

Species Relationship and Structural Prediction Based on Lipase Gene of A Thermohalophilic Bacterium PLS A

Diana Eka Putri¹, Teuku M. Iqbalsyah¹, and Febriani^{1*}

¹Biomolecules Application Research Group, Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Syiah Kuala, Banda Aceh, Indonesia

Corresponding Author:
Febriani
tettykemala@apps.ipb.ac.id

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Abstract

*Lipase is a hydrolase enzyme that catalyses the hydrolysis of triacylglycerol into glycerol and free fatty acids. It has broad industrial applications, including in the detergent, papermaking, leather, and food industries. This study aimed to predict the structure of the lipase enzyme encoded by a gene from a thermophilic bacterial isolate, Pria Laot Sabang A (PLS A), obtained from undersea fumaroles in Pria Laot Sabang, Weh Island, Aceh, Indonesia. The lipase gene was amplified using the Polymerase Chain Reaction (PCR) method with a specific primer pair (Flip2-Rlip2), resulting in a gene of ± 1300 bp. Homology analysis of the same genes in NCBI showed that the PLS A lipase gene was highly similar to that of *Geobacillus thermoleovorans* strain ARTRW1. However, phylogenetic analysis indicated that PLS A formed a distinct branch. The lipase structure was predicted using the SWISS-MODEL server, which showed the highest structural similarity to the lipase of the *G. zalihae* strain T1. The predicted protein structure revealed the presence of Zn^{2+} and Ca^{2+} ions, suggesting that the lipase from PLS A is a metalloenzyme. The protein structure of PLS A also indicated high stability, highlighting its potential for industrial applications.*

Keywords: PLS A, lipase gene, thermo-halophilic.

Introduction

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is a hydrolase enzyme that catalyses the hydrolysis of triacylglycerol to produce glycerol and free fatty acids [1]. In addition to hydrolysis, lipases catalyse various chemical reactions, including esterification, transesterification, interesterification, and acidolysis [2]. Owing to its catalytic versatility, lipase is widely used in various industries, including detergents, papermaking, cosmetics, leather, food, and biodiesel production [3]. Lipases can improve oil stain removal in detergent formulations and serve as bio-additive [4]. In the pharmaceutical sector, it has been applied to oral biofilm degradation [5]. Furthermore, lipase-catalysed hydroesterification of waste soybean cooking oil can be utilised to produce ethylene glycol in the cosmetics industry [6]. Lipase from lipolytic bacteria isolated from domestic waste compost has also demonstrated potential for biodiesel

production [7]. Consequently, the industrial demand for lipases continues to increase.

Lipase can be derived from animals, plants, and microorganisms, such as bacteria, fungi, and yeasts [8]. However, microbial lipases are generally preferred for industrial applications because of their greater stability under various environmental conditions. Some industrial processes require lipases with high-temperature resistance. Thermophilic bacteria can produce thermostable lipases capable of surviving at temperatures ranging from 40°C to 80°C [9]. Thermostable lipases exhibit high catalytic activity at elevated temperatures, maintain stability during storage, and help reduce production costs [3]. The natural habitat of thermophilic bacteria is geothermal areas, such as volcanic craters and hot springs [10]. An example of a thermophilic lipase-producing bacterium is *G. stearothermophilus* FMR12 [11].

Thermostable lipases have attracted significant attention in biotechnology due to their ability to maintain catalytic activity and structural stability under extreme environmental conditions such as high temperature, organic solvents, and variable pH. These enzymes exhibit enhanced conformational rigidity and improved intermolecular interactions, enabling sustained enzymatic performance compared to mesophilic counterparts [12]. Recent studies have demonstrated that thermostable lipases derived from thermophilic microorganisms show strong industrial potential in biodiesel production, food processing, and pharmaceutical synthesis because elevated temperatures improve substrate solubility and reduce microbial contamination risks [13].

One of the geothermal areas producing thermostable lipase is the undersea fumaroles of Pria Laot Sabang, located on Weh Island in the Aceh province. Previous studies have successfully isolated several thermophilic bacterial strains, including the PLS A isolate, from this location. Based on 16S rRNA and Pol I DNA gene analyses, PLS A was identified as *Geobacillus* and *Bacillus caldolyticus* strain XM, respectively [14]. This isolate has demonstrated the ability to produce protease enzymes at 65°C [15] and polypeptide antibiotics, suggesting its potential to produce other thermostable enzymes, such as lipase.

A major challenge in utilising lipase from PLS A is the difficulty in culturing this organism under laboratory conditions, as it originates from an undersea fumarole with conditions that are difficult to replicate in the laboratory. One solution to overcome this limitation is to clone the lipase gene into a more easily cultured host organism. However, the gene must first be identified before cloning. Therefore, this study aimed to amplify and translate the lipase gene from PLS A and predict its structural properties. The uniqueness of the structure was determined by studying its distinct characteristics, which will enable future cloning studies.

The main limitation in the development of PLS A lipase lies in its low availability due to the difficulty of cultivating isolates from

underwater fumaroles under laboratory conditions, thus often limited in industrial utilization. The molecular identification and amplification of the PLS A lipase gene as an alternative strategy prior to the cloning process. This study offers through a Polymerase Chain Reaction (PCR)-based gene amplification approach using specific primers. Specifically, this study aims to identify and amplify the PLS A lipase gene to produce sufficient copies of the gene as a basis for the cloning process in more easily cultured hosts and the development of further industrial applications.

Experimental

Methods

Bacterial Strain

The PLS A strain was previously isolated from an undersea fumarole in Pria Laot Sabang, Weh Island, Aceh, Indonesia. It has been preserved as a glycerol stock in the Biochemistry Laboratory, Department of Chemistry, Universitas Syiah Kuala.

Regeneration of PLS A

PLS A was regenerated on a modified ½ LB solid medium containing 0.5% tryptone, 0.5% NaCl, 0.25% yeast extract, 3% Bacto agar, and supplemented with 0.25% glucose. The medium was then dissolved in distilled water. An aliquot of the glycerol stock was spread on the medium and incubated at 70°C for 24 h. A single colony was then transferred to ½ LB liquid medium containing 0.25% glucose and incubated in a shaking water bath at 70 °C for 24 h at 150 rpm.

Isolation of Chromosomal DNA

Chromosomal DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega). Briefly, the PLS A liquid culture was centrifuged at 13000 × g for 2 min. The resulting pellet was resuspended in 600 µL of nuclear lysis solution and incubated at 80°C for 5 min. After cooling, 3 µL of RNase was added, and the mixture was incubated at 37 °C for 60 min. Subsequently, 200 µL of protein precipitation solution was added and vortexed for 20 s. The

sample was incubated on ice for 5 min and centrifuged at $13000 \times g$ for 3 min. The supernatant was precipitated using 600 μ L of isopropanol and centrifuged at $13000 \times g$ for 2 min. The DNA pellet was washed with 600 μ L of 70% ethanol and centrifuged at $13,000 \times g$ for 2 min. The DNA pellet was air-dried for 15 min, dissolved in 100 μ L of DNA rehydration solution, and incubated at 65°C for 1 h.

Amplification and Sequencing of Lipase Gene

The lipase gene was amplified using polymerase chain reaction (PCR) with the primer pair Flip2-Rlip2. The PCR products were sequenced at PT. Genetika Science Jakarta.

Agarose Gel Electrophoresis

Chromosomal DNA and PCR products were analysed using 1% agarose gel electrophoresis. Electrophoresis was performed at 85 volts and 400 mA for 60 min. DNA fragments on the gel were visualised using a Gel Doc System on an agarose gel.

Phylogenetic Analysis of Lipase Gene

Sequence homology analysis was performed by comparing the base sequence of the lipase gene with sequences in the National Centre for Biotechnology Information (NCBI) Gene Bank database using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed using the Molecular Evolutionary Genetic Analysis (MEGA) 11 software.

Protein Structure Prediction

The base sequence of the lipase gene was used to predict the protein structure using the homology method with SWISS-MODEL software. Previously, the sequenced lipase gene was translated into a protein using the ExPASy Translate software. The protein structure was predicted using SWISS-MODEL to identify a protein sequence that matched the PLS A protein, which was used as a template. The modelling results were then downloaded in PDB format and visualised using Visual Molecular Dynamics (VMD) software.

Table 1. Primer base sequence ^[16]

No.	Primer	Base sequence	Amplicons size (bp)
1	Flip2	5' -ATGTGAGGGGAGAAGG- 3'	± 1300
2	Rlip2	5' -GAGCCATCCGATCGAGATG- 3'	

Table 2. The PCR conditions for the amplification of PLS A lipase gene

PCR step	Temperature (°C)	Time	Cycle
Initial denaturation	95	1 minute	1
Denaturation	95	15 second	1
Annealing	54	1 minute	30
Elongation	72	15 second	30
Final elongation	72	5 minute	1
Cooling	4	15 minute	1

Results and Discussion

Regeneration of PLS A

The regeneration process was conducted to rejuvenate the PLS A cells. The modified $\frac{1}{2}$ LB medium used in this study contained tryptone as the nitrogen source, NaCl as the mineral source, yeast extract as the source of carbohydrates, and glucose as an inducer¹⁷. Figure 1 shows the growth of PLS A in solid and liquid $\frac{1}{2}$ LB media.

Isolation of Chromosomal DNA

Chromosomal DNA isolation from PLS A involves three main stages: cell wall destruction

(lysis), precipitation, and purification^[18]. Lysis was achieved through chemical and mechanical methods using Sodium Dodecyl Sulfate (SDS) in the lysis buffer and sonication^[19]. Electrophoretic analysis revealed that the size of PLS A chromosomal DNA was greater than 10 Kb (Figure 2A). The size of bacterial chromosomal DNA is generally in the megabase (Mb) range, typically between 0.5 and over 10 Mb depending on the bacteria species. Bacteria from the genus *Geobacillus* have been reported to have genomes of around 3-4 Mb^[20]. The size indicates that the chromosomal DNA was successfully isolated and could be used as a template for amplification.

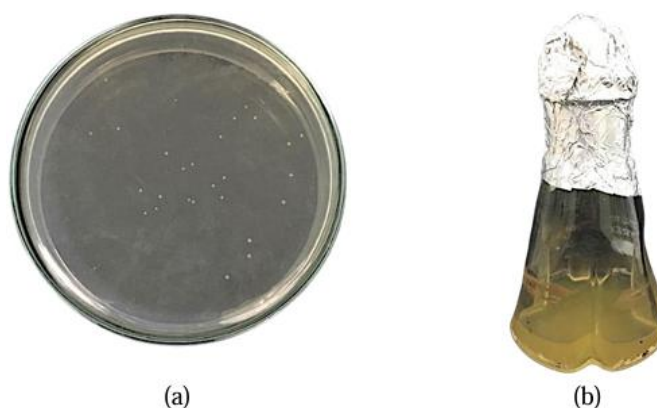


Figure 1. Growth of PLS A after regeneration on (A) $\frac{1}{2}$ LB solid medium and (B) $\frac{1}{2}$ LB liquid medium.

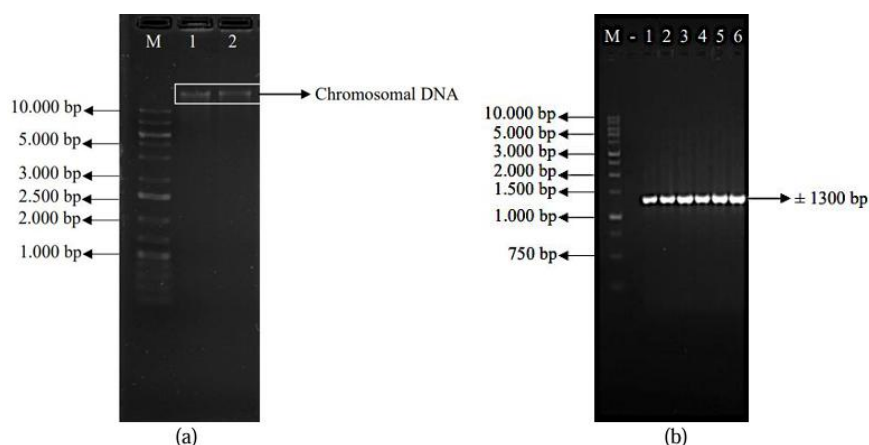


Figure 2. Electropherogram of (A) Chromosomal DNA of PLS A. M = marker, 1 & 2 = DNA chromosome samples, and (B) Amplicon of lipase gene. M = marker, - = negative control, 1-6 = amplicons. Electrophoresis conditions: Agarose 1%, 85 V, 400 mA, 60 mins.

Amplification of the Lipase Gene

The lipase gene was amplified using the polymerase chain reaction (PCR) method [21] with the primers Flip2 and Rlip2. Flip2 is a forward primer that binds approximately 20 bases upstream of the start codon, whereas Rlip2 is a reverse primer that binds approximately 50 bases downstream of the stop codon [16]. The PCR protocol consisted of three main steps [22]. First, denaturation was performed at 95°C for 15 s to separate double-stranded DNA into single strands. Second, annealing was performed at 54°C for 1 min to allow the primer to hybridise with the template DNA. Third, extension was conducted at 72°C for 15 s to synthesise the complementary DNA strand. The cycle was repeated 30 times, followed by a final extension step at 72°C for 5 min.

Figure 2B shows that the Flip2-Rlip2 primer pair successfully amplified the lipase gene from PLS A, producing a band of approximately 1300 bp. A lipase gene of similar size ($\pm$ 1300 bp), previously amplified using the same primers, exhibited the highest sequence similarity [16]. A single, distinct band at approximately 1300 bp in Figure 2B confirms the successful amplification of the lipase gene from PLS A. The success of PCR amplification is strongly influenced by primer design, including primer length, GC

content, melting temperature, and primer interaction [23]. DNA sequencing revealed that the lipase gene from PLS A was 1188 bp long.

Phylogenetic Analysis of Lipase Genes

Phylogenetic analysis determines the evolutionary relationships among microorganisms and is typically represented as a branched tree structure [24]. The lipase gene sequence from PLS A was compared with homologous sequences from other microorganisms in the NCBI GenBank database using the BLAST search tool. Homology analysis of the PLS A lipase gene showed the strain related to *Geobacillus thermoleovorans* strain ARTRW1 and *Geobacillus thermoleovorans* CCB US3 UF5 (Table 3) with highest homology 99.52%. This result suggested that the lipase gene was closely similarity to previously reported lipase genes. However, the novelty of this study lies in the isolation and molecular characterization of the lipase gene from the local PLS A strain and the successful amplification of a previously unreported gene fragment. This study provides additional information on lipase gene variation in the genus *Geobacillus* sp and expands the genetic data of lipases from local isolate, potentially developing them as thermostable enzymes for biotechnological applications.

Table 3. Homology of lipase genes from PLS A with the best ten lipase genes from several other microorganisms.

No.	Strain name	Access code	% similarity
1	<i>G. thermoleovorans</i> strain ARTRW1	CP042251.1	99.52
2	<i>G. thermoleovorans</i> strain ARTRW1	CP003125.1	99.52
3	<i>Geobacillus</i> sp. EPT9	KF470812.1	97.50
4	<i>G. stearothermophilus</i>	AY786185.1	97.44
5	<i>G. thermoleovorans</i> strain V0	CP046250.1	97.37
6	<i>Geobacillus</i> sp. SP22	JN711121.1	97.04
7	<i>Geobacillus</i> sp. ‘Domas’ lipase ITB.1	HQ398859.1	96.96
8	<i>Geobacillus</i> sp. ‘Papandayan’ lipase ITB2.1	HQ398860.1	96.88
9	<i>Geobacillus</i> sp. Y412MC52	CP002442.1	96.82
10	<i>B. thermoleovorans</i> lipase (ARA)	AF134840.1	96.65

Phylogenetic analysis of the PLS A lipase gene was conducted using the Neighbor-Joining (NJ) method in MEGA 11 software, based on a multiple sequence alignment with 25 reference lipase gene sequences from the NCBI database. The NJ method constructs a phylogenetic tree based on the degree of difference or genetic distance between sequences, thereby allowing for the assessment of evolutionary relationships between genes [25]. The reliability of the relationships at each tree branch was tested using bootstrap analysis with 1000 replicates. Bootstrap values greater than 70% generally indicate that the relationship at a given branch is well-supported [26]. The analysis results show that the PLS A lipase gene sequence falls within the *Geobacillus* genus and forms a distinct branch among the lipase sequences of that genus (Figure 3). However, the branch indicating the relationship between PLS A and its closest reference sequence has a bootstrap value of 35%. This value indicates that the relationship

between PLS A and that sequence is not strongly supported. Nevertheless, the placement of PLS A within the *Geobacillus* genus demonstrates an evolutionary relationship between the PLS A lipase gene and that genus. Several other branches on the tree exhibit high bootstrap values 97%, 96%, and 98% indicating strong evolutionary relationships within those sequence groups. The fact that the PLS A branch is distinct from the reference sequences suggests genetic differences in the PLS A lipase gene compared to the others. These results align with studies, which demonstrated variations in evolutionary relationships and lipase sequence characteristics among members of the *Geobacillus* genus [27]. Furthermore, these findings support the results of a previous 16S rRNA gene analysis, which indicated that PLS A is potentially a new species within the genus *Geobacillus* (NCBI accession number MK606068) [14].

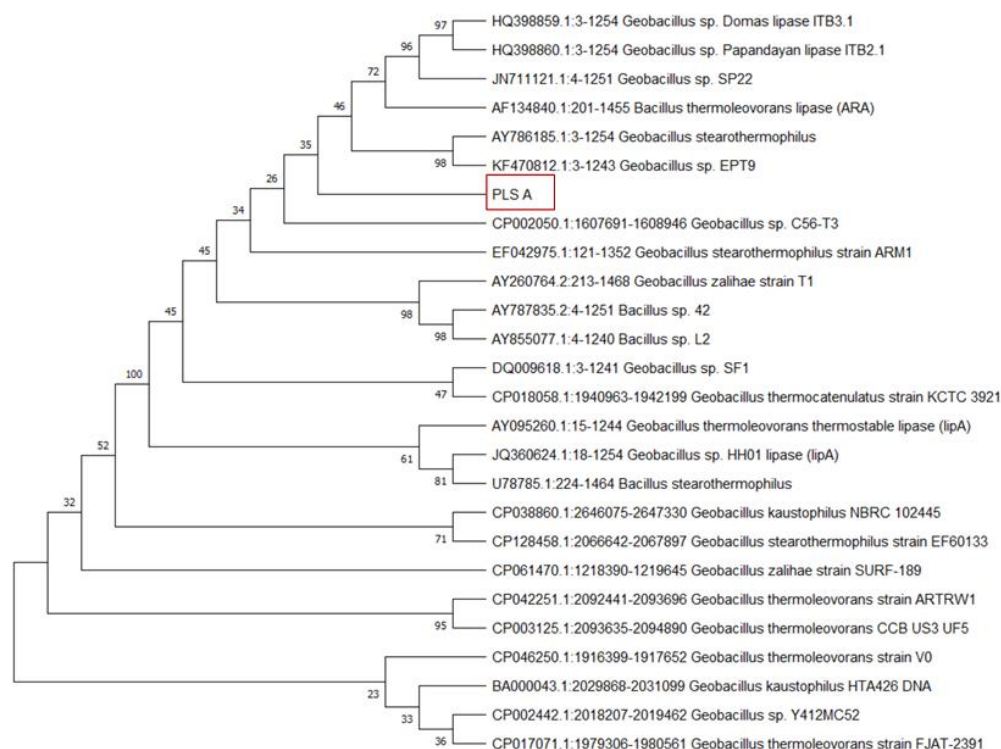


Figure 3. Phylogenetic tree of the PLS A lipase gene constructed together with 25 reference lipase gene sequences obtained from the NCBI database. The tree was generated using the Neighbor-Joining (NJ) method implemented in MEGA 11 based on multiple sequence alignment. The reliability of tree topology was evaluated using bootstrap analysis with 1000 replicates, and bootstrap values $\geq 70\%$ were considered to indicate strong statistical support for the corresponding branches. The PLS A sequence is positioned within the *Geobacillus* branch, highlighting its evolutionary relationship within thermophilic bacteria members of the genus

Lipase Structure Prediction

The structure of the lipase gene translated from PLS A was predicted using the SWISS-MODEL software. The software employs a homology approach to generate a three-dimensional protein structural model. The homology modelling technique predicts the three-dimensional structure of a target protein based on its amino acid sequence using that of a similar protein with a known three-dimensional structure in the laboratory (template) [28]. The greater the similarity between amino acid sequences, the more likely their three-dimensional structures are also similar [29]. Three-dimensional structural modeling of the PLS A lipase protein was performed using SWISS-MODEL, based on the protein sequence obtained from the analysis [30]. The modeling results provided a visualization of the PLS A lipase protein's three-dimensional structure, serving as the basis for analyzing its structural characteristics [31].

Translation of the lipase gene produced a protein consisting of 424 amino acid residues. The resulting amino acid sequence of the lipase gene from PLS A was analysed using SWISS-MODEL software to identify the best template for protein structure modelling. Template selection was based on several parameters, including Global Model Quality Estimation (GMQE), Quality Model Energy Analysis (QMEAN), sequence identity, alignment coverage, and structural resolution [28].

The best template for modelling was the *G. zalihae* strain T1 (PLS A-2dsn_A.pdb). The GMQE, QMEAN, sequence identity, coverage, and resolution values were 0.90, 0.94, 98.18%, 0.91, and 1.50 Å, respectively (Table 4). High GMQE and QMEAN values, both approaching 1, indicate that the predicted model is of high quality and closely resembles the native structure of the target protein [32]. The sequence identity reflects the similarity between the target protein and the template. A sequence identity above 70% generally suggests that the resulting model is a reliable approximation of the true protein structure [28].

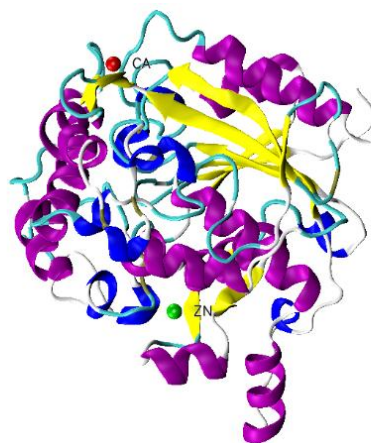
Although the sequence identity between the *G. zalihae* strain T1 and *Geobacillus* sp. SBS-4S (PLS A-3auk_A.pdb) is identical (98.18%). The resolution value also plays a critical role in protein structure modelling. Resolution reflects the quality and stability of the crystal structure. While structures with a resolution below 2.5 Å are generally considered stable, lower resolution values indicate a higher quality structure [33]. Therefore, the lipase structure from the *G. zalihae* strain T1 was selected as the template for modelling the translated lipase protein from PLS A.

The visualisation of the predicted protein structure (Figure 4) shows the presence of metal ions Ca^{2+} and Zn^{2+} , indicating that the lipase from PLS A is a metalloenzyme. The metal ions Ca^{2+} and Zn^{2+} contribute to the catalytic activity and structural stabilisation of the enzyme. Previous research [34] reported that lipase from *G. thermocetanulatus* BLT2 is stabilised by Ca^{2+} and Zn^{2+} ions. Therefore, the PLS A-derived lipase is likely to possess a high structural stability. The Ca^{2+} and Zn^{2+} ions observed in the modeled structure were inherited from the homologous template during homology modeling and therefore represent putative structural features rather than experimentally confirmed metal-binding sites [35].

The evaluation of the protein structural model was performed by examining the sequence identity value between the target sequence and the template, which reflects the overall quality of the generated model. A higher percentage of sequence identity indicates that the predicted model more closely resembles the native protein structure. High sequence identity values represent a strong structural similarity between the target protein and the selected template. Structural modeling analysis of the protein translated from the lipase gene of PLS A demonstrated a close approximation to the expected native structure, as evidenced by the high sequence identity of 98.18% between the target protein and the template structure.

Table 4. Template structure for modelling the result of lipase gene translation from PLS A in SWISS-MODEL

No.	Template structure	Microorganisms	GMQE	QMEAN	Seq identity (%)	Coverage	Resolution (Å)
1	PLS A-3auk.A.pdb	<i>Geobacillus sp.</i> SBS-4S	0.90	0.94	98.18	0.91	1.66
2	PLS A-2dsnA.pdb	<i>G. zalihae</i> strain T1	0.90	0.94	98.18	0.91	1.50
3	PLS A-4fdm.A.pdb	<i>Bacillus sp.</i> L2	0.89	0.94	97.92	0.91	1.60
4	PLS A-7ey3.pdb	<i>G. zalihae</i>	0.89	0.93	97.92	0.91	2.04
5	PLS A-4fkbA.pdb	<i>Bacillus sp.</i> 42	0.90	0.94	97.92	0.91	1.22
6	PLS A-3umjA.pdb	<i>G. zalihae</i>	0.89	0.93	97.92	0.91	2.10
7	PLS A-5xpxA.pdb	<i>Pseudomonas sp.</i> A3	0.89	0.93	97.67	0.91	2.77
8	PLS A-1ji3.pdb	<i>G. stearothermophilus</i>	0.89	0.93	96.61	0.91	2.20

**Figure 4.** Model of the protein structure resulting from the translation of the lipase gene from PLS A

Validation of the PLS A lipase protein structure model was performed by comparing the root mean square deviation (RMSD) value between the predicted model and the template structure. RMSD is used to assess the degree of structural similarity between the model and the template following structural alignment [36]. A lower RMSD value indicates smaller structural differences and a higher degree of similarity.

Analysis results show that the RMSD value between PLS A and the template is 0.154 Å. Structural modelling results are generally considered accurate when the RMSD value is ≤ 2 Å [37]. This value is well below the 2 Å threshold generally used to indicate good structural similarity. These results demonstrate that the three-dimensional structure model of PLS A shares a very high degree of similarity with the

template structure, with minimal structural differences. Thus, the PLS A model exhibits good structural quality based on the RMSD comparison results.

Conclusions

Amplification of the PLS A lipase gene using the Flip2-Rlip2 primer pair successfully produced an amplicon of 1188 bp. Sequence analysis against the NCBI GenBank database showed the highest similarity to *G. thermoleovorans* strain ARTRW1, while phylogenetic reconstruction positioned the sequence within a closely related lineage, indicating genetic relatedness within the *Geobacillus* group. Structural prediction analysis revealed similarity between the modeled PLS A lipase and the lipase structure of *G. zalihae* strain T1. The presence of Ca^{2+} and Zn^{2+} ions in the predicted model was inferred based on the selected structural template and therefore requires experimental validation. Although in silico analyses suggest structural features consistent with thermophilic lipases, conclusions regarding structural stability and potential industrial applications should be confirmed through further biochemical and experimental characterization.

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Declaration of Generative AI and AI-assisted Technologies

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

Author Contributions

Conceptualization, F.; methodology, F.; formal analysis, D.E.P.; investigation, D.E.P and T.M.I.; writing—original draft, D.E.P. and F.; writing—review and editing, D.E.P., T.M.I., and F.; funding acquisition, F.

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Declaration of Conflicts of Interest

The authors declare no conflict of interest.

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