Characterization of Thermophilic Microorganisms in the Geothermal Water Flow of El Chichón Volcano Crater Lake

Sandy Luz Ovando-Chacon [1,*,†], Veymar Guadalupe Tacias-Pascacio [1,†], Guillermo Efren Ovando-Chacon [2,*], Arnulfo Rosales-Quintero [1], Abelardo Rodriguez-Leon [2], Victor Manuel Ruiz-Valdiviezo [1] and Alberto Servin-Martinez [2]

[1] Tecnológico Nacional de México/Instituto Tecnológico de Tuxtla Gutierrez, Carretera Panamericana km 1080, Tuxtla Gutierrez, Chis 29050, Mexico; vey_pascacio@live.com (V.G.T.-P.); arnol122@gmail.com (A.R.-Q.); bioqvic@hotmail.com (V.M.R.-V.)

[2] Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, Calzada Miguel Ángel de Quevedo 2779, Veracruz, Ver 91860, Mexico; abelardo.rl@veracruz.tecnm.mx (A.R.-L.); alservin@gmail.com (A.S.-M.)

* Correspondence: ovansandy@hotmail.com (S.L.O.-C.); guillermo.oc@veracruz.tecnm.mx (G.E.O.-C.)

† Both authors contributed evenly in this paper.

Abstract: This study reports for the first time the isolation, identification and characterization of lipase-producing thermophilic strain from the geothermal water of the El Chichón volcano crater lake. Two strains were identified by 16S rRNA sequencing as *Geobacillus jurassicus* CHI$_2$ and *Geobacillus stearothermophilus* CHI$_1$. Results showed that *G. jurassicus* CHI$_2$ is Gram-positive, able to ferment maltose, fructose and sucrose and to hydrolyze starch and casein; while *G. stearothermophilus* CHI$_1$ showed to be Gram-variable, able to ferment maltose and fructose and to hydrolyze starch. Colonies of both strains presented irregular shape, umbilicated elevation of gummy texture and cells presented flagellar movement to survive in fluids with high temperature and mass gradients due to complex phenomena of heat and mass transfer present in the geothermal fluids. Lipase production for *G. stearothermophilus* CHI$_1$ was also evaluated. It was found that this strain possesses a growth associated with extracellular lipase production with a high activity of 143 U/mL at 8.3 h of incubation time, superior to the activities reported for other microorganisms of genus *Geobacillus*; for this reason, it can be said that the thermal flow of the El Chichón volcano crater lake can be a useful source of lipase-producing thermophilic bacteria.

Keywords: thermophilic bacteria; geothermal flow; lipase; temperature gradient; thermal biotechnological processes; hydrolysis; volcanic crater lake

1. Introduction

The need to synthesize value-added products in a more sustainable way led biocatalysis to be regarded as a competitive option against chemical processes [1–4]. However, due to the fact that operating conditions of industrial processes are generally very different from those required by biocatalysts, the application of biocatalysts is still limited. For this reason, nowadays there is a growing need for new enzymes that are stable under severe operating conditions, in order to replace or at least complement traditional chemical processes [2]. One possible solution to this drawback is the use of extremozymes produced by extremophilic microorganisms, which have developed a natural resistance to harsh conditions [3,4], being able to perform reactions at acidic and alkaline pH, at temperatures of up to 140 °C, or close to the freezing point of water, at high pressures or in non-aqueous environments and water/solvent mixtures, making them an excellent biotechnological

tool to catalyze reactions in severe conditions [4]. Extremophilic microorganisms are a rich source of extremozymes; these microorganisms are capable of surviving in extreme habitats under very high or low pH (alkaliphiles or acidophiles) and temperatures (psychrophiles and thermophiles), low humidity (xerophiles) or elevated pressure (piezophiles) and salt concentrations (halophiles) or ionizing radiation levels (radiophiles) [5]. Particularly, thermophilic microorganisms have gained importance in "white biotechnology", which is defined as the use of organisms and enzymes for industrial processing and the production of materials, chemicals and energy [6,7]. Thermophiles have optimum growth temperature of >55 °C and in general, many of them are polyextremophiles, which means that they are capable of living in other extreme environmental conditions, such as those related to pH, redox potential, salt concentration or the presence of a wide spectrum of toxic compounds [8–10]. Thermophiles are mainly found in hot springs [11–13], hot springs adjacent to volcanic environments [14–16], upper soil layers [17]—and even in man-made thermal environments, such as compost facilities [18]. Therefore, different research groups have reported important works related with the characterization of microorganisms in aqueous media [19–22]. On the other hand, thermophiles have numerous thermal biotechnological applications, either using whole cells or their macromolecules or metabolites, among which are the bioremediation [4,23–25], bioenergy [26–28], biomining [29,30] and biosurfactant production [14,31]. In addition, there is a great interest on the study of thermophilic bacteria due to their use as a source of thermostable enzymes (thermozymes) [6,7,32–34], such as cellulases, xylanases, pectinases, chitinases, amylases, pullalanases, proteases, esterases and lipases [6,7,35]. Thermozymes are very important in industrial processes due to the fact that higher temperatures improve the solubility of many reaction components (mainly polymeric substrates) and reduce the risk of contamination [36].

Lipases are among the most used enzymes in the industry; they are defined as triacylglycerol acylhydrolases that biocatalyze the hydrolysis of long chain triacylglycerols and are widely found in animals, plants and microorganism, but those originated from bacteria are more stable and easier to cultivate and optimize than others [37,38]. Lipases are used in several industrial sectors like detergents, foods, cosmetics, pharmaceutics [39,40] and biodiesel production [41,42], and they are selected for each application according to their substrate specificity, position of fatty acid esters and stereospecificity, as well as their temperature and pH stability. Multiple industrial applications of lipases have stimulated a growing demand for lipases with more robust catalytic properties that are able to withstand harsh operating conditions. Because of that, the isolation and selection of new strains of lipase-producing microorganism have been intensively investigated [43–48]. It has been reported the isolation of these microorganisms from different sources such as agro industrial waste [43], saltworks [1], vegetable oil processing factory [44], dairy plants [45], soil contaminated with oils [46,47] and petrol spilled soil [48]. In this sense, the isolation of microorganisms from extreme environments, with the ability to produce lipolytic enzymes, becomes very important.

El Chichón volcano (17.36° N, 93.23° W; 1100 m above sea level, MASL) is located northwest of the state of Chiapas, southeastern México and it is part of the Chiapanecan volcanic arc [49,50]. On 25 April 1982, it was observed for the first time the presence of three small lakes, which in November of the same year, coalesced into one [49]. Since the formation of the crater lake, its level, shape and geochemical composition has changed constantly and drastic temperature and pH variations have been observed [50–53]. There are several reports related to the hydrology, hydrochemistry, geothermal potential [54], hydrogeochemical [53], geophysical characterizations [50] and geological evolution of the El Chichón volcano crater lake [55,56], but very few studies related to its microbiology. A study on the diversity and abundance of bacterial communities of the El Chichón volcano crater lake sediments, was reported [57]. In this study, fifteen phyla were found in the sediment at 50 °C but Actinobacteria (33.1%), Proteobacteria (29.1%) and Acidobacteria (20.1%) dominated; nine phyla were found in the sediment at 92 °C where Firmicutes (52.7%, mostly *Alicyclobacillus* and *Sulfobacillus*) and Proteobacteria (44.8%, mostly *Bradyrhizobium*, *Methylobacterium*, *Sediminibacterium*) were the most abundant. Rincón-Molina et al. [58] studied the diversity of the Plant Growth Promoting Bacteria

(PGPB) associated with pioneer plants that grow at the surroundings of the crater lake of El Chichón volcano. Strains were isolated using selective culture media and then characterized phenotypically. However, as far as we know, there are no reports dealing with the isolation and identification of thermophilic microorganisms from the Chiapanecan Volcanic Arc that produce thermostable enzymes with biotechnological potential. For this reason, the objective of this paper is the identification and characterization of lipase-producing thermophilic bacteria isolated from the El Chichón volcano crater lake.

2. Materials and Methods

2.1. Site Description and Sample Collection

The crater lake of the El Chichón volcano is located in the Chiapanecan Volcanic Arc (north–western Chiapas, south–eastern Mexico) at 17.36° N, 93.23° W with a maximum elevation of 1100 MASL, one km of wide, 160 m of deep and an average annual rainfall of 4000 mm [50,54].

Samples of geothermal water were collected from three different sites in the El Chichón volcano crater lake, where thermophilic microorganisms live subject to phase changes with high temperature and mass gradients; Site 1 (17°21′38″ N, 93° 13′28″ W), Site 2 (17°21′36″ N, 93°13′38″ W) and Site 3 (17°21′37″ N, 93°13′39″ W) with a distance between them of approximately 10 m (see Figure 1). Temperature and pH were measured in situ using a temperature and pH meter standard portable HANNA model HI-98128 (Merck, Kenilworth, NJ, USA), and their average values were 71.6 °C and 5.4, 80 °C and 4.2 and 83 °C and 5.5, for sites 1, 2 and 3, respectively. Samples were stored in 1-L sterile flasks with screw tops and were brought immediately to the laboratory. After 5 h, the microorganism present in the collected geothermal water were used as initial inoculum in two culture media. To prevent contamination, samples were stored in 50-mL sterile tubes washed 3 times with geothermal water, after that, tubes were placed in 1-L sterile flasks washed 3 times and filled with geothermal water.
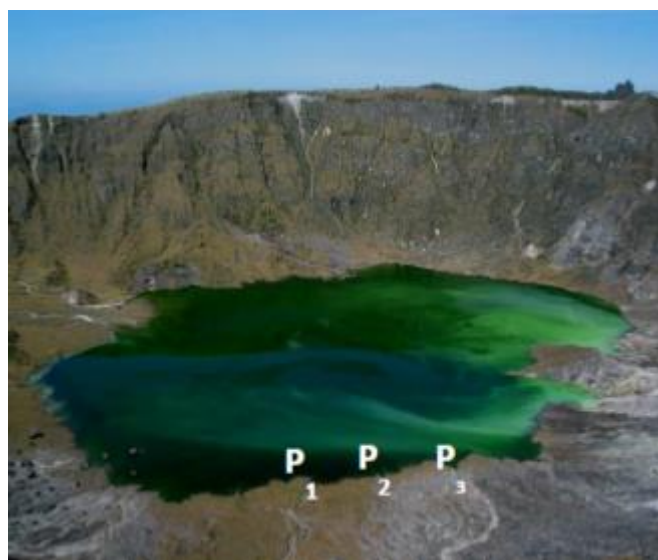
Figure 1. Sampling sites of the geothermal water in the El Chichón volcano crater lake.

2.2. Cultivation of the Thermophilic Microorganisms

Two enrichment liquid culture media were used for the growth of the thermophilic microorganisms. The first medium was a nutrient broth (NB) composed of 3-g/L meat extract and 5 g/L of peptone; the second one was a enrichment medium (EM) with the following composition (per liter): yeast extract, 1.0 g; olive oil, 5.0 mL; NaCl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.7 g/L; $CaCl_2 \cdot 2H_2O$, 0.5 g; KH_2PO_4, 0.3 g; K_2HPO_4, 0.3 g; $(NH_4)_2SO_4$, 0.5 g; Vitamin solution, 1.0 mL (catalog DSM No. 141) and

trace element solution, 1.0 mL (DSM catalog No. 141) [59]. Cultures of each sampling sites (in triplicate) were carried out in 1-L Erlenmeyer flasks containing 250 mL of each liquid culture media, which were inoculated with 3 mL of the collected geothermal water samples, under sterile conditions [60]. Incubation temperatures were 71.6, 80 and 83 °C and pH 5.4, 4.2 and 5.5 (adjusted with 1-M citric acid) for the samples of sites 1, 2 and 3, respectively. Microbial growth was monitored every 48 h (in triplicate) during 192 h by measurement of the optical density at 600 nm. Only the sample corresponding to sampling Site 1 showed microorganisms growth; for this reason, all the following experiments refer to these microorganisms. The culture temperatures were achieved using a programmable incubator shaker (New Brunswick, Eppendorf, Enfield, United States) with digital temperature microprocessor and shaking velocity control. A Hach UV-visible DR 6000 spectrophotometer (wavelength range: 190–1100 nm) was used to measure the optical density.

2.3. Isolation of Lipase-Producing Thermophilic Microorganisms

After enrichment culture, lipase producing thermophiles were screened on rhodamine B (Sigma) agar plates at 60 °C during 72 h. Medium rhodamine B (MRB) composition (per liter) was: peptone 10 g, NaCl 5 g, yeast extract 5 g, agar 17 g, rhodamine 0.001% and olive oil 31.25 mL (2.5% p/v). The rhodamine B was prepared in sterile distilled water solution, the resulting solution and olive oil were sterilized by filtration with a 0.22-μm pore filter, both oil and rhodamine B were poured onto the agar under aseptic conditions and then they were agitated [61,62]. Lipase production was monitored by irradiating plates with UV light at 350 nm (BIO 139 RAD-2000 UV transilluminator) [61]. Microorganisms with capacity to produce lipases were taken from the colonies with the highest fluorescent orange halos in plates [62]. The identification of lipase-producing bacteria was provided by molecular, morphological and biochemical characterization.

2.4. Identification of Lipase-Producing Bacteria

2.4.1. Molecular Identification

Samples of the isolated microorganisms were suspended in 2 mL triple distilled water in Eppendorf tubes. Aliquots of 600 μL were taken and centrifuged at 10,000 rpm for 10 min. DNA extraction was performed using the 'ZR fungal/bacterial DNA miniprep' kit according to the manufacturer's instructions. For PCR, the amplification solution contained 2.5 μL PCR buffer, 1.5 μL MgCl$_2$, oligonucleotides 1.25 μL (60 mM), 0.125 μL Taq polymerase, 0.5 μL dNTPs, 7.5 μL BSA, 9.125 μL deionized H$_2$O, 1.25 μL DMSO and 1 μL of DNA. The primers used were 27F (5′-AGAGTTTGATCMTG GCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The following conditions were used in the amplification of the gene 16S rRNA: 35 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min and a final extension of 72 °C for 10 min. PCR products were visualized on 1% agarose gel, purified and sequenced (Macrogen®, Korea). The sequences of the 16S rRNA gene were analyzed in the NCBI database [63]. The sequences were aligned using the CLUSTAL X (2.0) software with the default settings [64]. Phylogenetic and molecular evolutionary analyses were performed with MEGA v5.2. [65] The phylogenetic tree of the 16S rRNA gene sequences was constructed by neighbor-joining [66] and a Bootstrap analysis with 1000 pseudoreplicates using the Tamura–Nei model [67].

2.4.2. Morphologic and Biochemical Characterization

The macroscopic morphology of the isolated strains was evaluated using a Swift brand stereoscope series 80 [68]. For the determination of the microscopic characteristics the isolated strains were cultivated in lipase production medium (LPM) as described in the next section at 71.6 °C. Samples were taken from the flasks in the logarithmic stage and they were studied by light microscopy [69]. Gram stain was performed using a differential staining kit purchased from HICEL DE MÉXICO S.A. DE C.V. according to the method described by Mohan [69]. Biochemical characterization consisted of several tests such as consumption of different sources of carbon (lactose, maltose, raffinose, fructose and mannitol), urea,

MR-VP (methyl red Voges-Proskauer), citrate, SIM (sulfide indole motility), TSI (triple sugar iron), LIA (lysine iron agar), MIO (motility indole ornithine), hydrolysis of casein and hydrolysis of starch test. The tests for carbon consumption were performed in liquid media.

2.4.3. Growth of Lipase-Producing Thermophilic Microorganisms and Enzymatic Activity Determination

The selected strain was cultivated in MRB plates at 71.6 °C for 72 h; after that, samples of the colonies were taken and inoculated into 2-L Erlenmeyer flasks with 400 mL of LPM and incubated for 72 h. LPM composition per liter was: peptone 6 g, yeast extract 2 g, olive oil 15 mL, $CaCl_2 \cdot 2H_2O$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.1 g and $FeCl_3 \cdot 6H_2O$ (1% stock solution) 0.4 mL. Incubation temperature and pH were 71.6 °C and 5.4, respectively. Microbial growth was followed through monitoring of the optical density, taking samples every 20 min for 24 h.

Crude enzymatic extract was obtained simultaneously with the optical density measurement, taking aliquots of 2 mL of the culture in each monitoring. The samples were centrifuged at 10,000 rpm for 10 min at 4 °C and the cell-free supernatant was used as the crude enzymatic extract. Enzymatic activity of the extracts was determined by measuring the increase in absorbance at 410 nm produced by the release of *p*-nitrophenol in the hydrolysis of 0.4-mM 4-nitrophenol-palmitate (pNPP) in isopropyl alcohol (substrate solution) at pH 7 (*p*NPP molar extinction coefficient (ε) in these condition is 1.5×10^4 L mol^{-1} cm^{-1}). Reaction was started by adding 0.1 mL of the crude enzymatic extract to 0.9-mL of substrate solution at 60 °C, during 1 min. Spontaneous hydrolysis of *p*-NPP was monitored under identical conditions without enzyme. One international unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze 1 µmol of *p*NPP per minute [70]. The blank (non-enzymatic control) was prepared without adding any enzyme extract and the absorbance was measured under identical conditions. The activity was expressed in U/L. Protein concentration was determined by Bradford's dye binding method using bovine serum albumin as reference and recording the absorbance at 595 nm. The microbial growth and extracellular lipolytic activity were performed in triplicate.

3. Results and Discussion

3.1. Cultivation of the Thermophilic Microorganisms

Cultivation results of the geothermal microorganisms from sampling sites 1, 2 and 3 are presented in Figure 2a–c, respectively. As it can be seen, only microorganisms from Site 1 were able to grow in nutrient broth (NB) or enrichment medium (EM). In the case of microorganisms from sampling sites 2 and 3, no significant growth is observed in any of the two media used, even at 288 h of incubation and similar temperatures to those of the places where they were isolated. In NB, the maximum OD for microorganisms from Site 1 was 0.4397, while microorganisms from Site 2 and 3 reached a maximum OD of 0.0803 and 0.0463, respectively. In the case of EM, the maximum OD for microorganisms from Site 1 was 1.3947, while microorganisms from Site 2 and 3 reached a maximum OD of 0.0338 and 0.4820, respectively. This may be due to the fact that these microorganisms are classified as hyperthermophiles (growth temperatures of 80 °C and 83 °C, for microorganisms from Site 2 and 3, respectively), which are characterized by being mostly archaeal and anaerobic chemolithotrophs which use inorganic compounds as donors and electron acceptors, for this reason they were unable to grow in these two culture media that were supplemented with organic nutrients [71].

It can be observed in Figure 2a, that the microorganisms isolated from the sampling Site 1 showed higher and faster growth in EM than in NB; this can be attributed to fact that the bacteria from the crater lake of El Chichón volcano are adapted to the oligotrophic conditions of the volcanic environments, and for this reason they have a limited capacity to take advantage of complex nutrients such as those present in the NB [72]. In addition, they may require a specific nutrient or growth factor that was not present in this medium [73].

In the case of EM, although it is a specific media since it has olive oil as the only source of carbon, this medium was enriched with minerals, vitamins and traces of other elements, so this suggest that its composition was similar to the composition of the crater lake waters, thus promoting a greater and faster growth of microorganisms. In addition, it is important to mention that the microbial growth in the EM is indicative that in the crater lake of El Chichón volcano there are enzyme-producing microorganisms with lipolytic activity.
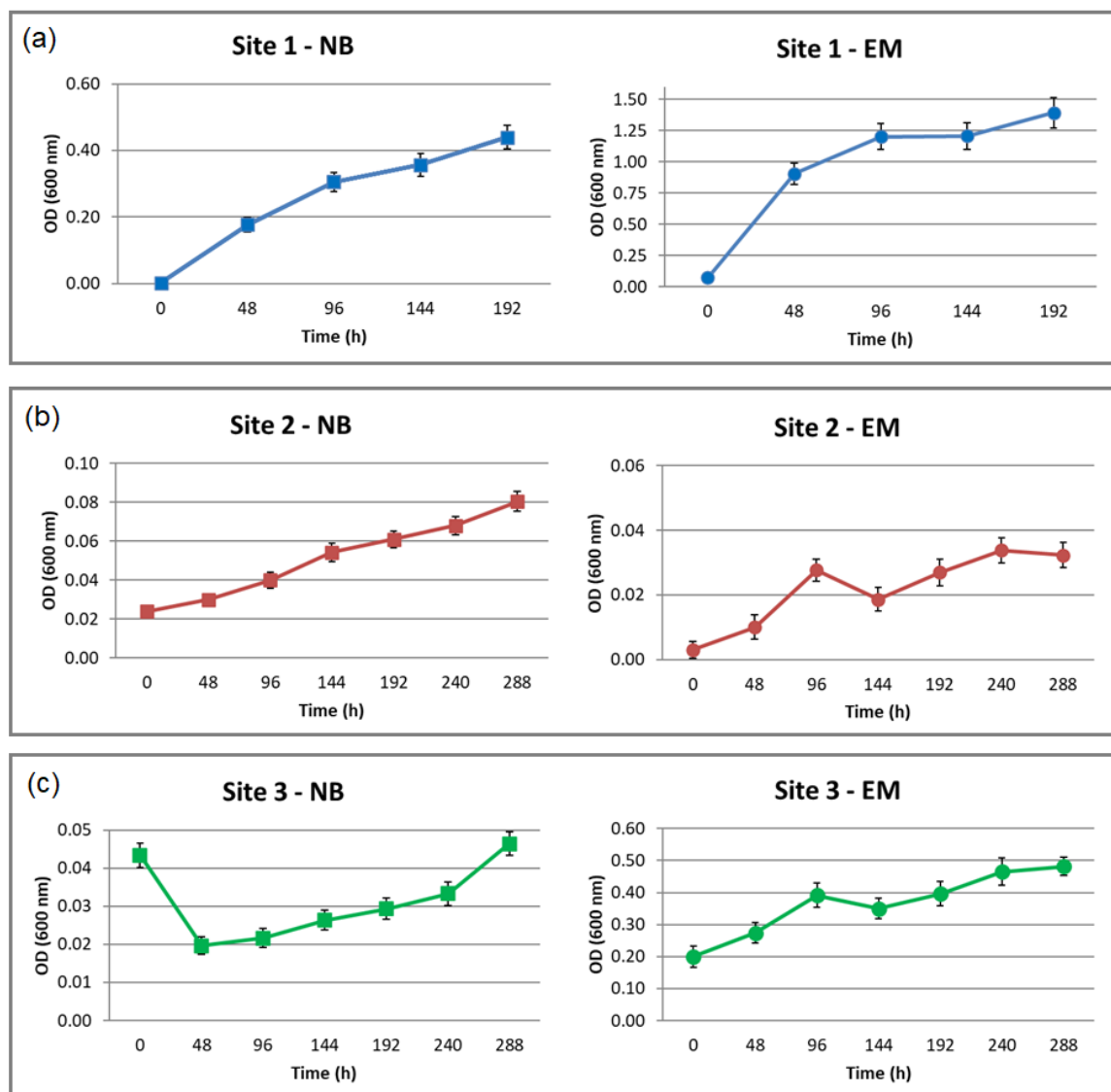

Figure 2. Growth of microorganisms isolated from El Chichón volcano crater lake in two culture media. (**a**) Microorganisms from Site 1, at pH 5.4 and 71.6 °C; (**b**) microorganisms from Site 2, at pH 4.2 and 80 °C and (**c**) microorganisms from Site 3, at pH 5.5 and 83 °C. Closed circles—enrichment medium (EM); closed squares—nutrient broth (NB).
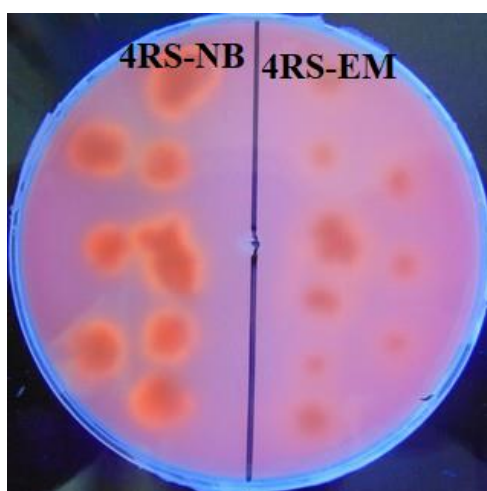
3.2. Isolation and Identification of Lipase-Producing Thermophilic Microorganisms

Isolation of lipase-producing microorganisms was carried out with the microorganisms from Site 1 cultivated in NB and EM, which were the only ones that showed significant growth during their culture. For isolation, consecutive reseeding was performed on plates of solid MRB (Medium rhodamine B) which evidence the lipolytic activity through the orange fluorescence at the border of the colonies formed due to the enzymatic hydrolysis of the triglycerides present in the olive oil [62]. In

Figure 3, it can be seen that lipolytic activity was observed in IS-NB and IS-EM plates (initial seeding of microorganisms cultivated in NB and EM, respectively) and in 4RS-NB and 4RS-EM (four reseeding of microorganisms cultivated in NB and EM, respectively), which were incubated at 60 °C and pH 5.4. According to these results, four potentially lipase-producing strains were isolated. In order to have a better understanding of the orange fluorescence color at the border of the colony, cultivation of lipase-producing microorganisms at colony level are presented in Figure 4. As it can be observed strains showed positive evidence of lipase production. The microorganisms were able to produce orange fluorescent halos (rhodamine B) at 60 °C and pH of 5.4. The 4RS-NB and 4RS-EM: four reseeding of microorganisms cultivated in NB and EM, respectively.
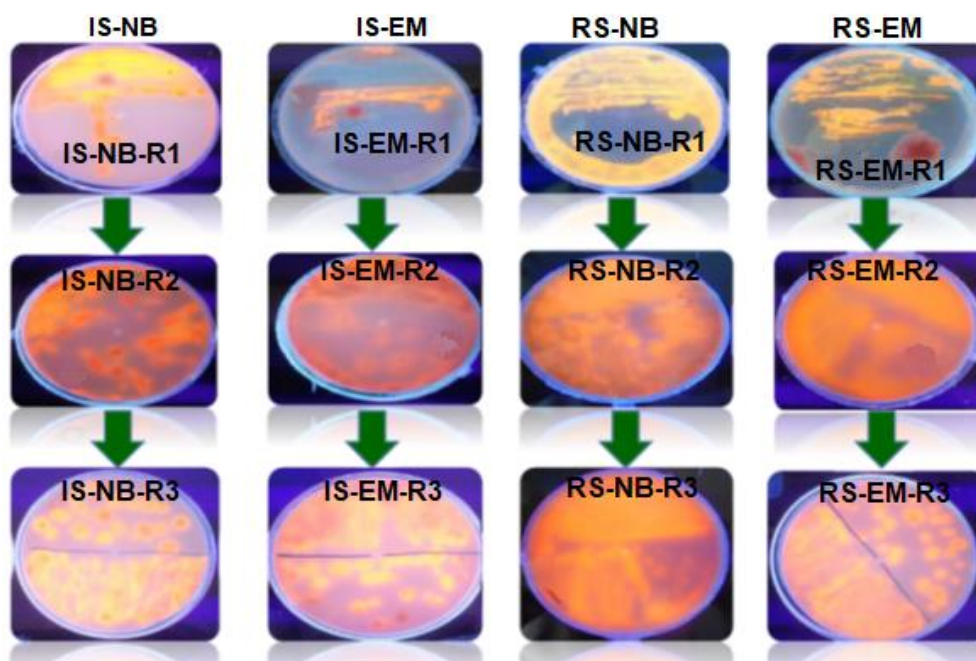
Figure 3. Isolation of lipase-producing microorganisms grown on plates with rhodamine B medium at 60 °C and pH of 5.4. IS-NB and IS-EM—initial seeding of microorganisms cultivated in NB and EM, respectively; 4RS-NB and 4RS-EM—four reseeding of microorganisms cultivated in NB and EM, respectively. IS: initial seeding.


Figure 4. Lipolytic activity on rhodamine B leading to the formation on a fluorescent halo. 4RS-NB and 4RS-EM—four reseeding of microorganisms cultivated in NB and EM, respectively.

3.3. Molecular Identification of Lipase-Producing Thermophilic Microorganisms

The potentially lipase-producing strains (CHI$_1$ and CHI$_2$) isolated from MRB plates were subjected to extraction of genomic DNA which was analyzed by agarose gel electrophoresis. Subsequently, PCR DNA amplification was carried out for the universal 16S rRNA gene. Amplicons showed approximately 1350 bp for the two isolated strains according to the molecular marker (M) that was of 50 to 2000 bp. This result indicates that the DNA of the two strains showed good amplification since the universal primers used for this study occupy regions from V1 to V9 of the 16S gene (1500 bp).

Genes sequencing results of the four strains were analyzed and compared to sequences in the NCBI database. Strains IS-NB and 4RS-NB corresponded to *Geobacillus jurassicus* CHI$_2$ with 98% identity, and strains of IS-EM and 4RS-EM to *Geobacillus stearothermophilus* CHI$_1$ with 99% identity. In Figure 5, it is showed the neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacterial species isolated of geothermal water in the El Chichón volcano crater lake. The phylogenetic analysis indicated that strain CHI$_2$ belonged to the genus *Geobacillus* with 98% identity with *G. jurassicus* DS1 and *G. uzenensis* U. The three strains have a relationship since they are thermophilic microorganisms that have been found in extreme environments. In fact, it has been reported that *G. jurassicus* DS1 was found in Dagang oil fields (China) with temperatures of 60 °C and 65 °C and *G. uzenensis* U was isolated from hot springs of oil fields with temperatures of 55 to 60 °C in Kazakhstan [68,74].

On the other hand, the strain CHI$_1$ showed 99% identity with *G. stearothermophilus* IFO12550 T. Characteristics of the *Geobacillus*, such as growth in several substrates, formation of endospores that allow them to survive in unfavorable conditions which facilitates their storage and transport [75] and high growth rates, make it an attractive genus as a potential source of many biotechnologically thermostable enzymes, such as proteases, amylases, lipases and reverse transcriptase [76].
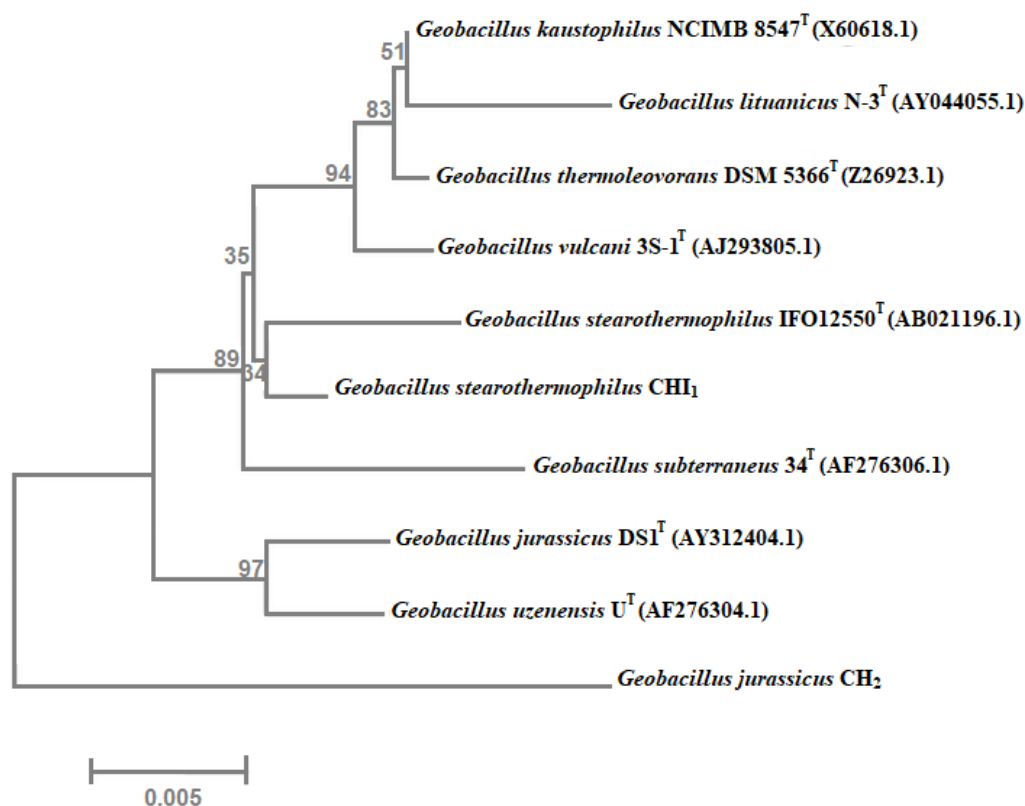

Figure 5. Phylogenetic tree on the basis of 16S rRNA data of the lipase-producing thermophilic bacteria (*G. jurassicus* CHI$_2$ and *G. stearothermophilus* CHI$_1$) isolated from the crater lake of El Chichón volcano. Bootstrap values calculated for 1000 replications are indicated. Bar—0.5% substitutions in nucleotide sequence. Accession numbers are given in parentheses.

3.4. Morphologic and Biochemical Characterization of G. jurassicus CHI₂ and G. stearothermophilus CHI₁

The colonies of the isolated strains identified as *G. jurassicus* CHI$_2$ and *G. stearothermophilus* CHI$_1$ were morphologically and biochemically analyzed. It was found that both strains are aerobic with a chemoorganotrophic metabolism. As it can be seen in Figure 6, the colonies presented an irregular shape, with a shiny wavy border and an umbilicated elevation of gummy texture and with a diameter of 2 mm, approximately. These results agree with those of other authors, who reported that these two species of the genus *Geobacillus* have a round, mucous and colorless morphology. The observed colony morphology is an indication that these strains are capable of grouping to form conglomerates and to migrate to where there is greater availability of nutrients, guaranteeing in this way their survival [68].

Cell of both strains *G. jurassicus* and *G. stearothermophilus* showed elliptical forms, with estimated measurements of 1.0- to 1.9-μm wide and 5.3- to 9.4-μm long and 0.8- to 1.4-μm wide and 2.4- to 4-μm long, respectively. Similar results were reported by Nazina et al. [68], who point out that the strains *Geobacillus jurassicus* DS1 and DS2 cultivated for 12 h at 60 °C in a medium with sucrose, presented an elliptical shape with cell sizes of 1.0- to 1.6-μm wide and 6.5- to 9.0-μm long and 1.3- to 1.6-μm wide by 9.0- to 14.0-μm long, respectively. In addition, regarding to Gram stain, *G. jurassicus* CHI$_2$ showed to be Gram-positive, while *G. stearothermophilus* CHI$_1$ showed to have a Gram stain variable, which is in agreement with results reported by other authors [74,77]. The variable Gram stain of *G. stearothermophilus* CHI$_1$ could be explained by a conformational change of components of the cell membrane caused by mechanisms of adaptation against complex phenomena of heat and mass transfer like the variation of temperature, concentration and phase change associated with the formation of gases on the shores of the El Chichón volcano crater lake. The above is related to the presence of a S-layer, which responds to the specific conditions of the environment [78].
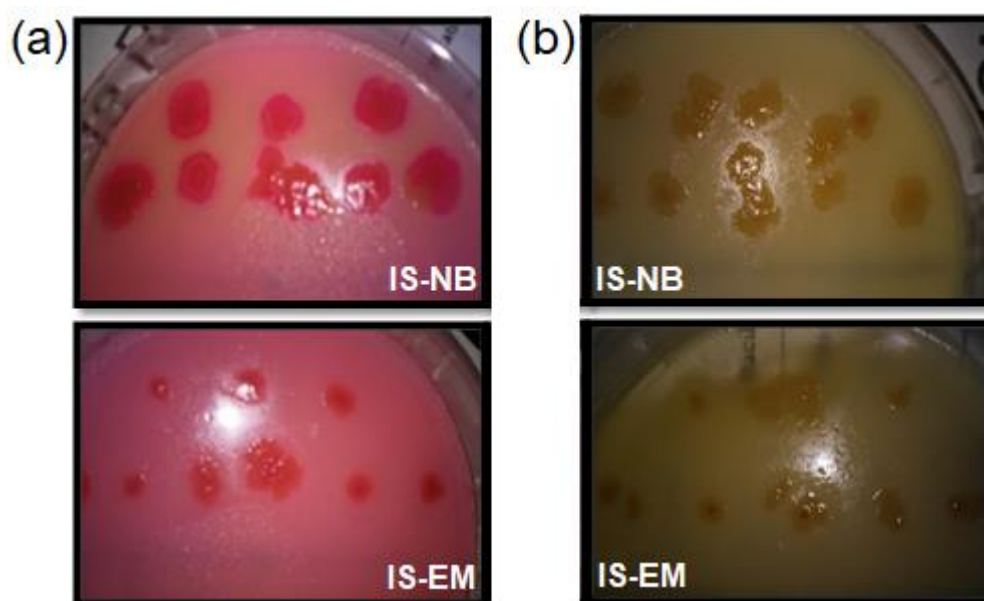
Figure 6. Colony formation of strains isolated from El Chichón volcano crater lake. (**a**) *Geobacillus jurassicus* CHI$_2$; (**b**) *Geobacillus stearothermophilus* CHI$_1$; IS-NB and IS-EM—initial seeding of microorganisms cultivated in NB and EM, respectively.

Biochemical tests for *G. jurassicus* CHI$_2$ and *G. stearothermophilus* CHI$_1$ are shown in Table 1. The two strains showed motility in the medium SIM (sulfide indole motility), similarly to what was reported for *G. jurassicus* DS1 and DS2 and *G. stearothermophilus* isolated from oil fields of Dagang (China) [68,74,77]. The motility presented by these strains is due to the formation of flagella, which possibly occurs in response to the need for movement to survive severe thermofluid flow processes in an environment with chemical, mass and temperature gradients which are due to the formation of

gases by sudden changes in the underwater chimneys of volcanic environments such as the crater lake of El Chichón volcano.

In Table 1 it can also be observed that for the tests of H_2S production, Simmons Citrate, urease, indole, Voges–Proskauer, methyl red, decarboxylation of lysine and ornithine and deamination of lysine, negative results were found for both studied strains. Negative results in the H2S production test suggest that these strains do not synthesize the enzymes cysteine desulfurase and thiosulfate reductase, possibly because these two strains are aerobic using oxygen as an electron acceptor instead of sulfates, which are electron acceptors in anaerobic conditions.

Table 1. Biochemical tests result of the strains *G. jurassicus* CHI_2 and *G. stearothermophilus* CHI_1.

Biochemical Test	*Geobacillus jurassicus* [68]	*Geobacillus stearothermophilus* [74,77]	*Geobacillus jurassicus* CHI_2	*Geobacillus stearothermophilus* CHI_1
Gram stain	+	±	+	-
Lactose	-	-	-	-
Mannitol	+	±	-	+
Raffinose	-	-	-	-
Maltose	+	+	+	+
Fructose	+	+	+	+
Starch	+	+	+	+
Casein	-	±	+	-
C. Simmons	-	±	-	-
Voges Proskauer	-	-	-	-
Methyl red	-	±	-	-
Urease	-	-	-	-
MIO				
Motility	+	+	+	+
Ornithine decarboxylation	+	+	+	+
Indol	-	-	-	-
Production of H_2S	-	-	-	-
TSI				
Glucose	-	-	-	-
Lactose	-	-	-	-
Sucrose	+	-	+	-
LIA				
Lysine decarboxylation	-	-	-	-
Lysine deamination	-	-	-	-
SIM				
Motility	+	+	+	+
Indole	-	-	-	-
Production of H_2S	-	-	-	-

In the case of the Simmons Citrate test, these strains could use citrate as a carbon source and inorganic ammonium as a nitrogen source, but when these reactions occur the medium is alkalinized, generating the death of these microorganisms because they are adapted to live in volcanic waters with pH between 2 and 5.5. Results of Voges–Proskauer and methyl red tests suggest that these two strains do not follow the route of acid fermentation, therefore there was no color change in methyl red due to no acid production; this result was corroborated in the glucose test that showed no acid production. Consequently, the tests of decarboxylation of lysine and ornithine and deamination of lysine also gave negative results since the enzymes responsible for these reactions do not show activity at neutral or alkaline pH. On the other hand, carbohydrate test results showed that *G. jurassicus* CHI_2 and *G. stearothermophilus* CHI_1 can ferment maltose, fructose and sucrose and mannitol, maltose and fructose, respectively. These results are similar to the reported by Nazina et al. [68,74], who mention that the strain *G. jurassicus* DS1 and DS2 and *G. stearothermophilus* 46 and 49, produce acids from cellobiose, glucose, fructose, maltose, mannose, mannitol, ribose, sucrose and trehalose.

G. jurassicus CHI_2 and *G. stearothermophilus* CHI_1 showed positive results in the starch hydrolysis test, which indicates that both strains have the ability to synthesize amylase, maltase and dextrinase enzymes to unfold the molecules of amylose, amylopectin, maltose and dextrin from the starch to obtain glucose. In the case of the casein hydrolysis test, *G. jurassicus* CHI_2 showed positive result, while the result for *G. stearothermophilus* CHI_1 was negative; this may be due to the fact that these

strains had different initial cultures, *G. jurassicus* CHI$_2$ grew in a medium rich in protein sources such as peptone and yeast extract (NB), which induced the synthesis of proteases in order to take advantage of its carbon source; on the other hand, *G. stearothermophilus* CHI$_1$ was cultured in an EM, in which its only carbon source was olive oil and for that reason the synthesis of proteases was not necessary. Hence, *G. stearothermophilus* CHI$_1$ was selected for further experiment regarding to lipolytic activity.

3.5. Microbial Growth and Extracellular Lipolytic Activity of G. stearothermophilus CHI$_1$

Geobacillus stearothermophilus CHI$_1$ was grown in lipase production medium (LPM), in order to evaluate the microbial growth and extracellular lipolytic activity. As it can be seen in Figure 7, this strain showed a growth-associated lipase production, as the lipolytic activity occurred in all growth phases, with a marked increase during the exponential phase. The adaptation and exponential phases lasted 3 and 17 h, respectively, with a maximum optical density (OD) of 0.98 at the end of the log phase (hour 20).

G. stearothermophilus CHI$_1$ showed a maximum extracellular enzymatic activity of 143 U/mL at 8.3 h (exponential phase) of incubation time. This activity is greater than that reported for other thermophilic microorganisms such as *Geobacillus thermoleovorans* (495 U/L) [79], *Geobacillus sp.* (1.2 U/mL) [80], *Geobacillus thermodenitrificans* strain AV-5 (330 U/L) [81], *Bacillus thermoleovorans* ID-1 (700 U/L) [59] and *Bacillus coagulans* BTS-3 (1.16 U/mL) [82]. Although it is difficult to make a direct comparison between the activities reported for these microorganisms due to the different conditions used during their study, the high enzymatic activity found for *G. stearothermophilus* CHI$_1$, which is due to the specific geothermal characteristics related to the heat and mass transport in the hot water of the El Chichón volcano, makes it an excellent thermal biotechnological tool for potential uses in industrial processes.
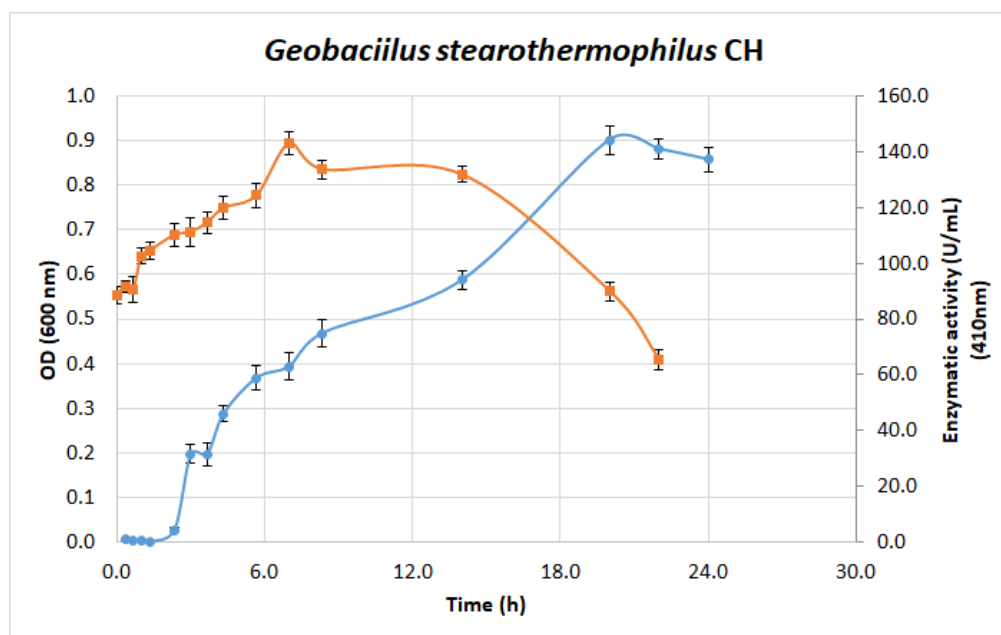
Figure 7. Biomass concentration (closed circles) and extracellular lipolytic activity (U/mL) (closed squares) of *G. stearothermophilus* CHI$_1$ culture in lipase production medium at 71.6 °C and pH 5.4.

4. Conclusions

In this study, two strains of thermophilic bacteria were isolated from the geothermal water of El Chichón volcano crater lake where microorganisms thrive in complex phenomena of heat and mass transfer. Gene sequence revealed that these strains corresponded to *Geobacillus jurassicus* CHI$_2$ and *Geobacillus stearothermophilus* CHI$_1$ with 98% and 99% of identity, respectively. *Geobacillus stearothermophilus* CHI$_1$ showed a growth associated with lipase production with a marked

increase during the exponential phase where a maximum lipase extracellular enzymatic activity of 143 U/mL was reached at 8.3 h of incubation. The high enzymatic activity found for *G. stearothermophilus* CHI₁ makes it an excellent biotechnological tool for potential uses in thermal industrial processes. In addition, it was demonstrated that the thermal water of El Chichón volcano crater lake can be an important source of native thermophilic bacteria producing extracellular lipases with a high lipolytic activity, which is of great importance for thermal biotechnological processes. Finally, it is important to point out that as far as we know, this is the first report dealing with the identification and characterization of lipase-producing bacteria isolated from El Chichón volcano crater lake.

Author Contributions: Conceptualization, S.L.O.-C., V.G.T.-P. and G.E.O.-C.; methodology S.L.O.-C., V.G.T.-P., G.E.O.-C. and A.R.-Q.; investigation, A.R.-Q., A.R.-L. and A.S.-M.; formal analysis, S.L.O.-C., V.G.T.-P., G.E.O.-C. and V.M.R.-V.; writing—original draft S.L.O.C., V.G.T.-P., G.E.O.-C. and A.R.-L.; writing—review and editing A.R.-Q., A.R.-L., V.M.R.-V. and A.S.-M. All authors have read and agreed to the published version of the manuscript.

References

1. Gutiérrez-Arnillas, E.; Rodríguez, A.; Sanromán, M.A.; Deive, F.J. New sources of halophilic lipases: Isolation of bacteria from Spanish and Turkish saltworks. *Biochem. Eng. J.* **2016**, *109*, 170–177. [CrossRef]
2. Woodley, J.M. Protein engineering of enzymes for process applications. *Curr. Opin. Chem. Biol.* **2013**, *17*, 310–316. [CrossRef] [PubMed]
3. Gomes, J.; Steiner, W. The biocatalytic potential of extremophiles and extremozymes. *Food Technol. Biotechnol.* **2004**, *42*, 223–235.
4. Elleuche, S.; Schäfers, C.; Blank, S.; Schröder, C.; Antranikian, G. Exploration of extremophiles for high temperature biotechnological processes. *Curr. Opin. Microbiol.* **2015**, *25*, 113–119. [CrossRef]
5. Rothschild, L.J.; Mancinelli, R.L. Life in extreme environments. *Nature* **2001**, *409*, 1092–1101. [CrossRef]
6. Elleuche, S.; Schröder, C.; Sahm, K.; Antranikian, G. Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. *Curr. Opin. Biotechnol.* **2014**, *29*, 116–123. [CrossRef]
7. Bergquist, P.L.; Morgan, H.W.; Saul, D. Selected enzymes from extreme thermophiles with applications in biotechnology. *Curr. Biotechnol.* **2014**, *3*, 45–59. [CrossRef]
8. Fütterer, O.; Angelov, A.; Liesegang, H.; Gottschalk, G.; Schleper, C.; Schepers, B. Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9091–9096. [CrossRef]
9. Giaveno, M.A.; Urbieta, M.S.; Ulloa, J.R.; González Toril, E.; Donati, E.R. Physiologic versatility and growth flexibility as the main characteristics of a novel thermoacidophilic acidianus strain isolated from copahue geothermal area in argentina. *Microb. Ecol.* **2013**, *65*, 336–346. [CrossRef]
10. Urbieta, M.S.; Toril, E.G.; Giaveno, M.A.; Bazán, A.A.; Donati, E.R. Archaeal and bacterial diversity in five different hydrothermal ponds in the Copahue region in Argentina. *Syst. Appl. Microbiol.* **2014**, *37*, 429–441. [CrossRef]
11. Amin, A.; Ahmed, I.; Khalid, N.; Zhang, Y.; Xiao, M.; Li, W.J. Insights into the thermophile diversity in hot springs of pakistan. In *Extremophiles in Eurasian Ecosystems: Ecology, Diversity, and Applications*; Springer: Singapore, 2018; Volume 8, pp. 1–28.
12. Ansari, I.; Kumar, R.; Sundararajan, M.; Maiti, D.; Rather, M.A.; Ali, M. Investigation on growth of oil degrading thermophilic bacteria isolated from hot spring. *Biosci. Biotechnol. Res. Asia* **2018**, *15*, 805–815. [CrossRef]
13. Slobodkina, G.B.; Baslerov, R.V.; Kostryukova, N.K.; Bonch-Osmolovskaya, E.A.; Slobodkin, A.I. *Tepidibaculum saccharolyticum* gen. nov., sp. nov. a moderately thermophilic, anaerobic, spore-forming bacterium isolated from a terrestrial hot spring. *Extremophiles* **2018**, *22*, 761–768. [CrossRef] [PubMed]
14. Urbieta, M.S.; Donati, E.R.; Chan, K.G.; Shahar, S.; Sin, L.L.; Goh, K.M. Thermophiles in the genomic era: Biodiversity, science, and applications. *Biotechnol. Adv.* **2015**, *33*, 633–647. [CrossRef] [PubMed]

15. Bendia, A.G.; Araujo, G.G.; Pulschen, A.A.; Contro, B.; Duarte, R.T.D.; Rodrigues, F. Surviving in hot and cold: Psychrophiles and thermophiles from deception island volcano, antarctica. *Extremophiles* **2018**, *22*, 917–929. [CrossRef] [PubMed]

16. Simanjuntak, S.; Samuel, M.Y. Isolation and identification of thermophilic bacteria, producer of amylase enzyme, from lake linow, north sulawesi. *J. Pure Appl. Microbiol.* **2018**, *12*, 543–554. [CrossRef]

17. Santana, M.M.; Gonzalez, J.M. High temperature microbial activity in upper soil layers. *FEMS Microbiol. Lett.* **2015**, *362*. [CrossRef]

18. Rastogi, G.; Bhalla, A.; Adhikari, A.; Bischoff, K.M.; Hughes, S.R.; Christopher, L.P.; Sani, R.K. Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains. *Bioresour. Technol.* **2010**, *101*, 8798–8806. [CrossRef]

19. Kim, H.; Lee, J.Y. Effects of a groundwater heat pump on thermophilic bacteria activity. *Water* **2019**, *11*, 2084. [CrossRef]

20. Kor-Bicakci, G.; Ubay-Cokgor, E.; Eskicioglu, C. Comparative analysis of bacterial and archaeal community structure in microwave pretreated thermophilic and mesophilic anaerobic digesters utilizing mixed sludge under organic overloading. *Water* **2020**, *12*, 887. [CrossRef]

21. Chen, X.; Wu, Y.; Zeng, L. Migration of gyrotactic micro-organisms in water. *Water* **2018**, *10*, 1455. [CrossRef]

22. Zerva, I.; Remmas, N.; Ntougias, S. Diversity and biotechnological potential of xylan-degrading microorganisms from orange juice processing waste. *Water* **2019**, *11*, 274. [CrossRef]

23. Guerrero-Barajas, C.; Ordaz, A.; Garibay-Orijel, C.; García-Solares, S.M.; Bastida-González, F.; Zárate-Segura, P.B. Enhanced sulfate reduction and trichloroethylene (TCE) biodegradation in a UASB reactor operated with a sludge developed from hydrothermal vents sediments: Process and microbial ecology. *Int. Biodeter. Biodegr.* **2014**, *94*, 182–191. [CrossRef]

24. Ilyas, S.; Lee, J.C.; Kim, B.S. Bioremoval of heavy metals from recycling industry electronic waste by a consortium of moderate thermophiles: Process development and optimization. *J. Clean. Prod.* **2014**, *70*, 194–202. [CrossRef]

25. Sen, S.K.; Raut, S.; Dora, T.K.; Mohapatra, P.K.D. Contribution of hot spring bacterial consortium in cadmium and lead bioremediation through quadratic programming model. *J. Hazard. Mater.* **2014**, *265*, 47–60. [CrossRef]

26. Talluri, S.; Raj, S.M.; Christopher, L.P. Consolidated bioprocessing of untreated switchgrass to hydrogen by the extreme thermophile *Caldicellulosiruptor saccharolyticus* DSM 8903. *Bioresour. Technol.* **2013**, *139*, 272–279. [CrossRef]

27. Lavania, M.; Cheema, S.; Sarma, P.M.; Ganapathi, R.; Lal, B. Methanogenic potential of a thermophilic consortium enriched from coal mine. *Int. Biodeter. Biodegr.* **2014**, *93*, 177–185. [CrossRef]

28. Li, X.; Qian, P.; Wu, S.G.; Yu, H.Y. Characterization of an organic solvent-tolerant lipase from *Idiomarina* sp. W33 and its application for biodiesel production using *Jatropha* oil. *Extremophiles* **2014**, *18*, 171–178. [CrossRef]

29. Abdollahi, H.; Shafaei, S.Z.; Noaparast, M.; Manafi, Z.; Niemelä, S.I.; Tuovinen, O.H. Mesophilic and thermophilic bioleaching of copper from a chalcopyrite-containing molybdenite concentrate. *Int. J. Miner. Process.* **2014**, *128*, 25–32. [CrossRef]

30. Li, S.; Zhong, H.; Hu, Y.; Zhao, J.; He, Z.; Gu, G. Bioleaching of a low-grade nickel–copper sulfide by mixture of four thermophiles. *Bioresour. Technol.* **2014**, *153*, 300–306. [CrossRef]

31. Bharali, P.; Das, S.; Konwar, B.K.; Thakur, A.J. Crude biosurfactant from thermophilic *Alcaligenes faecalis*: Feasibility in petro-spill bioremediation. *Int. Biodeterior. Biodegr.* **2011**, *65*, 682–690. [CrossRef]

32. Adiguzel, A.; Ozkan, H.; Baris, O.; Inan, K.; Gulluce, M.; Sahin, F. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *J. Microbiol. Methods* **2009**, *79*, 321–328. [CrossRef] [PubMed]

33. Bhalla, A.; Bansal, N.; Kumar, S.; Bischoff, K.M.; Sani, R.K. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresour. Technol.* **2013**, *128*, 751–759. [CrossRef] [PubMed]

34. Goh, K.M.; Kahar, U.M.; Chai, Y.Y.; Chong, C.S.; Chai, K.P.; Ranjani, V. Recent discoveries and applications of *Anoxybacillus*. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 1475–1488. [CrossRef] [PubMed]

35. Chai, Y.Y.; Rahman, R.N.Z.R.A.; Illias, R.M.; Goh, K.M. Cloning and characterization of two new thermostable and alkalitolerant α-amylases from the *Anoxybacillus* species that produce high levels of maltose. *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 731–741. [CrossRef] [PubMed]

36. Van den Burg, B. Extremophiles as a source for novel enzymes. *Curr. Opin. Microbiol.* **2003**, *6*, 213–218. [CrossRef]

37. Bornscheuer, U.T. Microbial carboxyl esterases: Classification, properties and application in biocatalysis. *FEMS Microbiol. Rev.* **2002**, *26*, 73–81. [CrossRef] [PubMed]

38. Hasan, F.; Shah, A.A.; Hameed, A. Industrial applications of microbial lipases. *Enzym. Microb. Technol.* **2006**, *39*, 235–251. [CrossRef]

39. Houde, A.; Kademi, A.; Leblanc, D. Lipases and their industrial applications. *Appl.Biochem. Biotechnol.* **2004**, *118*, 155–170. [CrossRef]

40. Ray, A. Application of lipase in industry. *Asian J. Pharm. Tech.* **2012**, *2*, 33–37.

41. Tacias-Pascacio, V.G.; Virgen-Ortíz, J.J.; Jiménez-Pérez, M.; Yates, M.; Torrestiana-Sanchez, B.; Rosales-Quintero, A.; Fernandez-Lafuente, R. Evaluation of different lipase biocatalysts in the production of biodiesel from used cooking oil: Critical role of the immobilization support. *Fuel* **2017**, *200*, 1–10. [CrossRef]

42. Tacias-Pascacio, V.G.; Torrestiana-Sánchez, B.; Dal Magro, L.; Virgen-Ortíz, J.J.; Suárez-Ruíz, F.J.; Rodrigues, R.C.; Fernandez-Lafuente, R. Comparison of acid, basic and enzymatic catalysis on the production of biodiesel after RSM optimization. *Renew. Energy* **2019**, *135*, 1–9. [CrossRef]

43. Salihu, A.; Alam, M.Z.; AbdulKarim, M.I.; Salleh, H.M. Lipase production: An insight in the utilization of renewable agricultural residues. *Resour. Conserv. Recycl.* **2012**, *58*, 36–44. [CrossRef]

44. Watanabe, Y.; Shimada, Y.; Sugihara, A.; Noda, H.; Fukuda, H.; Tominaga, Y. Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. *J. Amer. Oil Chem. Soc.* **2000**, *77*, 355–360. [CrossRef]

45. Hantsis-Zacharov, E.; Halpern, M. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Appl. Environ. Microbiol.* **2007**, *73*, 7162–7168. [CrossRef] [PubMed]

46. Sirisha, E.; Rajasekar, N.; Narasu, M.L. Isolation and optimization of lipase producing bacteria from oil contaminated soils. *Adv. Biol. Res.* **2010**, *4*, 249–252.

47. Veerapagu, M.; Narayanan, A.S.; Ponmurugan, K.; Jeya, K.R. Screening selection identification production and optimization of bacterial lipase from oil spilled soil. *Asian J. Pharm. Clin. Res.* **2013**, *6*, 62–67.

48. Bharathi, D.; Rajalakshmi, G.; Komathi, S. Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil. *J. King Saud Univ. Sci.* **2019**, *31*, 898–901. [CrossRef]

49. Armienta, M.A.; Vilaclara, G.; De la Cruz-Reyna, S.; Ramos, S.; Ceniceros, N.; Cruz, O. Water chemistry of lakes related to active and inactive mexican volcanoes. *J. Volcanol. Geotherm. Res.* **2008**, *178*, 249–258. [CrossRef]

50. Casas, A.S.; Armienta, M.A.; Ramos, S. Sulfur speciation with high performance liquid chromatography as a tool for El Chichón volcano, crater lake monitoring. *J. S. Am. Earth Sci.* **2016**, *72*, 241–249. [CrossRef]

51. Contreras, U.A.; Salgado, E.J. Evaluación geológico-estructural del complejo volcánico chichonal, chiapas, como fuente alterna de energía. *Geotermia* **2012**, *25*, 3–20.

52. Cuoco, E.; De Francesco, S.; Tedesco, D. Hydrogeochemical dynamics affecting steam-heated pools at El Chichón crater (Chiapas–Mexico). *Geofluids* **2013**, *13*, 331–343. [CrossRef]

53. Armienta, M.A.; De La Cruz-Reyna, S.; Ramos, S.; Ceniceros, N.; Cruz, O.; Aguayo, A.; Arcega-Cabrera, F. Hydrogeochemical surveillance at El Chichón volcano crater lake, Chiapas, Mexico. *J. Volcanol. Geotherm. Res.* **2014**, *285*, 118–128. [CrossRef]

54. Taran, Y.A.; Peiffer, L. Hydrology, hydrochemistry and geothermal potential of El Chichón volcano-hydrothermal system, Mexico. *Geothermics* **2009**, *38*, 370–378. [CrossRef]

55. Mazot, A.; Rouwet, D.; Taran, Y.; Inguaggiato, S.; Varley, N. CO_2 and he degassing at El Chichón volcano, chiapas, mexico: Gas flux, origin and relationship with local and regional tectonics. *Bull. Volcanol.* **2011**, *73*, 423–441. [CrossRef]

56. Paz, M.P.J.; Taran, Y.; Inguaggiato, S.; Collard, N. CO_2 flux and chemistry of El Chichón crater lake (México) in the period 2013–2015: Evidence for the enhanced volcano activity. *Geophys. Res. Lett.* **2016**, *43*, 127–134.

57. Rincón-Molina, C.I.; Hernández-García, J.A.; Rincón-Rosales, R.; Gutiérrez-Miceli, F.A.; Ramírez-Villanueva, D.A.; González-Terreros, E.; Peña-Ocaña, B.A. Structure and diversity of the bacterial communities in the acid and thermophilic crater-lake of the volcano "El Chichón", mexico. *Geomicrobiol. J.* **2019**, *36*, 97–109. [CrossRef]

58. Rincón-Molina, C.I.; Martínez-Romero, E.; Ruiz-Valdiviezo, V.M.; Velázquez, E.; Ruiz-Lau, N.; Rogel-Hernández, M.A. Plant growth-promoting potential of bacteria associated to pioneer plants from an active volcanic site of Chiapas (Mexico). *Appl. Soil Ecol.* **2020**, *146*, 103390. [CrossRef]

59. Lee, D.; Koh, Y.; Kim, K.; Kim, B.; Choi, H.; Kim, D. Isolation and characterization of a thermophilic lipase from *bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett.* **1999**, *179*, 393–400. [CrossRef]

60. Berekaa, M.M.; Zaghloul, T.I.; Abdel-Fattah, Y.R.; Saeed, H.M.; Sifour, M. Production of a novel glycerol-inducible lipase from thermophilic Geobacillus stearothermophilus strain-5. *World J. Microbiol. Biotechnol.* **2009**, *25*, 287–294. [CrossRef]

61. Kouker, G.; Jaeger, K.E. Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol.* **1987**, *53*, 211. [CrossRef]

62. Hou, C.T. PH dependence and thermostability of lipases from cultures from the ARS culture collection. *J. Ind. Microbiol.* **1994**, *13*, 242–248. [CrossRef]

63. Ruiz-Romero, E.; Coutino-Coutino Mde, L.; Valenzuela-Encinas, C.; Lopez-Ramirez, M.P.; Marsch, R.; Dendooven, L. Texcoconibacillus texcoconensis gen. nov., sp. nov., alkalophilic and halotolerant bacteria isolated from soil of the former lake Texcoco (Mexico). *Int. J. Syst. Evol. Microbiol.* **2013**, *63*, 3336–3341. [CrossRef] [PubMed]

64. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; Mc Gettigan, P.A.; Mc William, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948. [CrossRef] [PubMed]

65. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA 5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef]

66. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425.

67. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526.

68. Nazina, T.N.; Sokolova, D.S.; Grigoryan, A.A.; Shestakova, N.M.; Mikhailova, E.M.; Poltaraus, A.B. *Geobacillus jurassicus sp. nov.*, a new thermophilic bacterium isolated from a high-temperature petroleum reservoir, and the validation of the *Geobacillus* species. *Syst. Appl. Microbiol.* **2005**, *28*, 43–53. [CrossRef]

69. Mohan, S.K. *Gram Stain: Looking Beyond Bacteria to Find Fungi in Gram Stained Smear: A Laboratory Guide for Medical Microbiology*; AuthorHouse: Bloomington, IN, USA, 2009.

70. Poppe, J.K.; Costa, A.P.O.; Brasil, M.C.; Rodrigues, R.C.; Ayub, M.A.Z. Multipoint covalent immobilization of lipases on aldehyde-activated support: Characterization and application in transesterification reaction. *J. Mol. Catal. B Enzym.* **2013**, *94*, 57–62. [CrossRef]

71. Huber, R.; Huber, H.; Stetter, K.O. Towards the ecology of hyperthermophiles: Biotopes, new isolation strategies and novel metabolic properties. *FEMS Microbiol. Rev.* **2000**, *24*, 615–623. [CrossRef]

72. Tomova, I.; Lazarkevich, I.; Tomova, A.; Kambourova, M.; Vasileva-Tonkova, E. Diversity and biosynthetic potential of culturable aerobic heterotrophic bacteria isolated from Magura Cave, Bulgaria. *Int. J. Speleol.* **2013**, *42*, 65–76. [CrossRef]

73. Tomova, I.; Gladka, G.; Tashyrev, A.; Vasileva-Tonkova, E. Isolation, identification and hydrolytic enzymes production of aerobic heterotrophic bacteria from two Antarctic islands. *Int. J. Environ. Sci.* **2014**, *4*, 614–625.

74. Nazina, T.N.; Tourova, T.P.; Poltaraus, A.B.; Novikova, E.V.; Grigoryan, A.A.; Ivanova, A.E. Taxonomic study of aerobic thermophilic bacilli: Descriptions of Geobacillus subterraneus gen. nov., sp. nov. and Geobacillus uzenensis sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G.* th. *Int. J. Syst. Evol. Microbiol.* **2001**, *51*, 433–446. [PubMed]

75. Blanchard, K.; Robic, S.; Matsumura, I. Transformable facultative thermophile *Geobacillus stearothermophilus* NUB3621 as a host strain for metabolic engineering. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6715–6723. [CrossRef] [PubMed]

76. Qu, G.-R.; Xin, P.-Y.; Niu, H.-Y.; Jin, X.; Guo, X.-T.; Yang, X.-N.; Guo, H.-M. Microwave promoted palladium-catalyzed Suzuki–Miyaura cross-coupling reactions of 6-chloropurines with sodium tetraarylborate in water. *Tetrahedron* **2011**, *67*, 9099–9103. [CrossRef]

77. Priest, F.G.; Alexander, B. A frequency matrix for probabilistic identification of some bacilli. *J. Gen. Microbiol.* **1988**, *134*, 3011–3018. [CrossRef]

78. Sara, M.; Sleytr, U.B. S-layer proteins. *J. Bacteriol.* **2000**, *182*, 859–868. [CrossRef]

79. Abdel-Fattah, Y.R. Optimization of thermostable lipase production from a thermophilic Geobacillus sp. using Box-Behnken experimental design. *Biotechnol. Lett.* **2002**, *24*, 1217–1222. [CrossRef]

80. Mehta, A.; Saun, N.K.; Gupta, R. Purification and characterization of lipase from thermophilic Geobacillus sp. *Curr. Biotechnol.* **2016**, *5*, 81–89. [CrossRef]

81. Christopher, L.P.; Zambare, V.P.; Zambare, A.; Kumar, H.; Malek, L. A thermo-alkaline lipase from a new thermophile Geobacillus thermodenitrificans AV-5 with potential application in biodiesel production. *J. Chem. Technol. Biotechnol.* **2015**, *90*, 2007–2016. [CrossRef]

82. Kumar, S.; Kikon, K.; Upadhyay, A.; Kanwar, S.S.; Gupta, R. Production, purification, and characterization of lipase from thermophilic and alkaliphilic Bacillus coagulans BTS-3. *Protein Expr. Purif.* **2005**, *41*, 38–44. [CrossRef]