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Research article

Evaluation of alkali and thermotolerant lipase from an indigenous isolated *Bacillus* strain for detergent formulation

Rashmi Saraswat [a], Vijeshwar Verma [a], Srinivas Sistla [b], Indu Bhushan [a,*]

[a] Department of Biotechnology, Shri Mata Vaishno Devi University, Kakryal, Katra, 182320, Jammu and Kashmir, India
[b] GE Healthcare-Life Sciences, John F. Welch Technology Centre, Whitefield Road, Bangalore 560 066, India

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ABSTRACT

Background: Lipases are used in detergent industries to minimise the use of phosphate-based chemicals in detergent formulations. The use of lipase in household laundry reduces environmental pollution and enhances the ability of detergent to remove tough oil or grease stains.
Results: A lipase-producing indigenous *Bacillus subtilis* strain [accession no. KT985358] was isolated from the foothills of Trikuta mountain in Jammu and Kashmir, India. The lipase (BSK-L) produced by this strain expressed alkali and thermotolerance. Lipase has an optimal activity at pH 8.0 and temperature 37°C, whereas it is stable at pH 6.0–9.0 and showed active lipolytic activity at temperatures 30 to 60°C. Furthermore, lipase activity was found to be stimulated in the presence of the metal ions Mn^{2+}, K^{+}, Zn^{2+}, Fe^{2+} and Ca^{2+}. This lipase was resistant to surfactants, oxidising agents and commercial detergents, suggesting it as a potential candidate for detergent formulation. BSK-L displayed noticeable capability to remove oil stains when used in different washing solutions containing buffer, lipase and commercial detergent. The maximum olive oil removal percentage obtained was 68% when the optimum detergent concentration (Fena) was 0.3%. The oil removal percentage from olive oil-soiled cotton fabric increased with 40 U/mL of lipase.
Conclusions: This BSK-L enzyme has the potential for removing oil stains by developing a pre-soaked solution for detergent formulation and was compatible with surfactants, oxidising agents and commercial detergents.

1. Introduction

Lipases (EC 3.1.1.3) are serine hydrolases that evidently catalyse the hydrolysis of mono-, di- and triglycerides into glycerol and fatty acids in an aqueous medium. Lipases possess different properties, such as enantio-, chemo- and regioselectivity for use in industrial processes and have an advantage over chemical catalysts as they are eco-friendly, convenient, non-toxic and easily produced. Special consideration is given to lipases because of its stability at a broad range of pH and temperature conditions. Owing to their multi-functionality, lipases are used not only in detergent industry but also in various other industries, e.g., organic synthesis, leather degreasing, paper and pulp for removing pitch, biodiesel for alternative fuel production, food and dairy for flavour and aroma improvement, pharmaceutical for resolution of acids and alcohols, cosmetics for aroma generation, biodegradation of oil spills, and biosensors for lipid determination [1,2,3].

About 1000 t of lipase is estimated to be added to 13 billion t of detergent annually [4]. The use of lipase in household detergent diminishes or replaces synthetic detergents, making it eco-friendly, and improves the ability of detergent to remove tough oil or grease stains [5]. A cocktail of enzymes such as lipase, protease, amylase and cellulase have been used in many detergent products. Lipase used in detergent formulation should ideally be active and stable in a broad range of pH and temperature conditions and compatible with various detergent components such as surfactants, oxidising agents and metal ions [6]. Rathi et al. [7], reported that the lipase isolated from *Burkholderia cepacia* had better compatibility with commercial detergents and revealed superior activity in the presence of strong oxidants than commercially available lipase, i.e. lipolase. Lipase from other microorganisms such as yeast has been studied as a potential detergent additive [8]. In laundry, the aqueous medium is usually alkaline; thus, alkalophilic lipases are explored as detergent additives [9]. Lipases aid in the removal of grease or oil stains from clothes without much wear and tear.

Among several microorganisms, lipases from *Bacillus subtilis* is promising because it is non-pathological, 'Generally Recognised as Safe' (GRAS) and thus can be easily produced on the large industrial scale [10]. In 1992, Dartois et al. [11] cloned lipase gene from *B. subtilis*

* Corresponding author.
E-mail address: indu.bhushan@smvdu.ac.in (I. Bhushan).
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168, which expressed an extracellular enzyme called lipase A. The extracellular lipase A from *B. subtilis* 168 was genetically analysed [12]; its gene regulation was reported [13], and studies on enzyme activity by amino acid supply [14] were performed. Lipase A from *B. subtilis* 168 was studied as a model for detergent tolerance in 2015 by Fulton et al. [15]. In 2011, Adams et al. [16] reported recombinant lipase A from *B. subtilis* and filed a patent (US20120258900 A) for its application in detergent formulation.

Considering the immense potential of *B. subtilis* lipase for industrial applications; we investigated a thermotolerant, alkalophilic lipase (BSK-L) from indigenous *B. subtilis*. It was important to characterise and investigate lipase compatibility with surfactants, oxidising agents and commercial detergents. Additionally, BSK-L was evaluated for its washing efficiency of soiled cotton fabric for olive oil removal.

2. Materials and methods

2.1. Materials

BSK-L was produced in our laboratory. Analytical grade chemicals and organic solvents were procured commercially from Sigma, Himedia, Merck, Loba-Chemie and S. D Fine-chem Ltd. Commercial detergents were purchased from the local market, and MilliQ water was used for all experiments.

2.2. Methods

2.2.1. Screening and strain isolation

Potent microorganisms that expressed lipase were isolated from a soil sample of Jammu and Kashmir, India. The soil sample was suspended in 10 mL of sterile distilled water; the solution was mixed thoroughly, serially diluted and spread on nutrient agar plates composed of 0.5% (*w/v*) beef extract, 0.5% (w/v) yeast extract, 1% (w/v) peptone and 0.2% (w/v) NaCl and then incubated at 37°C. The colonies obtained were further qualitatively screened for lipase activity on tributyrin agar plates [17].

2.2.2. Lipase assay

2.2.2.1. The pH-stat method. Lipase activity was assayed in a buffer consisting of 1% (w/v) gum acacia, 2% (w/v) $CaCl_2$, 1 M NaCl, and 1% (v/v) olive oil as substrate at 37°C. The volume of 10 mM NaOH required to maintain pH at 7.0 for a specific reaction time was used to calculate the quantity of triglycerides hydrolysed by enzyme [18]. A unit of enzyme activity was considered equivalent to a unit of BSK-L activity and was defined as the amount of BSK-L required to release 1-μmol fatty acid per min from triglycerides under standard conditions.

2.2.3. Identification

The lipolytic microorganism was identified by 16S rRNA gene sequencing method. The culture was cultivated for 20 h before extracting the genomic DNA. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using a thermocycler (Master cycler pro-Eppendorf) with universal primers, forward primer 5′-AGAGTT TGATCCTGGCTCAG-3′ and reverse primer 5′-ACGGCTACCTTGTTAC GACTT-3′ [19]. The PCR conditions were optimised as follows: initial denaturation at 96°C for 4 min; denaturation at 94°C for 1 min, annealing at 59.8°C for 30 s and elongation at 72°C for 2 min for 35 cycles; and final elongation at 72°C for 4 min. PCR products were resolved on 1% (w/v) agarose gel in 1X TAE buffer. The amplified PCR products were sequenced by Sanger dideoxy sequencing by SciGenom Labs Private Ltd. and submitted to GenBank at National Centre for Biotechnology Information (NCBI). Basic Local Alignment Search Tool (BLAST) program was used for similarity analysis of 16S rRNA gene sequences.

2.2.4. Growth curve and BSK-L activity

The lipolytic microorganism was maintained on nutrient agar medium. The pre-culture nutrient medium containing 0.5% (w/v) yeast extract, 0.5% (w/v) beef extract, 1% (w/v) peptone and 0.2% (w/v) NaCl at pH 7.0 was inoculated with lipolytic microorganism and incubated at 37°C and 200 rpm for overnight in a refrigerated incubator shaker (Innova 4200, NBS). One percent (v/v) of overnight cultivated microorganism culture was then cultured in 100 mL of fermentation medium containing 0.5% (w/v) yeast extract, 0.5% (w/v) beef extract, 1% (w/v) peptone, 0.2% (w/v) NaCl and 1% (v/v) olive oil (pH 7). The culture media was sampled at regular time intervals of 2 h to 24 h for quantifying cell biomass and BSK-L activity. The BSK-L activity was quantified by obtaining the supernatant as crude enzyme after the sonication of cell biomass at 4°C for 5 cycles consisting of 1-min pulse and 4-min rest.

2.2.5. Characterisation of crude BSK-L

2.2.5.1. Effect of pH on BSK-L activity and stability. The optimum pH was determined by incubating BSK-L in a 100-mM buffer of different pH ranges from 2 to 12. The buffers used were glycin-HCl buffer for pH 2.0, acetate-acetic acid buffer for pH 3.0–5.0, phosphate buffer for pH 6.0 and 7.0, Tris base-HCl buffer for pH 8.0 and 9.0, glycine-NaOH buffer for pH 10.0, phosphate-NaOH buffer for pH 11.0 and KCl-NaOH buffer for pH 12.0. The maximum BSK-L activity was plotted as 100% relative BSK-L activity. Stability profile was determined by incubating the BSK-L at pH 6, 7, 8, and 9 and 37°C and examining its activity at 6, 12, 18 and 24 h. The initial activity was plotted as 100% relative activity for the stability profiling of BSK-L.

2.2.5.2. Effect of temperature on BSK-L activity and stability. The optimum temperature and stability were determined by incubating BSK-L at 30–60°C in 50 mM Tris buffer at pH 8 and 200 rpm. BSK-L activity was analysed after 6, 12, 18 and 24 h. The maximum activity was considered 100%, and the stability of BSK-L was considered 100% at the beginning of incubation.

2.2.5.3. Effect of metal ions. Effect of metal ions at 1 mM concentration was studied by incubating BSK-L with various metal ions at 37°C and 200 rpm. The BSK-L activity without the addition of metal ion was taken as 100%.

2.2.5.4. Effect of detergent, oxidising agents and surfactants. To determine the potential of BSK-L in the detergent formulation, its compatibility with various commercial detergents, oxidising agents and surfactants was investigated. One percent concentration of surfactants, 1% (v/v or w/v) concentration of oxidising agents and 7-mg/mL concentration of commercial detergents were incubated with BSK-L under standard conditions. The BSK-L activity without adding detergent, oxidising agents and surfactants was taken as 100%. Endogenous lipase in commercial detergents was inactivated by pre-heating at 65°C for 1 h.

2.2.6. Preparation of soiled olive oil cotton fabric

Cotton fabric was defatted by boiling in chloroform for 5 h. The soiling was done by spotting olive oil in 0.5 mL of 100-mg/mL benzene solution on cotton fabric.

2.2.7. Washing solutions, procedure and olive oil quantification

About 100 mL of four different compositions of washing solution were prepared (Table 1). Washing was done by soaking cotton fabric soiled with olive oil in different washing solutions in shaking flask at 37°C and agitating at 100 rpm for 30 min. Then, the fabric was rinsed in distilled water thrice for 2 min and air-dried. After the cotton fabric was washed, olive oil was extracted with petroleum ether for 6 h using a Soxhlet extractor. The quantification of olive oil was performed by weighing the residual olive oil after complete evaporation of petroleum

Table 1
Different washing solutions and their composition.

Components	Buffer	Buffer + Lipase	Buffer + Detergent	Buffer + Detergent + Lipase
Tris–HCL (0.05 M, pH 8)	40 mL	40 mL	40 mL	40 mL
Detergent (0.5%)	–	–	50 mL	50 mL
Lipase (50 U/mL)	–	10 mL	–	10 mL
Sterile water	60 mL	50 mL	10 mL	–

ether from the extract. The percentage of olive oil removed was calculated by

$$\text{Removal}\,\% = [(\text{Weight of total olive oil before washing (mg)} - \text{Weight of total olive oil after washing (mg)}) / \text{Weight of total olive oil after washing(mg)}] \times 100$$

3. Results and discussion

3.1. Screening and strain isolation

About 45 microbial colonies with different morphological characters such as opaque, round, smooth, convex, yellow and white were cultured on the nutrient agar plates. The lipase-producing microorganisms were screened by a plating method using tributyrin as substrate. Among 45 microbial colonies, only 18 colonies showed a zone of clearance, which indicated them as lipase-producing strains. The microorganism that produced the highest lipase activity was selected for further studies.

3.2. Identification

BLAST analysis of 16S rRNA gene sequence of the lipase-producing microorganism showed 99% sequence homology with *B. subtilis* and was thus referred as *B. subtilis* strain. The 16S rRNA gene sequence was deposited in GenBank and obtained accession no. KT985358.

3.3. Growth curve and BSK-L activity

Lipase (BSK-L) activity and cell biomass increased with increase in time up to 18 h of log phase (Fig. 1). After 18 h, a number of viable cells remained constant because of the reduction in optimal nutrient conditions, representing stationary phase, whereas the maximum lipase activity was observed in the stationary phase at 20 h. In the late stationary phase, decrease in both cell biomass and lipase activity was observed, representing the decline phase. Increase in lipase activity with an increase in cell biomass was also observed in studies conducted by Bora and Bora in 2012 [20] on *Bacillus* sp. The decline in cell biomass and enzyme activity occurred because of the exhaustion of nutrients and accumulation of toxic waste of bacterial cells or production of protease in the media.

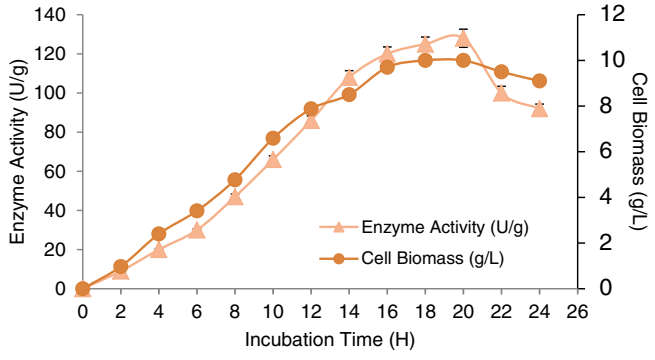

Fig. 1. Growth curve and BSK-L activity profile of *B. subtilis kakrayal*_1. All experiments were conducted thrice with calculated mean ± standard deviation.

3.4. Characterisation of crude BSK-L

Characterisation of biocatalyst plays an important role because BSK-L will be exposed to optimal/suboptimal/extreme physio-chemical environmental conditions to catalyse the industrial processes.

3.4.1. Effect of pH on BSK-L activity and stability

BSK-L was active in the pH range of 4 to 10, and optimum activity was observed in alkaline condition at pH 8 (Fig. 2a). However, the pH optima of *B. subtilis* Pa was observed at pH 7 [21] and *B. subtilis* 168 was reported at pH 10 [22]. The Relative pH stability of BSK-L is shown in Fig. 2b. At pH 8, BSK-L was found to be stable as it retained more than 85% of relative activity for 24 h, while at pH 6, 7 and 9, more than 60% of relative activity was retained. Because of its high activity and remarkable stability at alkaline conditions, BSK-L is not only applicable in detergent formulation but also in the leather industry in the degreasing process and sewage treatment [23]. The lipase produced from *Acinetobacter calcoaceticus* has shown stability in the pH range from 4 to 10 and was also used for detergent formulation [24].

3.4.2. Effect of temperature on BSK-L activity and stability

The optimum temperature for BSK-L was 37°C (Fig. 2c). Conversely, the temperature optima of *B. subtilis* 168 lipase was approximately 35°C and was stable for 30 min at 40°C [22]. BSK-L was active in the temperature range 30 to 60°C, whereas lipase from *B. subtilis* Pa was reported to be active at 30 to 50°C [21] and lipase from *Staphylococcus aureus* was active at 55 to 60°C [25]. The thermostability of BSK-L was examined at 37°C (Fig. 2d), and it was observed that 80% of lipase activity was retained after 24 h of incubation. More than 60% of BSK-L activity was retained on incubation at 35°C, 40°C and 45°C for 24 h, which represents it as a highly thermotolerant enzyme. However, 40% of lipase activity was retained at 50°C, and more than 20% was observed on incubation at 55°C and 60°C. This study presents the thermo-tolerant lipase, thus justifying its application in detergent formulation.

3.4.3. Effect of metal ions

In this study, relative BSK-L activity was stimulated when treated with Mn^{2+}, Ca^{2+}, K^{+}, Zn^{2+} and Fe^{2+} ions and partially reduced on treatment with Mg^{2+}, Na^{+}, Cu^{2+} and Co^{2+} ions (Table 2). Similarly, Ca^{2+} and K^{+} ions increased lipase activity in studies conducted by Wang et al. [24] Additionally, in 1993, Lesuisse et al. [22] reported that treatment with Ca^{2+} ions stimulated the activity of *B. subtilis* 168 lipase. However, Shah et al. [21] reported that Zn^{2+} inhibited and Mg^{2+} stimulated the activity of *B. subtilis* Pa lipase. In the presence of 1 mM EDTA (metal ion chelator) with Mn^{2+} and Ca^{2+}, the biocatalytic activity of BSK-L decreased to 62.8% and 71.5% respectively, suggesting a significant role of metal ions in BSK-L activity. Metal ions play a critical role in the maintenance of enzyme structure and activity [26]. Improved biocatalyst activity by treatment with Ca^{2+} ions would be due to the formation of long-chain insoluble fatty acid Ca^{2+} ions during the hydrolysis of the substrate and also because the bridge formation at the active site, which aids enzyme stability [27]. The negative effect of metal ions results in the direct inhibition of the active site of a biocatalyst [28]. However, it is noteworthy that BSK-L was not completely inhibited by the metal ions and thus can be used in laundry applications where water is contaminated with metal ions.

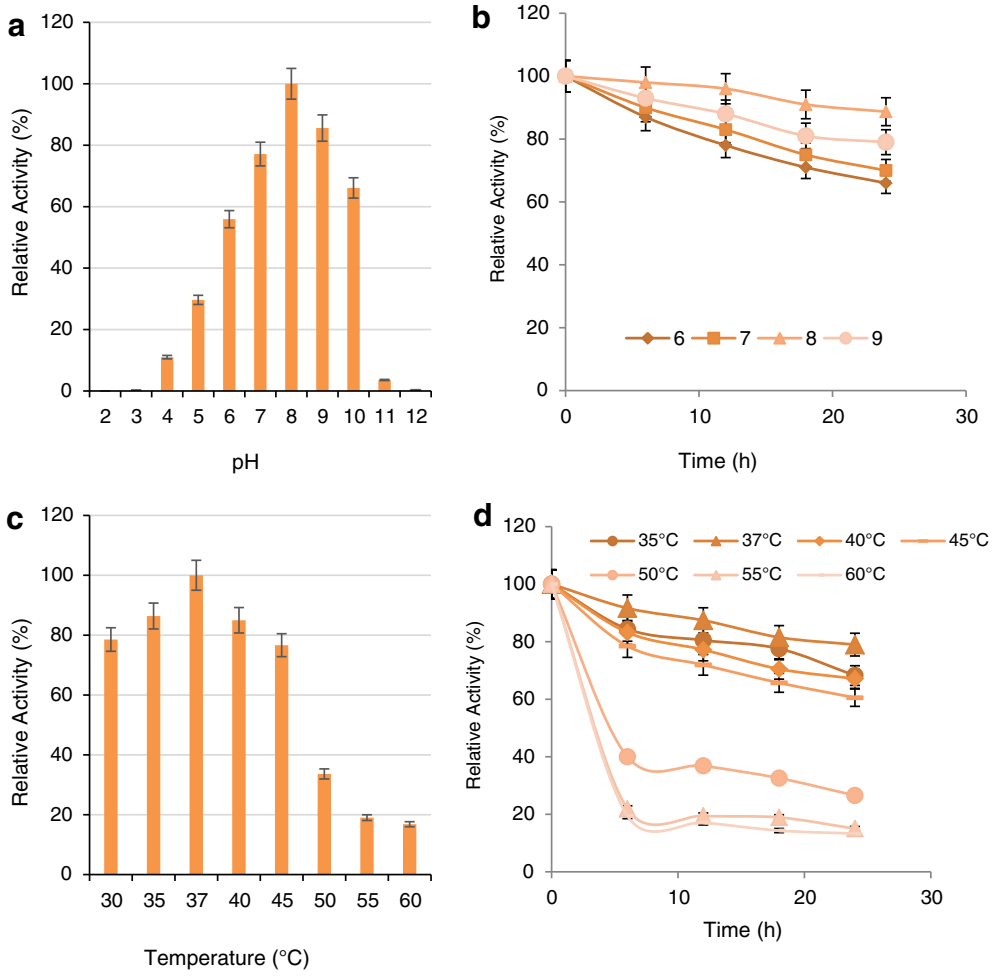

Fig. 2. Effect of pH on BSK-L activity (a) and stability (b) and effect of temperature on BSK-L activity (c) and stability (d). All experiments were conducted thrice with calculated mean ± standard deviation.

3.4.4. Effect of surfactants, oxidising agents and commercial detergents

A good detergent lipase should exhibit better activity and stability in the presence of surfactants. The effects of various detergents, oxidising agents and surfactants on BSK-L activity are depicted in Table 3. The relative BSK-L activity retained after 6 h in Tween 20, Tween 80 and Triton X-100 were 103%, 98% and 84%, respectively, whereas biocatalytic activity significantly dropped and reached below 1% when treated with SDS. Similarly, when lipase was treated with Tween 20 and Tween 80 in studies by Golaki et al. [26], the relative activity obtained was 92% and 42%, respectively, suggesting that Tween 80 has long acyl ester chains, which makes them more eligible candidates to be a substrate for competitive inhibition than Tween 20, and signifies that lipase prefers C18 (Tween 80) over C12 (Tween 20). SDS was reported to decrease the lipase activity in *Cohnella* sp. [26] and *Ralstonia pickettii* [29], while the activity was enhanced in the case of lipase from *Staphylococcus* sp. [5], *Aspergillus* sp. [30] and *Rhizopus* sp. [31]. In parallel to our results, non-ionic surfactants attributed to higher lipase activity, while inhibition of lipase activity was observed by anionic surfactants [32]. BSK-L activity was found to be stable and showed relative activity from 90% to 98% at 37°C in 1% concentration of oxidising agents such as sodium perborate, hydrogen peroxide and sodium hypochlorite, and commercial detergents such as Aerial, Tide, Fena and Henko at 7-mg/mL concentration (Table 3). The noticeable resistance of BSK-L towards oxidising agents makes this lipase capable

Table 2
Effects of various metal ions on BSK-L activity.

Metal ions	Relative activity (%)	Metal ions	Relative activity (%)
Control	100 ± 0.4	Mg^{2+}	85.2 ± 1.3
Mn^{2+}	146.3 ± 1.53	Na^{+}	83.3 ± 1.37
Ca^{2+}	138.3 ± 1.53	Cu^{2+}	69.2 ± 1.81
K^{+}	134.6 ± 1.51	Co^{2+}	66.4 ± 1.71
Zn^{2+}	126.7 ± 1.31	EDTA + Mn^{2+}	62.8 ± 1.55
Fe^{2+}	121.9 ± 0.51	EDTA + Ca^{2+}	71.5 ± 0.85

Table 3
Effects of various surfactants, oxidising agents and commercial detergents on BSK-L activity.

Surfactants (1%)	Relative activity (%)	Oxidising agents (1%)	Relative activity (%)	Commercial detergents (7 mg/mL)	Relative activity (%)
Tween 20	103.10 ± 1.07	Control	100 ± 0.4	Ariel	93 ± 1.9
Triton X-100	84.10 ± 0.60	Sodium perborate	90 ± 0.8	Tide	97 ± 1.4
Tween 80	98.31 ± 1.55	Hydrogen peroxide	95 ± 0.32	Fena	98 ± 2.1
SDS	1.40 ± 0.02	Sodium hypochlorite	92 ± 1.2	Henko	88 ± 1.5

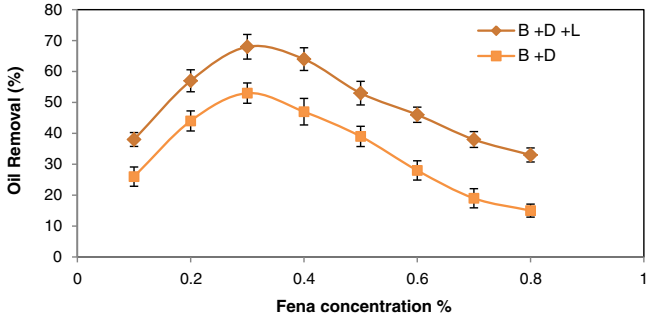

Fig. 3. Effect of detergent (Fena) and its concentration on oil removal percentage (B, Buffer; D, detergent; L, lipase). All experiments were conducted thrice with calculated mean ± standard deviation.

of being incorporated in laundry formulations. Comparable results were reported when lipase from *Acinetobacter calcoaceticus* [24] was treated with oxidising agents and lipase from *Staphylococcus aureus* was treated with commercial detergents [25].

3.5. Effect of detergent concentration and lipase concentration on olive oil removal percentage

Effects of different concentrations of Fena detergent on olive oil removal percentage was investigated as shown in Fig. 3. The cotton fabric was treated with washing solutions for 30 min. The maximum olive oil removal percentage obtained on treatment with buffer, detergent and lipase (B + D + L) (different detergent concentration) washing solution was 68% and with B + D washing solution was 53%. The optimum Fena concentration obtained was 0.3% with both washing solutions. The washing solution B + D + L improved oil removal percentage by 15% compared to the washing solution B + D. The percentage of oil removal with B + D + L washing solution was higher than with buffer and detergent washing solution, thus showing the advantage of adding lipase in detergent formulations.

The effect of different lipase concentrations on olive oil removal percentage is shown in Fig. 4. The maximum olive oil removal percentage obtained on treatment with B + D + L (different lipase concentration) washing solution was 66% and with B + L washing solution was 50%. The optimum lipase (BSK-L) concentration obtained was 40 U/mL with both washing solutions. The B + D + L washing solution improved oil removal percentage by 16% when compared to the B + L washing solution. The enhancement in oil removal percentage from olive oil-soiled cotton fabric in combination with detergents, buffer, and lipase was also reported in *Pseudozyma* sp. [32] and *Pseudomonas* sp. lipase [33].

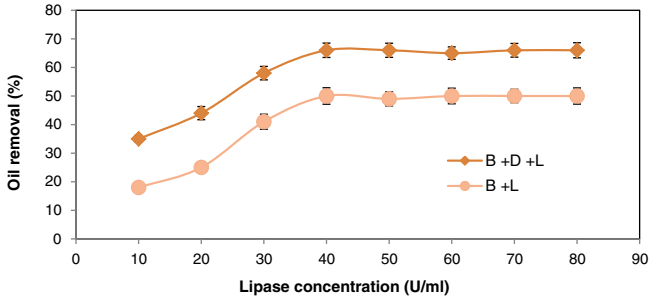

Fig. 4. Effect of BDK-L lipase concentration (U/mL) on oil removal percentage (B, Buffer; D, detergent; L, lipase) after incubation for 30 min. All experiments were conducted thrice with calculated mean ± standard deviation.

4. Conclusion

In the present study, lipase-producing indigenous *B. subtilis* strain was isolated from the soil sample. Lipase produced from this strain was characterised and evaluated for detergent formulation. Naturally adapted, indigenous microbes are not only stable and economical but also better adapted to environmental abiotic and biotic stress. The characterisation of crude BSK-L revealed that it was stable in a broad range of pH, 6 to 9, with optimal lipase activity at pH 8. Moreover, it was active in the temperature range from 30 to 60°C, with optimal activity at 37°C. BSK-L was stimulated by metal ions such as Mn^{2+}, Ca^{2+}, K^{+}, Zn^{2+} and Fe^{2+}, and it was partially inhibited by Mg^{2+}, Na^{+}, Cu^{2+} and Co^{2+} ions. Furthermore, BSK-L was resistant and retained activity in the presence of surfactants, oxidising agents and commercial detergents. The additional effect of a washing solution containing BSK-L, detergent (Fena) and buffer for the removal of olive oil on soiled cotton fabric makes it a potential additive in detergent formulations. The oil removal ability of the detergent was enhanced by 15% with the addition of BSK-L. Thus, BSK-L can be used as a possible candidate for eco-friendly detergent formulation. However, further study is required to ascertain the kinetic properties and molecular structure to determine the full potential of BSK-L from *B. subtilis* strain for industrial applications.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Casas-Godoy L, Duquesne S, Bordes F, et al. Lipases: an overview. Methods Mol Biol 2012;861:3–30. https://doi.org/10.1007/978-1-61779-600-5_1.
[2] Gurung N, Ray S, Bose S, et al. A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. Biomed Res Int 2013;329121:1–18. https://doi.org/10.1155/2013/329121.
[3] Sharma S, Kanwar SS. Organic solvent tolerant lipases and applications. Sci World J 2014;625258:1–15. https://doi.org/10.1155/2014/625258.
[4] Sanchez S, Demain AL. Enzymes and bioconversions of industrial, pharmaceutical, and biotechnological significance. Org Process Res Dev 2011;15(1):224–30. https://doi.org/10.1021/op100302x.
[5] Chauhan M, Chauhan RS, Garlapati VK. Evaluation of a new lipase from *Staphylococcus* sp. for detergent additive capability. Biomed Res Int 2013;374967:1–6. https://doi.org/10.1155/2013/374967.
[6] Cherif S, Mnif S, Hadrich F, et al. A newly high alkaline lipase: an ideal choice for application in detergent formulations. Lipids Health Dis 2011;10:221. https://doi.org/10.1186/1476-511X-10-221.
[7] Rathi P, Saxena R, Gupta R. A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. Process Biochem 2001;37(2):187–92. https://doi.org/10.1016/S0032-9592(01)00200-X.
[8] Thirunavukarasu K, Edwinoliver NG, Durai Anbarasan S, et al. Removal of triglyceride soil from fabrics by a novel lipase from *Cryptococcus* sp. S-2. Process Biochem 2008;43(7):701–6. https://doi.org/10.1016/j.procbio.2008.02.011.
[9] Hemlata B, Uzma Z, Tukaram K. Substrate kinetics of thiol activated hyperthermostable alkaline lipase of *Bacillus sonorensis* 4R and its application in bio-detergent formulation. Biocatal Agric Biotechnol 2016;8:104–11. https://doi.org/10.1016/j.bcab.2016.08.008.
[10] Ma J, Ma Y, Wei W, et al. *In vivo* functional expression of an extracellular Ca^{2+}-independent *Bacillus pumilus* lipase in *Bacillus subtilis* WB800N. Ann Microbiol 2015; 65(4):1973–83. https://doi.org/10.1007/s13213-015-1035-z.
[11] Dartois V, Baulard A, Schanck K, et al. Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. Biochim Biophys Acta Gene Struct Expr 1992;1131(3):253–60. https://doi.org/10.1016/0167-4781(92)90023-S.
[12] Dartois V, Coppée JY, Colson C, et al. Genetic analysis and overexpression of lipolytic activity in *Bacillus subtilis*. Appl Environ Microbiol 1994;60:1670–3.
[13] Eggert T, van Pouderoyen G, Dijkstra BW, et al. Lipolytic enzymes LipA and LipB from *Bacillus subtilis* differ in regulation of gene expression, biochemical properties, and three-dimensional structure. FEBS Lett 2001;502(3):89–92. https://doi.org/10.1016/S0014-5793(01)02665-5.
[14] Eggert T, Brockmeier U, Dröge MJ, et al. Extracellular lipases from *Bacillus subtilis*: Regulation of gene expression and enzyme activity by amino acid supply and external pH. FEMS Microbiol Lett 2003;225(2):319–24. https://doi.org/10.1016/S0378-1097(03)00536-6.
[15] Fulton A, Frauenkron-Machedjou VJ, Skoczinski P, et al. Exploring the protein stability landscape: *Bacillus subtilis* lipase a as a model for detergent tolerance. Chembiochem 2015;16(6):930–6. https://doi.org/10.1002/cbic.201402664.
[16] Adams C, Schmidt B. Detergent compositions containing *Bacillus subtilis* lipase and methods of use thereof. Available from Internet: https://patentscope.

wipo.int/search/en/detail.jsf?docId=WO2011084599&recNum=103&docAn=US2010060768&queryString=brazil&maxRec=4565; 2011. [cited date June 21, 2017].
[17] Bhushan I, Yadav A, Parshad R. Enhancement in the production of lipase from Arthrobacter sp. using fed-batch fermentation strategy. Asiat J Biotechnol Resour 2011;2(5):522–34.
[18] Bakir ZB, Metin K. Purification and characterization of an alkali-thermostable lipase from thermophilic *Anoxybacillus flavithermus* HBB 134. J Microbiol Biotechnol 2016; 26(6):1087–97. https://doi.org/10.4014/jmb.1512.12056.
[19] Abdel-Fattah YR, Soliman NA, Yousef SM, et al. Application of experimental designs to optimize medium composition for production of thermostable lipase/esterase by *Geobacillus thermodenitrificans* AZ1. J Genet Eng Biotechnol 2012;10(2):193–200. https://doi.org/10.1016/j.jgeb.2012.08.001.
[20] Bora L, Bora M. Optimization of extracellular thermophilic highly alkaline lipase from thermophilic *Bacillus* sp. isolated from hotspring of Arunachal Pradesh, India. Braz J Microbiol 2012;43(1):30–42. https://doi.org/10.1590/S1517-83822012000100004.
[21] Shah R, Bhatt S. Purification and characterization of lipase from *Bacillus subtilis* Pa2. J Biochem Technol 2011;3(3):292–5.
[22] Lesuisse E, Schanck K. Purification and preliminary characterization of the extracellular lipase of Bacillus Subtilis 168, an extremely basic pH tolerant enzyme. Eur J Biochem 1993;216:155–60.
[23] Joseph B, Ramteke PW. Extracellular solvent stable cold-active lipase from psychrotrophic *Bacillus sphaericus* MTCC 7526: Partial purification and characterization. Ann Microbiol 2013;63(1):363–70. https://doi.org/10.1007/s13213-012-0483-y.
[24] Wang H, Zhong S, Ma H, et al. Screening and characterization of a novel alkaline lipase from *Acinetobacter calcoaceticus* 1-7 isolated from Bohai Bay in China for detergent formulation. Braz J Microbiol 2012;43(1):148–56. https://doi.org/10.1590/S1517-83822012000100016.
[25] Ben Bacha A, Al-Assaf A, Moubayed NMS. Evaluation of a novel thermo-alkaline *Staphylococcus aureus* lipase for application in detergent formulations. Saudi J Biol Sci 2016. https://doi.org/10.1016/j.sjbs.2016.10.006.
[26] Golaki BP, Aminzadeh S, Karkhane AA, et al. Cloning, expression, purification, and characterization of lipase 3646 from thermophilic indigenous *Cohnella* sp. A01. Protein Expr Purif 2015;109:120–6. https://doi.org/10.1016/j.pep.2014.10.002.
[27] Kaur G, Singh A, Sharma R, et al. Cloning, expression, purification and characterization of lipase from *Bacillus licheniformis*, isolated from hot spring of Himachal Pradesh, India. Biotech 2016;6:49–59. https://doi.org/10.1007/s13205-016-0369-y.
[28] Chen S, Qian L, Shi B. Purification and properties of enantioselective lipase from a newly isolated *Bacillus cereus* C71. Process Biochem 2007;42(6):988–94. https://doi.org/10.1016/j.procbio.2007.03.010.
[29] Hemachander C, Puvanakrishnan R. Lipase from *Ralstonia pickettii* as an additive in laundry detergent formulations. Process Biochem 2000;35(8):809–14. https://doi.org/10.1016/s0032-9592(99)00140-5.
[30] Saisubramanian N, Edwinoliver NG, Nandakumar N, et al. Efficacy of lipase from *Aspergillus niger* as an additive in detergent formulations: a statistical approach. J Ind Microbiol Biotechnol 2006;33(8):669–76. https://doi.org/10.1007/s10295-006-0100-9.
[31] Derewenda U, Swenson L, Wei Y, et al. Conformational lability of lipases observed in the absence of an oil-water interface: crystallographic studies of enzymes from the fungi *Humicola lanuginosa* and *Rhizopus delemar*. J Lipid Res 1994;35:524–34.
[32] Sajna KV, Sukumaran RK, Jayamurthy H, et al. Studies on biosurfactants from *Pseudozyma* sp. NII 08165 and their potential application as laundry detergent additives. Biochem Eng J 2013;78:85–92. https://doi.org/10.1016/j.bej.2012.12.014.
[33] Grbavčić S, Bezbradica D, Izrael-Živković L, et al. Production of lipase and protease from an indigenous *Pseudomonas aeruginosa* strain and their evaluation as detergent additives: Compatibility study with detergent ingredients and washing performance. Bioresour Technol 2011;102(24):11226–33. https://doi.org/10.1016/j.biortech.2011.09.076.