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ORIGINAL PAPER

Impact of orientation and flexibility of peptide linkers on *T. maritima* lipase Tm1350 displayed on *Bacillus subtilis* spores surface using CotB as fusion partner

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Abstract Fusion protein construction often requires peptide linkers for prolonged conformation, extended stability and enzyme activity. In this study a series of fusion between *Thermotoga maritima* lipase Tm1350 and *Bacillus subtilis* coat protein CotB, comprising of several peptide linkers, with different length, flexibility and orientations were constructed. Effects of temperature, pH and chemicals were examined, on the activity of displayed enzyme. The fusion protein with longer flexible linkers L9 [$(GGGGS)_4$] and L7 (GGGGS-GGGGS-EAAAK-EAAAK-GGGGS-GGGGS) possess 1.29 and 1.16-fold higher activity than the original, under optimum temperature and pH respectively. Moreover, spore surface displaying Tm1350 with L3 (EAAAK-GGGGS) and L9 ($(GGGGS)_4$) showed extended thermostably, maintaining 1.40 and 1.35-fold higher activity than the original respectively, at 80 °C after 5 h of incubation. The enzyme activity of linkers with different orientation, including L5, L6 and L7 was determined, where L7 maintained 1.05 and 1.27-fold higher activity than L5 and L6. Effect of 0.1% proteinase K, bromelain, 20% ethanol and 30% methanol was investigated. Linkers with appropriate Glycine residues (flexible) showed higher activity than Alanine residues (rigid). The activity of the displayed enzyme can be improved by maintaining orientation and flexibility of peptide linkers, to evaluate high activity and stability in industrial processes.

Keywords *Bacillus subtilis* · Enzyme activity · Linkers · Surface display · Tm1350

Introduction

In protein engineering research, linkers are the obvious necessity to keep domains apart and allow their movement as a part for catalytic function. The average residue length of linkers are found to be 10.0 ± 5.8, however they are subdivided to small (4.5 ± 0.7), medium (9.1 ± 2.4) and large (21.0 ± 7.6) linkers (Richard and Jaap 2003). Linkers are natural domains to maintain the necessary function, distance and enhance interaction between two protein moieties and conserve consistent linkage between fusion protein without altering their function (Karginov and Hahn 2011). The two main linker types, namely helical (separating two domains by acting as rigid spacers) and non-helical (Prolines-rich, structurally rigid and isolating linkers from attached domains) act like scaffolding, preventing unfavorable interaction of folding protein domains. Helical linkers are 51% of the total linkers and show a high propensity for proline, phenylalanine, arginine, threonine and histidine while non-helical for leucine, methionine, glutamine and aspartic acid (Richard and Jaap 2003). The linkers are ubiquitous multi-domain structures, used increasingly in the research of fusion protein construction, to control interactions between adjacent proteins domains such as display of

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passenger proteins on bacterial spores (Chen et al. 2013; Wriggers et al. 2005). *Bacillus subtillis* is among one of the non-pathogenic spore forming bacterial species, with the ability of entering to culmination and differentiation phase during nutrient deprivation (Potot et al. 2010). Bacterial spores are dormant, hard and non-reproductive structures, encased in a protective shell known as outer coat proteins (Ghosh et al. 2009; Martins et al. 2002). The three main common outer and one inner anchor proteins used for display of passenger proteins are CotB, CotC, CotG and OxdD respectively (Ning et al. 2011; Potot et al. 2010), However, some new spore coat anchor proteins CotZ, CotY (Wang et al. 2015) and CgeA (Iwanicki et al. 2014) were also identified which can be selected on the basis of relative abundance and has tolerance for relatively large passenger proteins. Displaying proteins on *B. subtillis* spore surface is becoming a fundamental tool in biotechnological research (Kim and Schumann 2009), which allow a variety of proteins and antigens on their surface. In recent years many researchers reported display of enzymes, antibodies, antigens and human albumens (Feng et al. 2013) on the surface of spore surface for industrial purposes (Chen et al. 2015b, 2016a), vaccine development (Song et al. 2012) and drug delivery (Nguyen et al. 2013). Fusion of multiple proteins is a promising technology by combining multiple proteins in one complex, used in biotechnological, biochemical and multifunctional protein production researches, for enhancing expression and activity of proteins (Yu et al. 2015). Fusion of multiple proteins with the help of peptide linkers, play an important role in maintaining the correct conformation and necessary function (Wriggers et al. 2005), to reduce steric hindrance and increase domain–domain interaction (Bhaskara et al. 2013). The nature of linkers and properties of passenger and anchor proteins play a crucial role in fusion protein stability (Hinc et al. 2013), such as CotZ as anchor allowed full length UreA, but was unsuccessful with CotB and CotC (Krzysztof et al. 2010). The risk of failure for finding functional constructs is obvious to design a fusion protein, as some of them expressed successfully but many of them are not due to poor expression and display of resulting proteins (Hinc et al. 2013). In the current study, a fusion protein complex was constructed, by inserting different linkers N- and C-terminally of lipase Tm1350 from *Thermotoga maritime* MSB8 and CotB respectively. Ten linkers with various length and orientation of amino acid were selected to construct CotB-Tm1350 fusions. The fusion constructs were displayed on *B. subtillis* DB403 spore surface. The suitability of peptide linkers and its impact over fusion proteins was investigated, by analyzing the results of different chemicals, temperature and pH.

Materials and methods

Bacterial strains, vectors, chemicals and antisera

Bacterial strains used during experimental procedures are recorded in (Table 1). DNA polymerase, restriction

Table 1 Bacterial strains, recombinant plasmid and primers

Bacterial strain, recombinant plasmid and PCR primers	Depiction	Restriction sites and references
Strain		
E. coli	DH5α	Lab stock
B. subtilis	DB403	Lab stock
Plasmid		
PHS	pLJ derived recombinant plasmid	Current study
PHS-CotB	Plasmid with CotB and pHS gene	Current study
PHS-CotB-Linkers-Tm1350	pHS-CotB-Tm1350 with various Linkers	Current study
Primers		
PHS-forward	5′-CCG**GAATTC**ACTGAGCGTCAGACC CCGTA-3′	*Eco*RI
PHS-reverse	5′-CCG**GAATTC**CTGCAGCCCGGGGGA T-3′	*Eco*RI
CotB-forward	5′-TAG**CCCGGG**ACGGATTAGGCCGTTTGTCCTCATG-3′	*Xma*I
CotB-reverse	5′-CGG**ACTAGT**GTAGGGATGATTGAT-3′	*Spe*I
Tm1350-forward	5′-CGG**ACTAGT-**Linkers0,1,2…0.10-ATGAGAATGAACATCCAGAAA CACG-3′	*Spe*I
Tm1350-reverse	5′-TGC**TCTAGA**TTATTTCCCTCCAGAT TTTTCAGAAC-3′	*Xba*I

endonucleases and T4 DNA ligase, used for ligation of desired genes, were bought from Takara Biotech (Dalian, China). The substrate *p*-nitrophenyl butyrate used for recombinant Tm1350 activity, was bought from Sigma Aldrich (USA). Other chemicals used during the study were bought from Nanjing Chemicals (China) and Shanghai Lingfeng Chemicals (China). All the chemicals used were of experimental grade. The high replica shuttle vector PHS was obtained as described previously (Chen et al. 2015a).

Recombinant plasmid construction

For the construction of recombinant plasmid, PHS-CotB-Tm1350 with various peptide linkers (Table 2), a *B. subtillis–E. coli* shuttle vector PLJ was used as a sample. A 1184-bp fragment with promotor and *cotB*-gene coding region (GenBank:CAB07789.1), was amplified through standard PCR using *B. subtillis* chromosomal DNA. The plasmid PHS and amplified cotB was digested with *Xma*I, and then ligated using T4 DNA ligase to obtain PHS-*cotB* plasmid. The gene of *tm1350* with various peptide linkers, was PCR amplified using *T. maritima* genome as template. *Spe*I and *Xba*I was used for restriction digestion, and then ligated in such a way that the linkers were positioned between the C-terminus and N-terminus of *cotB* and *tm1350* respectively, into PHS-*cotB* recombinant plasmid. The recombinant vector comprising of *cotB* and *tm1350* with various linkers was obtained, known as PHS-CotB-Lx-Tm1350. The primers used, designed for gene manipulation are listed in (Table 1).

Transformation and devising of recombinant spores

Transformation of recombinant plasmids was operated by two step (GMI, GMII) method, defined by (Nicholson and Setlow 1990). For sporulation recombinant *B. subtillis* was grown in Difco Sporulation medium for 36 h. Spores were then collected by centrifugation at 8000 rpm and treated with lysozyme to remove the residual cells for 1 h at 37 °C. Washed with 1 M KCl, NaCl and 50 mM Tris–HCl sequentially and finally resuspended in 50 mM Tris–HCl, (pH 7.5) without addition of phenylsulfonyl fluoride.

Western blotting

The correct expression of CotB-Lx-Tm1350 was confirmed, through western blot analysis. Specifically used mouse antiserum was acquired from immunized mice. The specificity of used mouse antiserum was prokaryotically determined, by expressing Tm1350 lipase as antigen. Spores were subjected to coat extraction buffer for 15 min at 65 °C, for spore coat protein extraction (Lam et al. 1998). Western blotting was accomplished according to standard protocol. Horse radish peroxidase goat anti-mouse IgG antibodies purchased from Takara (Takara, China), were habituated for immunodetection. HRP-DAB Chromogenic color substrate kit purchased from Tiangen (Tiangen, China), was applied for coloring reaction following declared protocol.

Enzyme activity assay

To determine the enzyme activity analysis with substrate *p*-nitrophenyl butyrate, optical density (OD) 405 nm was used. The reaction mixture consisted of 50 mM Tris–HCl (pH 7.5) and 2 mM of *p*-nitrophenyl butyrate. The purified spore suspension was pre-incubated and then the reaction was initiated by adding *p*-nitrophenyl butyrate. The mixture was centrifuged (up to 30 s at room temperature and 10,000×*g*) after 2 min of incubation. The reaction was stopped by keeping on ice. The activity of the recombinant enzyme was calculated by absorption of light, at 405 nm using UV 1000 Spectrophotometer (Techcomp, China).

Table 2 Peptide sequence of used linkers and biological activity

Linkers	Peptide sequence	Enzyme activity
L0	None	Yes
L1	GGGGS-GGGGS	Yes
L2	EAAAK-EAAAK	Yes
L3	EAAAK-GGGGS	Yes
L4	GGGGS-EAAAK	Yes
L5	EAAAK-EAAAK-EAAAK-GGGGS-GGGGS-GGGGS	Yes
L6	GGGGS-GGGGS-GGGGS- EAAAK-EAAAK-EAAAK	Yes
L7	GGGGS-GGGGS-EAAAK-EAAAK-GGGGS-GGGGS	Yes
L8	GGGGS- EAAAK-EAAAK-GGGGS	Yes
L9	GGGGS-GGGGS-GGGGS-GGGGS	Yes
L10	EAAAK-EAAAK-EAAAK-EAAAK	Yes

The enzyme activity for recombinant lipase was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenyl butyrate in 60 s under above mentioned conditions. Extinction coefficient for *p*-nitrophenyl butyrate (ε405) is 16,540/mol cm. The amount of spore were calculated on direct plate count method, using serial dilutions and the concentration was determined by the Bradford method.

For determination of optimum temperature, enzyme activity assay was performed, at numerous temperatures extending from 40–80 °C in 50 mM Tris–Hcl buffer (pH 7.5). To conclude the thermostability, recombinant spores were subjected to 80 °C for up to 5 h. Optimum pH was investigated at 75 °C (optimum temperature). in 50 mM Sodium citrate buffer (pH 4.0), Sodium acetate buffer (pH 4.0–6.0), Sodium phosphate buffer (pH 6.0–7.5) and Tris–Hcl pH (7.5–10.0).

Effect of different chemicals on Tm1350, displayed on recombinant spore surface was observed in 0.1% proteinase K, 0.1% Bromelain, 20% ethanol and 30% methanol. Recombinant spores were incubated with all the four chemicals, for 1 h at 37 °C and then relative enzyme activity was measured by addition of pre-heated *p*-nitrophenyl butyrate, at a final concentration of 2 mM, keeping the respective activity as 100%. To eliminate the circumstantial hydrolysis at various conditions, the subsequent controls were applied. (1) Reaction conditions and components of the corresponding reaction were kept same: (2) non-recombinant *B. subtillis* (DB403) spores were used instead of recombinant spores: (3) isopycnic buffer was used without spores. The data was presented as the mean ± deviation.

Results

Construction of recombinant plasmid with different linkers

For the construction of recombinant plasmids PHS-CotB-Lx-Tm1350 a *B. Subtillis–E. coli* shuttle vector PLJ was used as a sample (Fig. 1). Each required gene for recombinant plasmid was amplified through standard PCR using precisely designed primers, restriction endonucleases were used to cut it on desired sites, and then ligated with DNA ligase. Different peptide linkers with various length and orientation were positioned between the C- and N-terminus of CotB and Tm1350 respectively, for the facilitated movements of essential domains and prevent the disturbance in domain function. Each plasmid was sequenced to ensure the correct construction of recombinant plasmid and unintended mutation.

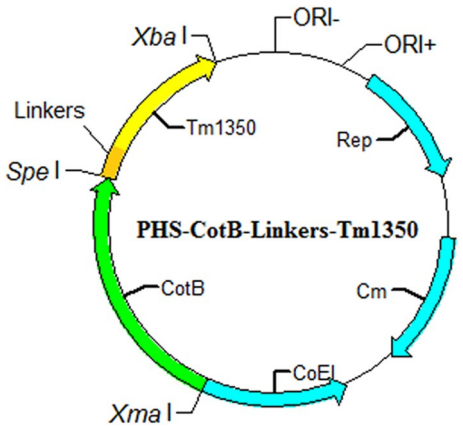

Fig. 1 Construction of recombinant plasmid PHS-CotB-Linkes-Tm1350. ORI− and ORI+ represents single and double strand origin of replication. CoEI represents *E. Coli* replicon. *Cm* chloramphenicol resistant gene, *XmaI, SpeI and XbaI* site for restriction digestions. CotB and encode *B. Subtillis* spores cote protein and Tm1350 lipase from *T. maritima*

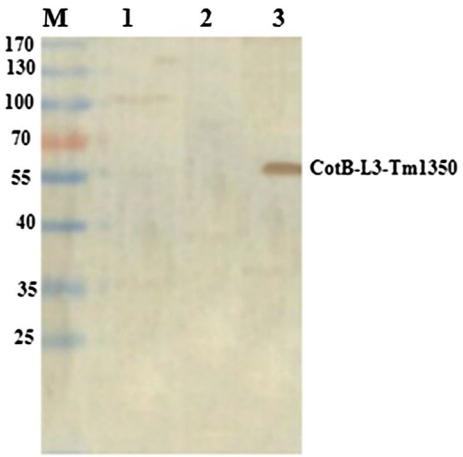

Fig. 2 Western blot analysis of CotB-L3-Tm1350 gene expression. Spore coat protein was extracted using decoating buffer and exposed to SDS-PAGE following western blotting using standard protocol. Where *M* protein marker, *1* non-recombinant *Bacillus subtilis* spores, *b* loading buffer as control, *3* CotB-L3-Tm1350

Expression of CotB-Tm1350 on recombinant spore surface via western blotting

To confirm the correct fusion of CotB-Lx-Tm1350 (with different linkers), on *B. subtillis* spores surface, Western blotting was performed using specific mouse antiserum. Expression of displayed CotB-L3-Tm1350 on spore surface was affirmed by detecting a band near 70 kDa in recombinant spores extracts (Fig. 2). The estimated molecular weight calculated for CotB-Tm1350 was 66 kDa. The

result suggests successful expression of CotB-L3-Tm1350 on the spore surface.

Confirmation of CotB-Tm1350 displayed on spore surface using different enzymes and solvents

To further confirm the correct expression of CotB-Tm1350 on spore surface, the effect of different enzymes and solvents, proteinase K, bromelain, ethanol and methanol were checked, by using the substrate *p*-nitrophenyl butyrate. The activity of recombinant spores was reduced to (L0) 30.09%, (L1) 37.92%, (L2) 38.94%, (L3) 29.59%, (L4) 35.65%, (L5) 38.15%, (L6) 32.05%, (L7) 40.61%, (L8) 35.44%, (L9) 37.34% and (L10) 39.73% (Fig. 3A), where L7, L2, L10, L5 and L1 are highly significant by means of statistics respectively, and the most suitable linkers to enhance Tm1350 stability, in the presence of 0.1% Proteinase K, followed by L9, L4, L8, L6, L0 and L3.

The relative activity of spores was calculated after treatment with 0.1% Bromelain. In the presence of 0.1% Bromelain the recombinant spores, with L3, L1 and L2 showed maximum activity, and are statistically highly significant, among all the linkers and without linker which is 70.05, 67.15 and 65.91% respectively (Fig. 3B). Based on statistical significance the activity of other linkers are as follows (L5) 61.73%, (L4) 57.99%, (L9) 55.56%, (L10) 54.75%, (L7) 52.15%, (L6) 48.51%, (L0) 42.71% and (L8) 40.87.

To evaluate the influence of Ethanol on spore surface displayed CotB-Tm1350 with various peptide linkers, 20% Ethanol treatment was used. Spore surfaced displayed

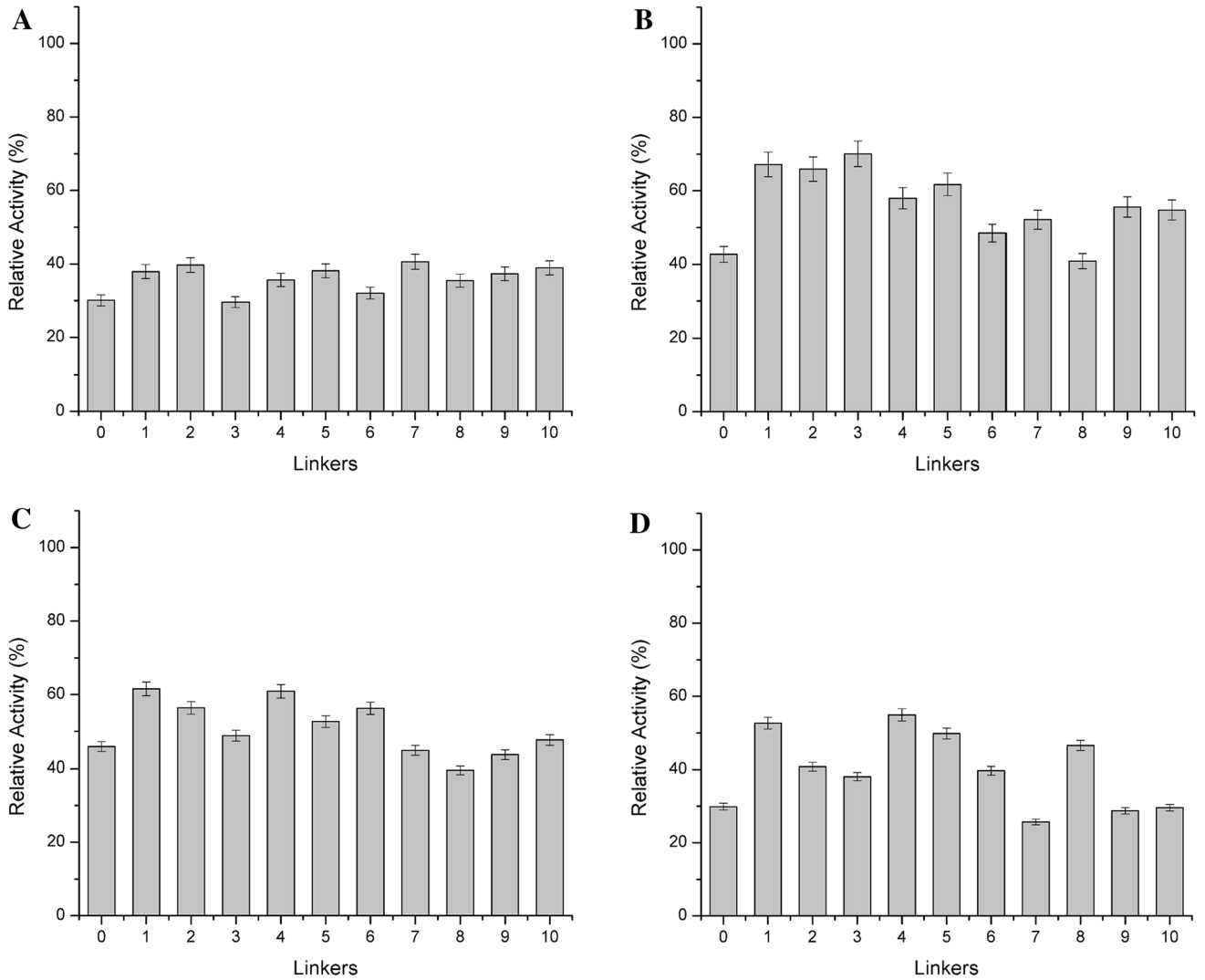

Fig. 3 Effect of different enzymes and solvents **A** 0.1% Proteinase K, **B** 0.1% bromelain, **C** 20% ethanol and **D** 30% methanol on spore surface displayed Tm1350 with various linkers was investigated. Recombinant spore were incubated for 1 h at 37 °C with enzymes and solvents and the activity was determined by adding *p*-nitrophenyl butyrate as substrate at a concentration of 2 mM keeping the original (untreated) activity as 100%. The data were presented as mean of three experiments ± deviation

CotB-Tm1350, with L1 and L4 are statistically highly significant linker followed by L2 and L6, and have the ability to enhance Tm1350 stability, by maintaining 61.57, 60.90% and (L2) 56.39%, (L6) 56.31% of activity respectively. The activity of spore surface displayed with other linkers are as follows (L5) 52.65%, (L3) 48.85%, (L10) 47.65%, (L0) 45.88%, (L7) 44.84%, (L9) 43.66% and (L8) 39.56% (Fig. 3C).

Effect of 30% methanol was investigated on recombinant spores. Where L4 is statistically highly significant linker followed by L1, and showed 54.93, 52.71% of activity respectively. The activity of other linkers are (L5) 49.86%, (L8) 46.59%, (L2) 40.79%, (L6) 39.70%, (L3) 38.06%, (L0) 29.98%, (L10) 29.61%, (L9) 28.71%, and (L7) 25.71% (Fig. 3D). All the treatments were performed at 37 °C for 1 h and calculate the relative activity, by keeping the original (untreated) activity as 100%.

Optimum temperature and thermostabilty of recombinant spores

To detrmine the optimum temperature of CotB-Tm1350 with various linkers, displayed on spore surface, the recombinant spores were assayed, at various temperatures ranging from 40–80 °C in Tris–HCl (pH 7.5). Recombinant spores showed maximum activity at 75 °C (Fig. 4A), consistent with *T. maritima* optimal growth temperature (75–80 °C), and calculated it as 100% activity. The relative activity calculated at optimum temperature was presented in (Fig. 4A-S). Where the activity of displayed Tm1350 with linker, presenting maximum activity was taken as 100%.

For concluding the thermostability of recombinant spores, the activity was determined at 80 °C, by increasing incubation time up-to 5 h. The residual activity of L3 and L9 calculated, after 5 h of incubation time was the highest, among all linkers and are highly significant linkers by means of statistics. The activity of L3 and L9 was reduced to 39.66 and 38.41%, of its original activity (Fig. 4B). The activity of other linkers, displayed on *B. subtillis* spore surface, after 5 h of incubation are as follow, L0 (28.41%), L1 (35.66%), L2 (32.43), L4 (33.42%), L5 (34.11%), L6 (32.48%) L7 (33.33%), L8 (34.25%), L10 (30.25%) (Fig. 4B-S).

Effect of pH on recombinant spores

The optimum pH of CotB-Tm1350 with various linkers, displayed on the spores surface was determined in various buffers, sodium citrate (pH 4.0), sodium acetate (pH 4.0–6.0), sodium phosphate (pH 6.0–7.5) and Tris–HCl (pH 7.5–10.0). L8 and L10 showed maximal enzyme activity at (pH 8.5), L0, L5, L6 and L9 at (pH 9.0) while L1, L2, L3, L4 and L7 showed max activity at (pH 9.5) (Fig. 5). The spore surface displayed CotB-Tm1350 with all the linkers, showed

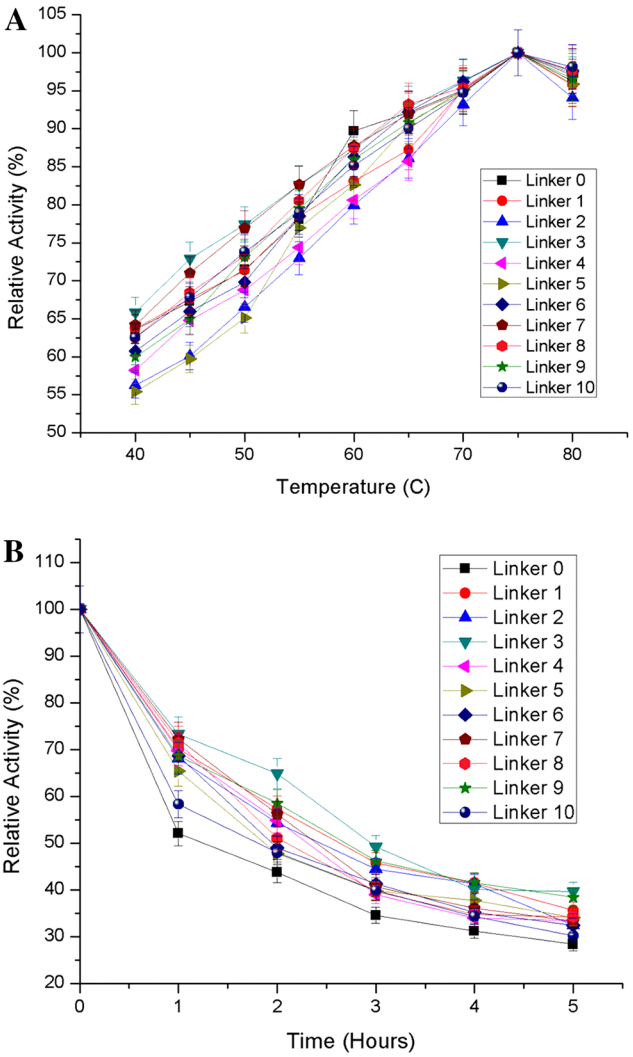

Fig. 4 **A** Optimum temperature. Optimum temperature of spore surface displayed Tm1350 with various linkers was checked in 50 mM Tris–Hcl (pH 7.5) form 40–80 °C using *p*-nitrophenyl butyrate as substrate at a concentration of 2 mM. **B** Thermo-stability. Thermo-stability was calculated after incubation for 5 h at 80 °C, keeping the original activity as 100%. The data were presented as mean of three experiments

maximal activity in the range from pH 8.5–9.5, which indicate that the displayed enzyme is alkali-stable and able to maintain maximum activity in alkaline environment. The relative activity calculated at optimum pH was presented in (Fig. 5-S).

Discussion

Fusing biologically active protein is an indispensable technology, by genetic fusion of two or more protein domains together, to get high expression and biological yields, in protein purification research (Terpe 2003), bio-pharmaceuticals

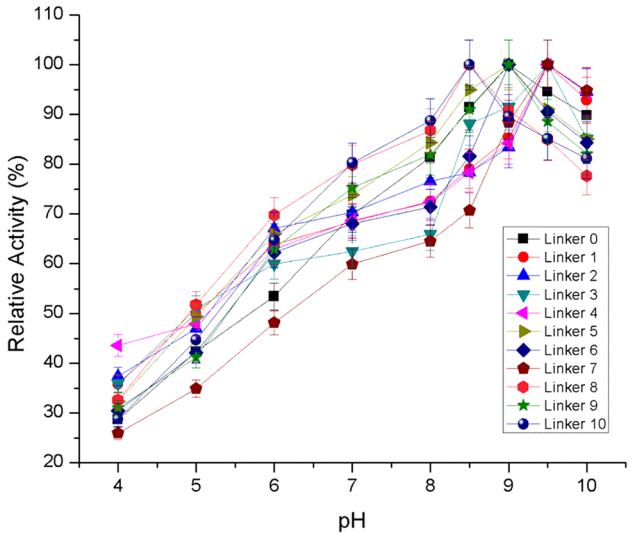

Fig. 5 Optimum pH. Optimum pH of spore surface displayed Tm1350 with various Linkers was measured at 75 °C (optimum temperature) in sodium citrate (pH 4.0), sodium acetate (pH 4.0–6.0), sodium phosphate (pH 6.0–7.5) and Tris–Hcl (pH 7.5–10.0). The data were presented as the mean of three experiments

(Stefan 2009) and bio-imaging (Rafeal 2005). Linkers are essential components for construction of fusion proteins, to achieve high expression, required pharmacokinetics and improved biological activity (Chen et al. 2013). The current study was conducted by constructing 11 recombinant plasmids, with 10 peptide linkers and one without linker, to analyze its impact over displayed enzyme Tm1350, using CotB as a fusion partner. The impact of 10 peptide linkers was investigated, according to their length, degree of flexibility and orientation of amino acid sequence. To maintain a certain distance, hydrolysis and flexibility, a specific inter domain positioning is required for the movement of domains interaction, and to avoid disturbance in domain function because linkers of very large or small length are detrimental to catalysis (Bhaskara et al. 2013; Ruiz et al. 2016). Linker length can also affect crystal packing in crystallography and help to reveal flexible regions of fusion proteins (Vastermark et al. 2017). In our previous study, the optimum temperature for spore surface-displayed Tm1350, with a short flexible linker GGGGS was 75–80 °C, which is consistent with the optimum temperature of *T. maritima*. In this study, the optimum temperature for all the fusion proteins, with various linkers of different length, degree of flexibility and orientations, were almost same, and showed the maximal activity at 75 °C, which demonstrates that the rigidity, flexibility, length and orientation of linkers have no impact on optimal temperature activity of displayed lipase Tm1350, but have a relative impact on the enzyme activity and optimum pH of displayed enzyme. The displayed lipase Tm1350 had maximum activity, with L8 and L10 at pH 8.5, L0, L5, L6, L9 at pH 9.5 and L1, L2, L3, L4, L7 at pH 9.5, which conclude that it might be attributed to some structural or charge changes (Chen et al. 2017), because some linkers contains ionizable groups such as lysine (K) and glutamic acid (E). The pK values of ionizing and non-ionizing amino acids varies. The number of hydrogen ions in the linkers are different, and the increase and decrease in pH depends upon hydrogen ions. The linkers with more flexible residues present a certain effect on the enzyme activity of displayed enzyme, as surface-displayed Tm1350 with L9 and L7 contain more flexible residues and present maximal activity in the relevant conditions, by controlling the distance between functional domains. The following results validate that flexibility and orientation of linkers, have more impact over displayed Tm1350 than length, because L9 is smaller in length and possess 1.12-fold higher activity than L7. Furthermore, L7, L6 and L5 have an equal amount of amino acid residues, but with different orientation and composition. Therefore, L7 showed 1.05 and 1.27-fold higher activity than L6 and L5. Further analysis showed that the length of L9 and L7 is different, with equal amount of glycine residues but higher than the other linkers. This show that glycine residues are the key amino acid to get higher enzyme activity (flexibility), of spore surface-displayed Tm1350. For thermostability, based on statistical significance, our results suggests that spore surface displayed with L3 and L9 are highly significant and showed extended thermostability by maintaining high activity, at 80 °C after 5 h of incubation, which may be due to lack of β-carbon and preference of glycine residues to communicate with dihedral angles (Saravanan and Krishnaswamy 2014). Moreover, in both L3 and L9, the glycine residues are attached to the C-terminus of CotB in the fusion protein, and it is the presence of glycine residue on the C-terminus which provides some specific movement to the fusion partner (Chen et al. 2016b). A previous study (Chen et al. 2017) reported, that spore surface-displayed nitrilase with GGGGS-EAAAK possess high thermal stability, which demonstrate that the presence of glycine residues on the C-terminus, may enhance the thermostability of displayed enzyme. According to our results, longer flexible linkers can provide more stability to the displayed enzymes than short and rigid linkers, as the displayed Tm1350 with longer flexible linker L9 possess higher activity than L1, but possessed low activity with long rigid linker L10 than shorter L2. Our data suggests that flexible linkers can lead Tm1350 to higher thermostability, by relatively maintaining the desired distance, fine tune flexibility, folding and stiffness between the domains.

Effect of different enzymes and solvents on the displayed enzyme was investigated. In the existence of 0.1% Proteinase K, L7, L2, L10, L5 and L1 are highly significant linkers, by enhancing enzyme stability than original activity respectively. Moreover, L6, L0 and L3 are

non-significant to L7 and are the least suitable linkers. The activity of L3, L1 and L2 was reduced to 70.05, 67.15 and 65.91%, after incubation with 0.1% Bromelain, and are the most significant linkers to enhance Tm1350 activity. Moreover L0 and L8 are the statistically least suitable linkers. After amplification of 20% ethanol, the activity of L1 and L4 was reduced to 61.57 and 60.90% respectively, which is 1.34 and 1.32-fold higher than the original activity, and are the most significant linkers, followed by L2 and L6, which possess 56.39 and 56.31% of activity respectively. In the existence of 30% methanol, L4 was the most significant linker and showed 54.93% of activity, which is 1.83-fold higher than the original activity, followed by L1, which possesses an activity of 52.71%. Moreover, L0, L10, L9 and L7 are the least suitable linkers by means of statistics. Hydrophilic solvent has the tendency to remove protein bound water (Serdakowski and Dordick 2008), which is necessary for protein function, to promote lubrication, necessary for optimal catalysis. On the other hand, excess amount of protein bound water also lead to enzyme denaturation, due to high conformation mobility (Alexander 2001). So the linkers with sufficient hydrophilic amino acid residues and length, will be able to stabilize enzymatic activity, under hydrophilic solvents. Our results infer that Tm1350 with linkers having low hydrophilic residues has more stability to hydrophobic solvents.

In conclusion, our results suggests that orientation of amino acid, flexibility/rigidity and length of peptide linkers, have a robust impact over spore surface displayed Tm1350, to improve enzyme activity, expression and stability. The linkers with glycine residues, are more stable to temperature, pH, enzyme and solvents than α-helical residues, and have the ability to achieve higher activity for spore surface-displayed enzymes. Alanine affects the folding and serine affects the unfolding rates in peptide linkers. Therefore, it need to maintain the length and composition of glycine, serine and alanine in peptide linkers, for attaining desired flexibility, high expression yield and capability, to survive in harsh biochemical processes for industrial applications.

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Compliance with ethical standards

Conflict of interest All the authors approved the manuscript and have no conflict of interest.

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