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EXPLORING MARINE BACTERIA-DERIVED LIPASES FOR SUSTAINABLE DETERGENT FORMULATIONS: PRODUCTION, PURIFICATION, AND STABILITY ASSESSMENT

Yoonus Pariyarath[1*], Lali Growther[2]

[1,2] Department of Microbiology, Hindusthan College of Arts and Science, Coimbatore, 641028, Tamil Nadu, India
*Corresponding Author: Yoonus Pariyarath, E.mail: yoonuspth@gmail.com,

ABSTRACT
Lipases, versatile enzymes produced by microbes, find extensive applications in diverse industrial processes, with notable significance in the laundry industry where lipase-based detergents efficiently eliminate lipid-based stains from textiles, enhancing cleanliness and sustainability. The utilization of microbial lipases in detergent formulations offers significant potential for reducing environmental impact. Marine environments, renowned for their extreme conditions, harbor microorganisms with robust enzymatic capabilities, making them attractive candidates for enzyme production. In this study, lipase enzymes sourced from marine bacteria, particularly Brevibacillus borestelensis, were targeted for production and partial purification to assess their suitability as detergent additives. Through process optimization, lipase production was enhanced, and subsequent partial purification facilitated the evaluation of enzyme stability and activity in the presence of various commercially available detergents. The identification of the marine bacteria was achieved through sequencing the 16S rRNA gene, confirming their taxonomic classification. The purified lipase exhibited a molecular mass of approximately 35 kDa and a specific activity of 110.8 U/mg. Furthermore, the stability of the lipase with commonly used detergent powders was evaluated, demonstrating compatibility with various detergents, with more than 80% relative activity retained. These findings underscore the potential of microbial lipases, particularly those sourced from extreme environments, as environmentally friendly additives in detergent formulations, contributing to sustainable cleaning practices in the industry.

KEYWORDS: Lipases; Microbial enzymes; Marine bacteria; Detergent formulations; Sustainability

1. INTRODUCTION

Microbial enzymes offer several advantages over traditional chemical processes, primarily due to their greater process efficiency and environmental friendly nature. These enzymes are capable of functioning in diverse reaction conditions [1]. Substantial attention has been directed towards lipases, among the frequently used microbial enzymes like amylases and proteases, owing to their all-embracing applications [2].

Lipases are triacylglycerol hydrolases that can act on carboxylic ester bonds. They catalyse esterification as well as hydrolysis reactions in suitable conditions [3]. As they have diverse applications, lipases abides as a subject of enormous study [4]. Various researchers focused their work particularly on isolation, production, characterization, elucidation of mechanism of action, purification, kinetic studies and cloning of lipase gene [5]. Several industrial processes carried out with the aid of lipase enzymes. They include formulation of detergents, biosurfactant synthesis, bioconversions in dairy industry, manufacturing of paper and agricultural chemicals, cosmetics preparation and pharmaceutical processing. Lipases can also play major part in the processing of poly unsaturated fatty acid, γ- linoleic acid, methyl ketones and flavour molecules [6]. Simple glyceride interesterification into valuable forms of glycerides by lipase enzyme is also crucial in industrial processes. Lipases have immense role in laundry industry. Removal of lipids adsorbed in fabrics can be achieved by lipase added detergents [7]. Microbial based lipases are ecofriendly and it can be readily used along with commercially available detergents [8].

Enzyme producing microorganisms include bacteria, fungi and Actinomycetes, etc. They may be present in diverse habitats. Microbial enzymes isolated from extreme habitats have a prior importance due to the ability to withstand extreme conditions. Marine environment is one of such habitat as it constitutes extensive resources to provide ideal environment for microbial growth. Several enzyme producing bacteria were reported from marine ecosystems. Lipase producing bacteria reported from marine habitat constitutes Enterobacter sp, Klebsiella sp, Flavobacterium sp, Moraxella sp, Pseudomonas sp, Bacillus sp, and Arthrobacter sp. [9].

Lipase enzymes can be used in industrial processes both in homogeneous and heterogeneous conditions. Many commercial processes do not require homogenous preparations of lipase. However purity makes the enzyme more efficient in catalytic process. Purification of enzymes aids to study kinetic parameters, structural properties and primary

amino acid sequences. More purified enzyme preparations were employed in processing of pharmaceuticals, fine chemicals and cosmetics [6].

The conventional enzyme purification technique begins with the removal of cells through filtration or centrifugation. Additional cell lysis is carried out prior to centrifugation to release intracellular enzymes. Proteins in solutions can be concentrated using salts such as ammonium sulfate, and the salt can be removed through dialysis. More purified protein products can be obtained by employing techniques like gel filtration and chromatography. Gel filtration and anion exchange chromatography are valid chromatographic techniques.

The present study was designed in accordance with all the aspects discussed earlier. Lipase-producing bacteria were isolated from marine water, and submerged fermentation was exploited for enzyme production. Identification, molecular characterization, and enzyme purification were performed. The stability of the partially purified enzyme with commonly used powdered detergents was assessed, and the percentage of relative activity retained was determined.

2. MATERIALS AND METHODS

2.1. Sample collection and isolation of bacteria

Marine sediments and water samples were collected from the coastal areas of Cochin and Calicut, Kerala. These samples were serially diluted to obtain pure isolates, which were subsequently inoculated onto Zobell marine agar (Himedia) to encourage the growth of marine bacteria. The pure isolates were then stored at 4°C.

2.2. Screening of isolates for lipase production

The initial screening for lipase production involved the use of Tributyrin Agar (TBA). Pure isolates were inoculated onto nutrient agar emulsified with 1% tributyrin and then incubated for a period of 2 to 6 days at a temperature of 30°C. The presence of the lipase enzyme was indicated by the development of a zone of tributyrin hydrolysis. Isolates that exhibited lipase production were subsequently subcultured and stored.

2.3. Submerged fermentation for lipase production

Isolates with lipase-producing capability were inoculated into tributyrin broth and incubated at 37°C for 48 hours. The enzyme protein was recovered by centrifugation at 10,000 rpm for 15 minutes at 4°C. The cell-free supernatant was used as a crude protein source for further studies.

2.4. Quantitative analysis of lipase activity

The lipase activity of all isolates was quantitatively determined using a spectrophotometric method with p-nitrophenyl palmitate as the substrate [3]. For this assay, 0.25 ml of the crude enzyme solution was added to a reaction mixture consisting of 0.25 ml of 4mM pNPP and 0.5 ml of 50mM Tris HCl. Following 10-minute incubation at 25°C, the reaction was halted by adding 2 ml of 0.5N NaCO3. The yellow color produced by liberated p-nitrophenol was measured at 420 nm using a Shimadzu UV 1800 spectrophotometer. Standard p-nitrophenol solutions were prepared in the concentration range of 0-1 µg/ml. Enzyme activity was defined as one unit when it liberated 1 µmole of p-nitrophenol per minute.

2.5. Molecular Identification of lipase producing bacteria

The identification of specific bacterial isolates was achieved by analyzing the gene sequence of 16S rRNA. Genomic DNA from the selected bacteria was extracted using the NucleoSpin Tissue Kit from Macherey-Nagel, and its purity was assessed through agarose gel electrophoresis with a 0.8% agarose gel. The 16S rRNA gene was then amplified using the extracted genomic DNA as a template. This amplification process utilized a forward primer (5'CAGGCCTAACACATGCA AGTC3') and a reverse primer (5'GGGCGGGTGTACAAGGC3'). The PCR mixture consisted of 10X reaction buffer (2.5 µL), Taq polymerase (0.20 µL), DNA template (1 µL, 2ng), primers (1 µL each, forward and reverse), dNTPs (2.5 µL of 2 mM solution containing dATP, dTTP, dGTP, and dCTP), and nuclease-free water (16.8 µL). The PCR thermal profile began with an initial heating step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 1 minute. The reaction concluded with a final extension step at 72°C for 3 minutes, and the amplified product was verified using 1.2% agarose gel electrophoresis. The purification of the PCR product was carried out using the Ultraclean PCR Clean-up Kit from Mo Bio Laboratories, Inc., California. Subsequently, the amplified product was sequenced at the Rajiv Gandhi Centre for Biotechnology, Trivandrum, India, and the quality of the sequence was assessed using Sequence Scanner Software v1 from Applied Biosystems. The consensus DNA sequence was then used for analysis, including similarity searches using BLAST to identify the organism and evolutionary analyses conducted using the MEGA11 program.

2.6. Selection of suitable cultural conditions for enhanced lipase production

Various cultural conditions influencing bacterial growth and enzyme production were meticulously analyzed and optimized using the one factor at a time method. The impact of each factor was assessed while holding all other variables constant. The optimization process entailed determining the most suitable carbon source options (glucose, fructose,

sucrose, or maltose), nitrogen sources (soybean meal, beef extract, peptone, or ammonium sulfate), inducer oils (Tween 80, sunflower oil, tributyrin, or olive oil), pH levels (5, 6, 7, or 8), and temperatures (25°C, 30°C, 37°C, or40°C).

2.7. Purification of lipase enzyme

2.7.1. Ammonium sulphate precipitation and protein dialysis

The bacterial strain was inoculated into 600 ml of tributyrin broth with the appropriate concentration of additional carbon and nitrogen sources. It was then incubated at 30°C for 3 days under shaking conditions. The crude protein solution was obtained by centrifugation at 10,000 rpm for 10 minutes at 4°C. Protein molecules from the crude preparation were precipitated by saturating with ammonium sulfate. The optimum concentration of ammonium sulfate required to precipitate the lipase protein was determined by adding ammonium sulfate at various concentrations (20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% saturation) with continuous stirring at 4°C. Precipitated protein from each level of saturation was recovered by centrifugation at 10,000 rpm for 15 minutes at 4°C in 24-hour intervals. Ammonium sulfate associated with the precipitated proteins was removed using a dialysis technique. Precipitated protein fractions were suspended in the minimum quantity of Tris-HCl buffer (0.1 M, pH 8) and dialyzed in a pretreated dialysis tube (12,000 kDa) against a 0.01 M solution of Tris-HCl buffer at pH 8 at 4°C for 24 hours, with 4 changes of buffer. The samples were then assayed for lipase activity, and protein content, specific activity, and fold of purification were determined.

2.7.2. Purification by ion exchange chromatography

The most active fraction of dialysis was subsequently subjected to further purification through ion exchange chromatography, employing DEAE-cellulose as the column material. DEAE-cellulose was activated and meticulously packed into a glass column. The column was then equilibrated overnight using a phosphate buffer at pH 8.0 (100 mM). Subsequently, 3 ml of the dialyzed sample was applied to the cellulose column and eluted in a stepwise manner, with a flow rate of 1 ml/min, utilizing a linear gradient of 0.1 to 0.5 M NaCl in the same buffer and 1 ml fractions were collected. Lipase activity and protein concentration of the most active fraction were quantified via spectrophotometry. The specific activity, yield, and fold of purification were calculated.

2.7.3. SDS PAGE profiling of purified lipase

Following ion exchange chromatography, obtained fraction of purified lipase was analyzed by means of SDS-PAGE on a 12% (v/v) polyacrylamide gel stained with Coomassie Brilliant Blue R-250. To determine molecular weight of the purified protein, relative mobility in the gel was compared with standard marker proteins.

2.8. Evaluation of detergent additive property

The stability of a partially purified lipase with detergents was investigated by assessing lipase activity in the presence of commonly available powdered detergents in the market. Solutions of various detergents were prepared at a concentration of 7 mg/ml and were heated to 80°C for 1 hour to deactivate any preexisting lipase protein. Next, 2 ml of properly diluted enzyme was incubated with 50 ml of the detergent solution at 30°C for 1 hour. The reaction was then terminated, and enzyme activity was assessed using the previously described method. Relative activity was determined as a percentage by comparing it to the activity of the enzyme solution without any detergents.

3. RESULTS

3.1. Isolation of lipase producing bacteria

Water samples were analyzed for the presence of bacteria. A total of 32 isolates were obtained and screened for lipase production by using tributyrin agar and 8 best isolates were subjected for quantitative assay. Isolate showed largest zone of tributyrin hydrolysis and enzyme activity of 15.6 U/ml was selected for further studies (Figure 1 A and B).

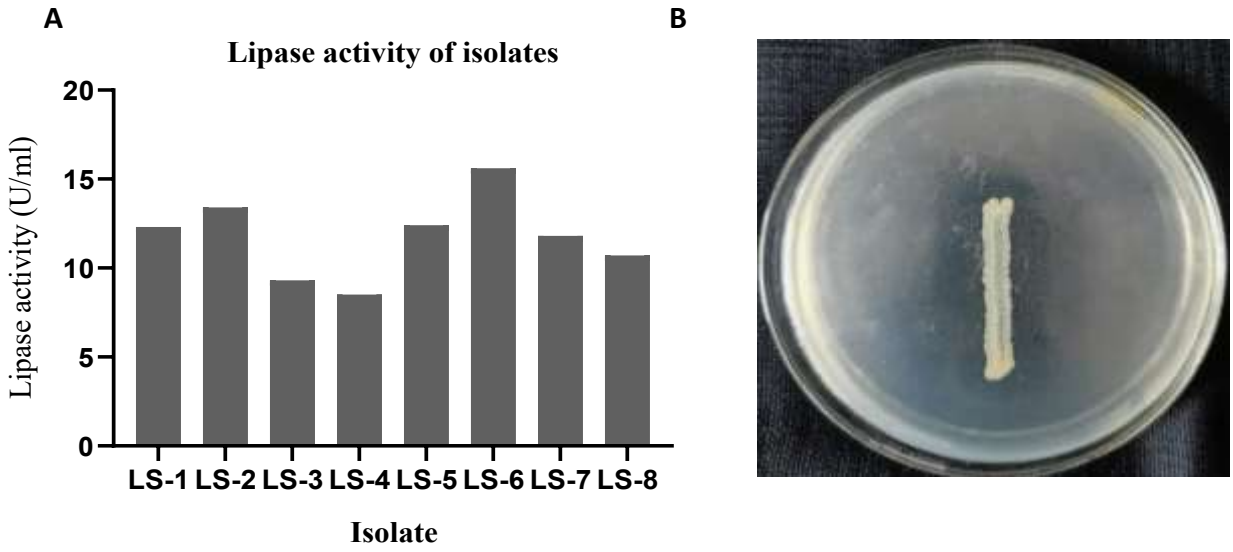

Figure 1: (A) lipase activity of isolates LS1-LS8. (B) Zone of hydrolysis by isolate LS-6 on TBA plate

3.2. Molecular Identification of bacteria

The chosen bacterial isolate underwent identification using the 16S rRNA gene sequence. A BLAST similarity search revealed a 100% identity match with Brevibacillus borstelensis. The identified bacterial gene sequence has been submitted to NCBI under the accession number OR591304. The Neighbor-Joining approach was used to infer the evolutionary history. The best tree is displayed (Figure 2). Next to the branches are the percentages of duplicate trees in which the related taxa clustered together in the bootstrap test (1000 replicates). The evolutionary distances were calculated using the Maximum Composite Likelihood technique and are in base substitutions per location. The final dataset contained a total of 635 locations. MEGA11 software was used to undertake evolutionary analysis.

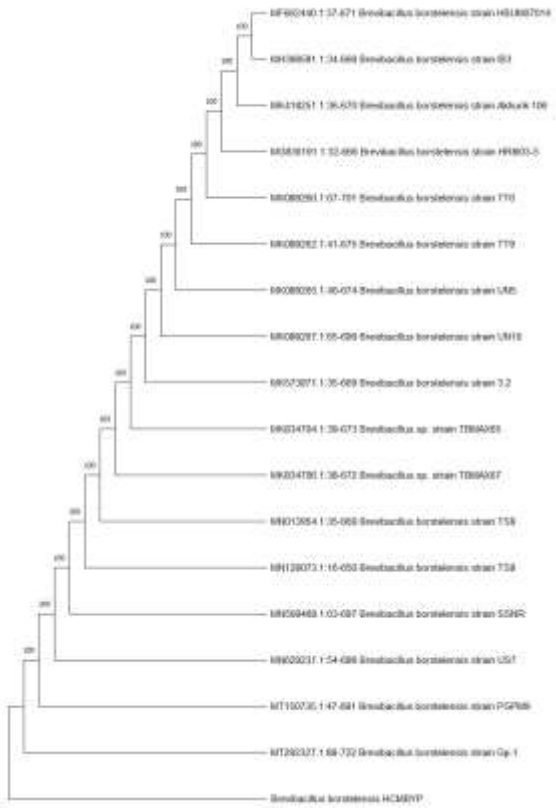

Figure 2. Phylogenetic tree of isolated bacteria

3.3 Optimization of lipase cultural conditions for lipase production

This study systematically looked at a number of factors affecting the synthesis of lipase. There was a noticeable increase in the production of lipase enzyme when glucose was added when compared with lipase production in the presence of fructose, sucrose, and maltose in the medium. (Figure 3A). Ammonium sulfate (Figure 3B) and Tributyrin (Figure 3C) were found as the best nitrogen source and inducer oil respectively. The isolated bacteria showed better production of enzyme lipase at pH 8 (Figure 3D) and the highest lipase production was observed at a temperature of 37°C (Figure 4).

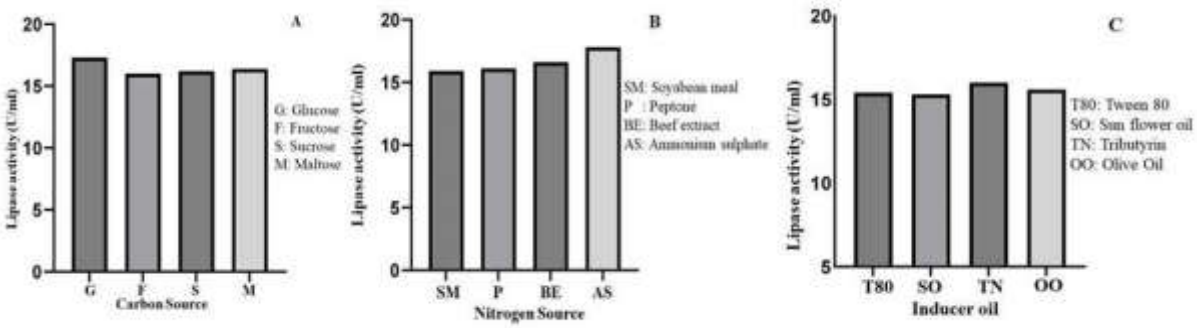

Figure 3: Effect of Carbon source (A) Nitrogen source, (B) and Inducer oil (C) on lipase production by the isolated bacteria

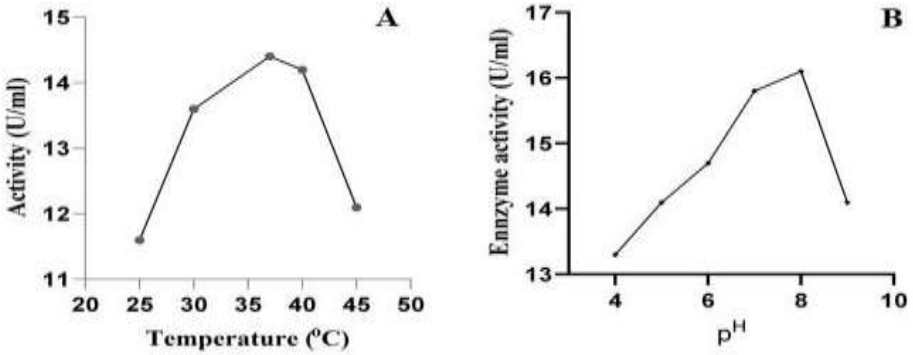

Figure 4. Effect of temperature and pH on the production of lipase by the isolated bacteria.

3.4 Purification of lipase

Salting out technique was found as acceptable for recovery of lipase. 70-80% ammonium sulfate saturation was observed as most effective concentration of salt. Specific activity was determined after dialysis and ion exchange chromatography and these techniques were found effective in purification of lipase from isolated bacteria B. borestelensis. Purification table is depicted in table 1. SDS-PAGE analysis was conducted to assess the molecular mass of the purified lipase enzyme and the effectiveness of purification procedures. The electrophoretic separation showed a distinct solitary protein band, as depicted in Figure 5, and the purified lipase showed a molecular mass of about 35 kilodaltons (kDa). The detected molecular mass bring into line well with the probable molecular mass of the lipase enzyme.

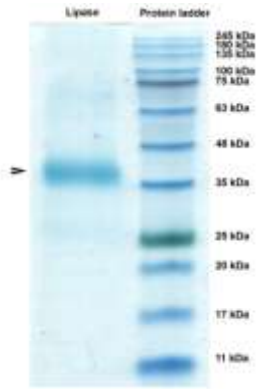

Figure 5. SDS-PAGE of the lipase purified from the B. borestelensis.

Table 1. Summary of purification of lipase from the B. borestelensis

Purification Step	Total Activity	Specific Activity	Fold of purification
Crude Extract	15.6	16	1
Dialysis	8.6	46.3	2.9
DEAE cellulose ion exchange chromatography	4.2	110.8	7

3.4 Stability of lipase with detergents

In the present study the lipase enzyme purified from isolated B. borestelensis found as compatible with detergents which are in common use. The enzyme retained its activity in the presence of all detergents used in the study. Elevated residual activity observed along with surf excel while with all other detergents, came by a minimum of 80% relative activity (Figure 5).

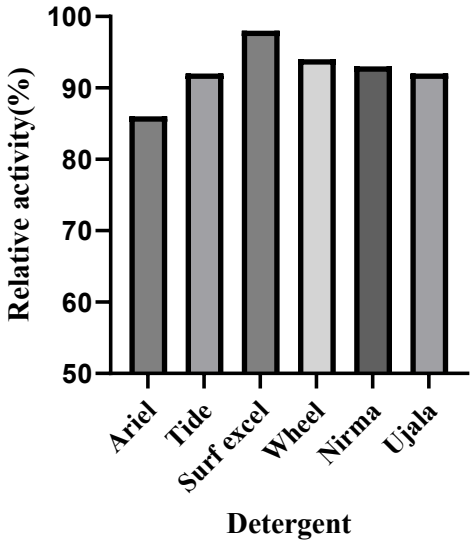

Figure 6. Relative activity of lipase in the presence of various detergents

4. DISCUSSION

Several researchers have demonstrated the potential of marine bacteria for the synthesis of lipase [10–12]. Glucose, a common carbon source, plays a crucial role in the production of lipase by bacteria [13, 14]. However, this phenomenon does not appear to be universal as some members of the Bacillaceae family was found to be repressing effect of glucose on lipase production [14]. Overall, the effect of glucose as a carbon source for bacterial production of lipase is complex and strain-dependent.

In this study Ammonium sulphate acted as best nitrogen source (Figure 3B). The use of Ammonium sulphate as a nitrogen source for bacterial lipase production has been a topic of investigation in several studies [15]. Ammonium sulphate has been found to enhance lipase production in various bacterial strains such as Bacillus sp. [16–19]. In a study [20], it was reported that the addition of Ammonium sulphate significantly increased lipase production by Penicillium camemberti.

Inducer oils play a crucial role in bacterial lipase production. They serve as inducers, stimulating the production and secretion of lipase enzymes by bacteria [21, 22]. Inducible extracellular lipases are produced in the presence of inducers such as fatty acids, oils, triacylglycerols, tween, bile salts, and glycerol [23]. In this study Tributyrin was served as best inducers for oil for the lipase production (Figure 3C). These oils provide the necessary carbon source for lipase production by bacteria, as lipases are inducible enzymes that are typically produced in the presence of lipids [24].

Various studies have investigated the effect of pH on lipase production by bacteria and have found that it has a significant impact [25]. One study found that bacteria prefer a neutral pH for optimal lipase production [26]. For example, the bacterial strain Pseudomonas sp. LSK25 showed maximum lipase production at pH 6-7 [27]. Similarly, another study demonstrated that maximum lipase activity by bacteria occurred at pH levels higher than 7 [25]. These findings suggest that pH plays a crucial role in regulating the production of lipase by bacteria. Furthermore, the effect of pH on lipase production can vary depending on the specific bacterial strain [27, 28].

Temperature plays a crucial role in the production of lipase by bacterial sources. Several studies have been conducted to investigate the effect of temperature on lipase production, and the results have shown that it is a significant factor.

According to one study in Candida tropicalis SD7, the best temperature for lipase production was found to be 30°C [26]. Another study by Al-Saleh and Zahran (1999) focused on the effect of temperature on growth and lipase production of psychrotrophic strains of Pseudomonas fluorescens. In their study, they observed that the optimum temperature for lipase production was 37°C. The production of lipase is generally observed within the temperature range of 20°C to 45°C [30, 31]. These findings indicate that different bacterial strains may have different optimal temperatures for lipase production. The effect of temperature on lipase production is closely related to the growth of the organism [26].
Lipid containing stains from fabrics can be removed by treating with lipase added detergents [7]. Various researchers proved that microbial lipases can withstand with commercially available detergents and can retain their activity in the presence of surface active agents. Lipase enzyme of Streptomyces fungicidicus retained 63-96 % of its hydrolytic activity with various detergents [7]. Lipase purified from Bacillus licheniformis achieved more than 50% relative activity after treating with various detergent powders [32]. Bacterial lipases have gained significant attention due to their ability to function in extreme environments, making them suitable for various industrial applications, including the laundry detergent industry [33]. Extensive industrial applications of bacterial lipases have been observed, including biodiesel production, oil degumming, synthesis of nutraceuticals, and their use in detergent formulations [34]. lipases from other bacterial species such as Pseudomonas mendocina, Pseudomonas alcaligenes, Pseudomonas glumae, and B. cepacia have also been utilized as detergent additives [34]. The findings of this study are in line with previous research that has highlighted the stability and potential of bacterial lipases in detergent formulations.

5. CONCLUSIONS

Marine Habitat is one of the richest sources of enzyme producing microorganisms. In the current study lipase producing bacteria was isolated from marine water and it was identified as B. borestelensis. Submerged fermentation with suitable substrates can exploit for lipase production and the produced enzyme can be purified employing techniques like precipitation with salts and anion exchange chromatography. Lipase from B. borestelensis was found as stable in the presence of commonly used detergent powders and shows a relative activity of more than 80% when treated with various detergent powders. Hence lipase from these bacteria can be utilized in production of detergents with ecofriendly lipase and it will be highly useful in laundry industry for removal of oil and grease like lipid containing stains from fabrics. Elevated cost of lipid substrates like tributyrin is one of the challenges that pose lipase production. Solid state fermentation with the utilisation of cheap solid substrates can overcome the cost challenge. Advanced production strategies along with genetic engineering techniques to develop lipase with improved properties will enhance the usefulness of microbial lipase enzyme.

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Competing interest

Authors have declared that no competing interests exist.

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