apply in the industrial production (paper induction, animal feed, biofuel) (Takayanagi *et al*., 1991; Tantimavanich *et al*., 1998; Kojima *et al*., 2006; Akanbi *et al*., 2010; Joo *et al*., 2011; Kumar *et al*., 2013). In addition, Morya and others (2018) reported that a thermo-tolerant bacterium strain *Bacillus* sp. ISTVK1 isolated from waste water treatment system can use glycerol as a sole carbon source to produce polyhydroxyalkanoate (PHA). Although Vietnam is one of the countries possessing high biological diversity in Asia, the research on thermophiles is still rare. The thermophilic bacterium species *Geobacillus caldoxylosilyticus* was isolated from sedimental sludge of My Lam hot spring in Tuyen Quang province, Vietnam (Tran Dinh Man *et al*., 2012). Furthermore, this strain became promising candidate in industry due to its capability of producing thermostable enzymes such as cellulase and amylase (Tran Dinh Man *et al*., 2012).

Natural stream in Truong Xuan hot spring (M' Dung village, Ninh Hoa, Khanh Hoa) was bubbled from the vein in the rock with temperature ranging from 37ºC to 67ºC. The pH was recorded in the range of 7.7 - 8.0 indicating alkaline environment. Binh Chau hot spring (Binh Chau commune, Xuyen Moc, Ba Ria – Vung Tau) is the largest hot spring (more than 1 $km^2$) in Vietnam. The temperature of water in the veins ranged from 43ºC to 65ºC with many air bubbles, and smell hydrogen sulfide ($H_2S$). Similarly, the pH was recorded in the range of 7.8 - 9.2 indicating alkaline environment. The temperature of the sampling site is unstable, normally, the temperature at the sampling sites was lower than that at the veins. This study aimed to isolate, identify thermophilic bacteria from Truong Xuan hot spring and Binh Chau hot spring in Vietnam. Results from this study are a preliminary step to apply thermophilic microorganism and their bioproducts in biotechnology. Indeed, thermophilic tolerance is one of the key factors that enables isolated strains become valuable host cells for producing chemicals, drugs, or polymers in industrial biotechnology.

## MATERIALS AND METHODS

### Sample collection

Soil, muddy, and water samples collected at Truong Xuan hot spring (12º31'20"N, 108º59'00"E, Ninh Hoa, Khanh Hoa), and Binh Chau hot spring (10º36'21"N, 107º33'29"E, Xuyen Moc, Vung Tau). In the morning, a total of 24 samples were randomly collected from different sites of off flow and stored in 500 mL sterile containers. Each sample was replicated at least 3 times. Samples were immediately brought into the laboratory and analyzed within 24 h. The samples (soil, muddy, and water) were collected separately in the vacuum flask, transported to laboratory and analyzed within 24 h.

### Isolation of thermophilic bacteria

Water and soil samples were inoculated in 10 mL of mineral salt basic (MSB) medium supplemented with 6 $gL^{-1}$ of yeast extract and incubated at 50ºC and 180 rpm in a thermal incubator for 24 h. The MSB medium used for the growth of strain TH-1 consisted of $(NH_4)_2SO_4$ 2.0 $gL^{-1}$, $KH_2PO_4$ 1.0 $gL^{-1}$, $K_2HPO_4$ 2.0 $gL^{-1}$, NaCl 0.5 $gL^{-1}$, $FeSO_4 \cdot 7H_2O$ 1.1 $mgL^{-1}$, $CaCl_2$ 30.0 $mgL^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.5 $gL^{-1}$ and trace element solution, pH 7.0 (Nguyen Huu Tri *et al*., 2017).

Cultured broths were then diluted down to concentration $10^{-5}$ and spread on MSB with agar 3% (w/v). A five tenfold serial dilution was performed, and then spread on MSBA plates (MSB medium supplemented with agar 3% (w/w)) and incubated at 50ºC for 72 h. Then, the isolates were classified by colony morphology and cellular characteristics including size, form,

color, margin, and elevation. Single colonies growing on plates were transferred into freshly prepared MSBA slants and kept at 4°C for further studies.

**Evaluation of thermal tolerance of thermophilic isolates**

In order to determine the optimal temperature for the growth of isolated thermophilic microorganisms, each isolate was inoculated in 5 mL of MSBY medium (pH 7) in a test tube in range of temperature from 45°C to 80°C, shaken at 180 revolutions per minute (rpm) for 12 h. Then, the optimum pH value was examined between 6 and 9 at the optimal temperature. The pH value of media was adjusted by using NaOH 1M. The microorganism growth was determined at 3 h intervals by measuring the optical density (OD) of the cultures at 540 nm and streaked onto freshly prepared MSBA plate. The mean value $OD_{540}$ of triplicates for each experiment was analyzed by using Microsoft Excel 2013 software. The high thermo-tolerance isolates were selected for further experiments.

**Genomic DNA extraction and 16S rDNA amplification**

DNA genome of the isolates was extracted and purified using phenol/chloroform method (Sambrook *et al*., 2006). Bacterial 16S rDNA was amplified from the extracted genomic DNA by polymerase chain reaction (PCR) using the following universal primer set: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane D J, 1991; Nguyen Huu Tri *et al*., 2011). The thermal cycles were performed with an initial denaturation step at 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2 min with a final extension at 72°C for 10 min. The PCR products were electrophorezed on 1% agarose gel.

**Analysis of the 16S rDNA sequence and phylogenetic tree**

PCR products were purified using QIAGEN PCR Purification KIT (QIAGEN, Inc.) and then sequenced by First Base Company, Singapore. The 16S rDNA sequences (~ 1,6 kb) were analyzed using Chromas Pro 1.34 software and BLAST on The National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). The phylogenetic tree was constructed by Lasergene 7.0 software.

## RESULTS AND DISCUSSION

**Isolation of thermophilic bacteria**

Thirty-three isolates that could grow at 50°C were isolated from 24 soil, muddy, and water samples from two hot springs in Khanh Hoa (16 isolates) and Ba Ria – Vung Tau (17 isolates) provinces. Among 33 isolates 11 isolates was obtained from soil (33.3%), 8 isolates from muddy (24.3%), and 14 isolates from water (42.4%) samples (Table 1-Supplementary). Most of isolates are Gram-positive and rod-shaped bacteria while other are Gram-negative and cocci bacteria. The colonies were appeared in various colors (beige, white, yellow, or pink) including 7 isolates were beige-colored, 11 were white, 14 were yellow, and 1 was pink on MSBA medium. The diversity of colonial morphology of isolated microorganisms were classified and presented in the previous article (Tran Mong Kha *et al*., 2018).

Regarding thermal tolerance, isolated strains were incubated at 55°C for 72 h. Consequently, the highest $OD_{540}$ values of six isolates including BM7, BS5, NS1, NS3, NS4, and NW6 were recorded from 0.4 to 0.6 that were significantly higher than the others. Thus by, these six isolates were selected for genome extraction and molecular identification by 16S rDNA sequencing.

**Examination of condition for thermophilic isolates growth**

In order to select the suitable temperature and pH for microorganism growth, the isolates were cultivated at temperature range from 45 to 80°C and pH range from 6 to 9. The result was shown detail in Table 4 - Supplementary. The aim of this study to isolate the moderate thermophilic microorganisms that were capable of growing from

50°C, therefore the intended study temperature range was 45, 50, 55, 60, 65, 70, 75, 80°C. However, the growth of isolated microorganisms is very weak when the temperature was over 55°C. That is reason why the isolated strains were evaluated from 50 to 55°C. At pH 9 the growth of microorganisms could not be observed then the data was not shown.

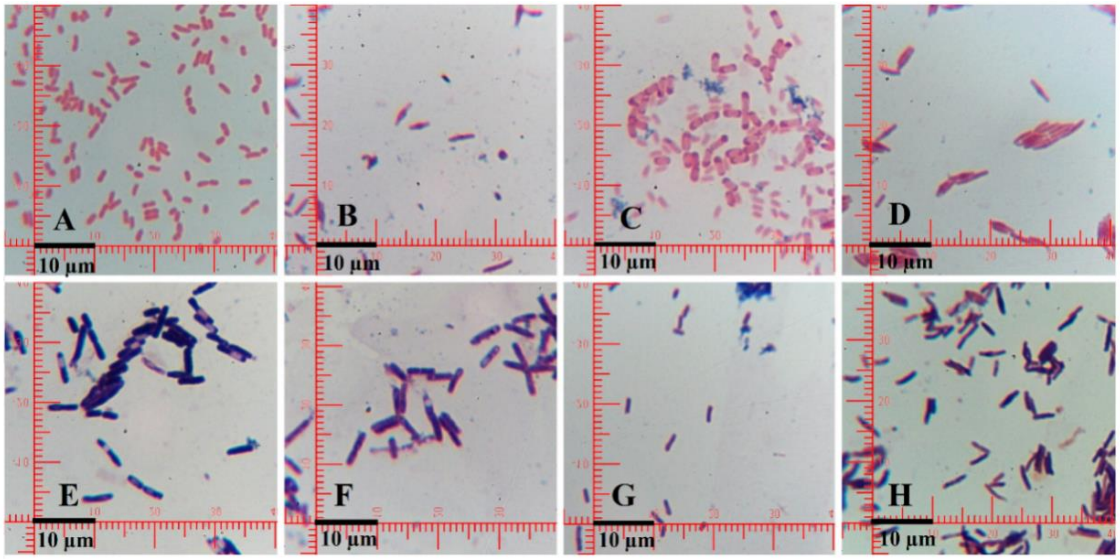


**Figure 1**. Gram stain of the isolates under microscope observation (magnificent 1000X). Bar, 10µm.

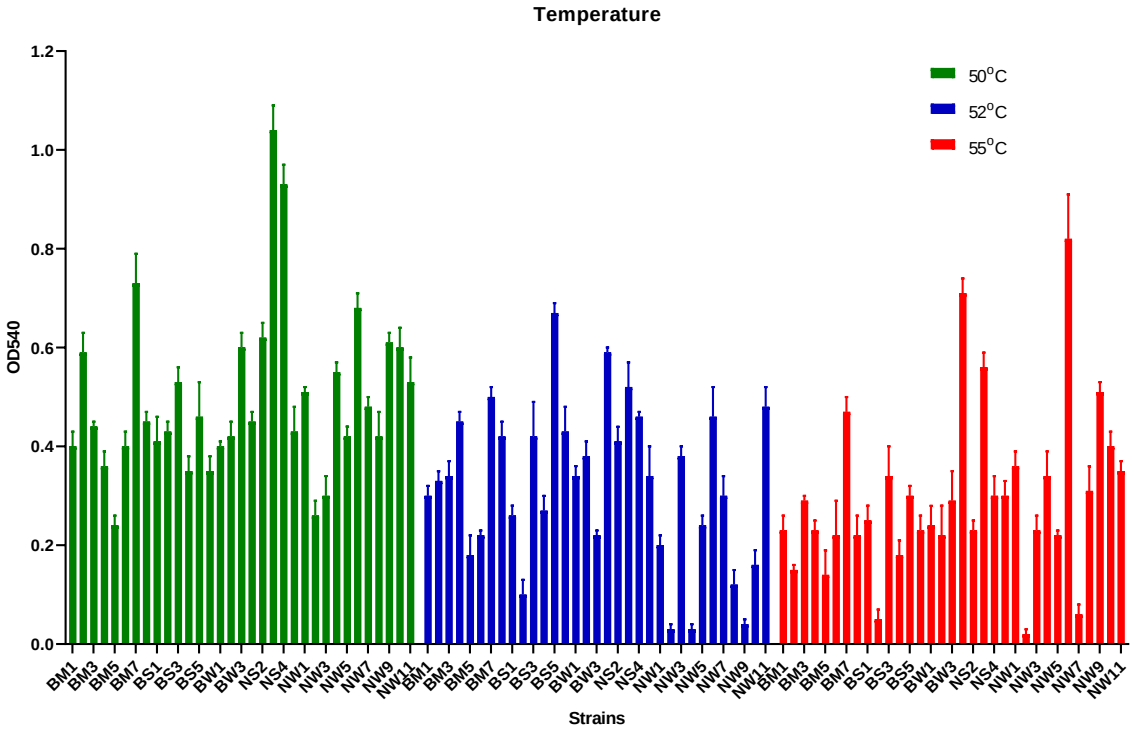


**Figure 2.** Effect of temperature on the growth of isolated microorganisms from hot springs.

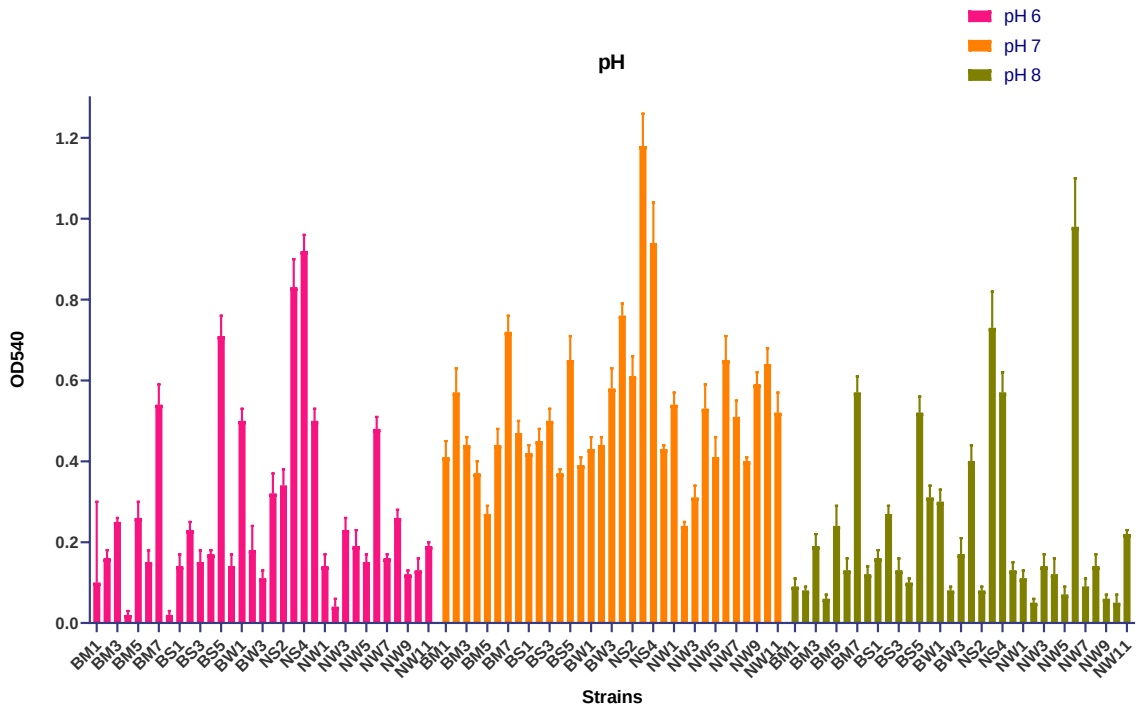


**Figure 3.** Effect of pH on the growth of isolated microorganisms from hot springs.

After 12 h of incubation, the $OD_{540}$ values of six isolates including BM7 (0.73 ± 0.06, at 50ºC), BS5 (0.67 ± 0.02, at 52ºC), NS1 (0.71 ± 0.03, at 55ºC), NS3 (1.04 ± 0.05, at 50ºC), NS4 (0.93 ± 0.04, at 50ºC), and NW6 (0.82 ± 0.09, at 55ºC) were higher than the others. Of these, isolates BM7, NS3, NS4 grew optimum at 50ºC with $OD_{540}$ from 0.73 to 1.04, while growth of isolate BS5 was optimal at 52ºC with $OD_{540}$ at 0.67 ± 0.02. Isolates NS1 and NW6 were optimal at 55ºC with high $OD_{540}$ at 0.71 ± 0.03 and 0.82 ± 0.09, respectively. The pH investigation also showed that isolate BS5 grew optimum at pH 6, isolates BM7, NS1, NS3, NS4 grew optimum at pH 7 while NW6 optimized at pH 8. Moreover, the highest $OD_{540}$ (1.18 ± 0.08) was recorded in isolate NS3 at pH 7. Generally, the collection of moderate thermophilic bacteria could be cultured optimally at 50ºC and pH 7 as shown in the Figure 2 and 3.

## 16S rDNA sequence analysis and phylogenetic tree construction

Genomic DNA of thermophilic bacteria were extracted and 16S rDNA sequences (~1,6 kb) were successful amplified by using universal primer set 27F – 1492R (Figure 4). The 16S rDNA sequencing results showed that six selected thermophilic isolates were highly similar to *Bacillus* spp..

In addition, the phylogenetic tree was analyzed and constructed through the 16S rDNA sequences of six selected thermophilic isolates and other *Bacillus* species for further analysis (Figure 5 and Figure 6). Six thermophilic isolated strains were identified and clustered of Group I (*B. cereus*, *B. licheniformis*, *B. subtilis*), the largest cluster. Strain NS1 was located closely link to *Bacillus depressus* BZ1 and *Bacillus gottheilii* ASG5-3 group, which bootstrap value was 88,8%. However, sequence similarity of strain NS1 with *Bacillus depressus* BZ1 was higher than that with *Bacillus gottheilii* ASG5-3, namely 99.4% and 99.2%. Base on the morphological characteristics and molecular data of strain NS1 in comparison with *Bacillus depressus* and

*Bacillus gottheilii*, strain NS1 was assigned as *Bacillus depressus*. Strain NS3 and NS4 were matched completely with *Bacillus licheniformis* to 100% and 99%, respectively. They were clustered in one group with *Bacillus licheniformis* L54 (96.5% bootstrap confidence) and *Bacillus licheniformis* DAS-1 (100% bootstrap confidence). In addition, biological characteristics of those strains were white-creamy colony, irregular shape, rough surface, positive Gram, spore forming suggested that strains NS3 and NS4 were *Bacillus licheniformis.* StrainBS5 was suggested as *Bacillus subtilis* with high similarity (100%), NW6 was suggested as *Bacillus cereus* (99%), and BM7 was *Bacillus tequilensis* (99%).

Genus *Bacillus* belong to the family *Bacillaceae* of the phylum *Firmicutes*, which includes Gram positive, spore forming rods, moderately thermophilic and aerobic or facultatively anaerobic species. These thermophiles have an optimum temperature range for growth from 50 to 70$^{o}$C and pH = 4.2 – 8.0, comprising *Bacillus, Aeribacillus, Anoxybacillus, Geobacillus, Cerasibacillus, Caldalkalibacillus, Alicyclobacillus, Sulfobacillus, Brevibacillus, Ureibacillus, Thermobacillus* và *Thermoactinomyces* (Kumar *et al.*, 2012). Previous studying of four hot springs in Morocco revealed that all thermophilic isolates were Gram positive, rod-shaped, spore forming. Depending on results of 16S rDNA sequence analysis, two hundred and forty isolated strains were dominated by the genus *Bacillus* (97.5%), for example, *B. licheniformis* (119 strains), *B. aerius* (44 strains) and *B. subtilis* (8 strains) (Aanniz *et al*., 2015).

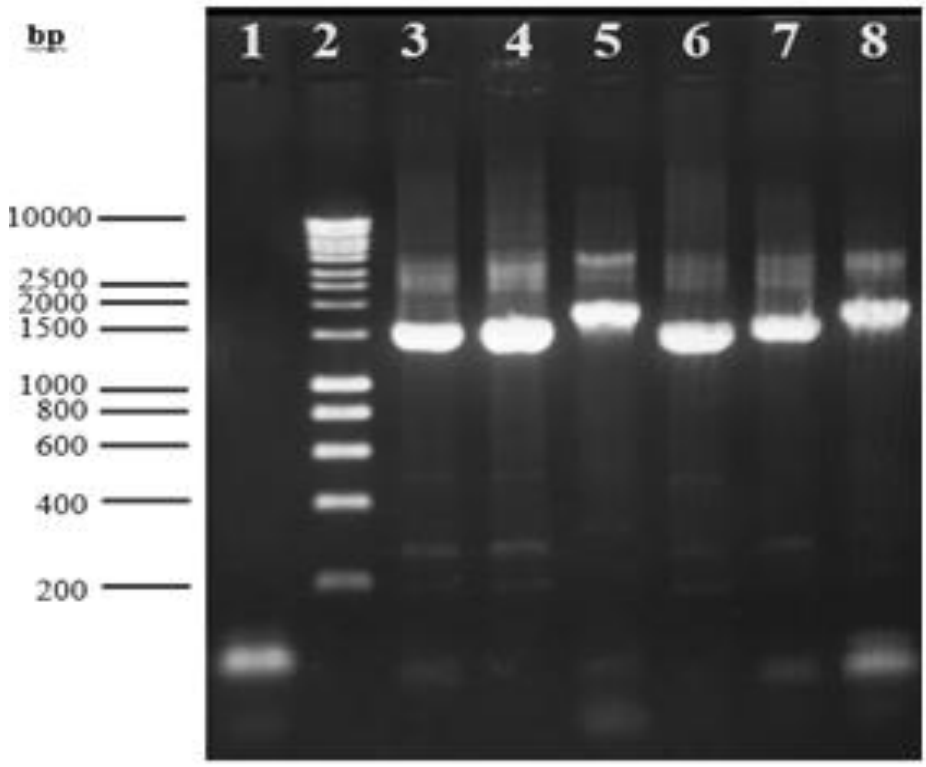


**Figure 4.** The electrophoresis image of the PCR products amplifying 16S rDNA genes from DNA genome of isolates using primers 27F-1942R. 1, Negative control; 2, Ladder; 3, NS1; 4, NS3; 5, NS4; 6, BS5; 7, NW6; 8, BM7.

Percent Identity

Divergence

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ID | 94.7 | 96.6 | 98.4 | 99.6 | 93.4 | 90.5 | 85.1 | 97.3 | 98.1 | 95.7 | 99.6 | 94.0 | 99.5 | 94.7 | 94.7 | 96.9 | 92.9 | 96.7 | 95.7 | 95.6 | 98.1 | 98.2 | 99.8 | 95.4 | 94.7 | 99.6 | 99.6 | 99.5 | 1 |
| 2 | 5.5 | ID | 93.3 | 95.2 | 94.7 | 92.5 | 90.4 | 85.2 | 95.5 | 95.1 | 92.7 | 94.7 | 99.1 | 94.6 | 93.8 | 100.0 | 95.9 | 92.3 | 95.8 | 94.5 | 92.3 | 95.1 | 95.2 | 94.7 | 96.0 | 93.9 | 94.7 | 94.7 | 94.6 | 2 |
| 3 | 3.5 | 7.1 | ID | 97.8 | 96.9 | 93.0 | 89.6 | 83.3 | 96.0 | 97.7 | 95.3 | 96.9 | 93.3 | 96.8 | 92.7 | 93.3 | 95.6 | 92.0 | 95.7 | 94.3 | 95.0 | 97.7 | 97.8 | 96.6 | 93.9 | 93.6 | 96.9 | 96.9 | 96.8 | 3 |
| 4 | 1.6 | 5.0 | 2.3 | ID | 98.6 | 94.6 | 91.3 | 84.9 | 97.6 | 99.7 | 96.9 | 98.6 | 94.4 | 98.7 | 94.6 | 95.2 | 97.1 | 93.5 | 97.0 | 96.2 | 96.8 | 99.7 | 100.0 | 98.6 | 95.3 | 95.5 | 98.6 | 98.6 | 98.7 | 4 |
| 5 | 0.4 | 5.5 | 3.1 | 1.4 | ID | 94.0 | 90.6 | 85.2 | 97.6 | 98.5 | 95.9 | 100.0 | 94.0 | 99.9 | 94.5 | 94.7 | 97.2 | 93.4 | 96.9 | 95.7 | 95.8 | 98.5 | 98.6 | 99.6 | 95.4 | 95.3 | 100.0 | 100.0 | 99.9 | 5 |
| 6 | 6.6 | 7.7 | 7.1 | 5.3 | 6.0 | ID | 91.7 | 84.0 | 93.9 | 94.6 | 92.2 | 94.0 | 91.7 | 94.0 | 92.7 | 92.3 | 94.1 | 92.5 | 94.0 | 92.8 | 91.6 | 94.6 | 94.6 | 93.6 | 92.8 | 94.7 | 93.8 | 93.9 | 94.0 | 6 |
| 7 | 10.2 | 10.3 | 11.3 | 9.3 | 10.1 | 8.5 | ID | 83.6 | 91.0 | 91.3 | 88.5 | 90.6 | 89.7 | 90.7 | 89.2 | 90.4 | 91.4 | 88.5 | 91.3 | 90.3 | 88.1 | 91.3 | 91.2 | 90.7 | 90.5 | 91.4 | 90.5 | 90.6 | 90.7 | 7 |
| 8 | 16.7 | 16.6 | 19.0 | 17.0 | 16.6 | 17.9 | 18.6 | ID | 85.3 | 84.9 | 82.3 | 85.2 | 84.2 | 85.2 | 84.7 | 85.2 | 84.9 | 84.2 | 84.9 | 84.7 | 82.3 | 84.9 | 84.9 | 85.2 | 84.9 | 84.0 | 85.2 | 85.2 | 85.2 | 8 |
| 9 | 2.7 | 4.6 | 4.1 | 2.5 | 2.5 | 6.1 | 9.6 | 16.5 | ID | 97.3 | 95.0 | 97.6 | 94.8 | 97.6 | 94.3 | 95.5 | 99.4 | 92.9 | 99.2 | 95.6 | 94.8 | 97.3 | 97.4 | 97.4 | 97.0 | 95.0 | 97.6 | 97.6 | 97.6 | 9 |
| 10 | 1.9 | 5.1 | 2.4 | 0.3 | 1.5 | 5.3 | 9.3 | 17.0 | 2.7 | ID | 96.8 | 98.5 | 94.3 | 98.6 | 94.4 | 94.9 | 96.9 | 93.3 | 96.8 | 95.9 | 96.7 | 100.0 | 99.7 | 98.3 | 95.1 | 95.5 | 98.3 | 98.4 | 98.6 | 10 |
| 11 | 4.4 | 7.7 | 4.8 | 3.2 | 4.2 | 8.0 | 12.5 | 20.3 | 5.1 | 3.2 | ID | 95.9 | 92.0 | 95.8 | 91.9 | 92.7 | 94.9 | 90.9 | 94.9 | 93.1 | 99.2 | 96.8 | 96.9 | 95.7 | 93.2 | 92.8 | 95.9 | 95.9 | 95.8 | 11 |
| 12 | 0.4 | 5.5 | 3.1 | 1.4 | 0.0 | 6.1 | 10.1 | 16.6 | 2.5 | 1.5 | 4.2 | ID | 94.0 | 99.9 | 94.5 | 94.7 | 97.2 | 93.4 | 96.9 | 95.7 | 95.8 | 98.5 | 98.6 | 99.6 | 95.4 | 95.3 | 100.0 | 100.0 | 99.9 | 12 |
| 13 | 6.3 | 0.9 | 7.1 | 5.8 | 6.3 | 8.6 | 11.2 | 17.8 | 5.4 | 5.9 | 8.6 | 6.3 | ID | 93.9 | 93.2 | 99.1 | 95.2 | 91.7 | 95.1 | 93.9 | 91.5 | 94.3 | 94.4 | 94.0 | 95.3 | 93.1 | 94.0 | 94.0 | 93.9 | 13 |
| 14 | 0.5 | 5.6 | 3.2 | 1.3 | 0.1 | 5.9 | 10.0 | 16.5 | 2.5 | 1.4 | 4.3 | 0.1 | 6.4 | ID | 94.6 | 94.6 | 97.1 | 93.5 | 96.8 | 95.8 | 95.7 | 98.6 | 98.7 | 99.7 | 95.3 | 95.3 | 99.9 | 99.9 | 100.0 | 14 |
| 15 | 5.3 | 6.4 | 7.5 | 5.5 | 5.5 | 7.3 | 11.6 | 17.1 | 5.7 | 5.6 | 8.5 | 5.5 | 7.0 | 5.4 | ID | 93.8 | 94.4 | 96.3 | 94.7 | 96.5 | 91.6 | 94.4 | 94.4 | 94.7 | 92.8 | 93.9 | 94.5 | 94.5 | 94.6 | 15 |
| 16 | 5.5 | 0.0 | 7.1 | 5.0 | 5.5 | 7.9 | 10.4 | 16.6 | 4.6 | 5.3 | 7.7 | 5.5 | 0.9 | 5.6 | 6.4 | ID | 95.9 | 92.1 | 95.8 | 94.5 | 92.3 | 94.9 | 95.0 | 94.7 | 96.0 | 93.8 | 94.7 | 94.7 | 94.6 | 16 |
| 17 | 3.1 | 4.2 | 4.5 | 2.9 | 2.8 | 5.9 | 9.2 | 17.0 | 0.6 | 3.1 | 5.3 | 2.8 | 5.0 | 2.9 | 5.6 | 4.2 | ID | 93.1 | 99.5 | 95.5 | 94.4 | 96.9 | 97.0 | 96.9 | 97.2 | 95.5 | 97.2 | 97.2 | 97.1 | 17 |
| 18 | 7.6 | 8.2 | 8.5 | 6.9 | 6.9 | 7.7 | 12.6 | 17.8 | 7.4 | 7.0 | 9.7 | 7.0 | 8.9 | 6.9 | 3.6 | 8.4 | 7.2 | ID | 93.4 | 95.8 | 90.5 | 93.4 | 93.3 | 93.0 | 91.6 | 93.4 | 93.2 | 93.3 | 93.5 | 18 |
| 19 | 3.4 | 4.3 | 4.4 | 3.0 | 3.1 | 6.0 | 9.3 | 17.0 | 0.8 | 3.2 | 5.3 | 3.1 | 5.1 | 3.2 | 5.3 | 4.3 | 0.5 | 6.9 | ID | 95.6 | 94.3 | 96.8 | 96.9 | 96.7 | 96.8 | 95.5 | 96.9 | 96.9 | 96.8 | 19 |
| 20 | 4.1 | 5.4 | 5.6 | 3.6 | 4.1 | 6.9 | 10.0 | 16.8 | 4.1 | 3.9 | 6.9 | 4.1 | 6.0 | 4.0 | 3.1 | 5.4 | 4.2 | 3.9 | 4.1 | ID | 93.0 | 95.9 | 96.0 | 95.9 | 93.9 | 94.9 | 95.7 | 95.7 | 95.8 | 20 |
| 21 | 4.5 | 8.2 | 5.2 | 3.2 | 4.3 | 8.7 | 13.1 | 20.4 | 5.4 | 3.3 | 0.8 | 4.3 | 9.1 | 4.4 | 8.8 | 8.2 | 5.8 | 10.2 | 5.9 | 7.0 | ID | 96.7 | 96.8 | 95.6 | 92.7 | 92.5 | 95.8 | 95.8 | 95.7 | 21 |
| 22 | 1.9 | 5.1 | 2.4 | 0.3 | 1.5 | 5.3 | 9.3 | 17.0 | 2.7 | 0.0 | 3.2 | 1.5 | 5.9 | 1.4 | 5.6 | 5.3 | 3.1 | 6.9 | 3.2 | 3.9 | 3.3 | ID | 99.7 | 98.3 | 95.1 | 95.5 | 98.3 | 98.4 | 98.6 | 22 |
| 23 | 1.8 | 5.0 | 2.3 | 0.0 | 1.4 | 5.3 | 9.4 | 17.0 | 2.6 | 0.3 | 3.2 | 1.4 | 5.8 | 1.3 | 5.6 | 5.2 | 3.0 | 7.0 | 3.1 | 3.8 | 3.2 | 0.3 | ID | 98.4 | 95.2 | 95.5 | 98.4 | 98.5 | 98.7 | 23 |
| 24 | 0.2 | 5.5 | 3.5 | 1.4 | 0.4 | 6.4 | 9.9 | 16.5 | 2.6 | 1.7 | 4.4 | 0.4 | 6.3 | 0.3 | 5.3 | 5.5 | 3.1 | 7.3 | 3.4 | 3.9 | 4.5 | 1.7 | 1.6 | ID | 95.2 | 94.9 | 99.6 | 99.6 | 99.7 | 24 |
| 25 | 4.8 | 4.1 | 6.4 | 4.8 | 4.7 | 7.4 | 10.3 | 17.0 | 3.0 | 5.1 | 7.2 | 4.7 | 4.9 | 4.8 | 7.5 | 4.1 | 2.8 | 8.9 | 3.3 | 6.1 | 7.7 | 5.1 | 5.0 | 5.0 | ID | 93.8 | 95.5 | 95.5 | 95.3 | 25 |
| 26 | 5.4 | 6.3 | 6.7 | 4.5 | 4.8 | 5.1 | 9.1 | 18.0 | 5.1 | 4.5 | 7.5 | 4.8 | 7.2 | 4.7 | 6.1 | 6.5 | 4.5 | 6.8 | 4.6 | 4.9 | 7.8 | 4.5 | 4.6 | 5.2 | 6.4 | ID | 95.1 | 95.2 | 95.3 | 26 |
| 27 | 0.4 | 5.5 | 3.1 | 1.4 | 0.0 | 6.2 | 10.2 | 16.6 | 2.5 | 1.7 | 4.2 | 0.0 | 6.3 | 0.1 | 5.5 | 5.5 | 2.8 | 7.1 | 3.1 | 4.1 | 4.3 | 1.7 | 1.6 | 0.4 | 4.7 | 5.0 | ID | 100.0 | 99.9 | 27 |
| 28 | 0.4 | 5.5 | 3.1 | 1.4 | 0.0 | 6.1 | 10.1 | 16.6 | 2.5 | 1.6 | 4.2 | 0.0 | 6.3 | 0.1 | 5.5 | 5.5 | 2.8 | 7.0 | 3.1 | 4.1 | 4.3 | 1.6 | 1.5 | 0.4 | 4.7 | 4.9 | 0.0 | ID | 99.9 | 28 |
| 29 | 0.5 | 5.6 | 3.2 | 1.3 | 0.1 | 5.9 | 10.0 | 16.5 | 2.5 | 1.4 | 4.3 | 0.1 | 6.4 | 0.0 | 5.4 | 5.6 | 2.9 | 6.9 | 3.2 | 4.0 | 4.4 | 1.4 | 1.3 | 0.3 | 4.8 | 4.7 | 0.1 | 0.1 | ID | 29 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | |

**Figure 5.** Matrix (%) of 16S rDNA genetic homogeneity of identified strains with other strains from *Bacillus* genus were analyzed by MegAlign program of Lasergene 7.0 software, DNASTAR.

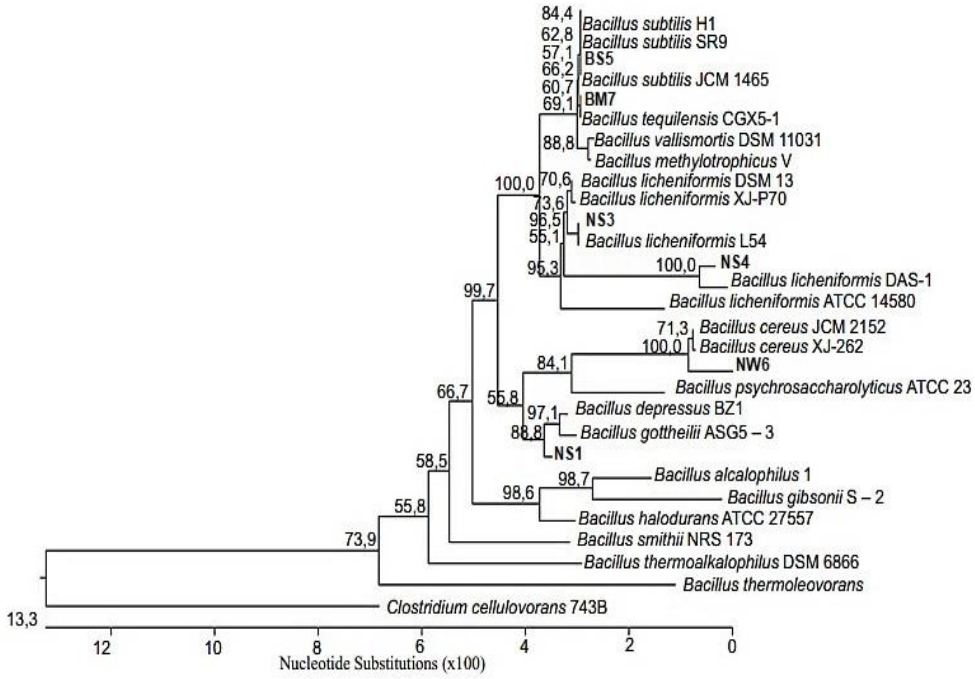


**Figure 6.** General phylogenetic tree showed that relationships of the collected strains and thermophilic bacteria belonging the *Bacillus* genus. Ruler indicates number of different nucleotides per 100 nucleotides. Bootstrap values (> 50%) after 1000 replicates are shown.

## CONCLUSION

In totally, 33 strains of thermophilic bacteria were isolated from the hot spring areas at Ba Ria – Vung Tau, and Khanh Hoa province of Vietnam and examined biological characteristics. In which, six thermophilic isolates able to survive and grow at 55°C were

chosen for identification to species level by 16S rDNA sequencing analysis. Since the identification rate of 16S rDNA sequence of isolated strains in comparison with published strains were not 100%, isolated strains NS1, NS3, NS4, BS5, NW6 and BM7 were identified belong to *Bacillus* genus, species as *Bacillus* sp.

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