CONCLUSION
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*Bacillus* sp. J33-A producing extracellular thermostable lipases, was isolated from hot springs of Himachal Pradesh, India. It produced maximum zone of hydrolysis and precipitation on tributyrin (1%) and Tween-80 plates respectively. The organism was unable to grow below 50°C and its lipase was found to retain 90% activity at 60°C up to 7 days. The enzyme was chosen for the study because of its higher thermostability.

In nutrient broth medium, the organism produced 26 U ml⁻¹ of lipase in the log phase of growth. The optimum pH and temperature for the growth and enzyme production of the organism were 8.0 and 60°C respectively. Amongst various nitrogen sources, peptone was required for the growth and lipase production of *Bacillus* sp. J33-A. Maximum lipase production was obtained with 1% D-galactose and sorbitol as carbon source and cod-liver and mustard oil (1%) as oil source in shake flask culture. Amongst different additives, there was no growth in presence of Tween but addition of starch and skim milk enhanced the enzyme production. Growth and enzyme production were found to be maximum at 0.2% starch concentration. Aerobic conditions were also found to be necessary for *Bacillus* sp. J33-A lipase production as growth and lipase production got enhanced with aeration.

Effect of various readymade media and agro-waste products on lipase production was studied and it was found that maximum amount of lipase was produced when wheat bran (1%) was taken with nutrient broth media (1%) under submerged liquid cultivation. The maximum activity observed was 366 U ml⁻¹ at 48 h with 2% inoculum. In case of solid substrate cultivation, use of wheat bran as solid substrate enhanced the lipase production of *Bacillus* sp. J33-A up to the extent of 14300 U gm⁻¹ of wheat bran after 72 h of cultivation. The organism was also immobilized on
polyurethane foam cubes to assess the lipase production and it was found that it increased with increase in the supplementation of number of cubes of polyurethane foam in wheat bran medium. Polyurethane foam was acknowledged to be a good support medium for immobilizing Bacillus sp. J33-A and enhancing the lipase production.

Production of thermostable lipase by parent Bacillus sp. J33 and earlier Bacillus J33-A was compared in wheat bran and nutrient broth medium and it was noticed that up to 96 h, lipase production by Bacillus sp. J33-A was 805 U ml\(^{-1}\) while the maximum level of production in case of Bacillus J33 was 450 U ml\(^{-1}\) at 36 h and it decreased after that. Therefore, Bacillus sp. J33-A was chosen for further studies.

The crude lipase was purified by ammonium sulphate precipitation, hydrophobic interaction chromatography, gel filtration on Sepharose-6B, Q-Sepharose ion-exchange chromatography and dialysis. Hydrophobic interaction chromatography revealed that the crude lipase preparation consisted of two lipases. These two lipases were then separately purified and later on checked for the purity on SDS-PAGE. The two lipases named peak I lipase & peak II lipase, were purified to 58-fold & 4-fold with 10\% & 0.38\% yield respectively and their specific activities were calculated to be 9730.5 & 669.57 U mg\(^{-1}\) respectively. Both the lipases showed a single band on SDS-PAGE and also the zone of clearance on tributyrin plate. By non SDS-PAGE, both the lipases showed the esterase staining bands at the same position. It was also observed that these lipases possessed same molecular weights i.e. 61 kDa. While doing gel permeation chromatography on Sephadex G-200, both the lipases eluted out in the same fractions and therefore had relative molecular weights of about 60 kDa.

Effect of growth stage on the production of peak I and peak II lipase was checked and it was observed that as the incubation time increased, the
production of peak I lipase increased. It was therefore concluded that peak I and II lipase production was growth phase associated. Both lipases were physico-chemically characterized and it was summarized that the optimum pH for peak I and II lipases were 9.0 & 8.0, whereas optimum temperatures were 65°C & 60°C respectively. The pH stability range for peak I and II lipases was pH 6.0-8.5 and 7.0-8.0 respectively and temperature stability range was 50-65°C & 50-60°C respectively.

Peak I lipase showed higher thermostability and greater shelf life (at 60°C) than peak II lipase. Half life of peak I lipase was also higher than peak II lipase. $K_m$ values of peak I and peak II lipases were found to be 0.50 & 0.19 mM respectively while $V_{max}$ were 0.139 and 0.032 µM ml$^{-1}$ min$^{-1}$ respectively. Mg$^{2+}$ caused the maximum enhancement in activity of both the lipases, while Hg$^{2+}$ completely inactivated it. Because of the potential utility of lipases in various organic reactions, several organic solvents were tried. Out of these, benzene and hexane showed highest stimulatory effect on both activity and stability of the lipases. These immiscible solvents are also needed during organic synthesis reactions catalyzed by lipases. Therefore, peak I and II lipases could be utilized for carrying out these synthetic reactions in the presence of water immiscible solvents.

Peak I lipase activity was completely inhibited by PMSF, SDS, β-ME, DTT and eserine. Thus indicating the involvement of a serine residue in the enzyme catalysis. The inhibition effect of EDTA was reversed by addition of Mg$^{2+}$ suggesting an important role of these divalent ions in the activity of peak I lipase. Addition of urea failed to exert any observable effect whereas DEPC caused a 50% loss of activity of both lipases. SDS, DTT and β-ME had no effect on peak II lipase while eserine at 5 mM concentration caused 33% inhibition of peak I lipase. Eserine caused no observable effect on the activity of peak II lipase. Thus both lipases differed in response to eserine. Proteolytic enzymes, trypsin and
chymotrypsin showed no effect on the activity of both lipases whereas thermolysin caused 50% inhibition.

Peak I lipase hydrolyzed small chain fatty acid more preferentially while peak II lipase hydrolyzed longer chain fatty acids. It points towards the greater esterase character in peak I lipase whereas peak II lipase had more of lipase character. With respect to positional specificity, both lipases attacked 3-positioned fatty acids with equal preference.

Immunological studies also confirmed that *Bacillus* J33-A was producing two lipases and that the concentration of second lipase was lower than the first lipase. Although both lipases had a great variation in their amino acid composition yet they partially shared their antigenic determinants. The antigenic epitopes of native peak I lipase was also completely accessible. ELISA, dot blot and western blot studies indicated that peak I and II lipases might be the different isoforms of one enzyme. Antibody binding site and active site were not overlapping in both the lipases.

On comparison of amino acid content, it was found that peak II lipase had very high content of alanine, which is known for causing the stabilization of the thermostable proteins. Both lipases had high percentage of hydrophobic amino acids that was also known for increasing the thermostability of proteins. Studies in protein alignments and amino acid composition comparison have now clearly shown that no single simple protein thermostabilization traffic rule can be defined for such enzymes.

Immobilization of peak I lipase was studied by using different matrices. Maximum adsorption was observed on CNBr-activated Sepharose 4B and Phenyl Sepharose. There was almost 100% adsorption of total lipase activity to these supports. Up to 50 days for 25 assay cycles after every two days, lipase activity was completely retained on CNBr-activated Sepharose 4B. Phenyl Sepharose was also found to have a good
retention capacity. Therefore both these supports can be used for immobilization of peak I lipase.

While trying for esterification of methanol with various acids, peak II lipase acted as a very good catalyst in the formation of methyl stearate while peak I was found to be a good catalyst for methyl oleate and caprylate. Therefore both these lipases can be utilized for esterification reactions in hexane.

The Thermophilic and alkalophilic isolate was found to be secreting two lipases which showed stability at high temperature and organic solvents. The production of lipase was found to be maximum and cost effective in agrowaste product (wheat bran). The unique properties exhibited by the lipase offers the opportunity to exploit the enzyme in oleochemical industry for biotransformation reactions. Apart from this, though standardization is required yet the enzyme can be used for dairy industry (for cheese ripening), as the enzyme can be easily immobilized with maximum stability and minimum leakage. The alkalophile nature of enzyme can be exploited in detergent industry also. In nutshell the lipase from *Bacillus* sp J33-A is another useful addition to ever expanding list of thermostable enzymes that hold promise for growing industrial enzyme market.