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Enzymatic Screening and Genotypic Characterization of Thermophilic Bacteria from the Hot Springs of Sarawak, Malaysia

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Abstract

Owing to their eccentric thermostable ability, thermophiles are among the most utilized extremophiles in various industries, such as manufacturing, and clinical research. Researchers believe that many unknown thermophiles are yet to be discovered. This study aimed to genotypically characterize the diversity of thermophiles and screen them for the potential production of enzymes in the recreational hot springs located at Northwest Coast of Borneo. Water samples were collected at 45 °C–50 °C from Annah Rais and Panchor hot springs during the sampling period from January 2018 to January 2019. Three samples (water and sediment) were collected twice in a 3-week interval from each pool of the sampling sites. Each water sample was diluted up to 10^{-3} and plated on thick nutrient agar at 55 °C for 24 h. Customized nutrient agar plus Bacto-agar plates were used for the optimum growth analysis of the isolates at 40 °C–90 °C for 24 h. The thermophiles were isolated, characterized biochemically, and amplified molecularly using DNA fingerprinting and 16S rRNA gene sequencing. Lipase, protease, gelatinase, amylase, catalase, and nitrate reductase enzymatic production was examined. Twenty-one thermophilic isolates were successfully characterized into seven clusters of *Amnoxybacillus* spp. and *Geobacillus* spp. by studying their phylogenetic dendrograms. Isolates AR10 and AR15 could produce most of the tested enzymes. All the isolates showed negative results in gelatinase and lipase production. PC14 was the only isolate that did not produce any of the enzymatic reactions in this experiment. The results showed that most of the thermophiles isolated from the two Borneo hot springs can synthesize enzymes and have potential to be thermostable. In conclusion, the search for the thermophilic producers of novel enzymes in Borneo is successful; further research must focus on their applications.

Keywords: Borneo, enzyme, genotypic, recreational hot springs, thermophiles

Introduction

The unique enzymatic potential of thermophilic and hyperthermophilic microorganisms was discovered two decades ago, prompting explorations on their potential biotechnological, industrial, and clinical applications. Thermophiles are the extremophiles that optimally grow in hot environments with temperatures ranging from 45 °C to 122 °C [1]. Thermophiles are grouped in three major clusters, namely, moderate thermophiles (survives at temperatures from 55 °C–65 °C), extreme thermophiles (survives at temperatures from 65 °C–80 °C), and hyperthermophiles (survives at temperatures higher than 80 °C) [2]. They exhibit normal function and metabolism in tremendously hot environments. Most thermophiles are classified as members of the *Bacillaceae* family, including *Thermobacillus*, *Coprobacillus*, *Anoxybacillus*, *Sulfobacillus*, *Halobacillus*, *Salibacillus*, *Marinibacillus*, *Virgibacillus*, *Amphibacillus*, *Alicyclobacillus*, *Gracilibacillus*, *Geobacillus*, *Jeotgalibacillus*, *Breviballicus*, *Paenibacillus*, *Aneurinibacillus*, and *Ureibacillus*, as identified using 16S rRNA gene sequences [3–5]. Thermophiles are found in habitats such as hot springs, deep-sea cores, petroleum reservoirs, deep-sea hydrothermal vents [6], and man-made facilities such as compost piles, slag heaps, and water heaters [7].

Researchers believe that these thermophiles have acquired certain thermophilic adaptation abilities, exhibit high metabolism, and secrete various physically and chemically stable enzymes [8] with special biotechnological interest. The thermostability of these organisms enables them to be utilized as biocatalysts in most manufacturing industries, such as biomass deconstruction, fuel-related compound manufacturing, and leaching or waste management industries.

Thermophilic bacilli are industrially important because they produce extracellular thermostable enzymes, such as proteases, amylases, lipases, pullulanase, xylanases, and others [9]. They can convert surrounding lipids and carbohydrates to energy and carbon sources. With these advantages, thermophiles can grow faster than mesophilic bacteria. Thus, these organisms can speed up fermentation; reduce microbial contamination risks, high diffusion rate, or mass turnover; decrease the viscosity of the liquid medium; and increase the solubility of polymeric substrates or fats [9]. Thermostable enzymes are resistant to proteolysis and chemical denaturation. Although thermophiles have many advantages in industrial application, they can also be harmful pathogens isolated from clinical samples and cause serious illness. A previous study identified two patients infected with septic meningitis caused by the thermophilic bacteria in their cerebrospinal fluid [10].

The biodiversity of thermophiles in our tropical habitat in Borneo has received huge attention from scientists globally. This study focused on two well-known recreational hot springs in Borneo, Annah Rais and Panchor hot springs. Annah Rais hot spring is an ancient 250-year-old hot spring surrounded by mountains and maintained by the early Bidayuh settlers, along with the longhouse. It is a natural feature formed by the geothermal forces heating the underground water to the surface; the water is drained into Sungai Semadang or Sungai Sarawak Kiri. It spans 71 km and is about 1 h and 20 min driving distance from the city. Panchor hot springs is a natural pool surrounded by forests and situated within them are settled by the Dayak natives. It spans 50 km and is about a 1h driving journey from the city. The early native people believed that hot springs are holy places to seek blessings and cures for various illnesses. Data profiling on the thermophiles in these hot springs is still limited and yet to be discovered. This study aimed to genotypically characterize the diversity of thermophilic bacteria in the hot springs of the Northwest Coast of Borneo and screen them for the potential production of extracellular enzymes.

Materials and Methods

Sampling sites. Water samples were collected from Annah Rais (latitude 1.1332°N, longitude 110.2676°E) and Panchor (latitude 1.2540°N, longitude 110.4508°E) hot springs located in the Northwest Coast of Borneo, Malaysia from January 2018 to January 2019.

Sample collection and processing. Water samples were collected from the major heat sources of each hot spring at different depths as surface water, sediment with 45 cm depth, sediment with 60 cm depth, and sediment with 120 cm depth. Three samples (water and sediment) were collected from each pool of the sampling sites and kept in thermostable flasks for further analysis. Samples were collected twice in a 3-week interval. The temperature and pH of different water layers were recorded. Each water sample was diluted up to 10^{-3}, plated on thick nutrient agar (20 g/L) (Merck, Germany) with the pH adjusted according to the pH level of the hot spring water using 1 M sterile Na-sesquicarbonate solution (Merck, Germany), and incubated at 55 °C for 24 h. The bacteria were kept in nutrient broth (OXOID, UK) containing 15% glycerol at −80 °C for further studies.

Biochemical characterization on thermophiles. The pure culture of each thermophilic bacterial strain was biochemically identified using Gram staining, sulfur indole motility (Merck, Germany), phenol red mannitol salt agar (Merck, Germany), McConkey agar (Merck, Germany), Simmons citrate agar (Merck, Germany), triple sugar iron agar (Merck, Germany), and Levine's eosin–methylene blue agar (Merck, Germany) tests.

Thermophile DNA extraction and purification. DNA was extracted from the pure culture of thermophilic bacterial strains using the cetyl trimethylammonium bromide (CTAB) DNA extraction method by Sahu *et al.* [11] with modification to obtain the maximum yield of DNA. In brief, 2 mL of the overnight pure culture was centrifuged at 10,000 rpm for 5 min. The procedure was repeated twice. The pellet was harvested and resuspended in 567 µL of Tris ethylenediaminetetraacetic (EDTA) (Merck, Germany) (TE) buffer, followed by the addition of 30 µL of 10% sodium dodecyl sulfate (SDS) (Merck, Germany) and 3 µL of 20 mg·mL^{-1} proteinase K (Merck, Germany) to achieve the final concentration of 100 µg·mL^{-1} proteinase K in 0.5% SDS. The mixture was then mixed thoroughly and incubated for 1 hour at 37 °C. After incubation, the mixture was added with 100 µL of 5 M sodium chloride (NaCl) (Merck, Germany) and 80 µL of CTAB/NaCl (Merck, Germany) solution and incubated at 65 °C for 10 min. Afterward, 780 µL of chloroform/isoamyl alcohol (Merck, Germany) was added and centrifuged at 10,000 rpm for 5 min. The aqueous supernatant was harvested and mixed with an equal volume of phenol/chloroform/isoamyl (Merck, Germany) alcohol. The mixture was then mixed well and centrifuged at 10,000 rpm for 5 min. The supernatant was harvested again and added with 0.6 mL of isopropanol (Merck, Germany). DNA was precipitated by spinning briefly at room temperature. Lastly, the DNA pellet was washed with 70% ethanol to remove residual CTAB,

centrifuged at 10,000 rpm for 5 min, and resuspended with 100 µL of TE buffer for further analysis.

DNA fingerprinting. The DNA was amplified by PCR to determine the strain diversity of the thermophiles using oligonucleotide probe (GTG)5 (5′-GTGGYGGYGGYGGYG-3) (Promega, USA) as stated by Sien *et al.* [12]. A PCR master mix was prepared with 5 µL of 5X Buffer (Promega, USA), 3 µL of 25 mM magnesium chloride (MgCl2), 1 µL of 10 mM of deoxynucleotide triphosphate (dNTP) (Promega, USA), 1 µL of 10 µM (GTG)5 primer (Promega, USA), 9.5 µL of sterilized distilled water, 5 µL of the isolate's DNA, and 0.5 µL of Taq polymerase (Promega, USA). Amplification was performed using the LabCycler (SensoQuest GmbH, Germany) with the following PCR profile: preliminary denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 1 minute, annealing at 50 °C for 1 minute, and an extension at 72 °C for 1 minute; and a final extension at 72 °C for 5 min. The amplified products were viewed using 1.2% agarose gel agar in Tris-borate-EDTA (TBE) (Merck, Germany) buffer at 90 V and 400 mAh for 105 min. The 1 kb DNA ladder (Promega, USA) was used as the standard DNA size marker for each run. The DNA fingerprint gel images were then analyzed using GelJ software Version 2.0. The dendrogram was constructed to look at the relationship between the isolates.

16S rRNA sequencing. The identified isolates were analyzed with 16S rRNA PCR by amplifying their 16S ribosomal RNA genes [13]. Primers 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 519R 5′-GWATTACCGCGGCKGCTG-3′ (Promega, USA) were used in the PCR amplification. A PCR master mix was prepared with 10 µL of 5X Buffer (Promega, USA), 6 µL of 25 mM MgCl2, 3 µL of 10 mM of dNTP (Promega, USA), 1 µL of each primer (Promega, USA), 8 µL of sterilized distilled water, 20 µL of the isolate's DNA, and 1 µL of Taq polymerase (Promega, USA). The amplification was repeated for 30 cycles with the following PCR profile: preliminary denaturation step at 95 °C for 10 min, denaturation at 99 °C for 30 seconds, annealing at 55 °C for 1 min, and extension at 72 °C for 80 seconds, followed by a final extension at 72 °C for 10 min. The samples were cooled at 4 °C. The amplified products were viewed using 1.0% agarose gel in TBE buffer at 90 V and 200 mAh for 30 min. The 100 bp DNA ladder (Promega, USA) was used. The purified PCR product was sent to Apical Scientific Sdn Bhd (Malaysia) for further DNA sequencing to identify the closest identity matches by comparing the product with other sequences from the data bank in the NCBI website (http://www.ncbi.nlm.nih.gov) using BLAST.

Optimum temperature determination. The bacterial isolates were plated on customized nutrient agar plus Bacto-agar (15 g/L) (Difco Laboratories, USA) plates in duplicate and incubated at 40 °C, 45 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, and 90 °C for 24 h. All colony forming units that grew on the plates were counted and recorded [14].

Assessment of enzymatic production. Screening for enzymes such as lipase, protease, gelatinase, amylase, catalase, and nitrate reductase was performed in the bacterial cultures grown for 24–48 h at their optimum temperature. Each enzymatic test was performed in triplicate. *Pseudomonas* sp. identified in the laboratory was used as the control.

Lipolytic enzyme. Lipolytic activity was assessed using tryptone soy agar (Lab M, United Kingdom) supplemented with 1% of Tween 20. The presence of a turbid halo around the inoculum indicated positive lipolytic activity.

Protease test. Protease activity was assessed using Mueller–Hinton agar (Himedia, India) supplemented with 3% of skimmed milk powder. The appearance of a transparent zone around the inoculum indicated caseinase activity.

Gelatinase test. Gelatinase activity was assessed by stabbing the isolate culture onto nutrient agar (Merck, Germany) supplemented with 3% gelatin. After the incubation, the medium was cooled at 4 °C for 30 min. Gelatin liquefaction indicated gelatinase activity.

Amylase test. Amylase activity was assessed using Difco™ Starch Agar (BD, United States). After staining with 1% iodine solution (Merck, Germany), the appearance of a clear halo zone around the colonies indicated amylase activity.

Catalase test. Catalase test was assessed using hydrogen peroxide (H2O2) (Merck, Germany). Rapid bubbling upon dipping the isolates into the H2O2 solution indicated catalase activity.

Nitrate reductase activity. Nitrate reductase activity was assessed using Difco™ Nitrate Broth (BD, United States). Nitrate A reagent containing 0.5 mL of 0.8% sulfanilic acid in 5N acetic acid and Nitrate B reagent containing 0.5 mL of 0.6% N, N-dimethyl-alpha-naphthylamine in 5N acetic acid were added into the bacteria cultured in nitrate broth. The appearance of a red color indicated nitrate reductase activity. Meanwhile, a colorless broth was added with Zn powder to test the nitrate reductase activity. The presence of a red color upon the addition of Zn powder indicated the lack of nitrate reductase activity. If the broth remained colorless after the addition of Zn powder, then it had nitrate reductase activity.

Results

Thermophile profiling. The sampling details, thermophilic bacteria isolates, and their thermal tolerance properties are stated in Table 1. The water samples collected from both hot springs had the same moderate temperature (45 °C–50 °C) but different pH levels. A total of 50 bacteria was successfully isolated from the hot springs and grouped into 8% (n = 4) mesophiles (40 °C), 48% (n = 24) thermophiles (55 °C–65 °C), and 44% (n = 22) hyperthermophiles (higher than 80 °C). The optimum temperature growth of the bacterial isolates was determined as shown in Figure 1.

Table 1. Thermophilic Bacteria Profiling Details Collected from Annah Rais and Pachor Hot Springs in the Northwest Coast of Borneo, Malaysia

Sampling Sites	Code	Sources	Layer of Water	Temperature (˚C)	pH	Bacteria Isolates	Optimum Growth Temperature (˚C)	Thermal Tolerance
Annah Rais	G	Small pond	Sediment (45 cm depth)	45	8.63	AR01	60	Thermophile
						AR02	60	Thermophile
						AR03	60	Thermophile
						AR04	90	Hyperthermophiles
	J	Small pond	Sediment (45 cm depth)	45	8.63	AR08	60	Thermophile
						AR09	60	Thermophile
						AR10	60	Thermophile
						AR11	90	Hyperthermophiles
						AR18	60	Thermophile
						AR19	40	Mesophile
	H	Big pond	Surface Water	45	8.17	AR05	90	Hyperthermophiles
						AR06	60	Thermophile
	I	Big pond	Sediment (60 cm depth)	50	8.17	AR07	90	Hyperthermophiles
						AR17	90	Hyperthermophiles
	K	Big pond	Surface Water	45	8.17	AR12	90	Hyperthermophiles
						AR13	90	Hyperthermophiles
	L	Big pond	Sediment (60 cm depth)	50	8.17	AR14	60	Thermophile
						AR15	90	Hyperthermophiles
						AR16	90	Hyperthermophiles
						AR20	90	Hyperthermophiles
						AR21	90	Hyperthermophiles
Panchor	A	Pond	Surface Water	45	6.62	PC01	90	Hyperthermophiles
						PC02	90	Hyperthermophiles
						PC03	90	Hyperthermophiles
						PC15	60	Thermophile
	B	Pond	Sediment (45 cm depth)	47	6.62	PC04	60	Thermophile
						PC05	40	Mesophile
						PC06	40	Mesophile
						PC07	40	Mesophile
						PC08	90	Hyperthermophiles
						PC16	90	Hyperthermophiles
						PC29	90	Hyperthermophiles
	C	Pond	Sediment (120 cm depth)	50	6.62	PC09	90	Hyperthermophiles
						PC17	60	Thermophile
						PC18	60	Thermophile
	D	Pond	Surface Water	45	6.62	PC10	60	Thermophile
						PC19	60	Thermophile
						PC20	60	Thermophile
						PC21	60	Thermophile
						PC22	60	Thermophile

Table 1. *Continue*

Sampling Sites	Code	Sources	Layer of Water	Temperature (˚C)	pH	Bacteria Isolates	Optimum Growth Temperature (˚C)	Thermal Tolerance
	E	Pond	Sediment (45 cm depth)	47	6.62	PC11	60	Thermophile
						PC12	60	Thermophile
						PC13	60	Thermophile
						PC23	90	Hyperthermophiles
						PC24	60	Thermophile
	F	Pond	Sediment (120 cm depth)	50	6.62	PC14	90	Hyperthermophiles
						PC25	90	Hyperthermophiles
						PC26	60	Thermophile
						PC27	60	Thermophile
						PC28	90	Hyperthermophiles

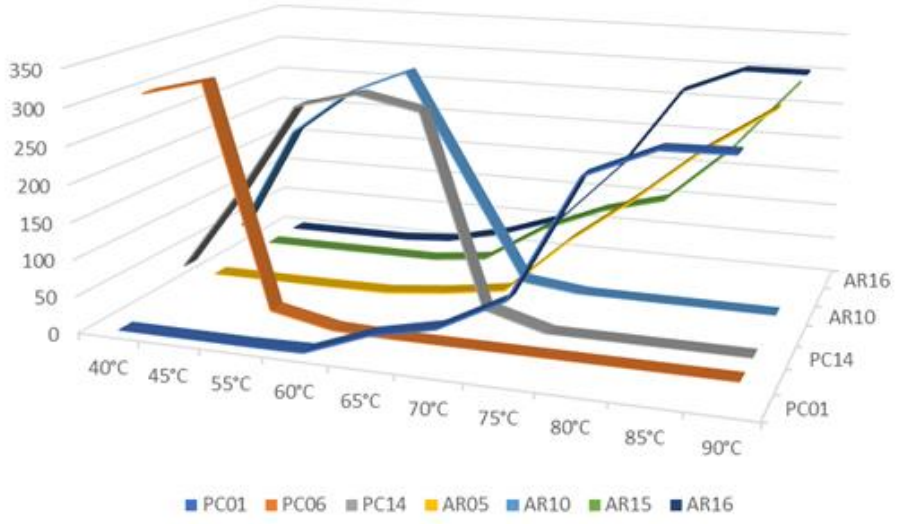

Figure 1. Optimum Temperature Growth of the Thermophiles Isolated from Annah Rais and Panchor Hot Springs in the Northwest Coast of Borneo, Malaysia

Phylogenetic analysis and thermophile identification. The gel electrophoresis image of the $(GTG)_5$ fingerprinting analysis and the constructed phylogeny tree are demonstrated in Figures 2 and 3. All the isolates were clustered into seven groups. Based on the analysis of colony morphology and phylogenetic tree, one isolate from each group (AR05, AR15, PC06, AR16, AR10, PC01, and PC14) was chosen for further characterization by amplifying its 16S rRNA gene sequence. The amplified DNA product was detected at 600 bp. Sequence homology was observed between the partial sequences of *A. rupiensis* strains 1–35 and isolate PC14 and between *G. stearothermophilus* strain WSUCF-035C and isolate AR10 in the NCBI Genbank database. BLAST sequencing result revealed that most of the isolated thermophiles fall in two main genera (Table 2).

Assessment of biochemical and enzymatic production. The seven isolates were biochemically characterized and tested for the production of several enzymes (Table 3). All the bacteria were Gram-positive and had rod shapes. All the seven isolates tested negative on all the selective and differential agars and were unable to produce gelatinase and lipase enzyme. PC14 was the only isolate that did not produce any enzymatic reaction and showed negative results on all the tested enzyme activities. AR10 and AR15 showed positive results in most of the tested enzyme activities except gelatinase and lipase activity. The isolates from the Borneo hot springs were able to synthesize most of the enzymes with high thermostability.

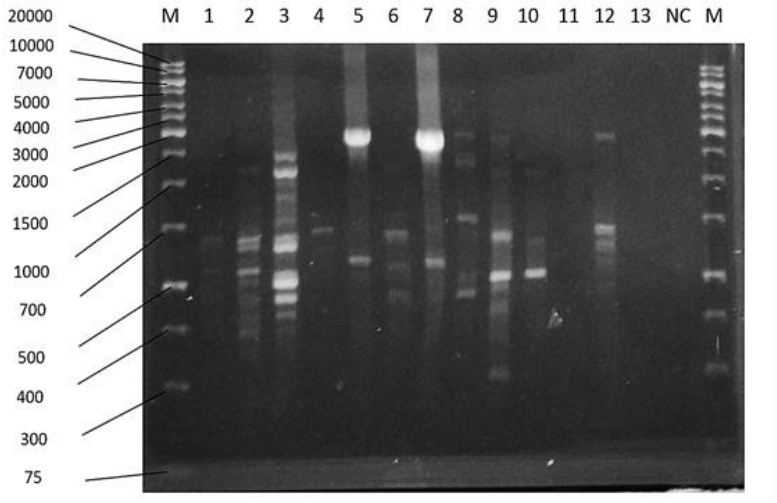

Figure 2. Gel Image Showing the $(GTG)_5$ Fingerprinting DNA Amplification Products of Eleven Isolates. The PCR Products were Loaded Onto 1.2 % (w/v) Agarose Gel and Run at 90 V for 105 min. M 1kb DNA Ladder. 1–12 Selected Thermophiles Isolates, Namely, AR02, AR03, AR05, AR07, AR08, AR09, AR10, AR11, AR12, AR13, and AR16. NC Negative Control

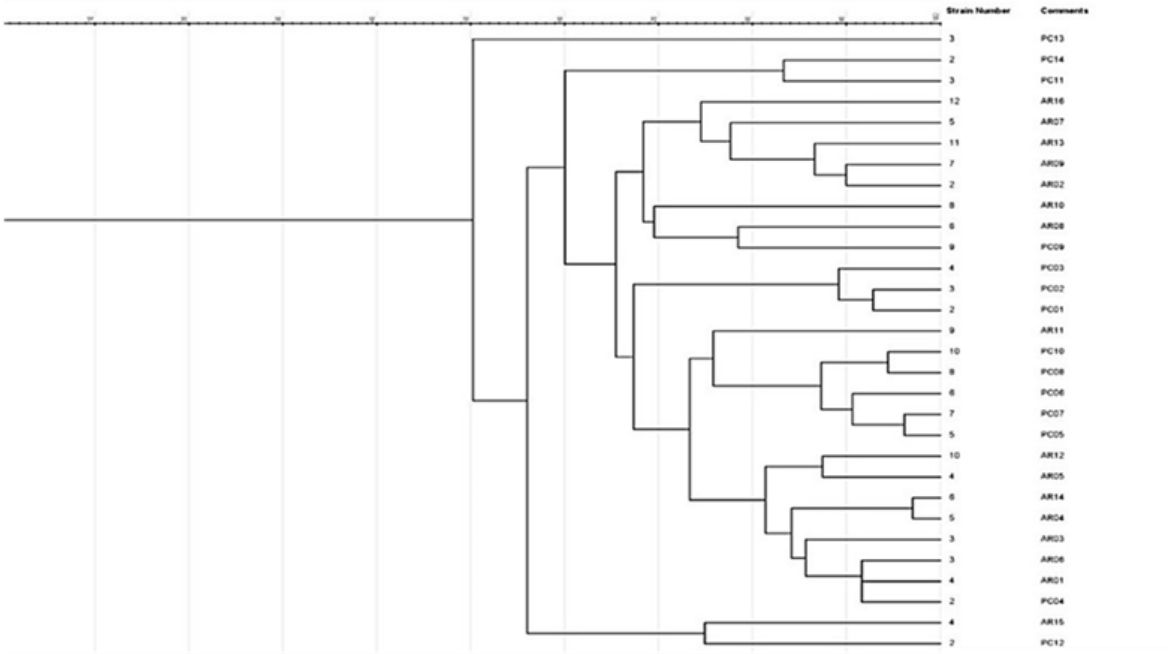

Figure 3. Phylogenetic Tree Constructed using GelJ Software Version 2.0 Showing the Relationships of the Thermophiles Isolated from the Hot Springs of the Northwest Coast of Borneo. Comments Showed All Thermophile Isolates

Table 2. Isolated Thermophiles Isolated from the Hot Springs of the Northwest Coast of Borneo

Isolates	Identification	Gene bank access number	Closest related species	Similarity %
PC01	*Anoxybacillus rupiensis*	MF037808	*A. rupiensis* strain T7	100
PC06	*Anoxybacillus flavithermus*	MH005096	*A. flavithermus* strain SS6_1017_022	100
PC14	*Anoxybacillus rupiensis*	KF583689	*A. rupiensis* strain 1-35	100
AR05	*Anoxybacillus gonensis*	MH005096	*A. gonensis* strain Lhs-8	100
AR10	*Geobacillus stearothermophilus*	MF965163	*G. stearothermophilus* strain WSUCF-035C	100
AR15	*Geobacillus stearothermophilus*	KP338613	*G. stearothermophilus* strain IR4	100
AR16	*Geobacillus stearothermophilus*	CP016552	*G. stearothermophilus* strain DSM 458	100

Table 3. Biochemical Characteristics of the Isolates Selected Due to Their Potential as Enzyme Producers

Isolates	Biochemical								Enzymatic					
	Gram staining	Morphology	TSI	Citrate	SIM	EMBA	MacConkey	MSA	Lipase	Protease	Gelatinase	Amylase	Catalase	Nitrate reductase
PC01	+	Rod	−	−	−	−	−	−	−	−	−	+	+	−
PC06	+	Rod	−	−	−	−	−	−	−	−	−	−	+	+
PC14	+	Rod	−	−	−	−	−	−	−	−	−	−	−	−
AR05	+	Rod	−	−	−	−	−	−	−	−	−	−	−	+
AR10	+	Rod	−	−	−	−	−	−	−	+	−	+	+	+
AR15	+	Rod	−	−	−	−	−	−	−	+	−	+	+	+
AR16	+	Rod	−	−	−	−	−	−	−	−	−	−	−	+
Con-trol									+	+	+	−	−	+

Note: − absent; + present; Control: *Pseudomonas* sp.

Discussion

The profile and diversity of thermophilic bacterial communities in the two recreational hot springs distributed in the Northwest Coast of Borneo were investigated (Table 1). Our study categorized the bacteria into 8% mesophiles, 48% thermophiles, and 44% hyperthermophiles. Temperature is an important growth parameter for bacterial diversity [15], and these two factors exhibit a positive linear correlation [16]. An increase in temperature, especially in harsh environments such as hot springs, remarkably decreased bacterial diversity and species richness, allowing only dominant extremophiles to grow. Fingerprinting analysis clustered the thermophiles into seven bacterial groups based on their genetic closeness. Based on the colony morphology and phylogenetic tree analysis, one isolate from each phylogenetic group was identified using 16S rRNA gene sequence. The thermophiles isolated from the hot springs in Borneo were unique. They could grow in the water and sediment of the hot springs at moderate temperature (45 °C–50 °C). However, as the optimum growth temperature for each isolate was reported high, they possess a substantially potential for thermostable enzyme production. They can be used in industrial processes, such as the degradation of environmental pollutants. BLAST sequencing result revealed that most of the isolated thermophiles fall in two main genera, i.e., *Anoxybacillus* spp. and *Geobacillus* spp. (Table 2).

Anoxybacillus spp. and *Geobacillus* spp. are thermophiles that can survive and abundantly multiply in these high-temperature hot springs. In 2000, *Anoxybacillus* spp. was first isolated from manure samples and categorized as anaerobic, alkaliphilic, fermentative, moderately thermophilic, endospore-forming rod-shaped bacteria [17]. Our findings showed that *Anoxybacillus* spp. are found in the alkaline and acidic hot springs of Borneo and concurred with the study of Goh *et al.* [18], who explained that *Anoxybacillus* spp. are well adapted to the environmental pH. Isolates PC01 and AR05, both detected as *Anoxybacillus* spp., can grow in temperatures up to 90 °C, although most studies [8, 17, 18] have described their optimum growth to be 65 °C. *Geobacillus* spp. are endospore-forming obligate rod-shaped thermophiles that can grow in temperatures up to 80 °C [19]. Both thermophiles are widely isolated found the hot springs. We detected *Anoxybacillus flavithermus*, which was also successfully isolated by Caspers *et al.* [20] from the dairy-processing plants in New Zealand and the Netherlands, and *Anoxybacillus rupiensis*, which was first discovered by Derekova *et al.* [21] in 2007 as a novel thermophilic bacterium isolated from Rupi Basin, Bulgaria. Similar results were obtained by Chai *et al.* [22], who successfully isolated *Anoxybacillus* spp. from Klah River and Dusun hot springs in Malaysia. Most of the isolated thermophiles have vast potential for industrial and agricultural functions. Isolate AR05, identified as *Anoxybacillus gonensis*, can secrete amylase enzyme for poly-3-hydroxybutyrate degradation [23]. Beris *et al.* [24] believed that the G2ALT gene in *Anoxybacillus* spp. in PP-loop ATPase superfamily may be responsible for aluminum tolerance. Kambourova *et al.* [25] stated that *A. flavithermus* is widely used in agriculture to produce a thermostable multienzyme xylanase complex in the hemicellulosic fraction of terrestrial plants to improve the yield of sugar production from oat-spelt xylan. Neumann *et al.* [26] stated that

Geobacillus stearothermophilus, a hyperthermophile, is often applied in sterilization because it can survive after the process in autoclaves. Similar results were obtained by Sharma *et al.* [27], who found 13 *Geobacillus* spp. isolated from the Soldhar hot spring site of Garhwal Himalaya in India. *Geobacillus* spp. has a broad spectrum of carbohydrate-utilization ability, which is yet to be discovered because this bacterium is still new to scientific research.

Among the thermophiles, the genera *Geobacillus* and *Amoxybacillus* have received considerable attention due to their thermostable adaptation and potential applications in producing industrially important enzymes. Hot springs are considered harsh environments with low nutritional status and sometimes high mineral content. Only the toughest extremophiles can endure these conditions for growth. The natural selection of most extremophiles varies with different ecological habitats. Thus, the thermophile population in most hot springs is dominated mainly by bacilli, including *Geobacillus* spp. and *Amoxybacillus* spp. On the basis of the results, all seven isolates were unable to produce gelatinase and lipase enzymes. Thus, most of the bacilli were unable to use gelatin and oil as their food sources. However, most of the reported thermophiles are lipase and gelatinase producers [28]. Pimpliskar *et al.* [29] detected gelatinase enzyme in the thermophiles isolated from the hot water in Vajreshwari, India. Most of the isolates showed positive nitrate reductase activity except PC01 and PC14, which are *Anoxybacillus rupiensis*. The nitrate reductase enzyme is responsible for nitrogen assimilation in most plants because it provides an enzymatic source of nitric oxide. AR10 and AR15 were identified as *G. stearothermophilus*, which produces most of the tested enzymes including amylase, catalase, protease, and nitrate reductase. However, AR16 was also identified as *G. stearothermophilus* but did not show as much enzymatic activity as AR10 and AR15 possibly due to the different adaptations of different bacterial strains. In addition, *G. stearothermophilus* can produce thermostable and thermoactive α-amylase [30], oxidase, catalase [31], and highly thermostable protease [32].

Conclusions

Our study reported 4 mesophiles, 22 thermophiles, and 24 hyperthermophiles with seven clusters of *Amnoxybacillus* spp. and *Geobacillus* spp. isolates from the two hot springs. Isolates AR10 and AR15 could produce most of the tested enzymes except gelatinase and lipase. We believe that other potential thermophiles are yet to be identified. Thus, further studies are crucial to discover the full potential of the thermophiles in the industrial and commercial sectors. *A. flavithermus* isolated in this study can enhance the sugar production yield by facilitating the enzymatic hydrolysis of xylan. Meanwhile, *A. salavatliensis* can produce alpha-glucosidase, the catalyst used in starch hydrolysis that may aid in the digestion of dietary carbohydrates in the food industry.

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Conflict of Interest Disclosure

None declared.

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