Isolation and optimization of the growth conditions of thermophilic microorganism from hot springs

Kha M. Tran[1], Van T. T. Le[1], Duy D. Ngo[2], Khanh Q. Hoang[2], Phong V. Nguyen[3], & Tri H. Nguyen[1*]

[1]Department of Biology, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Institute of Tropical Biology, Ho Chi Minh City, Vietnam
[3]Department of Biotechnology, Nong Lam University, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research paper

Received: April 24, 2018
Revised: May 30
Accepted: June 17, 2018

Keywords

Bacillus spp.
Hot spring
Isolation
Spore staining
Thermophilic microorganism

***Corresponding author**

Nguyen Huu Tri
Email: nhtri@hcmuaf.edu.vn

ABSTRACT

The aim of this study was to isolate and optimize the growth conditions of thermophilic microorganism from hot springs. The isolation was conducted by using the mineral salt basal medium supplemented with 0.6% yeast extract at 50^0C. Totally, 33 isolates of thermophilic microorganism were isolated from hot springs at Truong Xuan (Khanh Hoa province) and Binh Chau (Ba Ria - Vung Tau province). The effects of temperature (45 - 80^0C), pH (pH 6 - 9) and carbon sources (malate, pyruvate, acetate, glucose, fructose, or carbon dioxide) on the growth of isolates were examined. In addition, the isolate morphology was also investigated by Gram and spore staining. The isolated thermophilic microorganism showed the diversity in colony morphology and color appearance. Most of them were rod shaped, spore-forming and most grew well at 50^0C and pH 7. The highest growth of all isolates was observed under malate, glucose, or fructose, as an organic carbon source and unable to use carbon dioxide. Six out of 33 thermophilic microorganism isolates (namely BM7, BS5, NS1, NS3, NS4, and NW6) grew rapidly under high temperatures from 50 - 55^0C and their morphology characteristics showed high similarity to *Bacillus* sp. The study evidenced the polymorphic diversity of thermophiles in the geothermal hot spring ecosystems.

Cited as: Tran, K. M., Le, V. T. T, Ngo, D. D., Hoang, K., Nguyen, P. V., & Nguyen, T. H. (2018). Isolation and optimization of the growth conditions of thermophilic microorganism from hot springs. *The Journal of Agriculture and Development* 17(3), 55-60.

1. Introduction

Hot springs, the emerged water bodies produced by geo-thermally heated groundwater, are scattered all over the globe, in every continent and even under the sea. In Vietnam, there are more than 287 hot springs and water containing dissolved minerals distributed in different regions of the country (Cao et al., 1998). There are many previous studies that focus on investigation about geological features of geothermal areas (Rastogi et al., 2010; Tran et al., 2012; Tulasi et al., 2013). Besides, the geothermal ecosystems such as hot springs and volcanic eruption areas are the habitat of thermophilic microorganisms. Based on the range of optimal growth temperature, thermophiles are classified into the following groups: moderate thermophiles (40-60^0C), extreme thermophiles (60-85^0C) and hyperthermophiles (>85^0C) (Tulasi et al., 2013). These thermophilic-derivative products could be applied in biotechnology as industrially valuable compounds. Extremophiles have provided an interesting and challenging platform for researchers since they were explorered. Besides growth under the extreme conditions, extremophiles could pro-

duce thermophilic enzymes, biogradable plastic, biofuel, etc. (Tulasi et al., 2013). Thermophilic microorganisms capable of biosynthesis of heat-resistant enzymes are widely used in the industry where production conditions require high temperatures (Gaughran et al., 1947). During the past few years, the interest in diversity, ecology, and physiology and biochemistry of thermophiles has increased rapidly in Vietnam. The thermophilic bacterium species Geobacillus caldoxylosilyticus was isolated from sedimental sludge of My Lam hot spring in Tuyen Quang province, Vietnam (Tran 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 et al., 2012).

The southern of Vietnam is very rich in hot springs. One is Truong Xuan hot spring (M' Dung village, Ninh Hoa, Khanh Hoa), and another is Binh Chau hot spring (Binh Chau commune, Xuyen Moc district, Ba Ria – Vung Tau) that is very famous in Vietnam. The diversity of microbial communities in these hot springs has not yet been fully studied. This study aimed to isolate, optimize, and evaluate the carbon utilization of thermophilic microorganism isolated from these locations. Results from this study were a preliminary step to apply thermophilic microorganism and their products in biotechnology.

2. Materials and Methods

Soil, muddy, and water samples were collected at Truong Xuan hot spring ($12^0$31'20"N, $108^0$59'00"E, Ninh Hoa, Khanh Hoa), and Binh Chau hot spring ($10^0$36'21"N, $107^0$33'29"E, Xuyen Moc, Vung Tau). Hot water in Truong Xuan hot spring was bubbled from the vein in the rock with temperature ranging from 37^0C to 67^0C. The pH was recorded in the range of 7.7-8.0 indicating alkaline environment. Binh Chau hot spring is the largest hot spring (more than 1 km^2) in Vietnam. Water temperature in the veins ranged from 43^0C to 65^0C with many bubbles, and smell hydrogen sulfide (H_2S). 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.

Samples were randomly collected from different sites of off flow and stored in 500 mL sterile containers (Hildur et al., 2011). They were immediately brought into the laboratory and analyzed within 24 hours. In total, 24 samples were collected from Truong Xuan (14 samples) and Binh Chau (10 samples) hot springs. The samples (soil, muddy, and water) were collected separately in the vacuum flask, transported to laboratory and analyzed within 24 hours.

The mineral salt basic (MSB) medium used for microorganism growth and trace element solution with the components are shown in Table 1 and Table 2 (Goto et al., 1977). The isolation medium was MSB supplemented with 0.6% yeast (w/v) so-called as MSBY medium, pH 7.

Table 1. The components of basic cultivation medium

Deionized Water	1.0 L
$(NH_4)_2SO_4$	2.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	2.0 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.0011 g
$CaCl_2$	0.03 g
Trace elements solution	0.5 mL
Final pH	7.0

Table 2. The components of trace elements solution

Deionized Water	1.0 L
MoO_3	0.004 g
$ZnSO_4.7H_2O$	0.028 g
$CuSO_4.5H_2O$	0.002 g
H_3BO_3	0.004 g
$MnSO_4.5H_2O$	0.004 g
$CoCl_2.6H_2O$	0.004 g

2.1. Isolation of thermophilic microorganisms from hot springs

The thermophilic microorganisms were isolated based on the possible growth at 50^0C. Fifty Celsius degree was chosen to make the initial isolation temperature to isolate those microorganisms that were capable of growing by 50^0C or more.

The procedure of enrichment was as follows: 1 gram of soil, sludge or 1 mL of water was diluted in 5 mL of MSB supplemented with 6 g/L of yeast extract and incubated at 50^0C for 48

hours. The growth of microorganisms was observed and recorded via estimation of the environmental opacity in test tubes. A five tenfold serial dilution was performed, and then spread on MSBA plates (MSB medium supplemented with agar 3% (w/w) and incubated at 50^0C for 72 hours. Single colonies growing on plates were transferred into freshly prepared MSBA slants and kept at -20^0C for further study. The isolates were investigated by observation of colony morphology, Gram stain, and sporulation (Goto et al., 1977).

2.2. Optimization of the growth condition of the isolated thermophilic microorganisms

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 45^0C to 80^0C, shaken at 180 rpm for 12 hours. Then, the optimum pH value was examined between 6 and 9 at the optimal temperature. The pH value was adjusted by using 1M NaOH solution. The microorganism growth was determined at 3-hour intervals by measuring the optical density (OD) of the cultures at 540 nm and streaked onto freshly prepared MSBA plate (Goto et al., 1977). The high thermo-tolerance isolates were selected for further experiments.

2.3. Investigation of the potential use of different carbon sources of the isolated thermophilic microorganism

The carbon sources were used in this study including organic substrates [acetate (C_2), pyruvate (C_3), malate (C_4), glucose (C_6), or fructose (C_6)] and inorganic substrate (CO_2). The concentration of carbon in the organic compounds was equivalent to 15 mM. In order to evaluate the use of CO_2, the isolates were cultured in MSB medium with the addition of H_2: O_2: CO_2 (80%: 10%: 10%) (Goto et al., 1977).

The cultures were incubated in a reciprocating shaker at the optimal temperature and pH. The initial OD value at 540 nm was 0.04-0.06. The microorganism growth in various carbon sources was recorded within 72 hours. The mean value OD_{540} of triplicates for each experiment was analyzed by using Microsoft Excel 2013 software.

3. Results and Discussion

3.1. Isolation of the thermophilic microorganisms from hot springs

Thirty three isolates that could grow at 50^0C 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. Of 33 isolates, 11 isolates were obtained from soil (33.3%), 8 isolates from muddy (24.3%) and 14 isolates from water (42.4%) samples (Table 3).

The colonies were appeared in various of color (beige, white, yellow, or pink) including 7 isolates were beige-colored, 11 were white, 14 were yellow, and 1 was pink on MSBA medium (Figure 1).

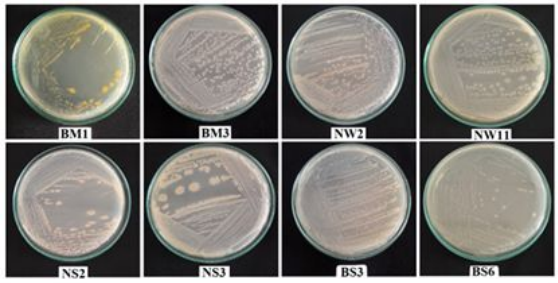

Figure 1. Diversity of colonial morphology of isolates microorganism from hot springs on MSBA.

The Gram-positive isolates were 18 isolates /33 (54.5%) of the collection. Of the 33 isolates, 31 (94%) were rod-shaped, with the size of cells in range of 0.16 – 0.8 ± m, 20 isolates (60.6%) were able to form oval endospores and had the size of the spores in range of 0.10 – 0.41 ± m (Figure 2). This result showed the diversity of the thermophilic microorganism communities in geothermal area.

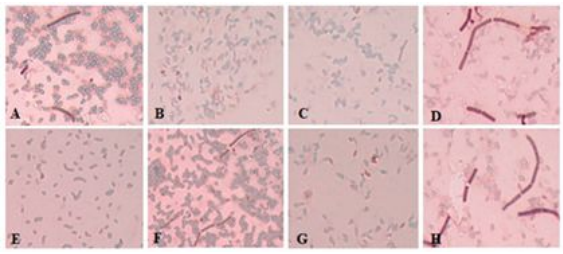

Figure 2. Spore shapes of the isolates under microscope observation (magnificent 1000X).
A: BS2; B: NW7; C: NW6; D: BM5; E: BS5;
F: BM5; G: BS4; H: BM8.

Table 3. Thermophilic microorganism isolated from hot springs

Hot spring	Sample	Isolate	Notation
Binh Chau (B) (Ba Ria – Vung Tau)	Water (W)	3	BW1,2,3
	Soil (S)	6	BS 1,2,3,4,5,6
	Mud (M)	8	BM 1,2,3,4,5,6,7,8
Truong Xuan (N) (Nha Trang, Khanh Hoa)	Water (W)	11	NW1,2,3,4,5,6,7,8,9,10,11
	Soil (S)	5	NS1,2,3,4,5
	Mud (M)	0	
Total		33	

3.2. Optimization growth conditions of thermophilic isolates

In order to optimize the temperature and pH for microorganism growth, the isolates were cultivated at temperature range from 45^0C to 80^0C and pH range from 6 to 9. The result was shown in details in Table 4. The aim of this study was to isolate the microbes that were capable of growing from 50^0C, therefore the intended study temperature range was 45, 50, 55, 60, 65, 70, 75, 80^0C. However, at the temperature higher than 55^0C the growth of microorganisms isolated was very weak. So, we focused on testing from 50 to 55^0C. At pH 9, the growth of microorganisms could not be observed, then the data were not shown in Table 4.

After 12 hours of incubation, the OD_{540} values of six isolates including BM7 (0.73 ± 0.06, at 50^0C), BS5 (0.67±0.02, at 52^0C), NS1 (0.71 ± 0.03, at 55oC), NS3 (1.04 ± 0.05, at 50^0C), NS4 (0.93 ± 0.04, at 50^0C), and NW6 (0.82 ± 0.09, at 55^0C) were higher than the others. Of these, isolates, BM7, NS3, NS4 grew optimal at 50^0C with OD540 from 0.73 to 1.04, while growth of isolate BS5 was optimal at 52^0C with OD540 at 0.67 ± 0.02. Isolates NS1 and NW6 were optimal at 55^0C with high OD_{540} at 0.71 ± 0.03 and 0.82 ± 0.09, respectively. The pH investigation also showed that isolate BS5 grew optimal at pH 6, isolates BM7, NS1, NS3, NS4 grew optimal at pH 7 while NW6 was optimized at pH 8. Moreover, the highest OD_{540} (1.18 ± 0.08) was recorded in isolate NS3 at pH 7.

3.3. Evaluation of the use of different carbon sources

Of the 33 isolates, 9 (27.3%) grew on acetate, 15 (45.5%) grew on pyruvate and 24 (72.7%) were able to use malate. All isolates were seen growth well in MSB medium with the supplement of glucose, or fructose. Under CO_2 condition, microorganism were grown in MSB medium (pH 7) with a final gas phase consisting of H_2: O_2: CO_2 (80%: 10%: 10%) at 50^0C. However, none of isolate was able to grow under autotroph condition. After 7 days of continuous observation, the turbidity environmental change was not found in the test tubes. This result suggested that there is no isolate could be fixed CO_2 or grown autotrophically. Experimental results showed that most isolates developing favorably in the presence of malate, glucose, or fructose. Malate acts as an intermediary helps the microbes produce energy as well as metabolite to produce amino acids via the tricarboxylic acid cycle, whereas glucose or fructose is easily metabolized via glycolysis pathway (Kim et al., 2008). Hence, the isolates could favorably utilize this organic substrate.

It is now very well-known that extreme thermophiles are mostly distributed among the genera of *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Thermotoga*, *Aquifex* (Tulasi et al., 2013). In which, Bacillus is a large and diverse genus that is widely distributed in soil and thermal water areas (Claus & Berkeley, 1986). During the past few decades, a great diversity of microorganisms has been discovered that exist in hot environments. In a previous report of Nguyen et al. (2015), 64 aerobic isolates of thermophilic microorganism were identified from muddy and hot water of Binh Chau hot spring. The percentage of microorganism with cellulase, amylase and protease activities is 19%, 67% and 24% of total 64 microorganism isolates, respectively.

Cellular structure and enzyme activities are deeply affected by temperature of habitat. For any microbe to grow at high temperature, their proteins must be able to resist heat. Hence, thermophiles have accumulated various thermostable enzymes that are high potential application in biotechnology. The thermophilic strains of *Bacil-*

Table 4. OD_{540} values of 33 isolates at different temperatures and pH

No.	Isolate	Temperature			pH		
		50^0C	52^0C	55^0C	6.0	7.0	8.0
1	BM1	0.40 ± 0.03	0.30 ± 0.02	0.23 ± 0.03	0.10 ± 0.02	0.41 ± 0.04	0.09 ± 0.02
2	BM2	0.59 ± 0.04	0.33 ± 0.02	0.15 ± 0.01	0.16 ± 0.02	0.57 ± 0.06	0.08 ± 0.01
3	BM3	0.44 ± 0.01	0.34 ± 0.03	0.29 ± 0.01	0.25 ± 0.01	0.44 ± 0.02	0.19 ± 0.03
4	BM4	0.36 ± 0.03	0.45 ± 0.02	0.23 ± 0.02	0.02 ± 0.01	0.37 ± 0.03	0.06 ± 0.01
5	BM5	0.24 ± 0.02	0.18 ± 0.04	0.14 ± 0.05	0.26 ± 0.04	0.27 ± 0.02	0.24 ± 0.05
6	BM6	0.40 ± 0.03	0.22 ± 0.01	0.22 ± 0.07	0.15 ± 0.03	0.44 ± 0.04	0.13 ± 0.03
7	BM7	0.73 ± 0.06	0.50 ± 0.02	0.47 ± 0.03	0.54 ± 0.05	0.72 ± 0.04	0.57 ± 0.04
8	BM8	0.45 ± 0.02	0.42 ± 0.03	0.22 ± 0.04	0.02 ± 0.01	0.47 ± 0.03	0.12 ± 0.02
9	BS1	0.41 ± 0.05	0.26 ± 0.02	0.25 ± 0.03	0.14 ± 0.03	0.42 ± 0.02	0.16 ± 0.02
10	BS2	0.43 ± 0.02	0.10 ± 0.03	0.05 ± 0.02	0.23 ± 0.02	0.45 ± 0.03	0.27 ± 0.02
11	BS3	0.53 ± 0.03	0.42 ± 0.07	0.34 ± 0.06	0.15 ± 0.03	0.50 ± 0.03	0.13 ± 0.03
12	BS4	0.35 ± 0.03	0.27 ± 0.03	0.18 ± 0.03	0.17 ± 0.01	0.37 ± 0.01	0.10 ± 0.01
13	BS5	0.46 ± 0.07	0.67 ± 0.02	0.30 ± 0.02	0.71 ± 0.05	0.65 ± 0.06	0.52 ± 0.04
14	BS6	0.35 ± 0.03	0.43 ± 0.05	0.23 ± 0.03	0.14 ± 0.03	0.39 ± 0.02	0.31 ± 0.03
15	BW1	0.40 ± 0.01	0.34 ± 0.02	0.24 ± 0.04	0.50 ± 0.03	0.43 ± 0.03	0.30 ± 0.03
16	BW2	0.42 ± 0.03	0.38 ± 0.03	0.22 ± 0.06	0.18 ± 0.06	0.44 ± 0.02	0.08 ± 0.01
17	BW3	0.60 ± 0.03	0.22 ± 0.01	0.29 ± 0.06	0.11 ± 0.02	0.58 ± 0.05	0.17 ± 0.04
18	NS1	0.45 ± 0.02	0.59 ± 0.01	0.71 ± 0.03	0.32 ± 0.05	0.76 ± 0.03	0.40 ± 0.04
19	NS2	0.62 ± 0.03	0.41 ± 0.03	0.23 ± 0.02	0.34 ± 0.04	0.61 ± 0.05	0.08 ± 0.01
20	NS3	1.04 ± 0.05	0.52 ± 0.05	0.56 ± 0.03	0.83 ± 0.07	1.18 ± 0.08	0.73 ± 0.09
21	NS4	0.93 ± 0.04	0.46 ± 0.01	0.30 ± 0.04	0.92 ± 0.04	0.94 ± 0.10	0.57 ± 0.05
22	NS5	0.43 ± 0.05	0.34 ± 0.06	0.30 ± 0.03	0.50 ± 0.03	0.43 ± 0.01	0.13 ± 0.02
23	NW1	0.51 ± 0.01	0.20 ± 0.02	0.36 ± 0.03	0.14 ± 0.03	0.54 ± 0.03	0.11 ± 0.02
24	NW2	0.26 ± 0.03	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.24 ± 0.01	0.05 ± 0.01
25	NW3	0.30 ± 0.04	0.38 ± 0.02	0.23 ± 0.03	0.23 ± 0.03	0.31 ± 0.03	0.14 ± 0.03
26	NW4	0.55 ± 0.02	0.03 ± 0.01	0.34 ± 0.05	0.19 ± 0.04	0.53 ± 0.06	0.12 ± 0.04
27	NW5	0.42 ± 0.02	0.24 ± 0.02	0.22 ± 0.01	0.15 ± 0.02	0.41 ± 0.05	0.07 ± 0.02
28	NW6	0.68 ± 0.03	0.46 ± 0.06	0.82 ± 0.09	0.48 ± 0.03	0.65 ± 0.06	0.98 ± 0.12
29	NW7	0.48 ± 0.02	0.30 ± 0.04	0.06 ± 0.02	0.16 ± 0.01	0.51 ± 0.04	0.09 ± 0.02
30	NW8	0.42 ± 0.05	0.12 ± 0.03	0.31 ± 0.05	0.26 ± 0.02	0.40 ± 0.01	0.14 ± 0.03
31	NW9	0.61 ± 0.02	0.04 ± 0.01	0.51 ± 0.02	0.12 ± 0.01	0.59 ± 0.03	0.06 ± 0.01
32	NW10	0.60 ± 0.04	0.16 ± 0.03	0.40 ± 0.03	0.13 ± 0.03	0.64 ± 0.04	0.05 ± 0.02
33	NW11	0.53 ± 0.05	0.48 ± 0.04	0.35 ± 0.02	0.19 ± 0.01	0.52 ± 0.05	0.22 ± 0.01

Table 5. Characteristics of six selected thermophilic microorganism isolates

Characteristic	BM7	BS5	NS1	NS3	NS4	NW6
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Color	Cream	Yellow	Orange	Cream	White	White
Gram/Spore	+/+	+/+	+/+	+/+	+/+	+/+
Optimum growth temperature (^{0}C)	50	52	55	50	50	55
Optimum growth pH	7	6	7	7	7	8
CO_2	–	–	–	–	–	–
Acetate	–	–	–	–	–	–
Pyruvate	–	–	–	–	–	–
Malate	+	+	+	+	+	+
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+

+: positive; –: negative

lus that synthesized cellulase, amylase and protease have a great significance for many fields of industry (Rastogi et al., 2010).

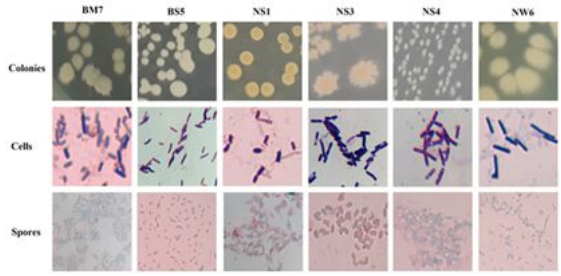

Figure 3. Image profiles of six selected thermophilic microorganism isolates.

Table 5 and Figure 3 described the profile of six selected thermophilic microorganism isolates that were selected from thermophilic microorganism collection in this study in details. In order to explore the potential application of six selected thermophilic microorganism isolates, the identification of microorganism to species as well as enzyme activity screening is required. Recently, the most effective approach to microorganism taxonomy may be analysis of 16S rDNA molecules by oligonucleotide sequencing. Detailed information of the molecular identification for six selected microorganism isolates will be announced very soon elsewhere.

4. Conclusions

From the sources of samples collected form the geothermal areas, we have successfully constructed the collection of thermophilic microorganism including of 33 isolates that are evaluated in terms of morphology, microscopy, and growth test on different substrates. Six selected isolates were Gram-positive, rod-shaped, and spore-forming. These characteristics of six selected isolates with the optimum growth temperature from 50-55^{0}C were found highly similar to *Bacillus* species. The achievement in collection of thermophiles is the preliminary step in effort to be able to apply the thermophilic microbes into the biotechnology sector.

Acknowledgements

We appreciate Nong Lam University – Ho Chi Minh City for the financial support under Research Project Code. CS-CB16-KH-03. We also thank our colleagues from Institute of Tropical Biology (Vietnam Academic of Sciences and Technology), who provided insight and expertise that greatly assisted the research.

References

Cao, T. D., Do, T. H., Nguyen, K. N., Chau, V. Q., & Vu, N. T. (1998). *Geographic Atlas of Vietnamese*. Ha Noi, Vietnam: Education Publishing House.

Claus, D., & Berkeley, R. C. W. (1986). The genus Bacillus. In Sneath, P. H. A. (Ed.). *Bergey's manual of systematic bacteriology* (1105-1139). Baltimore, USA: Williams and Wilkins.

Gaughran, E. R. (1947). The thermophilic microor ganisms. *Bacteriological reviews* 11(3), 189.

Goto, E., Kodama, T., & Minoda, Y. (1977). Isolation and culture conditions of thermophilic hydrogen bacteria. *Agricultural and Biological Chemistry* 41(4), 685-690.

Hildur V., Dagný B. R., & Jóhann O. (2011). *Hydrogenophilus islandicus* sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from an Icelandic hot spring. *International Journal of Systematic and Evolutionary Microbiology* 61, 290-294.

Kim, B. H., & Gadd, G. M. (2008). *Bacterial physiology and metabolism*. Cambridge, England: Cambridge University Press.

Nguyen, T. K., Tran, T. T., Tran, H. T., & Tran M. D. (2015). Potential of thermostable enzymes production from bacterial strains isolated in Binh Chau hot spring. *The 6th Scientific Conference on Biological Resources*. Ha Noi, Vietnam.

Rastogi, G., Bhalla, A., Adhikari, A., Bischoff, K. M., Hughes, S. R., Christopher, L. P., & Sani, R. K. (2010). Characterization of thermostable cellulases produced by Bacillus and Geobacillus strains. *Bioresource Technology* 101(22), 8798-8806.

Tran, M. D., Nguyen, T. K., Nguyen, D. T., & Nguyen, V. Q. (2012). Biological characteristics and classification of the thermophilic bacteria BML07 strain producing both thermostable amylase and cellulase isolated from My Lam hot spring. *Journal of Science and Technology* 50(1), 29-38.

Tulasi, S., Jennifer, L., & Yutaka, K. (2013). Thermophilic Microbes in Environmental and Industrial Biotechnology. In *Biotechnology of Thermophiles* (2th ed.). New York, USA: Springer.