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ORIGINAL PAPER

In silico characterization of thermostable lipases

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Abstract Thermostable lipases are of high priority for industrial applications as they are endowed with the capability of carrying out diversified reactions at elevated temperatures. Extremophiles are their potential source. Sequence and structure annotation of thermostable lipases can elucidate evolution of lipases from their mesophilic counterparts with enhanced thermostability hence better industrial potential. Sequence analysis highlighted the conserved residues in bacterial and fungal thermostable lipases. Higher frequency of AXXXA motif and poly Ala residues in lid domain of thermostable *Bacillus* lipases were distinguishing characteristics. Comparison of amino acid composition among thermostable and mesostable lipases brought into light the role of neutral, charged and aromatic amino acid residues in enhancement of thermostability. Structural annotation of thermostable lipases with that of mesostable lipases revealed some striking features which are increment of gamma turns in thermostable lipases; being first time reported in our paper, longer beta strands, lesser beta-branched residues in helices, increase in charged-neutral hydrogen bonding pair, hydrophobic-hydrophobic contact and differences in the N-cap and C-cap residues of the α helices. Conclusively, it can be stated that subtle changes in the arrangement of amino acid residues in the tertiary structure of lipases contributes to enhanced thermostability.

Keywords Thermostable lipases · Mesostable lipases · Motif · Conserved domains · Sub family · Tertiary structure

Abbreviations

MSA	Multiple sequence alignment
TBFL	Thermostable bacterial and fungal lipases
ML	Mesostable lipases
TBL	Thermostable bacterial lipases
TFL	Thermostable fungal lipases

Introduction

Lipases are known as glycerol ester or serine hydrolases and true lipases (E.C 3.1.1.3) as triacylglycerol ester hydrolases and are widely distributed among the five kingdoms of life. The basic catalytic activity of lipases being hydrolysis of triacylglycerols to free fatty acids, diacylglycerols, monoacylglycerol and glycerol. Structurally lipases have a canonical alpha/beta hydrolase fold (Schrag and Cygler 1997) comprising of eight parallel beta strands, second strand being anti-parallel and strands 3–8 are connected by alpha helices covering either side of the beta sheet (Cherukuvada et al. 2005). The active site is composed of the catalytic triad; Aspartate or Glutamate, Serine and Histidine residues. The active site Serine lies in the nucleophilic catalytic elbow and sequentially within the conserved pentapeptide GXSXG motif.

Lipases especially thermostable lipases from microbial origin are of high commercial interest as they are resistant

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to chemical denaturation (Lee et al. 1999), stable in organic solvents, do not involve cofactors, have broad substrate specificity and are highly enantioselective. Most importantly, they are cost effective and can be used in industries such as food processing, detergent (whose annual turnover being 1,000 tons of lipases for 13 billion tons of detergents) (Jaeger and Reetz 1998), organic synthesis, biopolymer synthesis, biodiesel production, pulp and oleochemical industries (Li et al. 2005; Haki and Rakshit 2003). Two well known commercially available thermostable lipases are from *Candida antarctica* (Novo Nordisk; Denmark, and Boehringer Mannheim) and *Burkholderia cepacia* (Amano, Fluka, and Boehringer Mannheim) application being organic synthesis and in detergent industries (Gunasekaran and Das 2005).

Thus to satisfy the global requirement of the industrial process, we should have thermostable lipases. In general lipases that are stable above 40°C are required for industrial applications (Wang et al. 1995). Thermophilic microorganisms are potential and alternative source of thermostable lipases (Brock 1985). However, it is often impractical to use them directly due to low yield of lipase. Alternatively, a direct approach could be screening of microorganisms for thermostable lipases and their sequence and structure analysis with comparison to that of mesostable counterparts with high expression level of lipases as this can pave the path for in vitro evolution from the mesophilic organisms with enhanced thermostability.

Significant research had been carried out in determination of thermostabilization factors for protein. Yano and Poulos 2003 and Trivedi et al. 2006, had carried out a detailed review for the same. Some of the common reported factors responsible for thermostability of proteins were increased in hydrophobicity of proteins (Haney et al. 1997; Sadeghi et al. 2006), better compactness of protein structure (Russell et al. 1997), increased rigidity due to Proline residues (Bogin et al. 1998), decreased occurrence of thermolabile residues (Russell et al. 1997), increment of hydrogen bonding (Vogt and Argos 1997; Gromiha 2001; Sadeghi et al. 2006) and salt bridges (Kumar et al. 2000; Sadeghi et al. 2006). It was also reported that protein thermo stabilize mainly by increment of ion pairs (Szilagyi and Zavodsky 2000). Gromiha et al. (1999) conclusively stated that a balance between better packing and solubility increases thermal stability. Grimsley et al. 1999 stressed that improving the coulombic interactions among charged groups on the protein surface can increase stability. Among other strategies analyzed Gromiha et al. 2002 and Chakravarty and Varadarajan 2002, stated that charge-to-charge and cation-Pi interactions among long chains of Tyrosine with Lysine in thermophiles are important contributors to thermal stability. Researchers also reported outer surface amino acid composition of folded protein, are important for the thermal stability (Argos et al. 1979; Fukuchi et al. 2003). To the contrary Ding et al. 2004 studied the influence of dipeptide composition on protein thermostability and found it to be larger than the influence of amino acid composition. Kannan and Vishveshwara (2000) gave a new insight to thermostability stating that large aromatic clusters of amino acids are responsible for the same. Another noteworthy study stated that the hydrophobic free energy plays pivotal role in conferring thermostability (Saraboji et al. 2000). It has been stated that in general, thermostable proteins exhibit rigidity that is lack of flexibility and thereby have a tendency to remain in the native folded state (Tyndall et al. 2002). However, no significant work has been carried out on determination of factors responsible for thermostability in sequence and structure level of lipases. Concentrating on lipases by comparing thermostable *Bacillus stearothermophilus* P1 and mesostable *Chromobacterium viscosum* lipases it was reported that ligand stabilization, salt bridges, aromatic clusters and Proline residues are responsible for its thermostability (Tyndall et al. 2002). However, they concluded that combinations of factors contribute to the thermostability of lipases as in other thermostable proteins.

In this paper, we intend an in silico study for unraveling additional factors leading to better thermostabilization of lipases correlating all the thermostabilization factors at sequence and structure level, laying special emphasis on the role of tertiary structure and distribution of amino acids in thermostabilization of lipases and to bring out possible ways that will assist in protein engineering of mesostable lipases (ML) to render them thermostable.

Methods

Sequence collection and characterization

Twenty-three thermostable lipase sequences were collected from UNIPROT Knowledgebase release 15.12. Respective families were assigned to the lipases through BLAST search in Lipase Engineering Database (LED) (Jurgen et al. 2000) (Table 1).

Seven well-characterized mesostable true lipase sequences were chosen for comparison with their thermostable counterparts. The lipases that were chosen were from the following organisms: *Rhizopus oryzae* (ROL; P61872; 1TIC; abH23.01), *Pseudomonas* sp. B-11 (PLB; O52270, abH04.01), *Bacillus sphaericus* 205y (BSL205y; Q8VQP2, abH04.04), *Staphylococcus epidermis* (SEL; Q9Z4M7; abH15.01), *Rhizomucor meihei* (RML; P19515; 4TGL; abH23.01), *Bacillus pumilus* (BPL; B8Y3H3; abH18.01), *Chromobacterium viscosum* (CVL; Q05489; 1CVL; abH15.02) and *Bacillus subtilis* (BSL; P37957; 1I6 W; abH18.01).

Table 1 List of organisms producing thermostable lipases with their properties

Sl. no.	Source	Lipases/(abbreviations)	Temperature stability/optimum range(°C)	pH stability range	Family (LED classification)	Accession No./PDB ID	Reference
1	Bacteria	*Bacillus stearothermophilus* P1 (BSLP)	30–65	8.0–9.0	abH15.1	Q9L6D3/1JI3	Joel et al. (2002)
2	Bacteria	*Bacillus thermocatenulatus* (BTL)	60–80	8.0–9.0	abH15.1	Q59260/2W22	Quyen et al. (2003)
3	Bacteria	*Geobacillus sp.* TW1 (GLTW1)	60–70	5.0–10.0	abH15.1	Q3T5M2/Nil	Li et al. (2005)
4	Bacteria	*Geobacillus thermoleovorans* YN (GThLYN)	60–70	5.0–10.0	abH15.1	Q2PWL8/Nil	Soliman et al. (2007)
5	Bacteria	*Bacillus stearothermophilus* L1 (BSLL)	60–65	9.0–10.0	abH15.1	O66015/IKU0	Ahn et al. (2004)
6	Bacteria	*Bacillus licheniformis* (BLL)	55–70	9.0–11.0	abH18.1	Q9K5F4/Nil	Horani (2004)
7	Bacteria	*Bacillus strain* 42 (BL42)	70–80	7.0–9.0	abH15.1	Q5U780/Nil	Eltaweel et al. (2005)
8	Bacteria	*Bacillus thermoleovorans lipase* ID-1 (BThLID)	75	7.5	abH15.1	Q9X6A4/Nil	Rathi et al. (2000)
9	Bacteria	*Geobacillus thermoleovorans* IHI-91 (GThLIH1)	65	6.0	abH15.1	Q9X6A4/Nil	Soliman et al. (2007)
10	Bacteria	*Geobacillus zalihae strain* T1(GZLT)	65	9.0	abH15.1	Q842J9/Nil	Leow et al. (2004)
11	Bacteria	*Staphylococcus xylosus* (SXL)	45–60	5.0–8.0	abH15.1	Q9F0Q9/Nil	Horchani et al. (2008)
12	Bacteria	*Staphylococcus aureus* (SAL3)	55	5.0–12.0	abH15.1	B4XGS3/Nil	Horchani et al. (2008)
13	Fungi	*Aspergillus terreus* (ATL)	15–90	4.0–10.0	abH23.1	Q0C912/Nil	Yadav et al. (1998)
14	Fungi	*Aspergillus niger* F044 (ANL)	45–60	7.0	abH23.1	B8YIE6/Nil	Yadav et al. (1998)
15	Bacteria	*Pseudomonas cepacia* (PCL)	60	5.0–7.0	abH15.2	P22088/3LIP	Sugihara et al. (1992)
16	Bacteria	*Pseudomonas sp.*KW1-56 (PLKW1)	60	7.0	abH15.2	P26504/Nil	Iizumi et al. (1990)
17	Fungi	*Candida antarctica* Lipase A (CALA)	>90	7.0	abH18.1	UPI000181D065 UniPrac/2VEO	Maria et al. (2005)
18	Fungi	*Thermomyces lanuginose* (TLL)	45	5.0–9.0	abH23.1	UPI000002F7C4/UniPrac/1TIB	Hayashi and Nagai (1987)
19	Fungi	*Rhizopus chinensis* (RCL)	30–50	6.0–9.0	abH23.1	A3FM73/Nil	Sun et al. (2009)
20	Bacteria	*Pseudomonas fluorescens* SIK W1 (PFLSIK)	60	7.0	abH24.1	Q9ZG91/Nil	Chung et al. (1991)
21	Fungi	*Candida Antarctica* Lipase B (CALB)	30–40	7.0	abH37.01	P41365/1TCA	Suen et al. (2004)
22	Bacteria	*Thermoanaerobacter thermohydrosulfuricus* (TTL)	75–90	8.0	abH27.01	UPI0000E45560 UniPrac/Nil	Royter et al. (2009)
23	Bacteria	Thermostable *Bacillus subtilis* lipase (TBSL)	65	10	abH18.01	P37957/3D2C	Ahmad et al. (2008)

Multiple sequence alignment of thermostable and mesostable lipases

Through multiple sequence alignment (MSA), we intend to bring out the possible amino acid residues conserved in thermostable lipases and absent in mesostable ones which may be responsible for their enhanced thermostability. Thus, thorough analysis of thermostable and mesostable lipases was carried out by MSA using Parallel PRRN; progressive (amino acid content) with iterative refinement, with default parameters (http://align.genome.jp/prrn/) using PAM 250 matrix due to the extensive differences in the length of the lipase sequences ranging from 181 to 453 amino acid residues.

Study of percentage amino acid composition of thermostable and mesostable lipases

A detailed comparison of thermostable and mesostable lipases were carried out with respect to the percentage amino acid composition of the sequences as this would shed light on the quantitative estimation of the 20 different amino acid residue composition of thermostable lipases in comparison to the mesostable lipases. This was performed using the webserver COPid which is composition based protein identification (Kumar et al. 2008) and can be accessed through http://www.imtech.res.in/raghava/copid/index.html.

Structural characterization by tree-based annotation of thermostable and mesostable lipases

To get a complete picture on the thermostabilizing factors of lipases, sequence analysis was inadequate, thus structural comparison was performed using the available PDB structures (eight for thermostable and four for mesostable lipases) (Table 1) by tree-based classification. The other 18 lipase sequence collected, unfortunately lack crystal structures in PDB till date.

Tree-based annotation was performed of the thermostable–mesostable lipase pairs for structural comparison as large differences were encountered in the length of the lipase sequences, altered alignment was observed in MSA for important lipase motifs with respect to bacterial and fungal lipases and poor secondary structure alignment was noticed for many thermostable–mesostable lipase pairs belonging to different classes as classified by LED. Moreover, PDB structures gave huge RMSD deviations as assigned by CE Calculate (Shindyalov and Bourne 1998) though majority of the thermostable lipases belonged to LED family abH15.1. Due to all the aforesaid we concluded that structural comparison of very divergent structures like that of fungal and bacterial thermostable–mesostable pairs would not solve the purpose of figuring out the possible minute structural differences which can lead to thermostability but comparison of very similar structures by classification of lipases into subfamilies would yield better results. So we tried classifying the thermostable and mesostable lipases into individual subfamilies for simplification of the task of structural annotation using SCI-PHY (Brown et al. 2007), PIRSF (Wu et al. 2004), SECATOR (Wicker et al. 2001) and CLUSS (Kelil et al. 2007). We used the latest CLUSS 2 version 1.0 (IJCBDD 2008) which can be accessed through http://prospectus.usherbrooke.ca/CLUSS/Server/Index.html. The criteria to be fulfilled for classification of thermostable and mesostable lipases was possible using only CLUSS because it utilizes nonaligned protein sequences as its input, however, the other classification programs utilized for the same purpose required MSA as their input for classification. This took care of the differences in length of the sequences leading to poor multiple alignments. Moreover, CLUSS having high sensitivity towards very similar and divergent sequences solved our problem of classifying the thermostable and mesostable lipases which show both properties of high similarity and divergence among themselves. From the resultant subfamilies, representatives for mesostable and thermostable lipases were chosen for further comparison on the basis of their least RMSD deviation from the rest of the family members and the availability of their PDB structures.

Structural analysis

To reach to a consummate conclusion of the structural factors leading to the thermostability of lipases and to further support our results, the different structural features of the representatives of each subfamily were compared by structural superimposition performed using PyMol v0.99 (DeLano 2002) and structural superimposition was performed using PyMol v0.99 (DeLano 2002). Adding to this qualitative and quantitative assessment of the representative lipase structures were performed using web server VADAR (Volume, Area, Dihedral Angle Reporter) (Willard et al. 2003) which can be accessed at http://redpoll.pharmacy.ualberta.ca/vadar/ and PDBsum database (http://www.ebi.ac.uk/pdbsum/).

Study of structurally important residues of thermostable–mesostable lipases

This study was intended to find out the most structurally important residues of thermostable–mesostable lipase pairs of subfamilies, lipases having greater than 80% sequence similarity were chosen because comparison of lipase structure having less than 80% sequence similarity yielded false positive results. SRide server (Gromiha et al. 2004;

Magyar et al. 2005) was employed to obtain information on structurally important residues which can be held responsible for thermostability.

Additionally, HotSpot Wizard 1.4 (Pavelka et al. 2009), which represent an easy way to perform several structural and evolutionary analyses (can be accessed at http://loschmidt.chemi.muni.cz/hotspotwizard/index.jsp) was employed for the purpose. Moreover, the mutational effect of the structurally important residues were checked with the CUPSAT server (Parthiban et al. 2006) which predicts protein stability changes upon point mutations.

Results

Sequence characterization

Multiple sequence alignment of thermostable and mesostable lipases have been presented in Supplementary Figure S1. MSA revealed some interesting facts about the active site, oxyanion hole and lid domain of thermostable lipases which could be contributory to temperature stability. The various conserved motifs observed after performing MSA of the lipases have been represented in Table 2.

Active site residues

In context to the active site pentapeptide sequence of lipases, we noticed that all *Bacillus* lipases from family I.4 and I.5 (Arpigny and jaeger 1999) whether thermostable or mesostable possess AXSXG motif questioning the role of Ala in thermostability as reported earlier for *Bacillus* thermoalkalophilic lipases (Jeong et al. 2002). Another interesting fact observed was that there are more titratable amino acid residues near the active site Ser of thermostable lipases. Moreover, we observed that mesostable lipases from *S. epidermis* and *Bacillus* (family I.4) lack Cys residues like that of thermostable *Thermoanaerobacter thermohydrosulfuricus* lipase (Royter et al. 2009).

Oxyanion hole

MSA revealed that the oxyanion hole consensus for lipases that was reported as [PNTSVL]-[VIF]-[VIFL]-[VIFLM]-[VLCAISQ]-H-G (Philip et al. 2002) whether thermostable or mesostable was highly conserved. No differences could be attributed to the sequence pattern to account for thermostability in relation to the oxyanion hole of lipases. However, all the thermostable lipases studied had the GX class of the oxyanion hole signature and unlike some mesostable *Bacillus* lipases never fell into the GGGX class. GX and GGGX class had been attributed the status of oxyanion hole class by the LED (Jurgen et al. 2000).

The lid of lipases

It was observed through MSA that in the lid of thermostable lipases *Staphylococcus* lipases lid domain possess less positively charged residues than mesostable *S. epidermis* lipase. Moreover, the presence of Trp residue in the lid domain of *Aspergillus terrus, Aspergillus niger, Rhizomucor meihei* and *Staphylococcus* lipases ensures the fact that not only thermostable but mesostable lipases also possess this Trp residue unlike the Trp89 in the lid of thermostable *Thermomyces lanuginosa* lipase (Zhu et al. 2001). By our analysis, Tyr224 cannot be strongly promoted to play a crucial role in thermostabilization of *Bacillus* lipases as given in a previous report (Wu et al. 2009) due to its presence in *Bacillus* thermoalkalophilic, thermostable *Staphylococcus* lipases along with mesostable *S. epidermis* lipase. Moreover, we found Val or Ala and Pro residues at comparable positions in thermoalkalophilic *Bacillus* lipases (Table 2) like Val137 and Asn138 in thermostable *Pseudomonas cepacia* lipase (Santarossa et al. 2005). Another notable fact from MSA was that Bacillus thermostable lipases have poly Ala in the form of di- or tripeptide in their lid domain.

Ion binding

It is well known that lipases coordinate with Ca^{2+} and Zn^{2+} ion for their structural stability (Tyndall et al. 2002; Invernizzi et al. 2009). The Zn^{2+} binding signature thus by comparison of the multiple sequence alignment can be assigned as GAAHAAKX where X can be either Tyr, Asp, or His residues and is present in thermoalkalophilic *Bacillus* lipases of family I.5, thermostable and mesostable *Staphylococcus* lipase but absent in thermostable *Bacillus* lipases of family I.4. Unlike Zn^{2+} binding residues Ca^{2+} binding residues (Figure S1) were conserved in all the lipase sequences whether thermostable or mesostable.

P-loop motif

P-loop like motif was observed in all *Bacillus* thermoalkalophilic lipases in line with the Zn^{2+} binding residues with the concensus [AG]XXXXGRT as reported in *Staphylococcus* lipases (Rosenstein and Götz 2000). The only difference being Arg replacing Lys (Figure S1). However, like mesostable bacterial lipases, thermostable minimum α/β hydrolase lipases of family I.4 and fungal lipases whether thermostable or mesostable lacked this motif due to the absence of Zn^{2+} coordinating residues in their sequences.

The AXXXA and GXXXG motifs

By manual counting of the 30 thermostable and mesostable lipase sequences for the AXXXA and GXXXG motifs

Table 2 Signature sequences of lipases showing conserved residues

Lipases	GXSXG motif residues	Oxyanion hole residues	Lid domain residues	P-loop motif	Zinc binding domain	No. GxxxG	No. AxxxA
BSLP1	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNV	GHKRYGRT	GAAHAAKH	3	5
BTL	AHSQG	PIVLLHGFTG	FFDLQKAVLKAAAVASNV	GHKRYGRT	GAAHAAKH	3	5
GLTW1	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNV	GHKRYGRT	GAAHAAKH	3	5
GThLYN	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNA	GHKRYGRT	GAAHAAKH	3	6
BSLL1	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNA	GHKRYGRT	GAAHAAND	3	5
BLL	AHSMG	PVVMVHGIGG	x	x	x	2	0
BL42	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNV	GHARFGRT	GAAHAAKH	3	6
BThlID-1	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNV	GHKRYGRT	GAAHAAKH	3	6
GThLIH1	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNV	GHKRYGRT	GAAHAAKH	3	5
GZLT1	AHSQG	PIVLLHGFTG	FFDLQKAVLEAAAVASNV	GHKRYGRT	GAAHAAKH	3	6
SXL	AHSMG	PIVLLHGFTG	ALGNEAIVRQLAFDYAKFKGNKNSKVDFGFGQWGLK	GHKRYGRT	GAAHAAKY	1	3
SAL	GHSMG	PVVFVHGFLG	KFGNTEAVRKIMFALNRFMGNKYSNIDLGLTQWGFK	GHKRYGRT	GAAHAAKY	1	3
ATL	GHSLG	IVLSFRG	SRSPANWIANLDFIF	x	x	3	4
ANL	GHSYG	IVLSFRG	SSDLSNWIADLDFGL	x	x	2	3
PCL	GHSQG	PIILVHGLSG	GLSSSVIAAFVNVFGILTSSSHNT	x	x	1	4
PLKW1-56	GHSQG	PIILVHGLSG	GLSSSVIAAFVNVFGILTSSSHNT	x	x	1	4
CALA	GYSGG	–	–	x	x	4	5
TLL	GHSLG	IVLSFRG	SRSIENWIGNLNFDL	x	x	1	1
RCL	GHSLG	IYVTFRG	TNSFRSAITDMVFTF	x	x	1	2
CALB	TWSQG	x	x	x	x	0	5
TTL	GESDG	PMVIMFHGF		x	x	3	1
ROL	GHSLG	IYLVFRG	TNSFRSAITDIVFNF	x	x	3	2
PLB	GHSMG	PLLVFFHGGGF	_	x	x	2	4
BSL205y	GESAG	PVIVQVHGGGW	_	x	x	2	1
SEL	GHSMG	PVVFVHGFVG	KIGGTKALDLELGFSQWGFK	GHKRYGRT	GAAHAAKY	3	3
RML	GHSLG	IYLVFRG	SSSIRNWIADLTFVP	x	x	2	1
BPL	AHSMG	PVVMVHG	_	x	x	1	0
CVL	GHSQG	PVILVHGLAG	GLSSTVIAAFVNVFGTLVSSSHNT	x	x	2	3
RML	GDSLG	IVFRG	SSSIRNWIAD	x	x	2	1
BSL	AHSMGG	PVVMVHGIGG	x	x	x	1	0
TBSL	AHSMGG	PVVMVHGIGG	x	x	x	1	0

x represents absence of the particular motif and – represents not found

which are involved in thermostable protein structure stabilization (Kleiger et al. 2002), we found that frequency of occurrence of AXXXA motif is more for thermostable *Bacillus* and *Pseudomonas* lipases than their mesostable counterparts (Table 2). However, nothing much could be concluded about the GXXXG motif showing distributed frequencies in bacterial and fungal thermostable and mesostable lipases.

Comparison of amino acid composition of thermostable and mesostable lipases

Differences in amino acid composition had been associated with the thermostable and mesostable proteins. We found some notable differences among thermostable and mesostable lipases on the basis of their percentage amino acid compositions (Fig. 1).

It was observed that thermostable bacterial lipases (TBFL) have lesser percentage of Cys (0.87%) when compared to mesostable lipases which is 0.98%, although the percentage of Cys is more for fungal thermostable lipases (TFL) i.e., 1.99% than bacterial thermostable lipases (TBL) i.e., 0.48%. Moreover, TBFL have more charged amino acid residues Glu, Arg, His (3.94%) than ML (3.23%). However, percentage of Asp and Lys residues is much less for TBFL (4.85%) than ML (5.33%) but TFL having 6.18% of Asp outnumbers both TBL (5.67%) and ML (5.92%). Aromatic amino acid residue for TBFL (3.26%) is greater than ML (2.95%). The percentage of Pro residue is more in TBFL (4.65%) than their mesostable counterparts (4.24%). Ala residues in TBFL (8.88%) are greater than the ML (7.93%), but TBL studied have lesser percentage of Ala residues than TFL 8.30 and 10.51%, respectively. Gly residue is comparatively higher for ML (9.98%) than TBFL (9.91); however, 10.55% of Gly residues in TBL is much higher than TFL. Labile amino acid residues Gln and Met are less in thermostable lipases (3.32%) than ML (3.73%). Average percentage of β-branched residues (Val, Leu and Ile) is less in TBFL (6.63%) than ML (7.37%). Hydrophobic residues show varying trend among TBL, TFL and ML, Val and Ile showing increment in mesostable lipases.

Structural analysis of lipases by subfamily tree annotation

Subfamily clustering by CLUSS 2 ver. 1.0 (IJCBDD 2008) separated thermostable and mesostable lipases into eleven subfamilies (Fig. 2). Thermoalkalophilic *Bacillus* lipases completely separated into a separate subfamily (subfamily 6 in our classification). Rest of the ten subfamilies showed mixed occurrence of thermostable and mesostable lipases (Fig. 2). Each individual subfamily showed marked differences in their thermostability, pH stability, substrate specificity, sequence and structure level (Table S1).

Subfamily 1 was represented by minimum α/β hydrolase lipases having two thermostable–mesostable pairs. Similar features were that all the family members lack Cys residues in their sequences, they lack lid domain, are alkalophilic with pH range of 9–11and show preference for medium to short chain length fatty acid substrate. Moreover, the thermostable lipases show temperature range of 55–70°C.

Subfamily 2 was dominated by *Pseudomonas* lipases; two thermostable and one mesostable. The thermostable lipases showed temperature stability range of 55–60°C. All the family members showed near neutral pH optimum, funnel shaped substrate binding cavity (Pleiss et al. 2000), varied substrate specificity, absence of Zn^{2+} binding motif.

Subfamily 3 and Subfamily 4 were fungal filamentous lipases with PDB structure available for only one mesostable and one thermostable member of each subfamily,

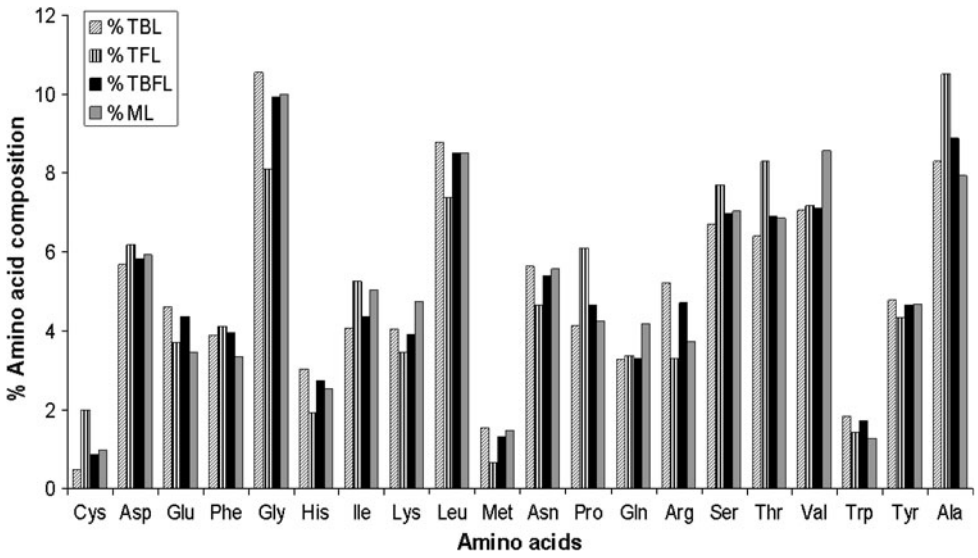

Fig. 1 Average % amino acid composition of bacterial, fungal thermostable and mesostable lipases. *% TBL* (Percentage of bacterial thermostable lipases); *% TFL* (Percentage of fungal thermostable lipases); *% TBFL* (Percentage of bacterial and fungal thermostable lipases); *% ML* (Percentage of mesostable lipases)

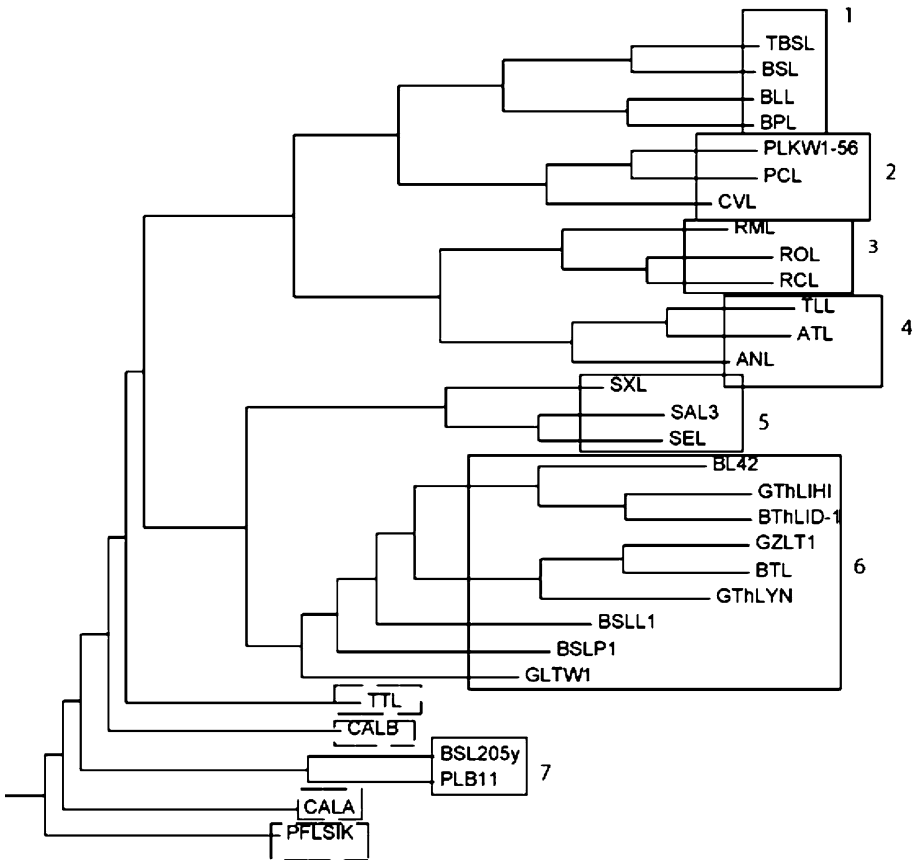

Fig. 2 Phenogram showing subfamily clustering of thermostable and mesostable lipases into 11 subfamilies. The *boxes* represent subfamily 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11. Subfamilies 8, 10, 11 are the orphan subfamilies represented by dashed box and subfamily 9 contains two bacterial mesostable lipase sequences

respectively. The former family showed slightly alkalophilic pH stability range, substrate specificity for medium chain fatty acids; hydrophobic, crevice-like binding site (Pleiss et al. 2000) and one member was moderately thermostable though the latter was dominated by all thermostable lipases having wide range of pH stability and showing 1,3 regiospecificity (Macrae and Hammond 1985) for their substrate.

Subfamily 5 comprises all *Staphylococcus* lipases; two thermostable and one mesostable. Sequences showed Zinc binding motif and P-loop motif in two members. Moreover, this subfamily was dominated with members showing short chain length substrate specificity.

Subfamily 6 was the largest of all comprising of all *Bacillus* thermoalkalophilic lipases showing medium chain length substrate specificity, and presence of Zinc binding.

Subfamily 7, 8, 10 and 11 contained orphan sequences and hence were not considered in our study.

However, subfamily 9 consisted of two mesostable *Bacillus* and *Pseudomonas* lipases lacking any structural information. Distinguishing characteristic from other subfamily being their GGGX type oxyanion hole signature (Figure S1).

Subfamily annotation clearly showed which thermostable–mesostable lipase structures were to be compared for further detailed analysis for structural role in thermostability. For this purpose, we performed structural superimposition of representative thermostable–mesostable lipase pairs from each intra and inter subfamily to grip concrete data for their differences in temperature stability (Figure S2a–d). Two intrasubfamily structures from subfamily 1 (BSL: 1I6 W with TBSL: 3D2C) and subfamily 2 (PCL: 3LIP with CVL: 1CVL) showed 95 and 84% sequence identity with RMSD of 0.217 and 0.32, respectively. Additionally, two intersubfamily structures from bacteria (BSLL1: 1KU0 with CVL: 1CVL) and fungus (TLL: 1TIB with RML: 4TGL) having 24 and 34% sequence similarity with RMSD of 14.49 and 0.97, respectively, were superimposed. The overall structural differences observed in thermostable lipases w.r.t mesostable ones were more γ-turns with difference in their structural positions being nearer to N or C terminus of helices or strands as the γ-turns near helix7 of TBSL. Moreover, in PCL Pro233 replaces Leu233 in CVL forming a γ-turn which may play a role to increase conformational stability. The other thermoadaptivity defining trends observed were lesser β-branched residues (Val, Ile, Thr) in helices, longer β-strands, shorter loops, frequent distribution of Ala residues in α-helices, e.g. Ala (dipeptide or tripeptide) residues in lid domain of Bacillus thermoalkalophilic lipases, more statistically favoured residues at N-cap and C-cap position (Asp, Asn, Ser, Glu, Gln, Ala, Arg, Lys) of α-helices, e.g. His in N-cap position of helix1 in CVL is replaced by Arg in

thermostable PCL and more Ser residue in N-cap end of helix1 in TBSL than BSL, greater preference for negatively charged amino acid residues in amino terminus and positively charged redsidues in carboxy terminus as seen in TBSL and BSL having Glu and Ala residues in amino terminus, respectively. Moreover, amino terminus Asp91 of helix6 in TBSL is not observed in BSL. Increase in protein conformational stability by optimizing β-turn sequences was also observed in thermostable lipases by increased preference for Pro, Gly and aromatic residues and avoidance of Ala residues in beta turns. This trend, however, was not noticed to be strictly followed in their mesostable counterparts. Structural analysis by VADAR program showed that thermostable lipases possess more charged-neutral hydrogen bonding pair and greater exposed polar surface are.

HotSpot Wizard and CUPSAT analysis of structurally important residues

HotSpot Wizard analysis of intrasubfamily thermostable–mesostable structures (TBSL-BSL and PCL-CVL) having greater than 80% sequence similarity highlighted the following interesting facts. The structurally important residues observed in TBSL and not in BSL with very low mutability rate were Leu143, Thr117, Ile169, Lys88, Asn138, Tyr86, and Asp118. These residues show maximum destabilizing mutation as predicted by CUPSAT and compared with BSL. The interatomic interaction of these residues as studied for both BSL and TBSL lipases in analysis of interatomic contacts of structural units (CSU) (Sobolev et al. 1999) accessed through PDB gave the following informations about the contribution of each of these residues towards thermostabilization. Leu143, Ile169, Lys88 showed enhanced hydrophic-hydrophobic contact. Thr117, Ile169, Lys88, Asn138, Tyr86 show increase in hydrogen bonding. Asp118 revealed differences in interaction with residues involved in hydrogen bond formation. Moreover, increase in charge-neutral interaction was observed for Leu143 and Ile169.

The structurally important residues in PCL with low mutability rate which may be responsible for enhancement of thermostability were observed to be Val267, Leu315, Pro304, Gly295, Thr245, Asn202, Gly60, Ala272, Thr280, Tyr282, Arg309, Lys316, and Ile232. Mutation of these residues can lead to very low to moderate stabilization of the lipase as predicted by CUPSAT. Only Thr245, Asn202, Arg309 which are buried in the core, will stabilize the structure when mutated with hydrophobic residues. Residues Val267, Leu315, Pro304, Ala272, and Ile232 increase hydrophobic-hydrophobic contacts. Gly295, Ala272 shows increment in hydrogen bonds. Moreover, greater charged neutral hydrogen bonding is observed for Gly60 and Lys316. The amino acid substitution in PCL responsible for increase in hydrogen bonding, hydrophobic contact and charged neutral hydrogen bonding are Ser280 → Thr280, Thr309 → Arg309, Ser267 → Val267, Lys315 → Leu315, Leu316 → Lys316, Ser202 → Asn202, Ser280 → Thr280, His282 → Tyr282. These mutations show stabilization of the structure when cross validated with CUPSAT. The residues predicted by SCride for structural stabilization of PCL absent from CVL are Ile11 and Ala105 which cannot lead to stabilizing mutation as predicted by CUPSAT and Hotspot wizard. These are hydrophobic and buried in the core, hence can be responsible for thermostability of PCL. Although no such substitution for the structurally important residues was observed in TBSL in comparison to BSL.

Discussion

The active site of lipases

Lipases belong to the α/β hydrolase family (Ollis et al. 1992) with the active site Ser in catalytic elbow defined by the conserved pentapeptide GXSXG where X may be any amino acid residue. Though it has been reported that Ala replacing Gly of the pentapeptide sequence leads to thermal stability of *Bacillus* lipases because the side chain of the Ala residue stabilizes the loop conformation by tight packing, contributing to thermostability (Jeong et al. 2002) we report here, the presence of the same signature sequence in mesostable *Bacillus* lipases ensuring that Ala residue alone cannot account for the thermostability of this class of lipases. It has been said that lack of Cys residues in hyperthermostable lipase from *T. thermohydrosulfuricus* may account for its thermostability (Royter et al. 2009) due to the high sensitivity of free Cys residues to oxidation at elevated temperatures. However, our finding indicates that mesostable lipases also lack Cys residues, it can be concluded that lack of free Cys residues cannot be the only factor leading to thermostabilization. It is known that the electrical potentials generated by the charged titratable residues have an important role in catalysis since they can enhance substrate binding, stabilization of the transition states of substrate and efficient product release (Petersen et al. 2001) leading to protein stability. This fact is also justified by our observation that the presence of more titratable amino acid residues near the active site Ser of thermostable lipases can lead to functional stability of the active conformation of lipases at elevated temperature.

The oxyanion hole

Though all the thermostable lipases studied were found to belong to the GX class of oxyanion hole. Nothing much could be conclusively said about the role of oxyanion hole

in thermostabilization of lipases which may be due to the high conservation of the amino acid residues forming the oxyanion hole in lipases, being critical for catalysis.

The lid of lipases

The lid of lipases was given special emphasis as it is involved in the interfacial activation in lipases generating a possibility that it can be a target site for enhancing thermostability of lipases. Our observations through sequence characterization justifies that the presence of less positively charged residues in the lid may be responsible for greater negative potential of the active site of thermostable *Staphylococcus* lipases leading to its stability. Even though it has been said by Zhu et al. that Trp89 in the lid of *T. lanuginosa* due to its hydrophobicity and π-cation interactions contributes to free movement of the lid at elevated temperatures and thus to thermostability (Zhu et al. 2001), by our study we can say that as Trp residue is present in lid of mesostable lipases, hence ruling out its sole contribution to thermostability of fungal and TBL. Another previous report stated that Tyr224 strengthens hydrophobic interaction between the helix7 and the lid helix6 and strengthens the tight packing and stability of the active site residues in *Geobacillus* sp. lipase (Wu et al. 2009). But MSA highlighted presence of Tyr224 residue near the lid domain of not only in thermoalkalophilic *Bacillus* and thermostable *Staphylococcus* lipases but also in mesostable lipase also not supporting that this is crucial for rendering the lipases thermoactive. As it is well known that hydrobhobicity and rigidity are among the two important factors which leads to stability of proteins, presence of Val or Ala and Pro at comparable positions in thermoalkalophilic *Bacillus* lipases like that reported for *P. cepacia* lipase in the lid helix (Santarossa et al. 2005) can enhance thermoadaptivity by making the active site more hydrophobic and the lid rigid.

Our result clearly supports this point as we noticed the presence of poly Ala residues in the lid helix in *Bacillus* thermoalkalophilic lipases which can lead to their thermostability to a large extent. It has been said that Ala residue is a good helix stabilizer as it leads to the formation of peptide hydrogen bonds due to its small side chain which is well accommodated in the helices (Rohl et al. 1999). Moreover, stability of lid helix at elevated temperature can be critical for thermo activeness of lipases.

Role of ion binding in lipases

It has been conclusively observed that Ca^{2+} and Zn^{2+} are tightly bound ions in lipases. The role of Ca^{2+} in increasing thermal stability of lipases was reported as it restricts conformational flexibility of certain helices and loops (Invernizzi et al. 2009) and stabilizes the catalytic His residue through hydrogen bonding as studied in *Pseudomonas* and *Burkholderia* lipases (Kim et al. 1996). It has also been reported that Calcium ion removal causes protein unfolding and aggregation (Invernizzi et al. 2009) though the role of Ca^{2+} is not as critical in *Bacillus* lipases as in *Pseudomonas* lipases (Jeong et al. 2002). However, in our study as we found Ca^{2+}coordinating amino acid residues in all thermostable and mesostable lipases thus Ca^{2+} coordination alone cannot render lipases thermostable. Similarly, Zn^{2+} coordination in lipases was considered to solely play a structural role not taking part in catalysis instead stabilizing the catalytic domain of lipases (Tyndall et al. 2002) and thus can lead to thermostability. Irrrespective of this fact, we found that Zn^{2+} coordinating residues are not only present in *Bacillus* and *Staphylococcus* thermostable lipases but also in comparable position in mesostable *Staphylococcus epidermis* lipase. Thus, it can be assumed that Zn^{2+} coordination, although playing a major role, cannot alone lead to thermoadaptivity. Interestingly however, it can be pointed out here that if both Zn^{2+} and Ca^{2+} coordination if present in lipases, as in TFBL, it can increase their stability at elevated temperatures due to their cooperative involvement in stabilization of lipase structures.

The P-loop motif

The P-loop or phosphate binding loop was first reported in *Staphylococcus* lipases. It has also been reflected in polynucleotide kinases and is a common motif in phosphotranferases and is termed as "Walker A box" (Wang and Shuman 2002). P-loop motif is present in many thermostable enzymes like T4 Bacteriophage polynucleotide kinase (Wang and Shuman 2001) and *Pyrococcus horikoshii* Clp1 (*Pho*Clp1); a thermostable 5′-OH polynucleotide kinase active at 55°C–85°C (Jain and Shuman 2009). Moreover, a P-loop like motif with Arg to Lys replacement is observed in protein tyrosine phosphatases (Zhang et al. 1998). We also witnessed the presence of such a P-loop like motif in thermoalkalophilic *Bacillus* lipases in line with the Zn^{2+} binding residues. As it was reported that Zn^{2+} binding causes thermostability of lipases (Fuji et al. 1996), this P-loop like motif can thus by our data analysis, be considered as a conserved pattern in thermoalkalophilic *Bacillus* lipases. So it can be theorized to lead to stronger coordination of the metal ion enhancing thermostability in *Bacillus* lipases.

The AXXXA motif

The pronounced role of AXXXA motif in thermostabilization of proteins had been attributed for the presence of this motif in helices which leads to improvement in interhelix interaction and stabilization of the folded state of

many proteins. As stated by Kleiger et al. (2002), this motif is enhanced in 24 fully sequenced genomes, *Aquifex aeolicus* having the greatest occurrence. This motif can lead to dimerization of proteins causing better stability by strong van der Waals interaction in thermostable proteins (Kleiger et al. 2002; Leonov and Arkin 2005). Thus, it can be concluded that the higher frequency of this motif in thermostable lipases stabilize lipases structures at elevated temperature.

Role of amino acids in thermostability of lipases

The exact role of amino acids in protein thermostability has been a long study. Studies carried out have shown the involvement of amino in thermostability of proteins and we stretch these findings being contributory to thermoadaptive properties of lipases. It had been shown that presence of more charged residues leads to thermostability of proteins as they are involved in electrostatic interactions which stabilize the secondary structure of protein (Fukuchi et al. 2001; Silver and Livesay 2003) like that of Arg as it shows charge resonance of the guanidium group which gives it the possibility to form more salt bridges and its side chain can form maximum of five hydrogen bonds preferably to carbonyl oxygen of peptide bond (Feller et al. 1997). We are in complete agreement with the same as our data showed the presence of more charged residues in thermostable lipases than mesostable ones. Cys residues are known to play a dual role by both increasing thermostability when present in disulphide bridges and decreasing thermostability when available in free form as it highly sensitive to oxidation at elevated temperature (Vieille et al. 2001). Though our data on thermostable lipases show lesser percentage of Cys residues the result becomes controversial due to the presence of conserved Cys residues in some thermostabe lipase sequence not involved in disulphide bridge formation and TFL having a much higher percentage of Cys residues. Among other amino acid residues, the relatively high percentage of Ala was noticeable in our data clearly shown in Fig. 1. Ala being a small non polar residue has been credited to be a good helix former (Shalongo et al. 1994) as the small side chain of Ala does not shield the backbone from solvent, allowing water to interact with the peptide carbonyl groups in a polyalanine helix (Luo et al. unpublished work). Thus, the higher percentage of Ala in thermostable lipases attribute towards thermostabilization of lipases. Moreover, thermolabile aminoacid residues like Asn, Gln and Met should be less in thermostable lipases as they tend to undergo oxidation and deamidation at elevated temperatures (Fukuchi et al. 2001, Russell et al. 1997). This is also endorsed by our result which shows lesser percentage of thermolabile amino acid residues in thermostable lipases. Gly is the smallest amino acid residue and is flexible which aids in relaxation of steric hindrance of thermophilic ezymes and increases stability (Vieille et al. 1996). The high percentage of Gly observed in TBL observed accounts for this fact but lesser percentage of Gly in thermostable lipases than mesostable ones shows its role as helix breaker. Thus, it can be said that Gly shows preference for TBL and further structural analysis showed that their frequency is much greater in loops than helices. Moreover, higher Pro percentage for thermostable lipases shows its pronounced role in enhancing thermostability as Pro is more rigid than other amino acids and reduces the entropy of the main chain polypeptide decreasing the chance of unfolding at elevated temperatures (Sælensminde et al. 2009). Greater percentage of hydrophobic: Val and Ile (Gromiha et al. 1999) and aromatic residues has been long assumed to lead to better thermostability of protein as weakly polar interaction made by the aromatic ring of residues like Phe are of an enthalpic importance compared to that of hydrogen bonding (Feller et al. 1997). However, contradicting the results of Gromiha et al. (1999), Val and Ile show increasing trend in mesostable lipases. Our data show greater percentage aromatic residues in thermostable lipases which is in agreement with the aforesaid.

Structural annotation of thermostable lipases

Structural comparison of thermostable and mesostable lipase pairs by tree-based annotation revealed many factors that can altogether be held responsible for thermostability of lipases. To the best of our knowledge, we report for the first time that increment in inverse γ-turn and their presence near the amino or carboxyl end of helices and strands plays an important role in enhancement of thermostability of lipases. To add to this our finding being based on the study of many different thermostable lipases also supports the assumption drawn long back by Paupitt et al. (1988) that a γ-turn may contribute to stability of Thermolysin when compared to neutral protease from *Bacillus* (Pauptit et al. 1988). As it was considered that one intramolecular hydrogen bond contributes about 2.1–6.3 kJ/mol to the free energy of stabilization (Stark et al. 1992) we can say that increase in inverse γ-turns can lead to enhancement of thermostability of lipases as they are sharp and tight turns comprising of three amino acid residues which leads increase in intramolecular hydrogen bonding because they induce hydrogen bonding between i ($C = O$) and $i + 2$ (N–H) residues in the polypeptide chain. Adding to the aforesaid, our report is also supported by the information provided by Milner that γ-turns near the amino or carboxyl terminal end of helices or strands are involved in stronger hydrogen bonding (Milner-White 1990). Thus, we can say that the presence of inverse γ-turns near the end of helices and strands in thermostable lipase structures leads to thermal

stabilization of lipases. In another report by Jong et al. it was said that γ-turns contribute very less to conformational entropy (Gong and Rose 2008). Based on this property of γ-turns we can say that increase in inverse γ-turns in thermostable lipases will lead to their lesser conformational entropy at elevated temperatures and thus to thermostability.

Lesser beta-branched residues in helices were another trend observed for thermostable lipases. Our result is in agreement with the report that beta-branched residues affects helix stability as their side chains are not well accommodated in the helix thus disturbing the helix propensity (Wang et al. 2003). Thus, we can say that as this can lead to decrease in thermostability of lipases, thermostable lipases through evolution have chosen to avoid beta-branched residues in their helices.

Increase in strand length up to seven residues near β-hairpins was considered to increase protein conformational stability (Stanger et al. 2001). Similar trend was observed in thermostable lipases thus longer β-strands near β-hairpins in thermostable lipases can be assumed to contribute to their temperature stability because they make the structure more compact leading to their conformational stability at elevated temperature. Moreover, it is believed that presence of shorter loops in thermostable protein structures plays a role in their thermoadaptive properties by increasing compactness and reduction of the entropy of unfolding in proteins leading to their stability (Li et al. 2005). We also came up with similar data in our analysis with regard to thermostable lipases, strongly supporting the aforesaid. Moreover, another observation which could be given credit for increasing thermostability of lipases was the presence of greater frequency of Ala residues in α helices of thermostable lipases and the absence of the same trend in mesostable lipases. We can say this because Ala is credited with the title of "best helix forming residue" (Vieille and Zeikus 2001) as it increases helix propensity because its side chain gets well accommodated in helices so can lead to stabilization of the structure of lipases at elevated temperatures. In addition to the above-mentioned reasons, it was reported that the occurrence of residues which can form hydrogen bonds in N-cap and C-cap positions like Asp and Glu in helices can stabilize protein structures by stabilization of helix dipole and hydrogen bonding (Li et al. 2005). This trend was observed in thermostable lipases and thus it can be said that N-cap and C-cap residues are important for making lipases thermostable. It is well known that negatively charged amino acid residues in amino terminus and positively charged residues in carboxy terminus in proteins stabilize their helix dipole (Eijsink et al. 1992). Therefore, stabilization of helix dipole leads to protein stability. This trend was followed by maximum of the thermostable lipases studied. This indicates that proper N- and C-terminal residues also play a crucial role in thermostabilization of lipases. Moreover, it is thought that some β-turn residues like Gly which relieves steric strain of the protein contributed by β-carbon of certain amino acid residues, aromatic residues which remain partially buried increases hydrophobic interactions and Pro as it has restricted φ-angle being entropically favorable at certain turn positions increases protein rigidity (Trevino et al. 2007). Between thermophilic and mesophilic lipase structures, we also observed a significant difference in the presence and distribution frequency of these residues which is more for the thermostable lipases. Thus, these residues and their distribution are another reason for increase in thermal stability of lipases. Besides all the above-mentioned factors which can be held responsible for thermostabilizing lipases, two other noticable trends in some thermostable lipases that differed from mesostable ones were increase in exposed polar surface area, increase in hydrophobic contact by amino acid substitution and change in torsion angle and surface exposure of amino acid residues. This observation can be rightly justified to also contribute to thermostability of lipases as it was showed that increase in the number of hydrophobic interaction (Branden and Tooze 1999) and increase in polar surface area leads to better protein stability by enhancement of ionic interactions and hydrogen bonding with surrounding water (Suvd et al. 2001).

Conclusion

By sequence and structure analysis of thermostable lipases with an added approach of tree-based annotation it can be concluded that each thermostable lipase adopts its own strategy in relation with the three dimensional arrangement of amino acid residues to increase its stability at elevated temperatures. Conclusively, we have come up with a set of strategies that can enhance thermostability of lipases. Increasing the titrable amino acid residues near the active site Ser can enhance thermostability. Increment of charged residues in surface and decreasing β-branched residues in helices can make mesostable lipases thermostable. However, the strategy that can be employed to increase thermal stability of bacterial lipases may not be applicable for fungal lipases as thermostabilization factors show a changing trend among these two distinct phylogenetic groups of lipase. An example being increase in frequency of Gly residues in loops of only *Bacillus* lipases leading to their thermostabilization. Decreasing the percentage of free Cys residues without disturbing the conserved ones can lead to enhanced thermostability. Increase of poly Ala residues in helices especially in the lid of *Bacillus* lipases, mutating thermolabile residues in helices with residues

having high helix propensity, more Pro residue in loops and turns and more aromatic residues in surface and core which increases hydrophobicity in lipases can also enhance their thermo stability. Increasing AXXXA motif in helices and increase in strand length up to seven residues near β-hairpins were found contributory to increase lipase stability. Moreover, mutating residues N-cap and C-cap positions with Asp and Glu which can stabilize helix dipole, increase in exposed polar surface area and increase in hydrophobic contact by amino acid substitution can cause lipase thermostabilization. The most important fact to be endorsed leading to enhancement of temperature stability is the increment in inverse γ-turn near the amino or carboxyl end of helices and strands which has been reported by us for the first time.

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