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ORIGINAL PAPER

Ability of T1 Lipase to Degrade Amorphous P(3HB): Structural and Functional Study

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Abstract An enzyme with broad substrate specificity would be an asset for industrial application. T1 lipase apparently has the same active site residues as polyhydroxyalkanoates (PHA) depolymerase. Sequences of both enzymes were studied and compared, and a conserved lipase box pentapeptide region around the nucleophilic serine was detected. The alignment of 3-D structures for both enzymes showed their active site residues were well aligned with an RMSD value of 1.981 Å despite their sequence similarity of only 53.8%. Docking of T1 lipase with P(3HB) gave forth high binding energy of 5.4 kcal/-mol, with the distance of 4.05 Å between serine hydroxyl (OH) group of TI lipase to the carbonyl carbon of the substrate, similar to the native PhaZ7$_{Pl}$. This suggests the possible ability of T1 lipase to bind P(3HB) in its active site. The ability of T1 lipase in degrading amorphous P(3HB) was investigated on 0.2% (w/v) P(3HB) plate. Halo zone was observed around the colony containing the enzyme which confirms that T1 lipase is indeed able to degrade amorphous P(3HB). Results obtained in this study highlight the fact that T1 lipase is a versatile hydrolase enzyme which does not only record triglyceride degradation activity but amorphous P(3HB) degradation activity as well.

Keywords Amorphous P(3HB) · Substrate specificity · PhaZ7$_{Pl}$ · Structure and function · T1 lipase

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Introduction

Protein structure studies are becoming more important nowadays. Relying only on proteins sequence affects the detection of proteins similarity [1] especially sequence with low identity. Thus, comparing protein structures helps in determining distant evolutionary relationships and function between proteins [2]. Previously, a thermophilic lipase termed T1 lipase with an optimum activity at 70 °C and pH 9 was isolated from *Geobacillus zalihae* [3]. This enzyme catalyzes the hydrolysis of long-chain triacylglycerides into fatty acids and glycerol at the lipid–water interface.

Substrate specificity study of T1 lipase showed preferences for lipids with the following carbon chain length; C8:0 > C18:1 > C18:0 in that order [4]. However, hydrolysis of several short-chain-length (SCL) PHAs by lipases had also been reported [5]. PHAs are basically polyesters of hydroxyalkanoates synthesized by various bacteria as intracellular carbon and energy storage compounds under imbalanced growth conditions, i.e., under limited oxygen, nitrogen, phosphorous, sulfur, or magnesium and an excess of carbon source [6].

Based on the number of their monomers, PHAs can be classified into three groups. Short-chain-length (SCL) PHAs consist of monomers with 3–5 carbon atoms, medium-chain-length (MCL) PHAs contain monomers with

6–14 carbon atoms, and SCL–MCL copolymers containing both SCL and MCL monomers [7]. They are accumulated as granules in the cytoplasm of cells which can attribute to more than 90% from the dry cell mass [8]. The ability to degrade PHA is widely distributed among bacteria and fungi and this depends on the production of specific PHA depolymerases based on the physical state of the polymer (amorphous or crystalline) [9].

Enzymes catalyzing intracellular degradation of PHA (amorphous or native granule) are intracellular PHA depolymerases (i-PHA depolymerases). Conversely, extracellular degradation is the utilization of an exogenous polymer (crystalline) by a not-necessarily accumulating microorganism that secretes extracellular PHA depolymerases (e-PHA depolymerases) [10]. Usually, most of the PHA-degrading bacteria studied so far produce only one PHA depolymerase except for *P. lemoignei* which was found to have seven PHA depolymerases to date. Of the entire seven PHA depolymerases in *P. lemoignei* (PhaZ7$_{Pl}$) is specific toward amorphous poly(3-hydroxybutyrate) [P(3HB)] [11] which is a type of SCL–PHA.

It is important to note that lipases and PHA depolymerase are very diverse in terms of their sequences but share a common α/β-hydrolase fold and catalytic triad which comprise of serine/cysteine [12–14], histidine, and aspartate [15]. Structure wise, the 1.5 Å crystal structure of T1 lipase showed that its overall structure was globular in shape, with a central β-sheet consisting of seven strands surrounded by 13 α-helices and 10 3_{10}-helices and loops, which resulted in an overall topology of a typical α/β-hydrolase canonical fold [16]. Meanwhile, the 1.90 Å crystal structure of (P(3HB) depolymerase) (PhaZ7$_{Pl}$) from *Paucimonas lemoignei* showed that the protein consists of a single domain with an α/β hydrolase fold in its core [17]. The difference between them is the substrate they catalyze. Lipases catalyze the hydrolysis of fats (lipid) [18, 19], while PHA depolymerases catalyze the hydrolysis of PHA's in general [20]. There is no report to date on the ability of T1 lipase to degrade any biopolymer. The important of the utilization of T1 lipase with biopolymer degrading abilities is to highlight the broad substrate specificity of T1 lipase. This information prompted us to study and compare in detail the structures and functions of both enzymes.

Materials and Methods

Bacterial Strains and Plasmids

pTrcHis2@TOPO TA harboring the T1 lipase gene was used as the source for T1 lipase enzyme while pTrcHis2@TOPO TA harboring the *phaZ7*$_{Pl}$ gene was used as the source for PHA depolymerase enzyme. *E. coli* TOP10 was used as cloning and expression host. *Paucimonas lemoignei* (AY026355) was purchased from American Type Culture Collection (ATCC).

T1 Lipase and PhaZ7$_{Pl}$ Sequence and Structural Comparison Studies

Possible structural homologs of T1 lipase with PHA depolymerases were searched among all existing PHA depolymerases from the protein database via NCBI PSI-BLAST. PHA depolymerase of *Paucimonas lemoignei* (PhaZ7$_{Pl}$) (PDB I.D: 2VTV) which has the highest similarity to T1 lipase (PDB I.D.: 2DSN) was identified and used for sequence and structural comparisons.

Amino acids sequences of both proteins were studied and compared using SSearch Smith–Waterman full-length alignments (http://pir.georgetown.edu/pirwww/search/pairwise.shtml). Both structures of T1 lipase and PhaZ7$_{Pl}$ were analyzed and compared via YASARA program [21]. For this purpose, both of the proteins' structures were superimposed using MUSTANG [22] in order to investigate in detail their global topology, geometry of their active sites, and oxyanion holes. Functional residues were mapped and the distances between all functional residues were calculated. Both structures were superimposed to evaluate their degree of similarity. Superimposition was carried out using the whole structure, active sites, and oxyanion holes, respectively.

Subsequently, both proteins were subjected to docking analysis using Autodock [23] whereby T1 lipase and PhaZ7$_{Pl}$ were docked with their native substrates, tryacylglyceride and P(3HB), respectively. The parameters were sets; the simulation cell was interactively placed around the active site to focus docking on the most important region, certain internal degrees of freedom of the ligand to perform anything from rigid to flexible docking was fixed, and typing of ligands and the assignment of pH dependent bond orders and hydrogen atoms were automatic. In order to determine the core fragment and its flexible attachments, automatic ligand structure analysis was used.

Selected active site residues were kept flexible during docking. The binding energy was compared. Following this, T1 lipase was then docked with P(3HB), and the binding energy generated was compared with that of involving its native substrate.

Cloning and Expression of *phaZ7*$_{Pl}$ in *E. coli* TOP10

For amplification of *phaZ7*$_{Pl}$ gene, the genomic DNA of *Paucimonas lemoignei* (ATCC: AY026355) was firstly extracted to be used as a template. The genomic DNA was

isolated using genomic extraction kit (Quick-Start Qiagen, Germany) following recommendations by the manufacturer. In general, the overnight cell culture (maximum of 5×10^6 CFU/mL) (colony forming units per milliliter) of *P. lemoignei* in Luria–Bertani broth with the optical density read at 600 nm was centrifuged at 190 rpm for 5 min and the pellet was suspended in 200 µL PBS. 20 µL of proteinase K and 200 µL of buffer AL were added. The mixture was then thoroughly mixed by vortexing and incubated for 10 min at 56 °C. 200 µL of ethanol (96–100%) was added and mixed thoroughly by vortexing.

The mixture was then pipetted into a DNeasy Mini spin column and placed into a 2-mL collection tube and centrifuged at 8000 rpm for 1 min. The spin column was placed in a new 2-mL collection tube, and 500 µL buffer AW1 was added. The mixture was then centrifuged again for 1 min at 8000 rpm. The spin column was placed in a new 2-mL collection tube, and 500 µl buffer AW2 was added into it. The sample was centrifuged again for 3 min at 14,000 rpm. The spin column was then transferred into a new 1.5-mL microcentrifuge tube. The DNA was eluted by adding 200 µL of buffer AE to the center of the spin column membrane and incubated for 1 min at room temperature. The mixture was then centrifuged for 1 min at 8000 rpm.

Amplification of $phaZ7_{Pl}$ was achieved using the following degenerate primers: 5′-AARGGNACNCARACICA RTAYGC-3′(Forward) and 5′-YTGDATDATNGCNCCIG TRTT-3′ (Reverse) with the following PCR conditions; polymerase activation and denaturation at 95 °C for 2 min and 20 s, respectively, followed by annealing at 55 °C for 10 s (repeated for 29 cycles) and extension at 70 °C for 6 min. The PCR product was then electrophoresed with 1% (w/v) agarose gel for viewing and was subsequently subjected to purification by using QIAquick Gel Extraction Kit (Qiagen, Germany) according to the protocol provided by the manufacturer.

The DNA fragment was excised from the agarose gel with a clean and sharp scalpel. The gel slice was weighed in a tube, and 3 volumes of Buffer QG were added to 1 volume of gel and were incubated at 50 °C for 10 min until the gel was completely dissolved. One gel volume of isopropanol was added to the sample. The mixture was transferred into a QIAquick column and centrifuged for 1 min (10,000×*g*). Then, 0.75 mL of Buffer PE was added to the column and subjected to 1 min of centrifugation (10,000×*g*). The column was then placed into a clean 1.5-mL microcentrifuge tube, and 50 µL of ultrapure water was added to the center of the membrane in the column and centrifuged for 1 min to elute the purified DNA.

The purified PCR product was later ligated into pTrcHis2 TOPO TA expression vector with the ligation mixture of 3 µL of purified PCR product with 1 µL of sterile distilled water and 1 µl vector. This mixture was mixed gently and incubated for 5 min at room temperature (24 °C). Two microliters of the mixture was added to 50 µl of *E. coli* TOP 10 competent cells and incubated for 30 min on ice. Then, 250 µL of Luria–Bertani broth was added into the mixture and incubated at 37 °C for 1 h 30 min. The cells was then plated onto Luria–Bertani agar plate containing 25 µL of 100 µg/mL ampicillin and 0.2% (w/v) amorphous P(3HB) substrate [11]. Plates were then incubated at 37 °C for 18 h. Besides qualitative screening for the confirmation of the cloned $phaZ7_{Pl}$ in pTrcHis2 TOPO TA expression vector, plasmid of a positive recombinant colony was extracted using QIAquick Plasmid Extraction Kit (Qiagen, Germany) as a template for PCR amplification using $phaZ7_{Pl}$ degenerate primers and the purified PCR product was sent for sequencing.

The recombinant plasmid was extracted by using QIAprep Spin Miniprep kit (Qiagen, Germany) according to the manufacturer's instruction. A single colony of the bacterium was inoculated into 10 mL Luria–Bertani broth supplemented with 50 µg/mL ampicillin and incubated at 37 °C for 16 h. Bacterial cells (3 mL) were harvested by centrifugation at 10,000×*g* for 1 min at room temperature. The pelleted bacterial cells were resuspended in 250 µL Buffer P1 and were transferred to a microcentrifuge tube. Buffer P2 (250 µL) was added, and the mixtures were mixed thoroughly by inverting the tube 4–6 times.

Next, 350 µL of Buffer N3 was added and the mixtures were mixed immediately and thoroughly by inverting the tube for 4–6 times. After that, the resulting mixtures were centrifuged for 10 min at 10,000×*g* to separate the supernatant and pellet. Then, the supernatant was transferred into QIAprep spin column and was subjected to centrifugation for 30 s. Buffer PB (0.5 mL) was added to wash the spin column followed by 30 s centrifugation. After that, 0.75 mL of Buffer PE was added to the spin column and subjected to centrifugation for another 30 s. The spin column was then placed into a clean 1.5-mL microcentrifuge tube, and 50 µL of ultrapure sterile water was added.

Additional centrifugation was conducted for 1 min to elute the DNA. The plasmid was then electrophoresed with 1% (w/v) agarose gel. The eluted plasmid was sent for sequencing and was also used as a template for PCR amplification using the degenerate primers as mentioned above. The PCR conditions used are as described above.

Screening for P(3HB) Depolymerase Activity

Positive recombinant colony identified from the $phaZ7_{Pl}$ transformation plate was used as a positive control for visual screening of PHA depolymerase activity on 0.2% (w/v) amorphous P(3HB) plate. Amorphous P(3HB) was

prepared from semicrystalline P(3HB) (Sigma-Aldrich, Germany) and detergents [24–27] and was later added into Luria–Bertani agar containing 100 μg/mL ampicillin. Recombinant *E. coli* TOP10 harboring pTrcHis2@TOPO TA carrying T1 lipase gene was also screened for PHA depolymerase activity using the same procedure as mentioned above.

Results and Discussion

T1 Lipase and PhaZ7$_{Pl}$ Sequence and Structural Comparison Studies

Based on sequence alignment, T1 lipase which consisted of 388 amino acids shows 21.4% sequence identity and 53.8% sequence similarity with PhaZ7$_{Pl}$ (Fig. 1). PhaZ7$_{Pl}$ amino acids sequence contained the motifs PXXXXHG and AHSMG, which are related to the oxyanion pocket and the lipase box pentapeptide (GXSXG) of many lipases and other serine hydrolases, respectively [28–30].

As shown in Fig. 1, the oxyanion pocket and lipase box pentapeptide sequences are present in both of these proteins. The oxyanion pocket is shown at the region PIVLLHG for T1 lipase while PVIFIHG for PhaZ7$_{Pl}$. Their lipase box pentapeptides are found at their respective active site serine region of T1 lipase and PhaZ7$_{Pl}$; AHSQ and AHSM, respectively. This central serine residue acts as a nucleophile, stabilized by a histidine and an aspartate (or glutamate) residues, with all three residues forming the catalytic triad. A tetrahedral transition state intermediate will be formed and stabilized by hydrogen bonding with residues of the so-called oxyanion hole.

Although they are low in sequence identity, their structures are highly similar. The structure of T1 lipase (PDB ID: 2DSN) with its active site consisted of Ser113, Asp 317, and His358 is shown in Fig. 2. All lipases have these residues as their catalytic triads, just differing in positions [31]. In closed conformation, this active site is buried under a long lid-helix (Fig. 2). The oxyanion hole of T1 lipase is constituted by Gln114 and Phe16, based on the open-form crystal structure of the BTL2 lipase (PDB ID: 2W22) [32]. It contains the metal ions Ca^{2+}, Zn^{2+}, Cl^{-} and Na^{+} as well as chain A and chain B in asymmetry [33]. The asymmetric unit of the crystal contains two monomers of the T1 lipase. The overall structure is globular in shape, with a central β-sheet consisting of seven strands surrounded by 13 α-helices and 10 3_{10}-helices and loops, which results in an overall topology of a typical α/β-hydrolase canonical fold. The distance between the active residues ranged in between 2 and 5.0 Å.

In the case of PhaZ7$_{Pl}$ (PDB ID: 2VTV), it is 342 amino acids in length and consists of a single domain with an α/β hydrolase fold at its core. PhaZ7$_{Pl}$ also has a similar active site compared to T1 lipase which consisted of Ser136, Asp242, and His306 but at a different position (Fig. 3). Its active site is similarly buried but devoid of the lid [34]. In order to lead the substrate to the buried active site, a degree of conformational changes have to occur [17]. The enzyme possesses an oxyanion hole which is constituted by Asn49 and Met137. PhaZ7$_{Pl}$ was chosen among all other PHA depolymerases enzyme to compare with T1 lipase because it shared the highest structural similarity among other PHA depolymerases with T1 lipase.

Superimposition of T1 Lipase and PhaZ7$_{Pl}$

Superimposition of T1 lipase and PhaZ7$_{Pl}$ proteins was carried out to see the degree of similarity and the overlapping parts of the proteins. The superimposition value for whole T1 lipase with PHA depolymerase (PhaZ7$_{Pl}$) was 1.981 Å with 53.8% sequence similarity (Fig. 4). From the figure, overlapping of the proteins' backbone at the α/β hydrolase fold and the excessive part of the lid domain in T1 lipase could be observed. The lid domain in T1 lipase is crucial for enzyme activation, termed "interfacial

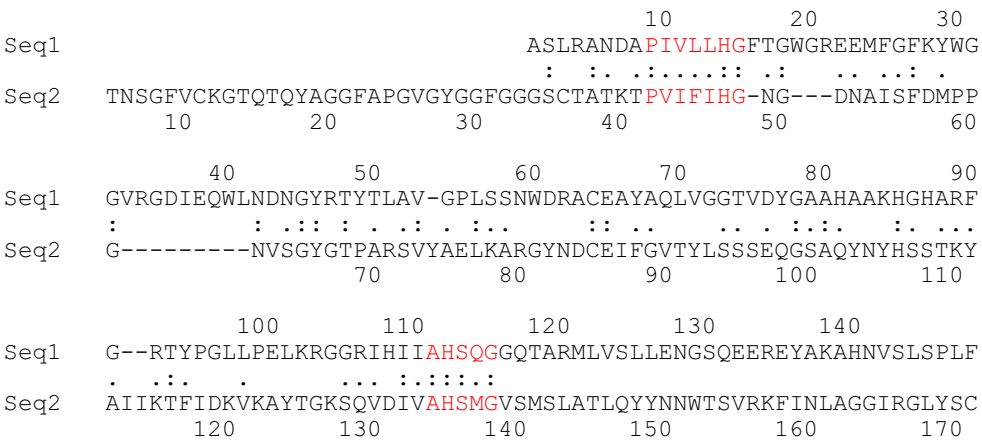

Fig. 1 Sequence alignment of PhaZ7$_{Pl}$ with T1 lipase using SSearch Smith–Waterman full-length alignments. The highlighted residues (*yellow*) show the conserved region between T1 lipase and PhaZ7$_{Pl}$. The alignment shows 21.4% sequence identity and 53.8% sequence similarity (Color figure online)

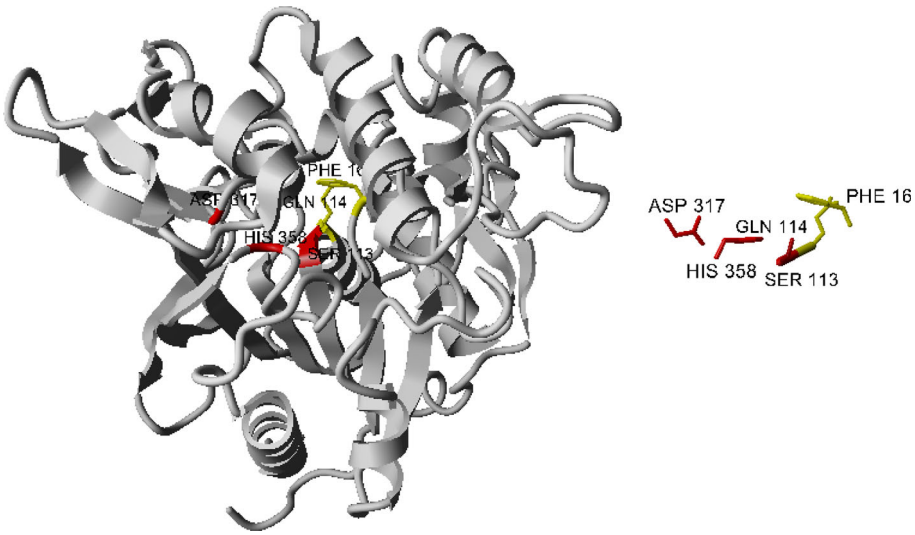

Fig. 2 T1 lipase structure (chain A) in closed conformations (PDB ID: 2DSN). Active site residues are shown in *red*, while residues of the oxyanion hole are shown in *yellow*) (Color figure online)

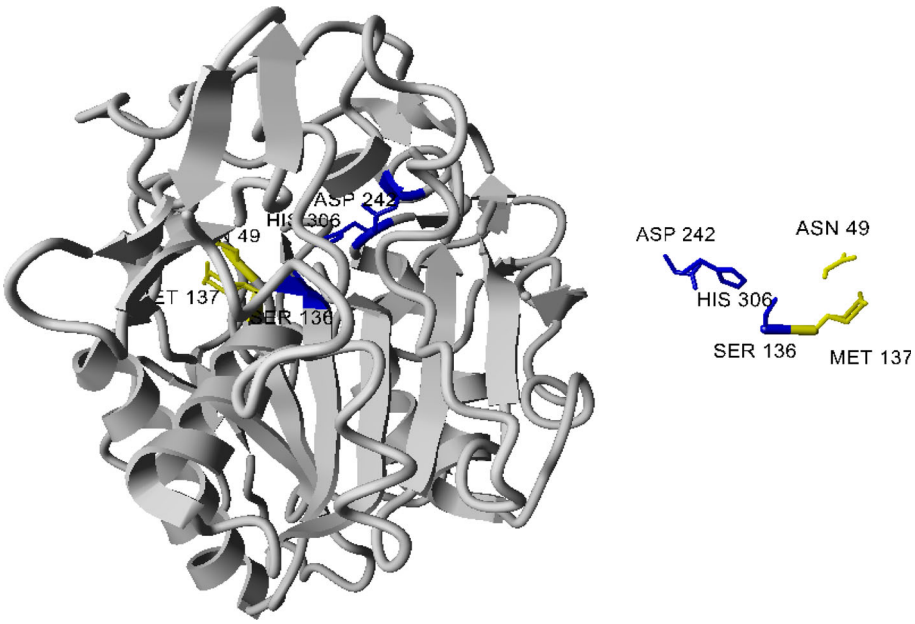

Fig. 3 PhaZ7$_{Pl}$ structure (PDB ID: 2VTV). Active site residues are shown in *blue*, while oxyanion hole residues are shown in *yellow* (Color figure online)

activation" at enzyme–lipid interphase. In the presence of lipid, the lid will be triggered to open [35].

Superimposition of their active sites generated an RMSD of 1.8 Å (Fig. 5), while superimposition of their oxyanion holes gave forth an RMSD of 1.76 Å (Fig. 6). As can be observed, both their active sites and oxyanion holes are structurally well aligned. Since their active sites are conserved, the RMSD value is lower than the superimposition of their whole structures. Active site plays an important role in protein function, thus the ability of T1 lipase to degrade the same substrate as PhaZ7$_{Pl}$ was hypothesized. The oxyanion holes for both T1 lipase and PhaZ7$_{Pl}$ are important for the stabilization of enzyme–substrate complex since these enzymes share the same catalytic mechanism [36]. These oxyanion holes are located near the carbonyl group of the substrate's scissile bond which stabilizes the tetrahedral enzyme–substrate intermediate [37]. These superimposition results certainly support the structure–function relationship between the proteins.

Docking of T1 Lipase with Triacylglycerides and PhaZ7$_{Pl}$ with P(3HB)

Docking of T1 lipase with its native substrate, triacylglycerides, gave forth a binding energy of 4.8 kcal/mol with the distance between active serine hydroxyl (OH) group to carbonyl carbon of the substrate to be 3.52 Å (Fig. 7). From Fig. 8 below, docking of PhaZ7$_{Pl}$ with its native substrate P(3HB) showed that the distance from

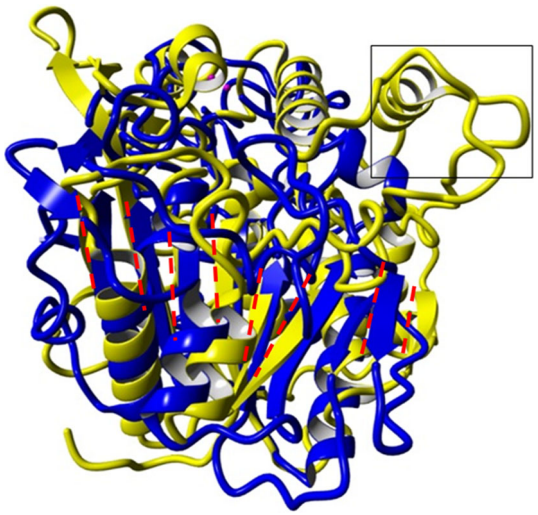

Fig. 4 Superimposition of T1 lipase (*yellow*) and PhaZ7$_{Pl}$ (*blue*) structures. The *box* indicates excessive part of the T1 lipase lid region while *red dots* indicate the α/β hydrolase fold (Color figure online)

Fig. 5 Superimposition of the active site residues of T1 lipase (*red*) and PhaZ7$_{Pl}$ (*yellow*) (Color figure online)

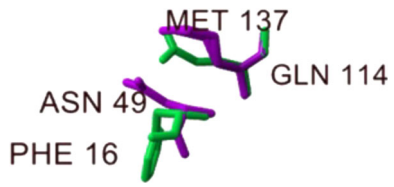

Fig. 6 Superimposition of the oxyanion hole forming residues of T1 lipase (*purple*) and PhaZ7$_{Pl}$ (*green*) (Color figure online)

hydroxyl group of active Ser136 of PhaZ7$_{Pl}$ to carbonyl carbon of the substrate P(3HB) was 4.16 Å. From the docking results, the binding energy of the substrate with the contacting receptor residues was calculated to be 3.93 kcal/mol.

This result gave the reference binding ability of the active site of PhaZ7$_{Pl}$ toward its specific substrate and its comparison with T1 lipase. With the docking results of PhaZ7$_{Pl}$ with its native substrate as reference, T1 lipase was then docked with P(3HB). This gave forth a binding energy of 5.4 kcal/mol with the distance of 4.05 Å between the active serine hydroxyl (OH) group to carbonyl carbon of the substrate. The readings obtained are similar to the binding energy and distance of native PhaZ7$_{Pl}$ to P(3HB) (Fig. 9).

Protein comparison study based on structure further strengthened our hypothesis that T1 lipase may be able to project degradative activity toward P(3HB), similar to PhaZ7$_{Pl}$ based on their highly conserved active residues and backbone structure.

Cloning and Expression of *phaZ7*$_{Pl}$ Gene in *E. coli* TOP10

Genomic DNA of *P. lemoignei* was successfully extracted as shown in Fig. 10. A total of 1141 base pair coding sequence of the *phaZ7*$_{Pl}$ gene was successfully amplified. The purified PCR product of *phaZ7*$_{Pl}$ was successfully cloned in pTrcHis2 TOPO TA expression vector and transformed into *E. coli* TOP10 as shown in Fig. 11a, b. The extracted plasmid for the positive recombinant transformant is shown in Fig. 11a while the amplification of the *phaZ7*$_{Pl}$ gene by using the extracted positive recombinant plasmid as a template and *phaZ7*$_{Pl}$ degenerate primer is shown in Fig. 11b.

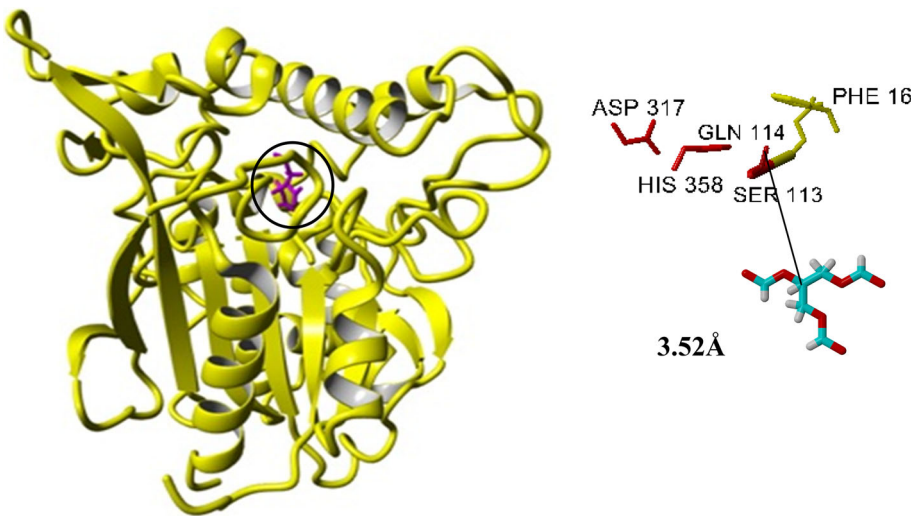

Fig. 7 Docking of T1 lipase with triacylglycerides. *Circled region* shows the substrate-binding region. The *line* indicates the distance between serine hydroxyl groups of T1 lipase with carbonyl carbon of triacylglycerides

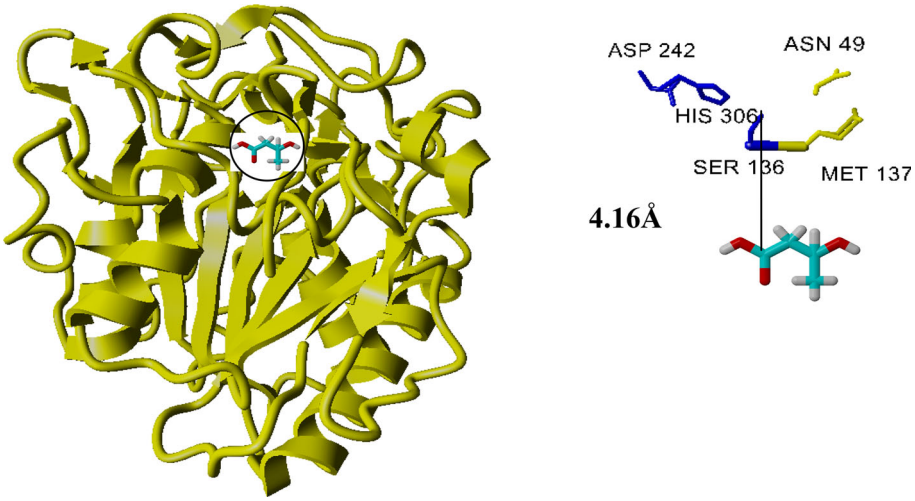

Fig. 8 Docking of PhaZ7$_{Pl}$ with P(3HB). *Circled region* shows the substrate-binding region. The *line* indicates the distance between serine hydroxyl groups with carbonyl carbon of P(3HB)

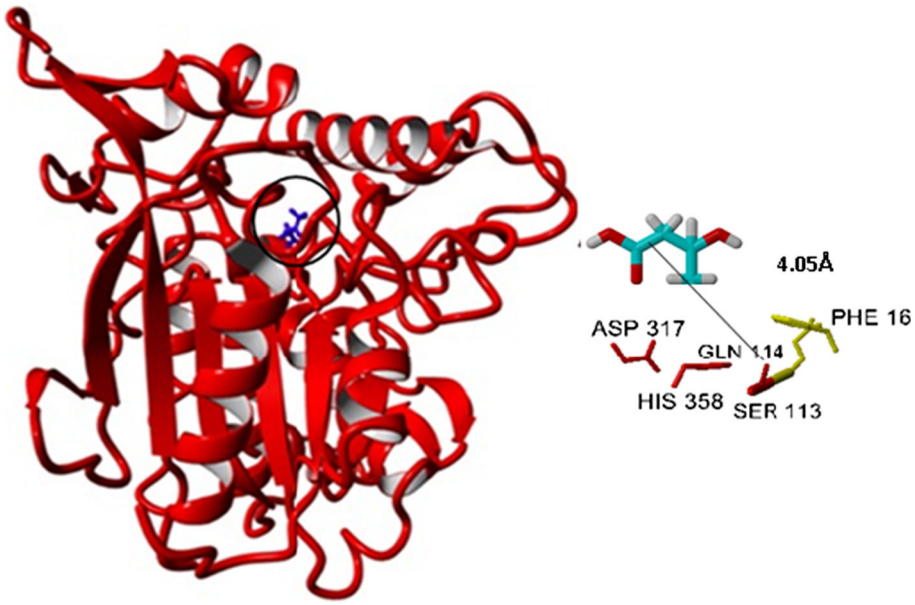

Fig. 9 Docking of T1 lipase with P(3HB). *Circled region* shows the substrate-binding region. The *line* indicated the distance between serine hydroxyl groups of T1 lipase with carbonyl carbon of P(3HB)

Screening for Amorphous P(3HB) Depolymerase Activity

Recombinant *E. coli* TOP10 harboring the constructed pTrcHis2::*phaZ7*$_{Pl}$ was used to screen for amorphous P(3HB) depolymerase activity. P(3HB) depolymerase activity of *E. coli* TOP10 recombinants containing PhaZ7$_{Pl}$ showed clear zones around the colonies (Fig. 12a). Such activity was also screened for recombinant *E. coli* TOP10 harboring the pTrcHis2 carrying the T1 lipase gene using similar plate screening method. Clear zones were indeed observed for recombinants harboring T1 lipase (Fig. 12a). This result showed that T1 lipase was able to degrade amorphous P(3HB). For comparison purposes, plate screening was also tested using crystalline P(3HB) as a substrate and results showed that both PhaZ7$_{Pl}$ and T1 lipase could not degrade crystalline P(3HB) (Fig. 12b). Negative control; *E. coli* TOP10 empty expression host for PhaZ7$_{Pl}$ and T1 lipase activity was shown in Fig. 12c. The negative control is to ensure that there is no depolymerase activity from native host.

These results showed that PhaZ7$_{Pl}$ has the unique property to recognize the physical state of the polymeric substrate by discrimination between amorphous PHA (good substrate) and denatured, partially crystalline PHA (no substrate). The ability and the rate of degradation of the enzyme toward the polymer were greatly affected by the nature of the polymer. The degree of crystallinity is a crucial factor affecting biodegradability, since enzymes mainly attack the amorphous domains of a polymer. The molecules in the amorphous region are loosely packed, thus making them more susceptible to degradation. The

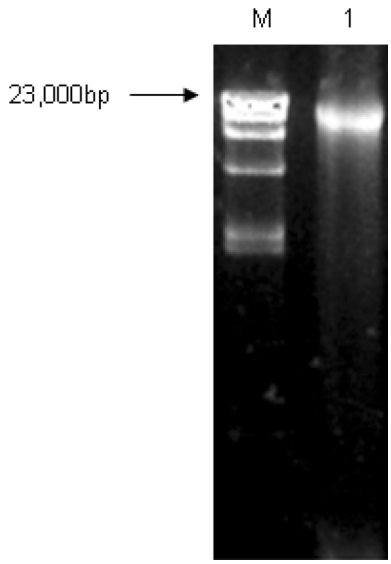

Fig. 10 Genomic DNA extracted from *P. lemoignei*. *Lane M* λ DNA-*Hin*dIII marker, *Lane 1* Genomic DNA of *P. lemoignei*

crystalline part of the polymers is more resistant than the amorphous region [38]. Thus, this fact support the ability of T1 lipase ability to degrade the amorphous state rather than the crystalline state of P(3HB).

The degree of crystallinity depends on the location of the PHA's. PHA's in vivo and outside the bacteria is present in two different biophysical states. In intracellular PHA granules the high molecular mass polymer (10^5–10^6 Da) is in the amorphous "rubbery" state (highly mobile chains in disordered conformation), and the granule surface layer consists of proteins and phospholipids [39].

Upon extraction from the cell, the granule surface layer is either damaged or lost [27] and the polyester chains tend to adopt ordered helical conformations [40] and develop a crystalline phase.

Interestingly, PhaZ7$_{Pl}$ was also reported to exhibit no significant amino acid homology to any other PHA depolymerases or to any other known proteins apart from the lipase box short-signature sequences around the catalytic triad amino acids. This explains the ability of this enzyme to degrade on different physical state of the polymer compared to other extracellular PHA depolymerases (PhaZ1$_{Pl}$–PhaZ6$_{Pl}$), which can only degrade the extracellular or the crystalline P(3HB) [34, 41]. T1 lipase could degrade only the amorphous P(3HB) similar to PhaZ7$_{Pl}$ since they share similar structure with very low RMSD and a conserved region at the serine active site residue in addition to the degree of crystallinity as mentioned earlier.

As a conclusion, based on sequence, structure, and plate assay analyses carried out in this study, T1 lipase was found to exhibit similarities with PhaZ7$_{Pl}$ initiated from their amino acid sequences (conserved region at the serine active site residue) that lead to their similarity in structures with overlapping of the proteins' backbone at the α/β hydrolase fold that consists all the functional residues (active site and oxyanion holes) thus finally the ability to degrade the same physical state of the amorphous P(3HB). Further study can be carried out to utilize this broad substrate specificity enzyme of T1 lipase for bioremediation purpose since it not only degrades oil but also amorphous plastic pollution (degrade polystyrene, PVC, and atactic polypropylene).

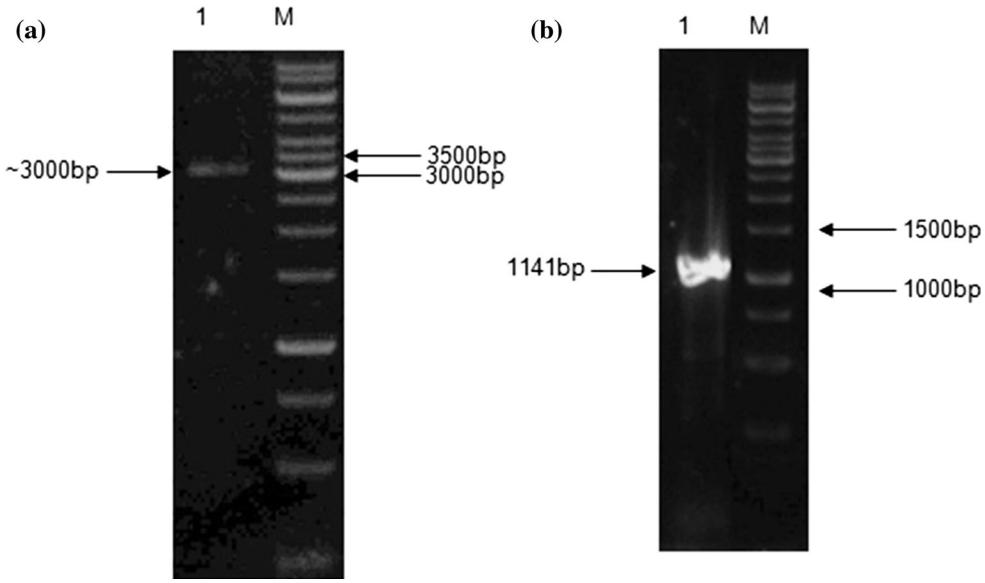

Fig. 11 **a** Recombinant plasmid pTrcHis2 TOPO TA::*phaZ7*$_{Pl}$ extracted from *E. coli* TOP10. *Lane M* 1 kb DNA marker, *Lane 1 phaZ7*$_{Pl}$ recombinant plasmid extracted from *E. coli* TOP 10. **b** *phaZ7*$_{Pl}$ gene amplicon amplified using extracted plasmid as a template. *Lane M* 1 kb DNA marker, *Lane 1* amplified *phaZ7*$_{Pl}$ gene

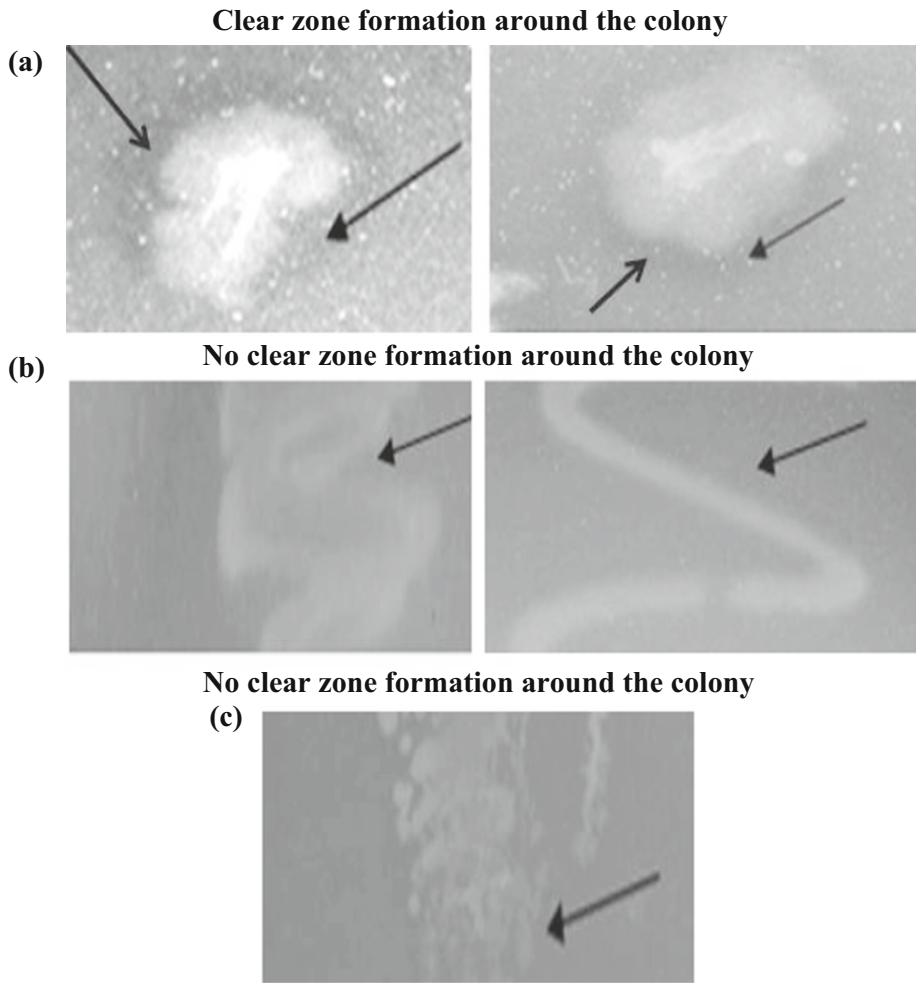

Fig. 12 **a** Plate screening of amorphous P(3HB) degradation by T1 lipase (*right*) and PhaZ7$_{Pl}$ (*left*); **b** plate screening of semicrystalline P(3HB) degradation by T1 lipase (*right*) and PhaZ7$_{Pl}$ (*left*); **c** plate screening of amorphous P(3HB) degradation by empty *E. coli* TOP10 host. *Arrows* indicate the clear zone (24a) and no clear zone (24b and 24c) around the colonies

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