DISCUSSION
Cloning, expression, purification and biochemical characterization of metagenomic lipase:

Enzymes have major appeal as catalysts because of their high turnover number and refined level of selectivity, particularly in the synthesis of single-enantiomeric compounds. One such biocatalyst, lipase exhibited remarkable broad substrate specificity which make them highly versatile biocatalyst. A variety of microbial lipases with different substrate specificities and biochemical properties have been reported. Sometimes these reactions are performed most efficiently at elevated temperatures and in organic solvents. The temperature stability of lipases has been regarded as the most important characteristic for use in industry (Rubin and Dennis 1997; Nawani et al. 2006). Attempts have been made to find thermostable lipases which would have advantages over thermolabile enzymes in such applications (Kim et al. 2004). The lipases scrutinized showed astonishing heterogeneity both in the catalytic properties and in the amino acid sequence. However, significant conservation of higher levels of protein structure was recorded, i.e. lipase possesses very low sequence identity but very high three-dimensional architectural similarity. In the field of biotechnology, new and improved technologies are needed to increase the speed and efficiency for discovering new molecules, especially from microorganisms. As only 1-4% of the microorganisms can be cultured in vitro by standard methods, vast majority of nature's microbial diversity is unexplored.

With the advent of metagenomic approach, the microbial diversity can be better explored. This resulted in exceptional increase in the number of potential candidates for biocatalysis. As enzymes are known to display habitat related characteristics, metagenomics can be an excellent strategy to obtain the enzyme with novel characteristics from the diverse environment. Till date only few thermostable lipases have
Discussion ...

been reported using metagenomic approach. Recently, Tirawongsaroj et al. (2008) cloned and characterized two lipolytic enzymes from a Thailand hot spring. One of the enzyme is a novel platin-like phospholipase (PLP) and the other one, an esterase (Est1). Both enzymes retained more than 50% of maximal activity in the temperature range of 50–75°C with optimal activity at 70°C. Chao et al. (2009) also reported esterase, EstY using metagenomic approach. The optimum temperature and pH for EstY was observed at 50°C and 9.0 respectively.

Keeping in mind that hot spring can be a good source of thermostable lipase, soil samples were collected from hot spring area of Manikaran in Kullu Distt. of Himachal Pradesh (India). The metagenomic DNA was isolated from the soil using Zhou et al. (1996) method with slight modifications. Extraction and purification of total metagenomic DNA was necessarily a compromise between the vigorous extraction required for the representation of all microbial genomes and the minimization of DNA shearing. Previously many methods for DNA purification were reported which either are expensive or took longer time for purification (Bertrand et al. 2005; Yeates et al. 1998; Roose-Amsaleg et al. 2001; Santos 2001; Lakay et al. 2006). The humic acid and polyphenol coextracted with the metagenomic DNA resulted in brown colour of the pellet. The developed method based on Q-Sepharose could purify high molecular weight unsheared DNA in less time without running any column chromatographic steps. The recovery of DNA after purification and reduction in humic acid was more than 80%. The purified DNA showed its efficacy for molecular manipulations i.e restriction enzyme digestion and PCR amplification.

Recently many methods for purification of metagenomic DNA were reported. Rojas-Herrera et al. (2008) reported a method for metagenomic DNA purification which was based upon silica. DNA purification took longer time for purification and the reduction of humic acid was also not satisfactory. Purohit and Singh (2009) reported assessment of various methods for extraction of metagenomic DNA
from saline habitats of coastal Gujarat (India) to explore molecular diversity. They evaluated the different methods based upon the enzymatic lysis, bead beating and sonication. All the methods evaluated by them had residual humic acids in the DNA while high molecular weight DNA got sheared.

In the present thesis a thermostable lipase gene JkP01 (Wt unless and other wise stated) was amplified and cloned from the metagenomic DNA by PCR. Sequence homology search for the cloned lipase gene against the protein database indicated that the encoding protein exhibits very high amino acid homology (90-95%) to number of lipases/esterases, especially to thermostable lipases of the *Bacillus* species, the *Geobacillus*. A comparison of the nucleotides and deduced amino acid sequence of this lipase with other lipase and esterase sequences in the EMBL and SwissProt database showed significant identity to the *Bacillus stearothermophilus* L1 (95%), *Geobacillus thermocatenulatus* (94%) and *Geobacillus species SF1* (93%). They all belonged to Family 1.5 lipolytic enzymes. The catalytic triad of the JkP01 lipase predicted based upon its multiple sequence alignment with the other similar reported lipase showed that serine 113, aspartate 346 and histidine 386 as a member of catalytic triad as reported earlier in the result section. In addition to the catalytic triad a conserved pentapeptide AHSQG was also observed in the amino acid sequence. Like other *Bacillus* lipases Ala replaces first Gly in conserved pentapeptide which is conserved amongst most lipases.

Lipolytic enzymes belonging to Family 1.5 also have a highly conserved dipeptide (HG) which flanks the catalytic pocket and plays a role in stabilizing the oxyanion intermediate. This dipeptide is also present in JkP01 lipase at position 43 and 44. On the basis of available crystallographic structure of the most similar reported lipase of *Bacillus stearothermophilus* L1 (Choi et al. 2005), it was predicted that the active site residues especially serine is protected by lid helix domain. The sequence alignment with *Bacillus stearothermophilus* L1 showed that
Discussion ...

out of total 388 amino acids, 16 amino acids were different in the mature polypeptide of the enzyme.

An expression system was developed using pQE vector with strong T5 promoter and six His-tag at N terminus of the cloned gene. It was observed that though the protein was expressed as His-tag along with the signal peptide, but the secreted lipase did not bind to Ni-NTA resin. It might be assumed that mature polypeptide was secreted into the media after cleavage of terminal signal sequence and therefore losing the His-tag present towards the N terminus site. The extracellular protein purified resulted in low yield of enzyme. The loss might be attributed to the loss of enzyme activity during ammonium sulphate precipitation as well as tight binding of lipase to hydrophobic column. It was observed further that significant amount of lipase activity never eluted from the column. Like most of Bacillus lipases, this lipase also form aggregates at high concentration. This could be another factor which in cumulative effect resulted in the low yield of the purified protein.

We have compared the biochemical properties of purified JkP0 lipase to the homologues lipases. It was observed that lipases showing more than 90% similarity at amino acid level displayed different biochemical properties to each other. The optimum temperature of all these lipases was in the range of 40-60°C. In comparison with JkP0 lipase, lipase from Bacillus stearothermophilus L1 demonstrated higher optimum temperature (60°C) with half life of 30 min at 60°C (Kim et al. 1998), while lipase from Geobacillus thermophilic sp. TW1 had comparatively low optimum temperature (40°C) with half life of 15 min at 60°C (Li and Zhang 2005). However the thermostable lipase reported from Bacillus stearothermophilus P1 had a temperature optimum of 55°C with half life for more than 1 h at 60°C (Sinchaikul et al. 2001). The T1 lipase reported by Leow et al. (2004) had quite high optimum temperature (65°C) and half life (30 min at 65°C). Recently Jiang et al. (2009) isolated a thermostable lipase gene from Geobacillus
stearothermophilus JC strain that was completely stable at 60°C for 30 min.

It was observed further that the JkP01 lipase exhibited more activity towards the long carbon chain substrates while no activity was observed with short carbon chain substrates (C₂, C₄). It displayed maximum activity with pNP-laurate (C₁₂) at pH 8.0, while the closely related lipases of *Bacillus stearothermophilus* L1 and thermophillic TW1 displayed maximum activity with pNP-caprylate (C₆) and pNP-deconate (C₁₀) respectively (Kim et al. 1998; Li and Zhang, 2005). Further it was observed that the enzyme displayed 100% activity each with SDS, sodium deoxycholate and β-Mercaptoethanol while 74% activity with Triton X-100. On contrary, lipase from *Bacillus stearothermophilus* L1 displayed only 2% activity with SDS and 80-90% activity each with sodium deoxycholate, β-Mercaptoethanol and Triton X-100 (Kim et al. 1998). The thermophillic TW1 lipase showed similar observation with SDS, Triton X-100 and β-Mercaptoethanol respectively when compared with JkP01 lipase (Li and Zhang 2005). The thermostable lipase from *Bacillus stearothermophilus* P1 showed 50% reduction in enzyme activity with SDS and 80-95% activity each with sodium deoxycholate, β-Mercaptoethanol and Triton X-100 respectively (Sinchaikul et al. 2001). Another lipase reported recently from *Geobacillus stearothermophilus* JC showed 76% inhibition in enzyme activity with Tween 80 comparable to the Wt lipase which also showed inhibition towards this detergent.

In presence of Triton X-100 the activity of the *Geobacillus stearothermophilus* JC lipase was increased by factor of 69%, in contrast the JkP01 lipase activity was decrease to 26% in presence of 1% V/V of Triton X-100 (Jiang et al. 2009). The lipase activity in these detergents might be due to the change in the interfacial properties or because of change in the conformation of the enzyme.

The inhibition study with chemical modifiers (DEPC PMSF eserine) of active site residue suggested histidine residue as an active site member.
Although all known lipases were reported to have catalytic triad of Ser-His-Asp, some lipases were reported to resist the inactivation by PMSF, a serine modifier specially with the lipases having lid domain. Like many other lipases, no effect of PMSF on enzyme activity was observed with Wt lipase even at 10 mM concentration (Li and Zhang 2005; Nakatani et al. 1992; Dannert et al. 1994). As with Wt lipase, BTL2 lipase was also not inhibited by PMSF. In contrast to this, *B. stearothermophilus* L1 lipase was different as it showed 90% and 77% enzyme activity after 10 min and 30 min incubation with PMSF. Similarly *B. subtilis* and L2 lipases (Schmidt-Dannert et al. 1997) demonstrated inhibition of enzyme activity in presence of PMSF (Sabri et al. 2009). It might be hypothesized that the serine residue was buried in the hydrophobic core and could not be accessed by the PMSF easily. On the other hand with DEPC, a histidine modifier, loss in enzyme activity was observed at very low concentration suggesting the easy accessibility of the catalytic histidine, an active member of the catalytic triad, to DEPC. The eserine, an esterase inhibitor, did not affect the enzyme activity suggesting Wt lipase to be a true lipase.

The effect of different metal ions on JkP01 lipase activity was studied and the results obtained were compared to the other closely reported lipases. It was observed that presence of the Ca++ increased JkP01 lipase activity. Similar enhancement was reported for the lipase of *Geobacillus stearothermophilus* (Jiang et al. 2009). It was suggested that Ca++ ion have generally found to stimulate the lipase activity by facilitating the removal of free fatty acids formed in the reaction at the water-oil interface. This is also in accordance with the lipase L2 reported recently from a thermophilic *Bacillus* sp. strain L2 where the lipase activity was increased 5-fold in the presence of Ca++ (Sabri et al. 2009). The Mn++ metal ion showed no inhibition towards the lipase JkP01 while Zn++ increased enzyme activity at 0.1 mM while inhibited the enzyme activity at 1mM. These reports are contradictory to report published by Sabri et al, (2009) where both these metal ions inhibited enzyme activity.
The inhibition of the lipase JkP01 with the Hg\textsuperscript{2+} at 0.1 mM concentration might be due to the involvement of cysteine amino acid in the catalytic activity.

Further it was observed in this study that the protein expressed and purified in presence of the terminal signal yielded less protein. To verify whether the transportation machinery of the host cell is able to process the over-expressed protein completely, amount of extra and intracellular lipase was determined. The comparison of production of intracellular and extra-cellular Wt lipase showed that nearly 35\% of total lipase activity was retained inside the host cell (data not shown). It suggested that nearly one third of lipase could not be transported out of the cell due to the high expression of protein followed by incomplete processing of the pre-lipase. Similar results were reported in earlier study also (Kim et al. 2000). Therefore an attempt was made to clone the lipase gene without terminal signal sequence. Intracellular expressed protein purified using Ni-NTA resin resulted in 26\% yield compared to 6\% yield with the extracellular purification and almost two fold increase in specific activity. This is in accordance with several reports that showed that removal of signal peptide in the lipases dramatically enhances expression levels (Kim et al. 2000; Leow et al. 2007; Rua et al. 1998). Similarly Jiang et al. (2009) also demonstrated that removal of the signal peptide greatly increased the enzyme's expression level by 4.3 times.

CD-spectrum and the intrinsic fluorescence of the protein is a sensitive reporter of the tertiary/ quaternary structural changes of a protein. Change in intrinsic fluorescence of the protein reports the polarity changes near the aromatic residues. nine tryptophans in the JkP01 lipase show conformation dependent fluorescence changes. It is further clear from the study that though this enzyme demonstrated optimum enzyme activity at 50\(^\circ\)C, Far-UV CD spectra indicate d significant change in lipase secondary structure at 35\(^\circ\)C and above. In terestingly, in spite of significant loss of secondary structure, the enzyme was still retaining its activity suggesting the intactness of the tertiary structure as supported
Discussion ...

by fluorescent spectroscopy data. The intrinsic fluorescence data suggested that there was not much loss in tertiary structure of the Wt lipase till 80°C.

In the present investigation we cloned a metagenomic lipase from hot spring. The enzyme was purified and characterized in detail. The present investigation indicated that the metagenomic lipolytic enzyme behaved like a lipase. Due to its thermal stability and tolerance for detergents, enzyme may be a good candidate for fat hydrolysis of solid lipids such as palm oil or beef extract in the production of fatty acids in the food industry or detergents. It was observed that in spite of the significant amino acid sequence similarity to other Geobacillus lipases, the biochemical properties of the lipases were quite different. The similarity of these lipases at structure and sequence level suggested that these might be closely related evolutionarily. The results further demonstrated that divergent arrangement of the amino acid had imparted different biochemical properties to these lipases.

Directed evolution/site directed mutagenesis towards enhanced thermostability

One important requirement for industrial application of enzymes is thermostability. Proteins from thermophiles often show higher thermostability as well as more stability toward organic solvents and show higher activity at elevated temperatures (Saxena et al. 1999). These proteins show low activity but higher thermostability at lower temperatures. Though all the information necessary for protein thermostability is encoded by amino acids of proteins (cloned thermostable protein has same properties when expressed in mesophilic host), no single traffic rule for correlation of thermostability with particular amino acids could be generalized. Structural factors that had been correlated with thermal stability in proteins are the hydrophobicity profile, the number of hydrogen bonds, and the amino acid composition, distribution and interactions in the protein (Vieille and Zeikus 2001). It
was further observed that the energy associated with the stability of the protein is small, as the difference between the native and denatured state of protein is only a matter of few weak interactions (Jaenicke 1996; 2000; 1998). The comparison of the protein from thermophilic and its counterpart mesophilic are used for the predictions of stabilizing mutation (Vogt et al. 1997; Cambillau et al. 2000). There are many biochemical reactions where enzymes with improved activity and enhanced thermal stability are required. Though the most of the discovered enzymes are potentially explored, however because of low stability and activity, its use in industrial processes is limited. Therefore need for improved enzymatic properties opened the door to tailor the existing enzymes. Although there might be multiple ways to manipulate gene for biocatalyst to obtain a different property, the approach that minimize the effort is preferred. Protein engineering helps in improvement of the existing enzyme either with rational designing or with directed evolution based approach. The rational designing is the information intensive and requires the three- dimensional structure information while directed evolution adds random mutations followed by further screening of lipases with desired properties.

Directed evolution is a method of protein engineering that utilizes the power of natural selection to evolve proteins with desired properties. Recently, in vitro evolution methods are routinely used to identify protein variants with novel properties that are difficult to achieve using rational design. The important properties for which protein engineering is being carried out includes the screening of the enzyme for thermostability, improved enzymatic activity or enantioselectivity. Since subtle variations in protein structure are responsible for the alteration of the biochemical properties therefore in the past several years, directed evolution has emerged as method of choice that enabled the improvement of the structural and functional properties of the enzymes. Previously the thermostability of many protein have been significantly improved using
Discussion ...

directed evolution based approach that included esterases, lipases, amylases and xylanases (Spiller et al. 1999; Zhang et al. 2003; Machius et al. 2003; Andrews et al. 2004). The multistep directed molecular evolution, a widely used, dynamic tool for protein engineering, draws its power from iterative cycles of random nucleotide-sequence mutagenesis, colony expression, and screening of mutants. Its feasibility depends upon a dynamic screening assay to identify mutant clones against a background of (tens of) thousands of normal and null clones. Error prone PCR (epPCR) is by far the most used approach to produce random mutants. Directed evolution provides a powerful tool to unveil mechanisms of thermal adaptation and is an effective and efficient approach to increasing thermostability without compromising enzyme activity. A relatively small number of amino acid substitutions were needed to convert a mesophilic enzyme into a variant as stable as its thermophilic counterpart, without sacrificing catalytic activity.

After careful observation it was observed that though the lipase JkP01 shared more than 90% identity at amino acid level with earlier reported lipases from thermophilic sp. of Bacillus, the JkP01 lipase was observed to be less thermostable at higher temperature. Since rational engineering approaches have been only marginally successful in improving the lipase stability, we decided to turn to directed evolution methodology which has been quite successful in improving functional properties of many enzymes including lipases (Arnold et al. 2001; Jaeger and Eggert 2002). To expand the functionality of metagenomic JkP01 lipase, attempts have been to create mutants with improved resistance towards irreversible thermal inactivation using error prone PCR based technology. The method was designed to alter and enhance the error rate of the polymerase. The error prone PCR with varying ratio of nucleotides in the reaction mix along with high concentration of MgCl₂ was performed in order to stabilize the non-complimentary pairs. After the PCR amplification under error prone conditions, the library of variant
Discussion

Clone was constructed in pGEM-T easy vector. A mutagenic library was screened for the clones that showed enhanced thermostability at 60°C. The selection process consisted of incubating the mutant libraries at an elevated temperature as described in Materials and Methods and then assaying for residual activity (RA). It should be emphasized that this approach does not directly select for mutants that are active at elevated temperatures and consequently have higher Tm, but rather identifies variants with increased propensity for refolding (as compared with the wild-type enzyme) or/and those that are able to retain their native structure at the elevated temperature.

The screening of the mutagenic library resulted in lip M1 variant with enhanced thermostability at higher temperature. Mutant lip M1 (N384K) was selected that satisfied both established selection criteria (RA mutant > RA parent and the activity prior to inactivation was at least 75% that of the parent). A remarkable observation was that the lip M1 mutant lipase showed no loss of enzyme activity even after incubation at 60°C for 1h. While JkP01 enzyme got inactivated at 60°C within 15 min. By sequence analysis, a transverse type of mutation was observed with a thymine nucleotide at 1152 position was replaced with guanine nucleotide in JkP01 ORF (open reading frame). The changed nucleotide sequence resulted in change of triplet codon AAT (Asparagine) to AAG (Lysine) and was observed near one of the catalytic triad residue histidine, present towards the C terminus of the protein. As there was only one amino acid change noted by sequence comparison to the Wt lipase, it might be assumed that the thermostability comes from the change of N384K. It was interesting to note here that though the optimum temperature of the mutant enzyme activity decreased slightly when compared to the Wt enzyme, the thermostability was enhanced several fold in comparison to the Wt enzyme. No change in optimum pH and pH stability was observed when compared with Wt. Similar results were reported by Khurana et al. (2010). In contrast, Eggert et al. (2003) observed the
Discussion …

change in pH stability in a lipB variant, A76G, that was thermo-labile at alkaline pH but stable at pH 5.0 when compared to the Wt.

The kinetic studies of the mutant N384K revealed interesting facts. The N384K mutation resulted in decreased $K_m$ and increased $V_{max}$ in comparison to Wt. The increase in the lipase activity of the mutant might be due to the increase in the $k_{cat}$ as well as due to its increased affinity for the substrate. The 20 fold increase in the $k_{cat}/K_m$ value of lip M1 in comparison the Wt lipase might be attributed to its increasing affinity for the substrate, though the possibility of a concurrent increase in the $k_{cat}$ value can not be ruled out. Therefore, the mutation at 384 could affect the lipase activity either by improving the affinity with the substrate (decreased $K_m$) or by increasing the catalytic efficiency ($k_{cat}$), but the latter was more interesting from a mechanistic point of view.

The single amino acid substitution N384K so close to the catalytic centre of the enzyme provide excellent opportunity to consider the probability of remodeling of the entry to the enzymes active site to better accommodate the broad range of substrate. In deed the increase in $k_{cat}/K_m$ of N384K could be the result of mutant’s more open conformation and high enzyme activity. Replacing polar amino acid residue to positively charge bulky amino acid might also change the geometry of its immediate vicinity presumably by changing the hydrogen bonding. The changed conformation might be playing a role in faster dispersion of product away from catalytic microenvironment, resulting in enhanced catalytic performance.

As CD spectrum of a protein was considered to be a sensitive indicator of tertiary/ quaternary structural changes in the protein, we investigated and compared the solution structure of the mutant (lip M1) and the wild-type (Wt) lipase by fluorescence and circular dichroism spectroscopy. We have followed changes in intrinsic fluorescence and far-UV CD upon heating. Far-UV CD spectra for lip M1 purified protein indicated that
there was no significant changes in lipase secondary structure up to 80°C as compared to the Wt enzyme where the secondary structure was distorted after exposing the protein above 35°C. These result indicated that the enzyme was able to retain its secondary structure even at high temperature. On the other hand intrinsic fluorescence data is suggesting that there was not much loss in tertiary structure observed both in Wt and lip M1 purified enzymes.

The error prone PCR and DNA shuffling approach has been utilized to improve the thermostability and optimum temperature of the *Rhizopus arrhizus* lipase enzyme (Ning et al. 2006). The optimum temperature of a mutant with three substitutions was increased to 10°C high as compared to Wt and the thermostability was increased 12 fold at 50°C. Ahmed et al. (2008) reported the improvement in the thermostability of mesophilic lipase enzyme of *Bacillus subtilis* using directed evolution based approach. A total of six stable mutations were introduced in two generation with three mutations per generation. Each of these mutations additively contributed to the thermostability of the enzyme. The combination of all the six mutations produced a variant that showed remarkable increase of 15°C in the melting temperature. This resulted in millionfold increase in the thermal inactivation of the enzyme. Most of the studies describe changes in more than one amino acid for getting reasonably good thermostability. In this study the lip M1 mutant lipase (N384K mutation) demonstrated more than 140 folds increase in thermostability at 60°C in comparison to Wt. Most of the other reported thermostable variants demonstrated the change in surface amino acids (Khurana et al. 2010; Ahmed et al. 2008). Acharya et al. (2004) reported nearly 10 fold increase in half life of lip A at 50°C by a single mutation N166Y. In another study Khurana et al. (2010) demonstrated 3 fold increase in half life of a mesophilic lipase at 50°C by changing an isoleucine on the protein surface with threonine. The existence of mutants that are significantly more thermostable without losing enzyme...
activity indicates that activity and thermostability are at least partially independent properties and therefore both of these properties can be optimized in the same enzyme. During the course of in vitro evolution, Wt lipase was evolved for thermostability without sacrificing its activity at lower temperature. It is interesting to observe that the catalytic efficiencies of the lip M1 mutants were not compromised, while the thermostability was dramatically enhanced. These results further support the observation that the structure of a protein has higher tolerance for change in structural amino acids than the functional ones (Schreiber et al. 1994). In several reports an inverse correlation between protein stability and enzyme activity has been indicated. Patkar et al. (1998) reported a mutant protein with single amino acid substitution had an increased half life at 60°C but only 50% of its original activity compared to the Wt. Similarly the maltogenic amylase M375T mutant, which was located near the active site of ThMA, probably caused a conformational or dynamic change that enhanced thermostability but reduced the specific activity of the enzyme (Kim et al. 2003).

Morley and Kazlauskas (2005) argued that the mutation altering the activity of the enzyme reside close to the active site. It was surprising to note by sequence comparison with other lipases that the N384K was already present in the Bacillus stearothermophilus L1 lipase with which the Wt lipase showed 95% amino acid similarity. L1 lipase demonstrated higher thermostability in comparison to Wt lipase. Therefore we tried to look for any other mutations near the active site residue present in Bacillus stearothermophilus. We noticed another change at residue 344 where a glutamic acid (E) near the active site aspartic acid (D) at position 346 was changed to glycine (G). Therefore E344 near the active site was our next target. By site directed mutagenesis we converted 344 E to G in Wt lipase. The variant clone so obtained was named as lip M2. Surprisingly contrary to our initial expectations, the thermal stability of lip M2 showed no alteration with respect to Wt.
However the enzyme showed higher $K_m$ and lower $V_{max}$. This observation was astonishing at first since the mutation E344G, that also was near the Aspartate residue (one of the catalytic member of the catalytic triad), behaved in contrary to the first observed mutation (N384K) near histidine residue. The lip M2 lipase showed almost same thermal denaturation profile comparable to the Wt. The optimum pH and pH stability of lip M2 shifted towards little lower side (around pH 8.0). The change of charged residue to hydrophobic residue near active site might be responsible for this. It was further observed that the affinity of the substrate (pNP laurate) with lip M2 lipase decreased resulting in higher $K_m$. The catalytic efficiency of the enzyme also decreased as represented by $k_{cat}$. Surprisingly in contrast to Wt, E344G mutants displayed enzyme activity towards short chain pNP-ester also. The single amino acid substitution E344G might be playing a role in remodeling the entry of the substrates to the active site of the enzyme. Indeed the decrease in $k_{cat}/K_m$ of E344G could be the result of changed confirmation of active site.

To further analyze the effect of double mutation, that was already present in *Bacillus steatorrheophilus* L1, on lipase activity and thermostability lip M1 (N384K) was used for site directed mutagenesis to create E344G mutation in it. The clone with these two mutations was named as lip M3. These mutations (N384K and E344G) were confirmed by sequencing. Thermal stability of the double mutant was several folds less than the lip M1 and was little higher than Wt. Since the thermal stability of the double mutant lip M3 sharply declined in comparison to lip M1, it was assumed that E344G mutation exerted a negative influence on enzyme thermal stability provided by N384K. Therefore amino acid substitutions at positions 384 was determined to be critical for lipase stability, while the residue at position 344 had only a marginal effect on its own but somehow interacting with 384K to exert the negative effect on thermostability. The substrate specificity of double mutant was shifted.
Discussion ...

towards short chain substrates in comparison to Wt and very similar to the *B. stearothermophilus* L1 substrate specificity.

In conclusion this study demonstrates how forced protein evolution was used to introduce changes resulting in enhanced thermal stability into industrially relevant enzymes. This study describes a stabilizing mutations not previously described. The directed evolution of the cloned gene successfully identified the mutation site 384 as hot spot for selectively enhancing the thermal stability as well as lipase activity. The mechanism of thermal stability of the mutant needs to be explored and pave a way to correlate structure function relationship. To the best of our knowledge there is no report available showing such enhancement in thermostability by single amino acid change that also near the active site. The mechanism by which E344G nullifies the effect of N384K on protein thermostability needs further investigation. By homology modeling we could not propose a relevant hypothesis. Attempts are being made to crystallize the protein for detail analysis.