Improved activity of α-L-arabinofuranosidase from *Geobacillus vulcani* GS90 by directed evolution: Investigation on thermal and alkaline stability

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Abstract

α-L-Arabinofuranosidase (Abf) is a potential enzyme because of its synergistic effect with other hemicellulases in agro-industrial field. In this study, directed evolution was applied to Abf from *Geobacillus vulcani* GS90 (*Gv*Abf) using one round error-prone PCR and constructed a library of 73 enzyme variants of *Gv*Abf. The activity screening of the enzyme variants was performed on soluble protein extracts using p-nitrophenyl α-L-arabinofuranoside as substrate. Two high activity displaying variants (*Gv*Abf L307S and *Gv*Abf Q90H/L307S) were selected, purified, partially characterized, and structurally analyzed. The specific activities of both variants were almost 2.5-fold more than that of *Gv*Abf. Both *Gv*Abf variants also exhibited higher thermal stability but lower alkaline stability in reference to *Gv*Abf. The structural analysis of *Gv*Abf model indicated that two mutation sites Q90H and L307S in both *Gv*Abf variants are located in TIM barrel domain, responsible for catalytic action in many Glycoside Hydrolase Families including GH51. The structure of *Gv*Abf model displayed that the position of L307S mutation is closer to the catalytic residues of *Gv*Abf compared with Q90H mutation and also L307S mutation is conserved in both variants of *Gv*Abf. Therefore, it was hypothesized that L307S amino acid substitution may play a critical role in catalytic activity of *Gv*Abf. Volume 66, Number 1, Pages 101–107, 2019

Keywords: *α-l-arabinofuranosidase, Geobacillus vulcani, directed evolution, catalytic activity, thermal stability, alkaline stability*

1. Introduction

Alpha-l-arabinofuranosidase (Abf) (E.C.3.2.1.5) catalyzes the hydrolysis of the terminal and non-reducing α-1,2-, α-1,3-, and/or α-1,5-L-arabinofuranoside substitutions of α-L-arabinosides such as arabinoxylan, 1-arabinan, and the other polysaccharides that include arabinose [1]. Recently, hemicellulases have attracted great attention in a wide range of industrial fields including the manufacture of medicinal substances, wine, acetic acid for bread quality, paper from pulp, feedstock for animals, and fruit juice and fermentable sugar for bioethanol [2]. In the agro-industrial field, Abf and the other hemicellulases play a promising role in a synergistic manner [3, 4]. It has been emphasized that specific activity of Abf and its cooperative enzymes ensure efficient degradation of hemicellulose [5]. Therefore, Abf as one of the important accessory enzyme is considered necessary to enhance the synergistic action of cellulases. Much effort has been devoted to isolate Abf from variety sources including microorganisms [6–11]. So far, Abf from thermophilic sources represent a good target because most of the related industrial processes operate at high temperature [9, 12, 13]. Thus far, Abf activity has been shown in a couple members of thermophilic microorganisms such as *Geobacillus stearothermophilus*, *Geobacillus thermoleovolerans*, and *Geobacillus caldoxylolyticus* [14–16]. We have previously characterized Abf from *Geobacillus vulcani* GS90 [17]. There is great need for a thermostable enzyme with improved activity and stability for large-scale industrial applications.

Abbreviations: *Abf, α-L-Arabinofuranosidase; GvAbf, Geobacillus vulcani GS90.*

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Directed protein evolution is an approach that simulates the Darwinian evolution in laboratory scale in order to improve the protein stability and their biochemical function. It is based on random mutagenesis for an increase of the gene diversity by iterative rounds [18]. The selection of the improved proteins is subjected by screening the random mutant library [19, 20]. The approach has been successfully applied for development of different properties of various industrial enzymes [21–23]. So far, there has been only one study associated with the improvement of some properties of Abf. This is GH51 Abf in *Pleurotus ostreatus* and the improved property is specific activity [21].

In this study, directed evolution approach was employed to enhance the catalytic action of Abf from *G. vulcani* GS90 (*Gv*Abf). We generated a library of *Gv*Abf with 73 variants using one round error-prone polymerase chain reaction (epPCR) and they were overexpressed in *Escherichia coli* BL21 (λDE). Active soluble extracts were screened, selected using *p*NP-α-L-arabinofuranoside, and compared with wild-type *Gv*Abf. The selected variants were purified, partially characterized, and structurally analyzed. In this study, we achieved to enhance the specific activity and thermal stability of *Gv*Abf by one round epPCR.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and growth conditions

Thermophilic *Geobacillus sp.* was grown as previously described [17] and its genomic DNA was extracted. λDE was grown at 37 °C as cloning and expression host. pET28a(+) and pG-Tf2 were used for expression vector and chaperon plasmid, respectively. Bacterial growth was carried out in Luria-Bertani (LB) (1% peptone, 0.5% yeast extract, and 0.5% NaCl) medium supplemented with kanamycin (50 μg/mL) and chloramphenicol (20 μg/mL) during cloning. Isopropyl β-d-1-thiogalactopyranoside (IPTG; 1 mM)) and tetracycline (10 ng/mL) were used for overexpression of *Gv*Abf and its variants and chaperon protein, respectively.

2.2. Identification of the bacterial strain

In this study, the environmental isolate (V90) was previously obtained from thermal vent of Balcova geothermal site (İzmir, Turkey) [24]. Single colony from V90 was isolated by streaking technique on LB agar medium and was grown at 55 °C for overnight. The strain was identified by 16S rRNA gene sequencing using universal bacterial primers E334F (CCAGACTCCTACGGGAGGCAG) as forward primer and E1115R (CAACGAGCGCAACCCT) as reverse primer. The nucleotide sequence of partial 16S rDNA was determined and deposited in the EMBL Nucleotide Sequence Databases and assigned the accession number as MG283275.

2.3. Error-prone PCR

*Gv*Abf gene was amplified by PCR over 32 cycles. The *Gv*Abf gene variants were generated by one round of epPCR using the forward primer (5′-ggaacttgcatatggctacaaaaaaagcaacc-3′, *Nde*I site underlined) and reverse primer (5′-ggaacatgaa gcttttatcgttttcctaaacg-3′, *Hin*dIII site underlined). Mutagenic conditions were constructed using 10× Mutagenic PCR Buffer, 10× Mutagenic dNTP mix (2 mM dATP, dGTP and 5 mM dCTP, dTTP) and 50 μM $MnCl_2$, adapted by Cadwell and Joyce [25]. Thermal cycler was used following conditions: initial denaturation at 95 °C for 3 Min, 30 cycles of denaturation at 95 °C for 30 Sec, annealing at 55.9 °C for 45 Sec, extension at 72 °C for 90 Sec, and a final extension at 72 °C for 10 Min. PCR products were purified from agarose gel, digested by *Nde*I and *Hin*dIII and ligated into pET28a(+) expression vector. The constructed plasmid was transformed to λDE with pG-Tf2 chaperon plasmid. The nucleotide sequence of the *Gv*Abf was determined and deposited in the EMBL Nucleotide Sequence Databases and the accession number was assigned as HE653772.

2.4. Screening of high activity displaying *Gv*Abf variants

The randomly selected colonies were grown in 10 mL of LB medium including 50 μg/mL kanamycin, chloramphenicol (20 μg/mL), and tetracycline (10 ng/mL) at 37 °C. Tetracycline was used to induce the chaperon protein precluding the protein aggregation. After reaching OD_{600} of 0.7, 1 mM IPTG was added into each culture and they were incubated at 37 °C, 225 *g* for 4 H. The soluble extract was obtained as following. The cells were harvested by centrifugation (4,500 *g* for 20 Min, 4 °C). The pellet was resuspended with breaking buffer [26] and sonication was applied to degrade the cell wall and membrane for 5 Min on ice (Sonopuls Ultrasonic Homogenizers, HD 2070; Bandelin, Berlin/Germany). Finally, the samples were centrifuged (12,100 *g* for 20 Min, 4 °C) and supernatant was used as soluble extract for screening assay.

The screening assay was performed using 5 μL of 2 mM *p*NP-α-L-arabinofuranoside (*p*NPA) substrate, 85 μL of Na-acetate (pH 5.0) buffer and 10 μL of soluble extract in 96-well plate. The mixture was incubated for 10 Min at 70 °C and then the reaction was stopped by supplementing 100 μL of 1 M Na_2CO_3. OD_{420} was measured as dual repetition and screening assay was applied twice for each of randomly selected *Gv*Abf variants. High activity displaying *Gv*Abf variants were selected for further analysis.

2.5. Purification of high activity displaying *Gv*Abf variants and SDS-PAGE analysis

*Gv*Abf and its variants were overexpressed in 500 mL LB culture and the soluble extracts were obtained as described above. Heat purification of *Gv*Abf and its variants was performed by heating the supernatants recovered after centrifugation as soluble extract. Samples were heated 15 Min at 50 °C and thereafter cooled on ice for 20 Min before pelleting the denatured proteins. Thermotolerant proteins were recovered from the supernatant and subjected to affinity chromatography for further purification. The thermotolerant proteins were loaded onto His-tag Nickel Affinity (HIS-Select™ HF Nickel

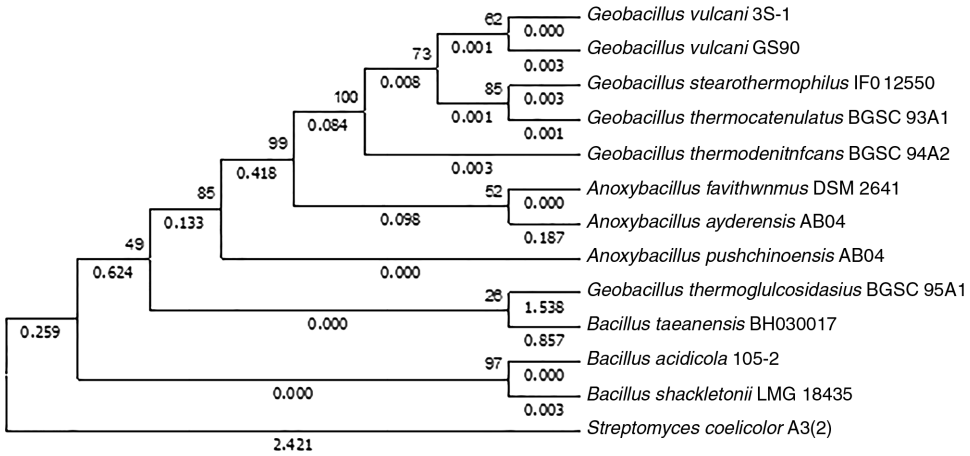

FIG. 1 *Evolutionary relationship of Geobacillus vulcani GS90 using maximum likelihood (ML) method.*

Affinity Gel; Sigma) column and washed two times with washing buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, and 1 mM imidazole). *Gv*Abf and its variants were recovered with elution buffer (20 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 250 mM imidazole). The selected fractions were combined and dialyzed using 20 mM Tris–HCl (pH 8.0) buffer to remove the salt. The enzyme concentration was determined by Bradford assay using bovine serum albumin as standard. 12% of SDS-PAGE gel was used to detect the purity of *Gv*Abf and its variants by staining with Coomassie Brillant Blue R-250.

2.6. Biochemical assays of high activity displaying *Gv*Abf variants

All enzyme-catalyzed reactions as standard activity assay were carried out using 2 mM of *p*NPA substrate in 50 mM Na-acetate in 96-well plate for 10 Min. Sodium carbonate (1 M) was added to stop the enzymatic reaction and the released *p*-nitrophenol was measured at 420 nm ($\varepsilon = 17{,}800\ M^{-1}\ cm^{-1}$). Each experiment was performed triple replicates.

The temperature effect on the activity of *Gv*Abf and its variants was investigated at 70 °C for 100 Min, 71 °C for 60 Min, and 72 °C for 45 Min at pH 5.0 (50 mM Na–acetate buffer). Alkaline pH effect on the activity of *Gv*Abf and its variants was investigated at pH 9.6 (50 mM glycine–NaOH buffer) at 70 °C for 80 Min. The purified enzymes were incubated over time and 20 μL from each sample was taken at time intervals. The residual activity was determined by the standard assay and then stability profiles were obtained.

2.7. Bioinformatics analysis

The 16S rRNA gene fragment (731 bp length) was sequenced in both direction and BLAST algorithm (http://blast.ncbi.nlm.nih.gov/) was utilized to assess the DNA similarities.

The DNA sequence of *Gv*Abf and its variants as well as 16S rRNA sequence were obtained using dideoxynucleotide chain termination method by 16 and 80 capillary, 3130XL (Applied Biosystem, CA/USA). Finch TV 1.4.0 version was used for sequence analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [27]. The deduced amino acid sequence was analyzed using Clustal Omega and BLAST algorithms. Homology modeling was carried out using I-Tasser online server [28–30]. The predicted three-dimensional (3D) structure of *Gv*Abf was modeled using I-Tasser online server [28]. They were analyzed using PyMOL Molecular Graphic System, Version 2.0 (Schrödinger, LLC, NY/USA). Amino acid sequence alignment between *Gv*Abf and templates from PDB library were performed by LOMETS, a meta-server threading program.

2.8. Statistical analysis

Unpaired and two-tailed *t*-test was performed for statistical data analysis using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) (www.graphpad.com).

3. Results and Discussion

3.1. 16S. rRNA analysis

Partial 16S rRNA gene (731 bp) from single colony of V90 environmental isolate [24] was amplified, cloned, and sequenced in order to identify the bacterial strain. According to the BLAST analysis, *G. vulcani* 3S-1 was found out as the closest one to our strain showing the query 99.7% sequence identity [31]. The phylogenetic tree was constructed considering partial 16S rDNA by maximum likelihood (ML) method to determine the evolutionary connection of this strain. Clearly, the strain had the closest connection with G. vulcani 3S-1 and thus it was named as *G. vulcani* GS90 (Fig. 1).

3.2. Selection and purification of high activity displaying *Gv*Abf variants

One round epPCR was applied to increase the variation on *Gv*Abf gene. The *Gv*Abf and its variants were cloned into λDE and 73 of colonies were screened to determine high activity displaying *Gv*Abf variants. Two constructs were determined as the high activity displaying *Gv*Abf variants. *Gv*Abf and its variants were overexpressed in large scale. Purification of protein samples was achieved via heat treatment and then

TABLE 1 *The concentration and specific activity of GvAbf and high activity displaying GvAbf variants*

Enzyme name	*Concentration (OD/mL)*	*Concentration (mg/mL)*	*Specific activity (mU/OD)*	*Specific activity (mU/mg)*
*Gv*Abf	9.55 ± 1.458	1.328 ± 0.238	10.018 ± 0.573	72.066 ± 4.12
*Gv*Abf L307S	4.35 ± 0.033	0.679 ± 0.004	25.144 ± 1.379	160.991 ± 8.83
*Gv*Abf Q90H/L307S	4.45 ± 0.730	0.695 ± 0.097	22.171 ± 1.024	142.024 ± 6.56

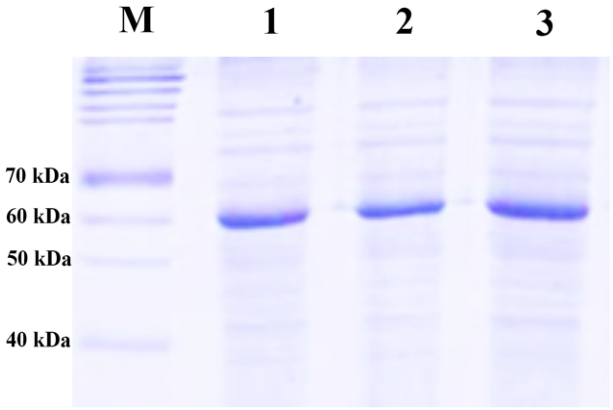

FIG. 2 *SDS-PAGE display of GvAbf and high activity displaying GvAbf variants (M: Protein marker, Line 1: GvAbf, Line 2: GvAbf Q90H/L307S, Line 3: GvAbf L307S).*

affinity chromatography. Bradford analysis was performed to detect the concentration *Gv*Abf and its variants (Table 1).

The purity of *Gv*Abf and its variants was assessed by SDS-PAGE analysis as shown in Fig. 2. The molecular weights of protein samples were around 60 kDa including six-histidine tag as previously characterized [17]. *Gv*Abf and its variants had similar purity profiles ensuring that they were pure and comparable (Fig. 2).

3.3. Sequence analysis of high activity displaying *Gv*Abf variants

Nucleotide sequences of *Gv*Abf and its variants were revealed using dideoxynucleotide chain termination method. The gene length of *Gv*Abf and its variants was 1,509 bp. *Gv*Abf sequence was confirmed as previously shown [17]. The mutations from *Gv*Abf variants were shown by Clustal Omega algorithm in reference to *Gv*Abf. Each of *Gv*Abf and its variants had 502 amino acids. *Gv*Abf variants were named as *Gv*Abf L307S and *Gv*Abf Q90H/L307S based on their amino acid substitution.

3.4. Specific activity of high activity displaying *Gv*Abf variants

Specific activity was investigated in optimum pH and temperature (pH 5 and 70 °C) using *p*NPA as substrate [17]. *Gv*Abf showed a specific activity of 10.0 ± 0.6 mU/OD. Both *Gv*Abf variants exhibited almost 2.5-fold higher specific activity compared with *Gv*Abf (Table 1). It has been shown that specific activity of GH51 Abf in *P. ostreatus* increases approximately as much as two-fold by directed evolution and it was 36.8 mU/OD [21]. Interestingly, comparing with *Gv*Abf Q90H/L307S, *Gv*Abf L307S exhibited relatively higher specific activity. Introduction of Q90H substitution in addition to L307S showed similar activity indicating that the L307S is responsible for the improved catalytic performances. Therefore, the main reason of dramatic increase of specific activities of *Gv*Abf variants could be L307S substitution that might have changed spatial arrangements of catalytic site residues.

3.5. Effect of temperature and alkaline pH on high activity displaying *Gv*Abf variants

Biochemical characteristics of *Gv*Abf variants were investigated. Effect of temperature and alkaline pH on *Gv*Abf variants were performed using *p*NPA as substrate. The equal volume of *Gv*Abf and its variants were used. Temperature effect was performed in three different temperatures (70, 71, and 72 °C) at pH 5. At 70 °C, residual activities of *Gv*Abf variants were relatively higher than that of *Gv*Abf. *Gv*Abf L307S had significantly more residual activity at 60, 80, and 100 Min, comparing with *Gv*Abf and *Gv*Abf Q90H/L307S (Fig. 3A). At 71 °C, *Gv*Abf L307S also conserved the highest residual activity at 20, 30, and 40 Min ($P < 0.0001$) and *Gv*Abf Q90H/L307S displayed more residual activity at 30 and 40 Min, compared with *Gv*Abf. After 40 Min, *Gv*Abf L307S and *Gv*Abf Q90H/L307S maintained 47.1% and 28.2% of the residual activities, respectively. On the contrary, this value for *Gv*Abf was only 19.8% (Fig. 3B). Although the residual activity of all enzymes is 100% at the beginning, *Gv*Abf variants conserved much more residual activity than that of *Gv*Abf. Thus, *Gv*Abf variants included more thermal stability than *Gv*Abf in this condition. L307S substitution could apparently lead to positive impact on *Gv*Abf stability. It has previously been reported that thermostability of an enzyme can be increased by promoting the formation of intra-loop hydrogen bond between serine and glycine [23]. Thus, it could be hypothesized that serine substitution on the 307 location might have increased thermostability of *Gv*Abf variants, enabling new hydrogen bond interactions. Previous study has also reported that the substitution of Gln for His in α-amylase from *Bacillus amyloliquefaciens* provides significant enhancement in temperature stability, compared with wild-type [32]. Similar results were obtained in this study. However, Q90H substitution

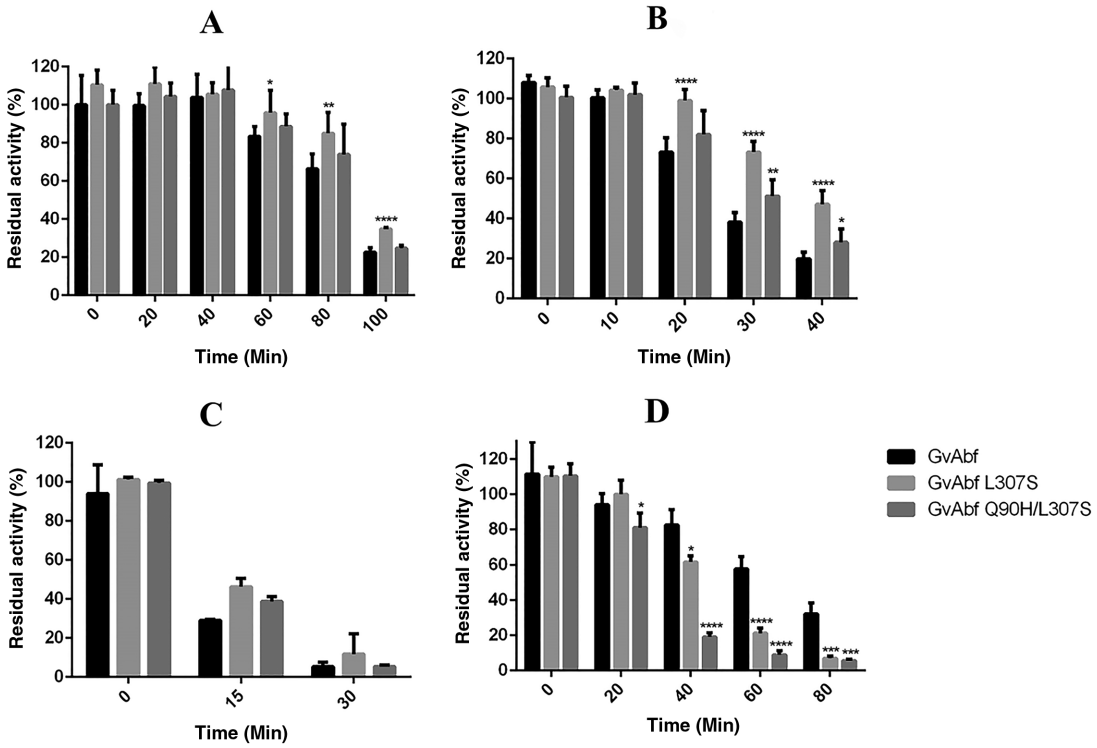

FIG. 3 *Residual activity of GvAbf and high activity displaying GvAbf variants against four conditions at specified time intervals. (A) 70 °C—pH.5. (B) 71 °C—pH.5. (C) 72 °C—pH.5. (D) 70 °C—pH.9.6. Statistical significance indicated *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.*

in addition to L307S partially removed the favorable impact of serine residue at 71 °C-pH 5. As a result, *Gv*Abf L307S exhibited the highest residual activity whereas the residual activity of *Gv*Abf Q90H/L307S was more than *Gv*Abf at the higher temperature. At 72 °C, *Gv*Abf and its variants almost completely lost the activity at the end of 30 Min. Indeed, *Gv*Abf L307S had the highest residual activity at 15 and 30 Min (Fig. 3C).

Furthermore, alkaline stability was studied incubating the enzymes at 70 °C and pH 9.6. Comparing with *Gv*Abf variants, *Gv*Abf had the highest residual activity at 40, 60, and 80 Min. It conserved 82.5% of residual activity at 40 Min, whereas these values were 61.5% and 19.4% for *Gv*Abf L307S and *Gv*Abf Q90H/L307S, respectively. Higher residual activity of *Gv*Abf significantly maintained at 60 and 80 Min. At minute 80, this value was 32.1%, whereas *Gv*Abf variants remained around 5%–7%. Among *Gv*Abf variants, residual activity of *Gv*Abf L307S was significantly more than that of *Gv*Abf Q90H/L307S. Thus, alkaline pH stability of *Gv*Abf was relatively better at the indicated pH value compared with *Gv*Abf variants (Fig. 3D). Even though L307S substitution caused to relative decline of alkaline stability of *Gv*Abf variants, actual dramatic decrease occurred because of the Q90H substitution. Thus, it could be concluded that Gln90 might have been a critical role on alkaline pH stability of the enzyme.

According to the results of this study, a couple of properties in *Gv*Abf enzyme were improved and these were specific activity and thermostability. This versatility has been similarly performed in GH51 Abf from *P. ostreatus* by developing the specific activity, pH stability, and thermostability [21]. This kind of development has been also shown in the other industrial enzymes. For example, Akbulut et al. [23] has achieved to increase thermostability and specific activity of *Bacillus pumilus* lipase by directed evolution. They have suggested that there may be a correlation between specific activity and thermostability [23].

3.6. Structural analysis of high activity displaying *Gv*Abf variants

LOMETS, a meta-server threading program, identified the structure templates with the highest statistical significance from PDB library and utilized many templates of GS51 family proteins for alignments in the I-Tasser modeling of *Gv*Abf. As a result, only three PDB hits were found out: 1QW9 from *G. stearothermophilus* T-6 (98% sequence identity), 2C7F from *Clostridium thermocellum* (69% sequence identity), and 2C8N from *Clostridium thermocellum* (69% sequence identity). *Gv*Abf model shares highly amino acid sequence identity with 1QW9 (Fig. 4A). The structural alignment of *Gv*Abf and AbfA using PyMOL Molecular Graphic System also followed similar structure and all folding pattern was almost the same between each other (Fig. 4B). 3D model structure of *Gv*Abf was acquired by I-Tasser homology modeling and the location of two mutations Q90H and L307S were shown on *Gv*Abf model structure using PyMOL Molecular Graphic System (Fig. 5). E175 acid/base, E294 nucleophile residues [33, 34] as well as E29 proposed as a third catalytic residue [35] were conserved in

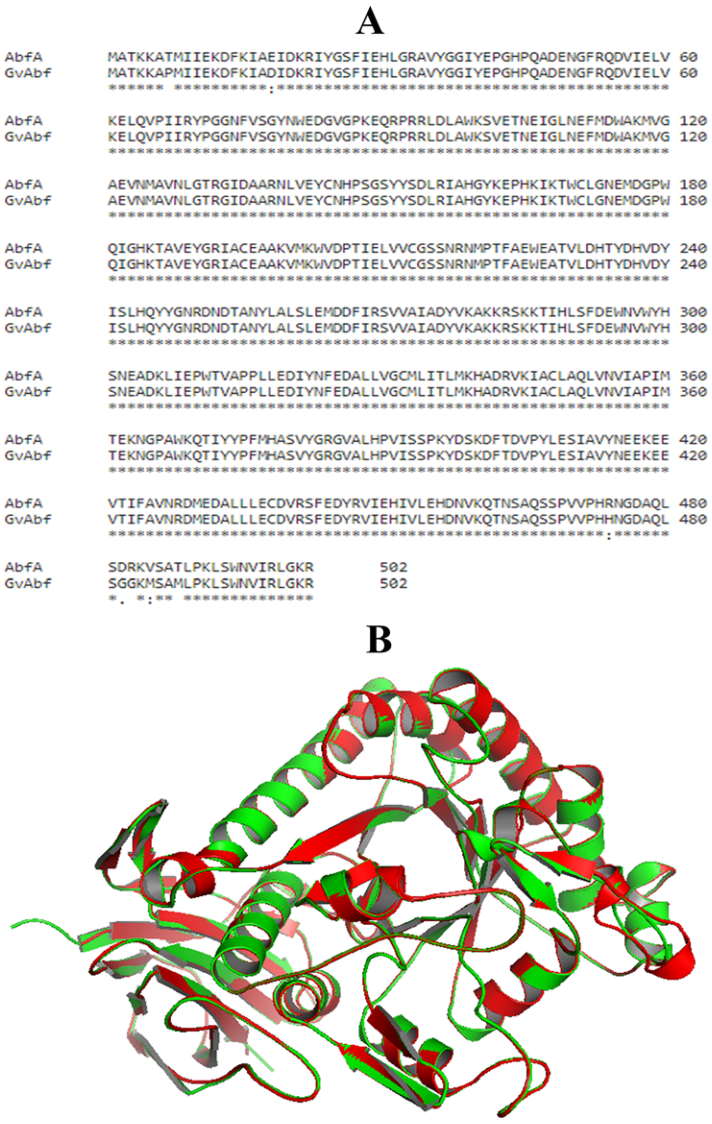

FIG. 4 *Structural alignment of GvAbf (green) and template AbfA (red) using PyMOL Molecular Graphic System.*

the *Gv*Abf model and overlapped well with the template AbfA from *G. stearothermophilus* T-6 (Fig. 5).

Amino acid sequence of *Gv*Abf is different in seven residues from the template of AbfA. These residues are T7, E18, R474, D482, R483, V485, and T488 for AbfA, whereas they are P7, D18, H474, G482, G483, M485, and M488 for *Gv*Abf. All residues are located in a 12-stranded β sandwich domain with jelly-roll topology. Kamitori et al. [36] have suggested that 12-stranded β sandwich domain stabilizes another domain called $(\beta/\alpha)_8$-barrel domain (TIM barrel) in the α-amylase from Thermoactinomyces vulgaris R-47. TIM barrel domain is responsible for catalytic action in many Glycoside Hydrolase Families including GH51 and is consisted of residues 20–383 [37]. Two mutations Q90H and L307S are located in residues

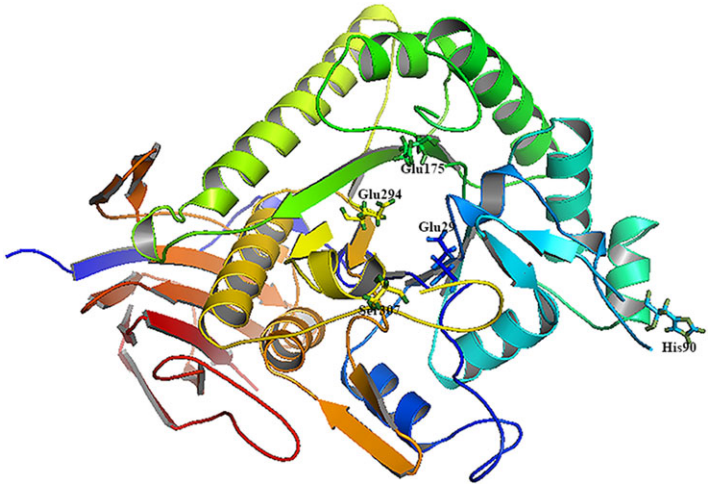

FIG. 5 *Cartoon representation of GvAbf model. The conserved catalytic residues Glu29, Glu175, and Glu294, as well as two mutations (His90 and Ser307), are shown as stick representation in the model structure.*

among TIM barrel domain. The structure of *Gv*Abf model indicates that the position of L307S mutation might be closer to the catalytic residues compared with Q90H mutation. Among catalytic residues, E294 nucleophile is the closest residue to L307S mutation. The helix structure including L307S is the bound to β-sheet structure including E294 via a loop. In this study, both single and double mutant variants of *Gv*Abf showed higher catalytic activity than *Gv*Abf and L307S mutation is conserved in both variants of *Gv*Abf. We hypothesized that L307S amino acid substitution may play a critical role in catalytic activity of *Gv*Abf (Fig. 5).

4. Conclusions

This study describes the first application of directed evolution to Abf from *Geobacillus sp.* We generated a library of 73 mutant enzymes of Abf using epPCR and selected two high activity displaying variants of the enzyme as *Gv*Abf L307S and *Gv*Abf Q90H/L307S. *Gv*Abf and two variants of *Gv*Abf were purified, partially characterized and structurally analyzed. The selection of *Gv*Abf variants and the characterization of the enzymes were performed using *p*NPA. Both *Gv*Abf variants exhibited more than twofold specific activity in optimum pH and temperature compared with *Gv*Abf. Because L307S mutation is conserved in both variants of GAbf, it is thought that L307S amino acid substitution may play a critical role in catalytic activity. Thermal stability analysis indicated that *Gv*Abf L307S and *Gv*Abf Q90H/L307S exhibit more stability at 71 °C compared with *Gv*Abf. Conversely, alkaline stability analysis showed that the variants of *Gv*Abf, especially *Gv*Abf Q90H/L307S, displayed lower stability than *Gv*Abf at pH 9.6. Based on these results, *Gv*Abf variants obtained here could be used as a template for new directed evolution rounds in order

to improve the hydrolytic properties of *Gv*Abf. Thus, *Gv*Abf could be utilized with a better efficiency in various industrial fields as an accessory enzyme.

5. Acknowledgements

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The authors declare no conflict of interest.

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