BIOLOGICAL CHARACTERISTICS AND CLASSIFICATION OF THE THERMOPHILIC BACTERIA BML07 STRAIN PRODUCING BOTH THERMOSTABLE AMYLASE AND CELLULASE ISOLATED FROM MY LAM HOT SPRING

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ABSTRACT

The thermophilic bacteria strain BML07 was isolated from sedimental sludge of My Lam hot spring (Tuyen Quang province, Vietnam). Furthermore, this strain became promising candidate due to its capable of producing thermostable enzymes such as cellulase and amylase. The biophysiological characteristics of this strain revealed the optimal conditions for growth at pH 7.5; 2 - 3% NaCl, and 70 ^{o}C, however it is still able to survive at 80 ^{o}C. Observation of colony resulted in round shape and white, its cells obtained rod-shape (0.4 - 0.6 µm × 1.3 - 1.5 µm), positive Gram. Therefore, this strain was classified continuously based on Kit API 50/CHB as well as sequence of 16S rRNA. According to the taxonomical results using APIWEB software, the strain BML07 is similar of 97 % as *Geobacillus caldoxylosilyticus* (AY608950), however the sequence of 1500 bp fragment coding for 16S rRNA indicated that the homologous sequence between this strain and *Geobacillus caldoxylosilyticus* (AY608950) strain raised up to 99.8 %, as hence its own named *Geobacillus caldoxylosilyticus* BML07.

Keywords: Thermophilic bacteria, thermostable enzyme, hot springs, *Geobacillus sp*

1. INTRODUCTION

The classification of living organisms based on their relation to temperature has always been considered as the most basic element of biological systematics [1]. Three major groups were created with respect to optimum growth temperature (T_{opt})**,** e.g., psychrophiles that have a T_{opt} below 20 °C, mesophiles that grow optimally between 20 °C and 45 to 55 °C and the microorganisms which have a T_{opt} higher than 55 °C belong to thermophiles [2]. Recently, the thermophile group is further divided into two more subgroups due to the expansion of the upper limit of life, i.e., the discovery of the archaeon *Pyrolobus fumarii* that can grow optimally at temperatures of 113 °C [3]. Nowadays, it is generally classified microbes growing optimally above 80 to 85 °C as hyperthermophiles [1]. Most hyperthermophiles belong to the archaeal domain. Among Bacteria there are only few species that can be called hyperthermophiles, such as *Thermotoga* and *Aquifex* which have a T_{opt} in the range of 90 to 95 °C [4]. Thermophiles have been isolated from high temperature terrestrial and marine habitats. The most common habitats are volcanically and geothermally heated hydrothermal vent systems such as hot springs and

submarine hot vents [5].

Extreme thermophiles are mostly distributed among the genera of *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Thermotoga*, *Aquifex*. Most hyperthermophiles, on the other hand, include the two kingdoms of *Archaea*, *Crenarchacota* (*Sulfolobus*, *Pyrodictium*, *Pyrolobus*), *Euryarchaeaota* (*Thermococcus*, *Pyrococcus)*, methanogenes (*Methanococcus*, *Methanobacterium*), sulfate reducers and halophiles [5]. The genus *Bacillus* is described as a large and heterogeneous collection of aerobic and facultatively anaerobic, rod-shaped, Gram (+) (to Gram variable), endospore forming bacteria [6, 7]. Many kinds of species which have thermophilic, psychrophilic, acidophilic, alkalophilic and halophilic properties are included in the genus. In 2001, moreover, Nazina et al. undertook thoroughly polyphasic examinations of thermophilic *Bacillus* group derived from "earth" to obtain the notion of a phylogenetically distinct, physiologically and morphologically consistent taxon. These results supported for the submission the validly-described genus name of *Geobacillus* [8]. The importance of thermophilic *Bacillus* have increased because of their biotechnological importance as sources of thermostable enzymes such as proteases, amylases, pullulanases, glucose-isomerases, lipases, xylanases, cellulases and DNA restriction endonucleases [9, 10].

Among the thermostable enzyme, amylases constitute a class of industrial enzymes having approximately a 25 % share in the world enzyme market [11]. Amylases is used as potential applications in a number of industrial processes such as food (baking, brewing, dairy industries), fermentation, textile, detergent and paper industries. They would also be useful in the pharmaceutical and fine chemical industries [12]. Alpha-amylases from *B. licheniformis* and *B. stearothermophilus* have been typically applied for those industrial steps [13, 14, 15]. Besides thermostable starch degrading enzymes, cellulase is an enzyme degrading cellulose which is used in textile industry for bio-polishing of fabrics and in house-hold laundry detergents for improving fabric softness and brightness. They are also used in animal feed, processing of fruit juice, and in baking and deinking of recycled paper [16]. Industrial ethanol production is based on corn-starch which is liquefied and saccharified. The oligosaccharide syrup is then used for ethanol fermentation. It has been described that the use of cellulases during starch saccharification and liquefaction increase the yields [15]. Since these steps are performed at high temperatures, thermophilic endoglucanases are important for these steps.

In Vietnam, more than three thousands hot springs speard out from North to South, however, assessment of the properties and capacity of geothermal resources involves their biodiversity is not known yet. Some studies focused on typical hot springs such as Binh Chau, Son La which isolated approximately 70 mesophilic bacteria. The mesophiles mainly belongs to *Bacilli* genus i.e. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and almost of them are able to produce thermostable enzymes [17, 18]. It is generally accepted that less than 1% of the naturally occurring microorganisms can be isolated and grown in pure culture, therefore only spore forming bacteria can be achieved from natural sources by the traditional techniques. Here, we report characteristics of the thermophilic bacteria which was isolated from My Lam hot springs as a novel source for producing thermostable enzymes.

2. MATERIALS AND METHODS

2.1. Materials

- Strain BML07 was obtained from the collection of Lab of Biomaterials, Institute of

Biotechnology, Vietnam by isolating from sedimental sludge of My Lam hot springs

- All of the chemicals and reagents were purchased having analytical grades.

2.2. Methods

- Physiological and chemical tests such as observation of cells and colony shape under microscope; determination of Gram property; starch, gelatin, pulp and CMC hydrolysis capacities, pH_{opt}, T_{opt} growth was followed Nguyen LD et al. [19].

- Total DNA extraction was described by Sambrock and Russell [20].

- Amplification of bacterial 16S rRNA gene: The total DNA of BML07 strain plays as a template to amplify the fragment coding for 16S rRNA gene. A pair of primers 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5-TACGGTTACCTTG TTACGACTT-3') was used in the following reaction: Taq polymerase buffer (10x) 5µl; dNTPs (10 mM) 2µl; Dream Taq polymerase (5000 U/ml) 0.3 µl; 16SF primer (10 pmol) 1µl; 16SR primer (10 pmol) 1 µl; DNA template (20 ng) 2µl; water 38.7 µl. The reaction was carried out in the Thermal cycler PT-100 (MJ Reasearch Inc.) with the following program: 95 ^oC 3 min; 95 ^oC 1 min; 55 ^oC 1 min; 72 ^oC 1 min 15 sec; 30 cycles repeat from step 2 to step 4; 72 ^oC 7 min; 4 ^oC: endless.

- The PCR product was purified by Kit "Wizard ® SV Gel and PCR Clean-Up System" (Promega) and subsequently, it was analyzed by the sequencing ABI-377 Perkin Elmer device in Institute of Biotechnology according to Sanger method [21]. Sequencing 16S rRNA gene result was blasted to identify the homology between BML07 strain with the others which were published in Gen Bank.

3. RESULTS AND DISCUSSION

My Lam hot springs locates at West-Northern mountain of Vietnam where is influenced by a strong monsoon and seasons changing. Four distinct seasons are most evident in the Northern provinces which the most noticeable seasonal changes in temperature are found a difference of 12 degrees Celsius is possible. The hydrothermal vent of My Lam hot springs has temperature of 65 – 67 ^oC and pH range 8.0 - 8.2 (table 1).

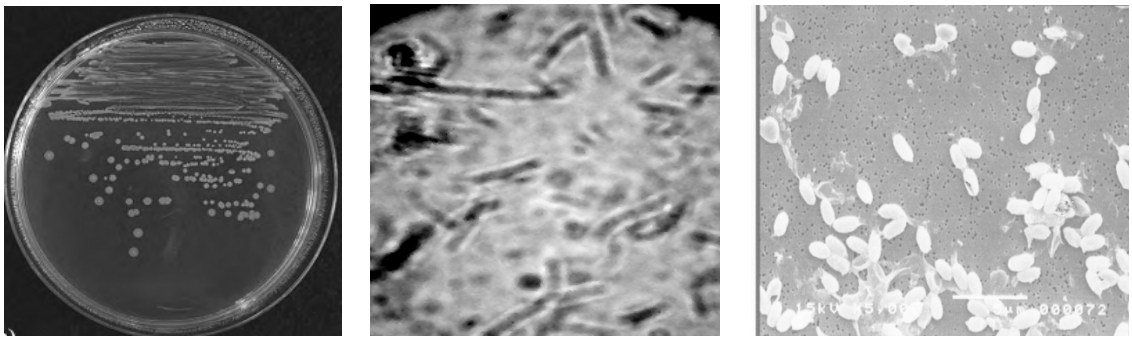
Table 1. The mineral components of My Lam hot springs

Cation (mg/l)	**Anion (mg/l)**	**Metal (mg/l)**
Na^+: 61.3	Cl^-: 17.5	Cu: 0.003
K^+: 2.045	SO_4^{2-}: 13.0	Zn: 0.0348
NH_4^+: < 0.01	NO_2^-: 0.18	Pb: 0.001
Ca^{2+}: 4.0	NO_3^-: 0.18	Ni: < 0.001
Fe^{2+}: < 0.01	HCO_3^-: 140.3	Co: 0.01
Other compotents		Mn: 0.05
H_2S: 3.5 mg/l		Al: 0.03
pH: 8.0 - 8.2		Cr: 0.012
Temperature: 65 - 67 ^oC		Ag: < 0.01

As high temperature and alkaline, natural conditions in My Lam hot springs are favourite environment for microorganisms growing which enhance ecological biodiversity. Here is also a prominent source for discovery of a varied thermophilic as well as alkaphilic bacteria which are promising candidates for production of thermostable enzymes.

3.1. Morphological and physiological characteristics of BML07 strain

Strain BML07 was isolated from sedimental sludge of My Lam hot springs. Cell morphology was examined under an Olympus AX70 microscope (magnification ×1000) and a JEM-100S electron microscope (magnification ×5000) after cultivation of the strains at 60 ^{o}C on LB agar for 17 – 24 h. Bacterial size was also determined by bright-field microscopy in living cell preparations from cultures grown on LB agar for 17 – 24 h. This strain produced small, round, mucous, non-pigmented colonies with a diameter of about 1 mm. The cells were 0.4 - 0.6 µm wide and 1.3 - 1.5 µm long. The endospore was terminal and ellipse-shaped, and placed in the middle of the mother cell. The Gram-positive staining of the cell wall was also demonstrated by light microscopy (figure 1).



A B C

Figure 1: Colony morphology and cells of BML07 strain. **A**: Strain grow aerobically on LB agar plate. **B** and **C**: Cell-shape of strain under optical microscope with magnification × 1000 and × 5000 times, respectively

Apart from these biophysiological characteristics optimal growth temperature and pH were determined. The highest cell yield was obtained at 70 °C and a pH of 6.5 to 9.0. Strain BML07 could grow between 45 °C and 75 °C and a pH of 6.0 to 9. Majority of *Geobacillus* members are unable to grow at pH 9.0 whereas strain BML07 could grow at this pH [22, 23]. When we were examining the requirement of a salt for growth of the microorganism we found that strain BML07 could survive up to 4 % NaCl for growth. No cell growth could be observed when NaCl was present at a final concentration of 5 % even after 30 h of incubation at 65 °C (figure 2). Physiological characteristics of BML07 showed the optimal conditions for BML07 strain growing are suitable completely with the natural conditions of My Lam hot springs where the strain was isolated.

In order to examine thermostable enzymes produced by strain BML07, it was inoculated for 48 hours at 65 ^{o}C on LB agar containing pulp, carboxymethylcellulose (CMC), starch and gelatin which are specific substrates for endo, exo-cellulase, amylase and protease, respectively. Interestingly, strain BML07 could be able to degrade all of those substrates and resulted in very clear zones (figure 3). Protease and endo-cellulolytic seems to be the strongest activities as

compare with starch degrading enzyme and exo-cellulase where shown the biggest hydrolyzed zones of 23 and 24 cm, respectively. These results suggest that the producing enzymes obtained from strain BML 07 can be thermophilic. However, to confirm their properties, we have to investigate further examinations.

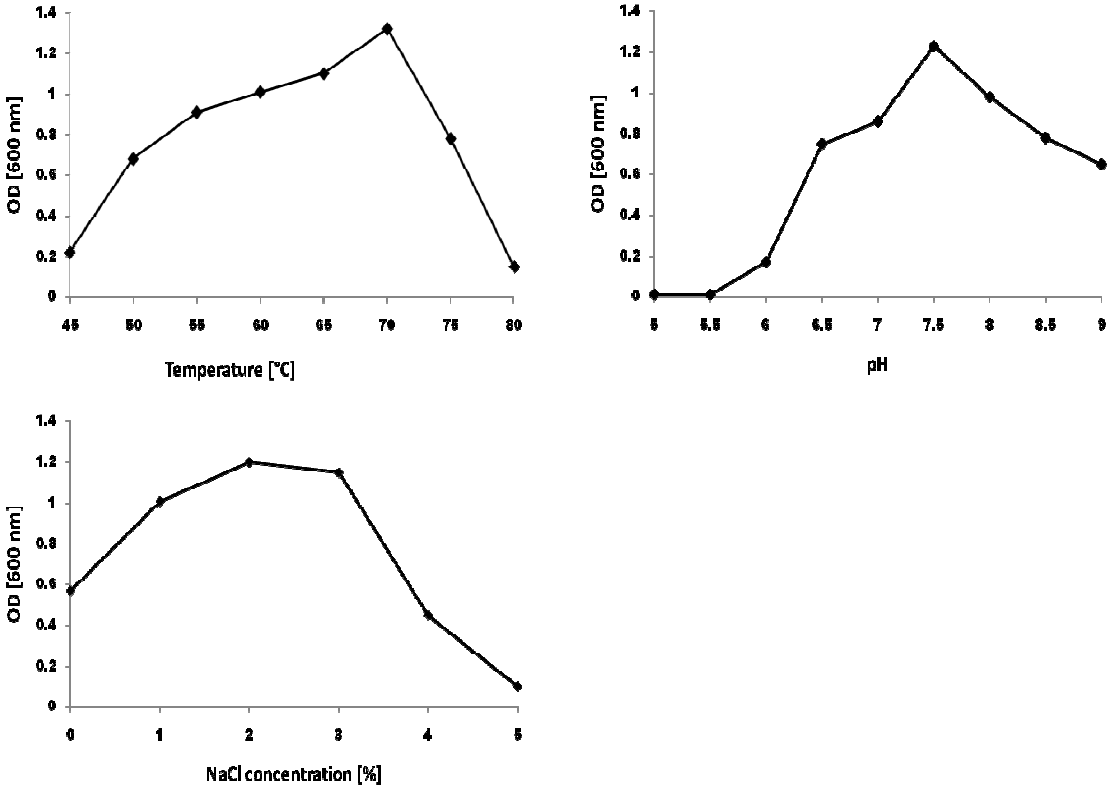
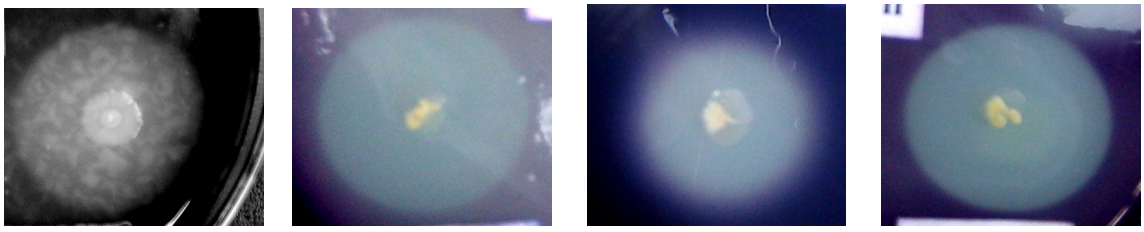


Figure 2. Effect of temperature, pH and NaCl concentration on the growth of BML07 strain



A B C D

Figure 3. Hydrolysis zone demonstrating the organism's ability to produce thermostable enzyme degrading pulp (A), CMC (B), starch (C), and gelatin (D). The diameter hydrolysed zone of 18 cm (A), 24 cm (B), 16 cm (C) and 23 cm (D)

3.2 Classification of BML07 strain

Because of the capacity producing the thermostable enzymes which become a promissing candidate for industrial biotechnological aspects, strain BML07 was continued studying to

identifiy it. According to morphological and physiological properties of BML07 strain obtained, this strain belongs to genus *Geobacillus* and among various *Geobacillus* species. In order to futher classify strain BML07, a specific Kit for genus *Bacilli* identification, API 50/CHB (bioMérieux, France), was used in approximately 48 hours. The analyzed results (*Table 2*) using APIWEB software indicated that the strain BML07 was similar of 97% as *Geobacillus caldoxylosilyticus* BGSC W9A36 (AY608950).

Table 2. Carbon hydrate metabolism of strain BML07 at 65°C in 48 hours. The results was continuously analyzed by APIWEB to determine the homology of strain BML07 with the reported species

	Substrate	**Reaction**		**Substrate**	**Reaction**
0	Control	-	25	Esculine	-
1	Glycerol	+	26	Salicine	-
2	Erythritol	-	27	Cellobiose	+
3	D-Arabinose	+	28	Maltose	+
4	L- Arabinose	+	29	Lactose	+
5	Ribose	+	30	Melibiose	+
6	D-Xylose	+	31	Saccharose	+
7	L-Xylose	+	32	Trehalose	+
8	Adonitol	-	33	Inuline	-
9	β-Methyl-xyloside	-	34	Melezitose	-
10	Galactose	+	35	D-Raffinose	-
11	D-Glucose	+	36	Amidon	+
12	D-Fructose	+	37	Glycogene	+
13	D-Mannose	+	38	Xylitol	-
14	L-Sorbose	±	39	β-Gentiobiose	-
15	Rhamnose	±	40	D-Turanose	+
16	Dulcitol	-	41	D-Lyxose	-
17	Inositol	-	42	D-Tagatose	-
18	Mannitol	+	43	D-Fucose	-
19	Sorbitol	+	44	L-Fucose	-
20	α-Methyl-D-mannoside	-	45	D-Arabitol	-
21	α-Methyl-D-glucoside	+	46	L-Arabitol	-
22	N-Acetyl glucosamine	+	47	Gluconate	-
23	Amygdaline	-	48	2-ceto-gluconate	-
24	Arbutine	-	49	5-ceto-gluconate	-

One of the most valuable methods to classify bacteria is the sequence of 16S rRNA gene. DNA extraction and amplification of the 16S rRNA gene were performed as described in the material and methods chapter. The 16S rRNA gene PCR product was extracted from agarose gel using a Kit "Wizard ® SV Gel and PCR Clean-Up System" (Promega) (figure 4). DNA sequencing was carried out directly on the purified PCR - products.

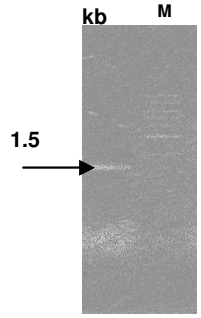


Figure 4. Product of 16S rRNA amplification from strain BML07

The sequence of strain BML07 was most similar to that of *G. caldoxylosilyticus* BGSC W9A36, having 99.8 % sequence similarity. A search of the BLAST database also revealed the highest level of similarity with sequences of different strains of *G. caldoxylosilyticus*. A high level of similarity was also determined for another species of *Geobacillus* genus such as *G. stearothermophilus* BGSC 9A5 (99.2 % sequence similarity). Lower sequence similarities were obtained for *G. toebii* SK-1 and *G. thermodenitrificans* (97.4 – 98.4 % sequence similarity). *G. thermoglucosidasius* and *B. subtilis* were the most distantly related to strain BML07 among *Geobacillus* species. The 16S rRNA gene sequences of the tested strains were aligned using the CLUSTAL_X program [24] and also manually. The size of the 16S rRNA gene used for alignment was 1470 nucleotides. A phylogenetic tree was constructed using the TreeView software, version 1.6.1 [25]. The phylogenetic tree (figure 5) shows the position of strain BML07 among the species of genetic *Geobacillus* species.

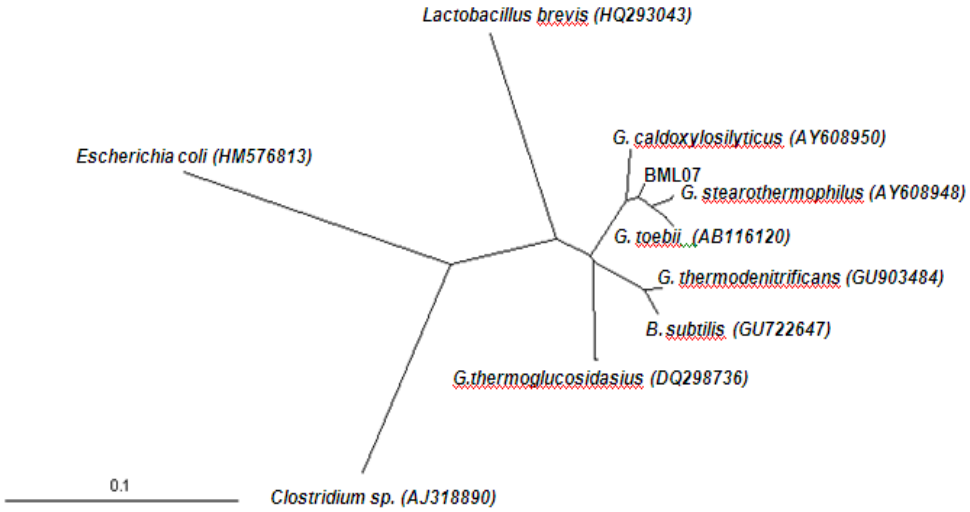


Figure 5. Phylogenetic tree showing the evolutionary position of strain BML07 among the genus *Geobacillus* and three different genera. GenBank accession numbers are given in *parentheses*. The *bar* represents one substitution per 10 nucleotides

4. CONCLUSION

Strain BML07 isolated from My Lam hot springs is a thermophilic bacteria which has T_{opt}

at 70 ^{o}C and pH_{opt} of 7.5. Morphological and other biocharacteristics as well as 16S rRNA sequence of strain BML07 reveals that this strain is most similar to that of *G. caldoxylosilyticus* (99.8 %), therefore it was called G. caldoxylosilyticus BML07.

This strain was expected to become a candidate to exploit the thermostable enzymatic resources such as cellulase, protease and amylase.

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TÓM TẮT

ĐẶC ĐIỂM SINH HỌC VÀ PHÂN LOẠI CỦA CHỦNG VI KHUẨN ƯA NHIỆT BML07 PHÂN LẬP TỪ SUỐI NƯỚC NÓNG MỸ LÂM CÓ KHẢ NĂNG SINH TỔNG HỢP AMYLASE VÀ CELLULASE BỀN NHIỆT

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Chủng vi khuẩn ưa nhiệt BML07 được phân lập từ mẫu cặn sa lắng của suối nước nóng Mỹ Lâm (Tuyên Quang, Việt Nam) có khả năng sinh tổng hợp các enzyme bền nhiệt như cellulase và amylase. Nghiên cứu đặc điểm sinh học của chủng này cho thấy chủng sinh trưởng tốt nhất ở điều kiện pH 7,5; 2 – 3 % NaCl. Nhiệt độ sinh trưởng tối ưu cho chủng ở 70 ^{o}C mặc dù ở điều kiện nhiệt độ 80 ^{o}C chủng vẫn có khả năng sống sót. Khuẩn lạc của chủng BML07 có hình tròn, màu trắng, tế bào hình que (0,4 – 0,6 µm × 1,3 – 1,5 µm), Gram dương. Phân tích kết quả phân loại chủng bằng Kit hóa sinh API 50/CHB và trình tự gen 16S rRNA bằng phần mềm APIWEB cho thấy chủng BML07 có độ tương đồng 97 % với chùng *Geobacillus caldoxylosilyticus* (AY608950), tuy nhiên khi so sánh trình tự gen 16S rRNA giữa hai chủng, độ tương đồng tăng lên 99,8 %, và do đó chủng được đặt tên là *Geobacillus caldoxylosilyticus* BML07.

Từ khóa: vi khuẩn ưa nhiệt, enzyme bền nhiệt, suối nước nóng, *Geobacillus sp*