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ORIGINAL ARTICLE

Biochemical Optimization of Lipase Enzyme and Its 16srrna Sequencing Produced by *Bacillus licheniformis* Isolated from Rock Lobster

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ABSTRACT
Lipases from a large number of bacterial, fungal and plant and animal sources have been purified to homogeneity. Lipases isolated from different sources have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc. In the present study to explore the diversity between different sources screening of lipase producing organism were isolated from the intestine of the rock lobster and the biochemical characterization were optimized. Phylogenetic analysis revealed that it belongs to Bacillus licheniformisan active strain for lipase production. The most suitable for lipase production is olive oil and the maximum amount of paranitrophenyl palmitate released is found to be 0.69µg/ml/mints at 72hrs of incubation. The optimum temperature and pH for that particular organism to produce lipase is seems to be 5 and 37°C. Also, it resembles 99% identity with that of other well-known characterized lipases; hence it is essential for large scale lipase production in industries.
***Keywords**: Rock lobster, Bacillus licheniformis, Lipase, Substrates.*

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INTRODUCTION

Lipases are considered to be the third largest group of industrial enzymes, subsequent to proteases and amylases. Lipases occur widely in bacteria, yeast and fungi [1]. Most of the lipase research focuses on the production of extracellular lipases through a wide variety of microorganisms. Studies on the production of extracellular lipases with *Bacillus* have shown variations among different strains. However, the requirement for lipid carbon source remains essential for enzyme production. Most of the lipase research focuses on the production of extracellular lipases through a wide variety of microorganisms. Studies on the production of extracellular lipases with *Bacillus* have shown variations among different strains. Lipases differ greatly as regards both their origins (which can be bacterial, fungal, mammalian, etc.) and their properties, and they can catalyze the hydrolysis, or synthesis, of a wide range of different carboxylic esters and liberate organic acids and glycerol. They all show highly specific activity towards glyceridic substrates [2]. However, the requirement for lipid carbon source remains essential for enzyme production. The tremendous potential of lipases in various fields shows the need to develop novel cost-effective technologies for increased production, scaling up and purification of this versatile enzyme. The properties of lipases are being improved by protein engineering and genetic engineering to wide their applications in extreme conditions. Now a day, microbial biotechnologists have shifted their importance to the commercial use of lipases from microbial origin, and a lot of strains from microbes have been screened and characterized for enzyme production. The most frequently used microbes for lipase production include *Penicillium sp., Candida rugose, Aspergillus niger, Rhizopus sp., and Pseudomonas sp.*, [18]. The range of microorganisms belonging to different genera of fungi, bacteria, and yeast have been isolated and characterized from different environmental conditions and functionally screened. Those

microbes are potential sources of lipases in many industries [19]. The enzymes of microbial origin have proved distinct physiochemical and biological characteristics that also showed their importance as prominent biocatalysts and have proved to be competent alternatives source to traditional organic techniques in the selective transformation of complex molecules in different industrial purposes. Generally, lipases are commonly used in the food industry for the manufacture of a variety of food products (baked food, juices, and fermented foods). Although lipases are extensively used in industrial cleaners, leather processing, cosmetics, paper, and detergent industries, while other applications of lipases include biosensors, biodiesel production, biomedical applications, pesticides, and bioremediation are of great importance [20].

MATERIAL AND METHODS

Sample collection: Lobster sample was collected in a sterile bag from the fish market near Kanyakumari, Tamil Nadu, India and brought to the laboratory within 15 minutes.

Isolation of microorganisms from the intestine of the Lobster:

The intestine of the lobster was dissected out aseptically and homogenized with 0.089% of Nacl solution aseptically. The homogenate was serially diluted and inoculated on sterilized nutrient agar plates and then incubated at 37^0C for 24 hours. The colonies were counted for TVC (Total viable Count). The dominant colonies were selected for further identification.

Media used for the isolation of organism:

Peptone	-	3% (w/v)
Yeast Extract	-	1% (w/v)
Sodium Chloride	-	0.5% (w/v)
Olive Oil	-	1% (w/v)
pH	-	7

Lipase assay:

One Unit (U) of lipase activity was defined as the amount of enzyme solution liberating 1µmol of p-nitrophenol released per min under standard assay conditions.

Optimization of fermentation media:

The fermentation media prepared was optimized with following factors such as Incubation period, pH, Temperature and Lipid Substrates.

Optimization by Incubation Period:

The organisms were subjected to different incubation periods such as 24, 48 and72 hours using production media.

Optimization by different pH:

The microorganisms isolated from the rock lobster were incubated in the production media containing different PH varied from 5, 7 & 9.

Optimization by different temperature:

The microorganism in the production media was incubated at different temperature 27◦C, 37°C, and 47°C.

Optimization by Lipid Substrate

In order to optimize the enzyme lipase different lipid substrates were used, such as, Glycerol, Olive oil, Coconut oil, Fish Bone and chicken intestine.

Enzyme Assay: Microbial Culture was assayed for lipase enzyme activity using Spectrophotometric method [3]and results were recorded.

Principle:

In the present investigation one unit (U) of lipase activity was defined as the amount of enzyme solution liberating 1ìmol of p-nitrophenol per minute under standard assay conditions.

Reagents Required:

To carry out the study following reagents were required such as, Reaction Buffer (500ml) (It contains the mixture of 50 mM Tris Hcl (PH 9.0) and Triton X-100), 2-Propanol (20ml) p-nitrophenylpalmitate (P^{NPP}) The spectrophotometric method, using P-nitrophenyl Palmitate as substrate was applied for rapid and routine measurement of the lipase activity. Enzyme or blank solution (480ml) was added to the reaction buffer (500ml) which has 50mM Tris-HCI (PH 9.0) with variable concentrations of Triton x-100. The content was incubated at 25◦C for 5 minute and 10mM P^{NPP} in 2-propanol (20ml) was added to the enzyme buffer solution and shakes well. The mixture was emulsified for 2 minutes at 50◦C. The progress of the reaction was followed by monitoring the change in the absorbance at 400nm over a period of 5 minutes at 500C using Perkin-Elmer Spectrophotometer. The molar extinction coefficient of P-nitrophenol (E=16.900M-1 Cm-1) was estimated from the absorbance measured at 400 nm of standard solutions of P^{NP}.

16SrRNA sequencing:
DNA isolation from bacteria culture using the phenol chloroform method of Genomic DNA isolation. Amplification of the 16SrDNA region using primers designed in the conserved region, the primers used were FD1 and RP2.Sequencing of the ~1.5kb region using internal sequencing primers and all the processes were processed with the help of Bioserve accelerating discovery system.

RESULT
Lipase activity was assayed by means of different substrates like Glycerol, Olive oil, Coconut oil and Fish bone. The production of lipase was observed at varying pH (5,7 & 9), temperature (27°C, 37°C & 47^{0}C), substrate concentration (0.1, 0.2, 0.3, and 0.4) and at different time intervals (24, 48, and 72 hrs). Lipase producing organisms was isolated from rock lobster and was noted as k2.It was identified as lipase producing organism by comparing with Bergey's Manual of Determinative Bacteriology and it was identified as *Bacillus licheniformis* by means of 16srRNA sequencing. The effect of lipase production by the *Bacillus licheniformis(k2) w*ith various substrates (glycerol, olive oil, coconut oil and fish bone) at different incubation periods (24hrs, 48hrs and 72hrs) were carried out and the results were indicated in Fig.1. The most suitable substrate for lipase production was identified as olive oil, and the maximum amount of paranitrophenyl palmitate released was recorded as0.69 µg/ml/min at 72hrs of incubation period. The minimum amount of paranitrophenyl palmitate was released with coconut oil was recorded as 0.08 µg/ml/min at 24hrs of incubation time.

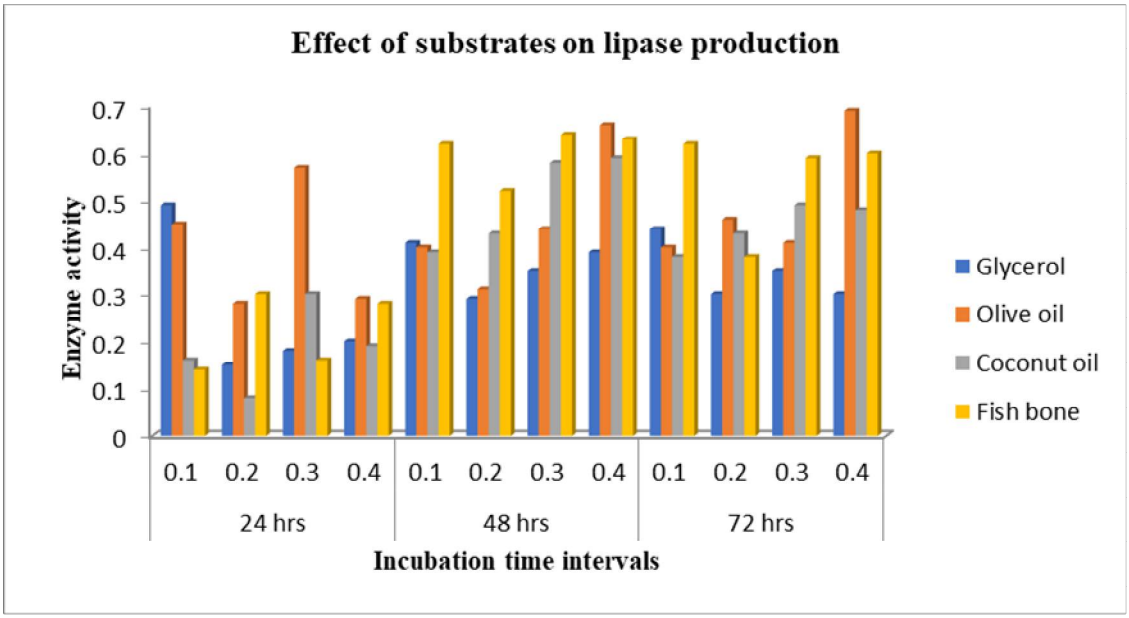

Fig.1 Effect of different substrates on lipase production by *Bacillus licheniformis (K2)*

The effect of pH on lipase production at various incubation time intervals have been carried out and the results were indicated in Fig. 2. At 48hrs of incubation at pH 5, the *Bacillus licheniformis (K2)* showed the maximum production lipase and the amount of paranitrophenyl palmitate released was recorded as0.79 µg/ml/min, whereas the minimum production was noted at pH 5 at 24hrs of incubation period and the amount of paranitrophenyl palmitate released was recorded as0.48 µg/ml/min.

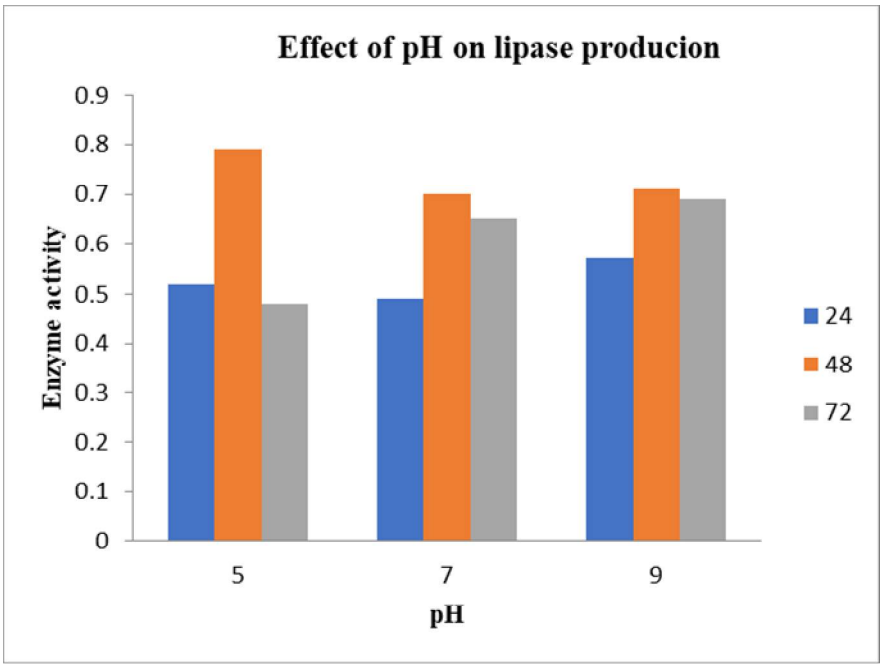

Fig.2 Effect of different pH on lipase production by *Bacillus licheniformis(k2)*

The effect of different temperatures on lipase production at various time intervals was shown in Fig.3. At 72hrs of incubation at 37°C, the *Bacillus licheniformis(k2)* showed a maximum production and the amount of paranitrophenyl palmitate released recorded was 0.59 µg/ml/min, whereas the minimum production observed at 47°C at 24hrs and the amount of paranitrophenyl palmitate released was recorded as 0.27 µg/ml/min.

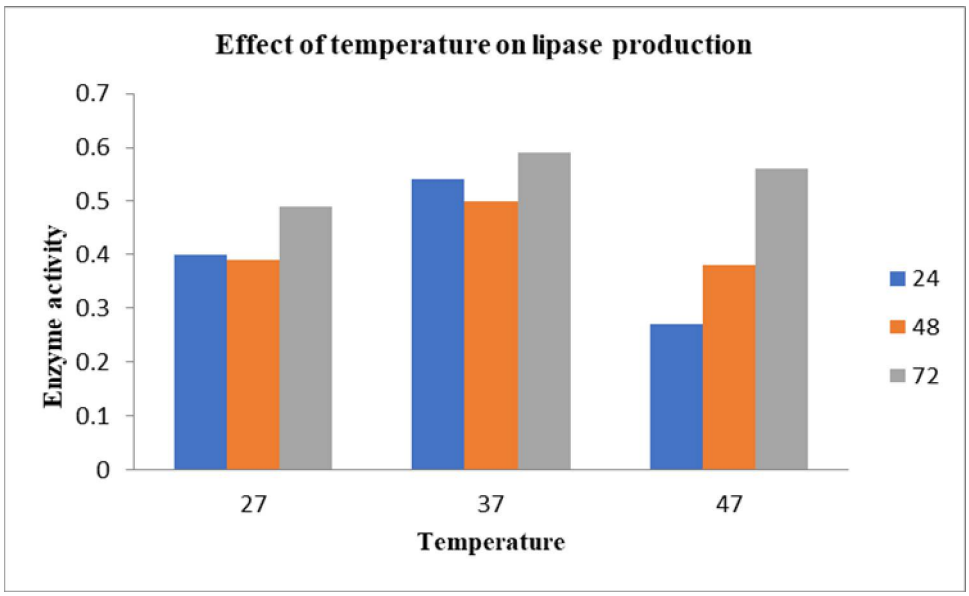

Fig.3 Effect of different temperature on lipase production by *Bacillus licheniformis(k2)*

16Sr DNA Sequence of Sample K2:

1 AGAGTTTGAT CCTGGCTCAG GACGAACGCG GGCGGCGTCC TTAATACATG CAAGTCGAGC
61 GGACCGACGG GAGCTTGCTC CCTTAGGTCA GCGGCGGACG GGTGAGTAAC ACGTGGGTAA
121 CCTGCCTGTA AGACTGGGAT AACTCCGGGA AACCGGGGCT AATACCGGAT GCTTGATTGA
181 ACCGCATGGT TCAATCATAA AAGGTGGCTT TTAGCTACCA CTTACAGATG GACCCGCGGC
241 GCATTAGCTA GTTGGTGAGG TAACGGCTCA CCAAGGCGAC GATGCGTAGC CGACCTGAGA
301 GGGTGATCGG CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTAG
361 GGAATCTTCC GCAATGGACG AAAGTCTGAC GGAGCAACGC CGCGTGAGTG ATGAAGGTTT

421 TCGGATCGTA AAACTCTGTT GTTAGGGAAG AACAAGTACC GTTCGAATAG GGCGGCACCT
481 TGACGGTACC TAACCAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA
541 GGTGGCAAGC GTTGTCCGGA ATTATTGGGC GTAAAGCGCG CGCAGGCGGT TTCTTAAGTC
601 TGATGTGAAA GCCCCCGGCT CAACCGGGGA GGGTCATTGG AAACTGGGGA ACTTGAGTGC
661 AGAAGAGGAG AGTGGAATTC CACGTGTAGC GGTGAAATGC GTAGAGATGT GGAGGAACAC
721 CAGTGGCGAA GGCGACTCTC TGGTCTGTAA CTGACGCTGA GGCGCGAAAG CGTGGGGAGC
781 GAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA GTGTTAGAGG
841 GTTTCCGCCC TTTAGTGCTG CAGCAAACGC ATTAAGCACT CCGCCTGGGG AGTACGGTCG
901 CAAGACTGAA ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGTGGAGC ATGTGGTTTA
961 ATTCGAAGCA ACGCGAAGAA CCTTACCAGG TCTTGACATC CTCTGACAAC CCTAGAGATA
1021 GGGCTTCCCC TTCGGGGGCA GAGTGACAGG TGGTGCATGG TTGTCGTCAG CTCGTGTCGT
1081 GAGATGTTGG GTTAAGTCCC GCAACGAGCG CAACCCTTGA TCTTAGTTGC CAGCATTCAG
1141 TTGGGCACTC TAAGGTGACT GCCGGTGACA AACCGGAGGA AGGTGGGGAT GACGTCAAAT
1201 CATCATGCCC CTTATGACCT GGGCTACACA CGTGCTACAA TGGGCAGAAC AAAGGGCAGC
1261 GAAGCCGCGA GGCTAAGCCA ATCCCACAAA TCTGTTCTCA GTTCGGATCG CAGTCTGCAA
1321 CTCGACTGCG TGAAGCTGGA ATCGCTAGTA ATCGCGGATC AGCATGCCGC GGTGAATACG
1381 TTCCCGGGCC TTGTACACAC CGCCCGTCAC ACCACGAGAG TTTGTAACAC CCGAAGTCGG
1441 TGAGGTAACC TTTTGGAGCC AGCCGCCGAA GGTGGGACAG ATGATTGGGG TGAAGTCGTA
1501 ACAAGGTAGC CGTAG

Phylogenetic analysis of k2:

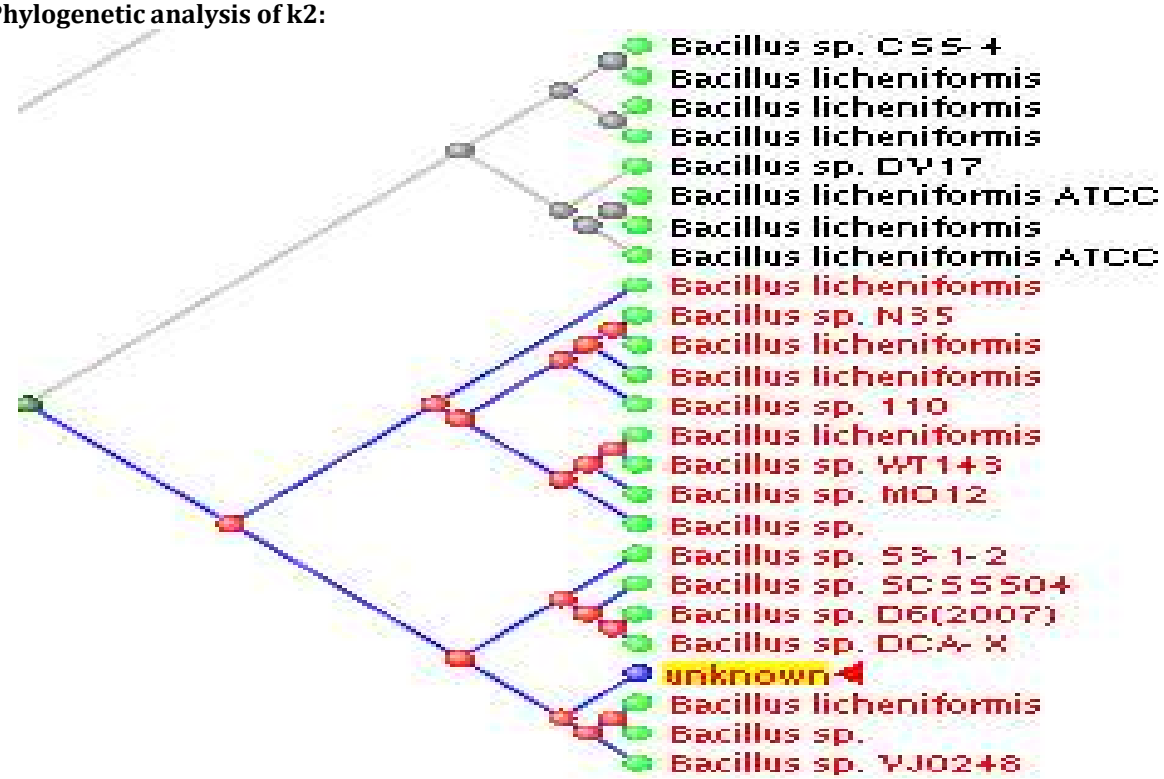

Fig 4: Phylogenetic analysis of *Bacillus licheniformis (*k2)

DISCUSSION

Lobsters harbor a diverse microbial flora and most of the microorganisms are able to produce enzymes. Lipase producing microorganism isolated from the lobster was identified as *Bacillus licheniformis(k2).*The lipase enzyme activity was assayed with varying pH , temperature and substrates at different time intervals .In the present study the effect of lipase enzyme activity with the olive oil as a substrate showed the maximum production (0.69 µg/ml/min) at 72hrs of incubation and the minimum amount of paranitrophenyl palmitate released in coconut oil as a substrate was recorded as 0.08 µg/ml/min at 24hrs of incubation. Among the various substrates, gingili oil cake and wheat bran were found to be the best substrates [4]. Whereas in the present investigation, the olive oil used as substrate was found to be the best with a high enzyme production. Among all the tested oils, the affinity results suit the best with rice bran oil as after oleic acid, the highest percentage of composition is linoleic acid and palmitic acid.

Like, in case of olive oil, the highest composition is of oleic acid but it has very low percentage of linoleic and palmitic acid).

At 48hrs of incubation with pH 9, the *Bacillus licheniformis(k2)* showed the maximum production and the amount of paranitrophenyl palmitate released was recorded was 0.89 µg/ml/min and the minimum production at pH 5 at 48hrs the amount of paranitrophenyl palmitate released was 0.79 µg/ml/mi. The lipase enzyme was active in the range of pH 7-9, and the maximal activity was observed at pH 9.0. It is a characteristic that the most microbial lipases, the optimum pH fall on the alkaline side. In present study the similar trend was observed that pH was optimum and it falls on the alkaline side [5]. Alkaline lipases are important in many industrial applications like leather processing, detergent formulations and sewage treatment [17]. At 72hrs of incubation at 37°C the *Bacillus licheniformis(k2)* showed the maximum production and the amount of paranitrophenyl palmitate released was observed as 0.59 µg/ml/min and the minimum production noted at 47°C at 24hrs, and the amount of paranitrophenylpalmitate released was recorded as 0.27 µg/ml/min. In contrast, in the present study, the PNPP-hydrolysing activity had a clear temperature which is "optimum" at 37°C even though the enzyme was fully stable up to 80°C for at least 15 min, which is much longer than the duration of the activity assay [6].The overall properties showed by *Bacillus licheniformis(k2)*indicated that the cloned lipase is a mesophilic enzyme acting on a broad range of pH that becomes inactivated only by a limited number of lipase inhibitors. In the present study, pH was varied using different substrates concentration [7]. In connection with their biochemical properties, both lipolytic enzymes display different substrate specificities [8]. In the present study the substrate specificities are done using Glycerol, Coconut Oil, Olive Oil, and Fish Bone as substrates. The cell produces lipases in order to obtain the energy required to form new cells from the available carbon sources and at the same time it does a normal metabolic activity irrespective of growth [9]. Comparing results of lipolytic activities with those of other research studies is difficult owing to the different methodologies used by different groups. However, comparing the results obtained in this work with those reported using the same methodology and reaction substrate. Glucose, Olive Oil, Peptone and Fecl3 6H2O were found to have more significance on lipase production by *Candida rugosa*.[10]. In the present study glucose, olive oil, coconut oil and fish bone are used as substrates and among that fish bone showed the highest enzymatic production. The lipase purified in the current study would be a better choice to use in the detergent formulation as it is active in alkaline pH ranging from 7 to 9 with optimum activity at pH 8 and temperature range of 5–65°C with an optimum at 37°C. Lipases with activity in the pH range of 7–9 with an optimum at 7 and temperature range of 0–60°C with an optimum at 15°C [15], pH range of 7–11 with an optimum at 8.5 and temperature range of 5–37°C with optimum at 20°C [16] were suggested as good detergent additives in previous studies. The compatibility of the purified enzyme with common commercial detergents was checked to see its effectiveness. The activity of purified lipase increased by ~50% in the presence of all the tested commercial detergents at a temperature ranging from 5 to 60°C.

Sequencing and phylogenetic analysis were processed with the bioserve and the organism which produced the lipase resembles 99% identity with that of the other well characterized lipases isolated from other natural samples. The amino acid sequences and the conserved areas are also well matched with other bacterial species. A new family of bacterial lipases from a Korean flat met library has been reported; an important feature of this family is an Arg-Ala sequence that can serve as an oxyanion hole [11]. It has also been reported that enzyme properties, including molecular weight, pH and temperature optima, stability, and substrate specificity, of about 30 bacterial lipases from different species existing in the different environment are significantly different [12]. Binding of detergent at the active sites of the enzyme leading to its conformational change has been reported to be the possible reason for such improved substrate accessibility [13]. The increase in the activity of ERMR1:04 lipase in presence of commercial detergents is in agreement with several earlier lipase studies [14]. The studied lipase with activities in a broad temperature and alkaline pH proves to be a suitable detergent additive for enhanced washing performance at different environments ranging from colder to moderately high-temperature regions.

CONCLUSION

Lipase producing microorganism isolated from the lobster was identified as *Bacillus licheniformis(k2).* The lipase enzyme activity was assayed with varying pH, temperature and substrates at different time intervals. Among the substrates olive oil shows the maximum production and also it is effective substrates for lipase production. The optimum temperature and pH for that particular organism to produce lipase is seems to be 5 and 37°C. Also, it resembles 99% identity with that of other well-known characterized lipases; hence it is essential for large scale lipase production.

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