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PURIFICATION AND CHARACTERIZATION OF LIPASE FROM THERMOPHILIC BACTERIAL CONSORTIUM

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Abstract

The purification and characterization of lipase from a thermophilic bacterial consortium is crucial for developing robust biocatalysts for industrial processes requiring high temperatures, spanning biotechnology, biofuels, detergents, and more. Thermophilic lipase from *Bacillus toyonensis* and *Bacillus thuringiensis* consortium was purified and characterized. Purification of lipases to homogeneity was conducted by precipitation with ammonium sulphate, dialysis and chromatography (G-75 gel filtration) with a final recovery of 27.09% with 39.27 fold purification. The molecular weight of *Bacillus consortium* was 41 kDa using SDS-PAGE. Maximum activity of extracellular lipase was observed at pH 7.8, and temperature of 70oC. Conclusively, the thermophilic bacterial consortium can be exploited for biotechnological applications for production of lipase on large scale, where alkaline conditions prevailed.

Keywords: Thermophiles, lipase, shake flask, purification.

Introduction

Nature's sustainable catalysts are enzymes. They are made from renewable resources, biodegradable, and biocompatible materials (Sheldon and Pelt, 2013). The huge biological globular protein molecule known as an enzyme is responsible for thousands of metabolic processes that make life possible and serves as a catalyst for particular chemical reactions within the cell (Zhegunov and Pogozhykh, 2023). All living things contain enzymes, which catalyze the metabolic processes required for life (Olempska-Beer *et al*., 2006). Enzymes do not affect the equilibrium of the reactions, they catalyze or are consumed by them. There are 4,000 known biological processes that enzymes can catalyze (Schnell *et al*., 2006). Due to the importance of lipolytic enzymes in biotechnology, they are currently intriguing attention of industries. According to Turner *et al*. (2007), the geothermal environment, such as hot springs, are typically preferred habitat for thermophilic bacteria. Thermophilic organisms refer to microorganisms with growth optima ranging from 50 to 80°C, thriving in high-temperature environments beyond the tolerances of typical mesophiles.

However, most of the bacterial lipases reported thus far demonstrate non-specific substrate selectivity and are constitutive, some are

thermostable (Sugihara *et al.*, 1991*;* Sharma *et al*., 2002). The most commonly encountered bacterial lipase include those from *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus coagulans*, and *Bacillus alcalophilus* (Hassan and Al-Jabory, 2006). Bacterial lipase is frequently used in the dairy and food industries to hydrolyze milk fat, ripen cheese, enhance flavour, and lipolyze butter. The primary substrates used by lipase are triacylglycerol's. Substrates such as fatty acid esters, synthetic triglycerides, and natural oils are used to make lipase (Ohnishi *et al*., 1994; Masurkar & Pathade, 2023). Although lipase is extensively distributed in nature and can be produced by a variety of bacteria and higher eukaryotes, the majority of lipase used in technology are derived from microbes, because their corresponding genes are reasonably accessible and can be expressed effectively (Olymon *et al.,* 2023). Microbial lipase offers advantages of high yield and cost-effectiveness in production, diverse catalytic activities, genetic modifiability, organic solvent stability, and broad substrate specificities (Shu *et al*., 2010).

Inducers like oils, tweens, hydrolysable esters, fatty acids, triacylglycerols, glycerol, and bile salts are commonly present during the production of these enzymes (Sharma *et al*., 2001). Microbial lipase has drawn the attention of industry due to presence of specificity in targeting of certain substrates, and stability. Extremophile organisms are those that can thrive in conditions with a high salt content, low pH, or high temperature. These organisms frequently produce enzymes with special qualities that make them valuable for industrial uses. In order to maximize enzyme yield, it is necessary to optimize the following physicochemical factors: pH, temperature, incubation time, inoculum level, and medium components (Gupta *et al*., 2004).

Enzymes produced by thermophiles are stable at high temperatures to sustain physiological functions and are in complete thermal equilibrium with their micro-environments (Poddar *et al.,* 2023). In the temperature range of 60 to 100 ºC, thermophiles thrive. Due to their unusual biochemical characteristics and distinctive protein sequence, thermophilic lipase from *Bacillus* sp. is currently focused substance for extensive research (Dutra *et al*., 2008; Griebeler *et al*., 2011). The objective of the present study to purify lipase from *Bacillus toyonensis* and *Bacillus thuringiensis* consortium, characterization and shelf stability determination.

Materials and Methods

Microorganism and isolation

Water samples were collected from hot springs of Azad Jammu and Kashmir and Karachi. Bacterial isolates were obtained by streak plate method. Pure cultures were confirmed as thermophilic by growth at 70 ºC. They were maintained at 4 ºC.

Lipase production medium

Thermophilic lipase was produced in medium containing ferrous chloride (1g), ammonium sulphate (2g), sodium chloride (0.5g), agro industrial waste (0.5g), brassica oil (1.5ml), triton X-100 (0.5ml), tryptone (1.5g), rate of agitation (250 rpm), volume of production medium (75ml) at pH 6 in shake flask system for 24 hours using submerged fermentation technique.

Lipase assay:

The activity of lipase was estimated through a titrimetric assay, as described by Iftikhar *et al.* (2003).

Protein estimation:

Protein in supernatant was measured using Lowry method, with BSA standard curve (Lowry, 1951).

Lipase purification:

Crude lipase was collected and purified using ammonium sulfate precipitation, gel filtration chromatography, and dialysis in a three-step process (Vivek *et al*., 2023)

Ammonium sulphate precipitation:

Ammonium sulphate was added at different concentration (0-80%) in crude enzyme extract. Enzyme extract was fractionated with ammonium sulfate and stirred on a magnetic stirrer to ensure complete dissolution of all the fractions. Then, the enzyme extract after mixing with ammonium sulphate was centrifuged for 20 minutes at 4^{o}C to get the desired precipitates of protein. After centrifugation, solution of 0.01 M potassium phosphate buffer with a pH of 7 was added to the pellet until the pellet dissolved completely.

Dialysis:

For dialysis of precipitated enzyme suspension, dialysis membrane was used. The membrane containing the enzyme suspension was suspended in the same buffer as suspension was dissolved. An enzyme suspension was incubated in a big beaker at 4^{o}C for 24 hours to extract ammonium sulphate ions from protein molecules using dialysis tubing. After six hours, the buffer was changed to improve desalting effectiveness, and the new buffer was replaced with a buffer to stop the development of equilibrium. Lipase activity and protein content were estimated for every fraction of the enzyme and were compared with crude enzyme extract.

Gel filtration chromatography:

Further purification after dialysis was conducted by loading the enzyme sample on Sephadex G-75 column (1.5 cm × 170 cm). Elution was performed with 0.02 M elution buffer (0.02M) by using FPLC system. The column of the sephadex was eluted with same buffer as earlier. Fraction collector was used for collection of various eluted fractions. The flow rate was 2mL per minute. The absorbance was monitored continuously at 260 nm. Fractions showing highest peak were pooled, dialyzed against distilled water, for improved lipase activity. All the fractions containing highest peaks were subjected to lipolytic activity. Among all the fractions the enzyme fractions with best lipase activity were dried by freeze drying, stored at -20^{0}C separately and selected, for further studies.

Molecular weight calculation:

SDS-PAGE, was used to determine the molecular weight of the purified (Laemmli, 1970).

Preparation of resolving gel:

Resolving gel was prepared at the concentration of 10%. The ingredients [distilled water (400mL), 30 % acrylamide (3.3 microliter),1.5 M tris chloride (pH.8) 2.5 microliter, 10 % SDS (0.1 microliter), and 10% APA (0.1 microliter), TEMED (0.004 microliter)] with variable quantity was mixed on vortex mixer. Afterwards, resolving gel was poured on plates and allowed to solidify at 37 ^{o}C. To prevent photo-oxidation either other reactions, distilled water or isopropanol was added. Then, the gel was polymerized in an oven at 37°C. After gel solidification, distilled water was removed from the plates (Laemmli, 1970)..

Preparation of stacking gel:

Stacking gel (6 %) was prepared by dissolving distilled water (4.1mL), tris pH 6.8 (0.75mL), 30 % polyacrylamide (1.0mL), 10 % APA (0.06mL), and TEMED 0.006 (10microlitre). The stacking gel was poured on the top of resolving gel and comb was added to produce wells. The gel was allowed to polymerize at 37°C. After the gel solidified, the comb was removed and the gel was fixed into the SDS-PAGE. Finally, 1X-Tris-glycine (SDS running buffer) was added to the upper and bottom sides of the gel (Laemmli, 1970)..

Characterization of purified lipase:

pH effect on activity and stability of purified lipase:

To study the effect of pH on the properties of the purified lipase, various buffers (3.4-9) were evaluated. These contained 100 mM buffers of phosphate (pH 5.4–7), acetate (pH 3.4-5), and tris HCl (pH 7.4–9). The pH stability of purified lipase was determined by pre-incubating for different time intervals such as 4, 8, 12 and 24 hours at different pH values (3.4 - 9). Following an incubation period under standard assay conditions, the enzyme activity of purified lipase was calculated in the reaction mixture (Balan *et al*., 2012).

Incubation temperature effect on enzyme activity and stability:

The effect of incubation temperature (40-90°C) on the characterization of the purified lipase was investigated. To evaluate the thermostability of the lipase enzyme, the purified enzyme was pre-incubated at different temperature ranging from 40°C to 90°C for various durations (4, 8, 12, and 24 hours) in a water bath. The enzyme activity was measured by incubating the reaction mixture under standard assay conditions (Balan *et al*., 2012).

Reaction time effect of on activity and shelf stability of lipase:

The effect of reaction time (30min-150min) was observed on activity of purified lipase. By pre-incubating the enzyme in 100 mM Tris HCl solution for a period of 10 days after every 12 hours at 4^{o}C, lipase shelf stability was evaluated (Balan *et al*., 2012).

Results and Discussions

Bacillus consortium of Tatta pani Azad Jammu and Kashmir and Manghoopeer Karachi hot spring bacterial strains was utilized for production of lipase enzyme. The *Bacillus* consortium was identified to belong to *Bacillus thuringiensis* and *Bacillus toyonensis* as confirmed by molecular studies. For purification of lipase using *Bacillus* consortium, chromatography (gel filtration), dialysis, and precipitation (ammonium sulphate) technique was used. Fraction nine had the highest activity (2.17 U/mL), when analyzed using gel filtration chromatography. A single 41 kDa molecular weight band was visible on the gel after SDS-PAGE analysis as illustrated in fig. 1. Conversely, the molecular weight of lipase extracted from *Geobacillus* sp. in earlier studies was determined to be 30 kDa and 43 kDa, (Druteika *et al*., 2020). Because smaller enzymes have fewer modifications (unfolding) in their tertiary structure, they are more stable, lipase with reduced molecular weight offer an advantage (Sharon *et al*., 1998). After three steps of purification, crude lipase was isolated from the culture supernatant with a final recovery of 27.09% and was refined to homogeneity by 39.27

fold (Table 1). Previous study reported molecular weight of *Geobacillus stearothermophilus* to be 61 kDa, with 8.8% recovery and 22.6 fold purification by using ion exchange chromatography (Q-Sepharose), gel filtration (G-100) and adsorption on hydroxyl apatite (Sifour *et al*., 2010). In 2005, Kumar *et al.* identified a 31 kDa *Bacillus coagulans* BTS-3 alkaline thermostable lipase.

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Table 1: Purification of lipases from bacterial consortium

Purification stage	lipases activity U/mL	Protein content mg/ml	specific activity (U/mg)	Purification fold	Percentage recovery/Yield (%)
Crude enzyme extract	8.01	3.04	2.63	1	100
Ammonium sulfate precipitation (60%)	4.5	1.03	4.36	1.67	56.17
DEAE Cellulose	3	0.096	31.25	11.88	37.45
Gel filtration (Sephadex G-75)	2.17	0.021	103.3	39.27	27.09

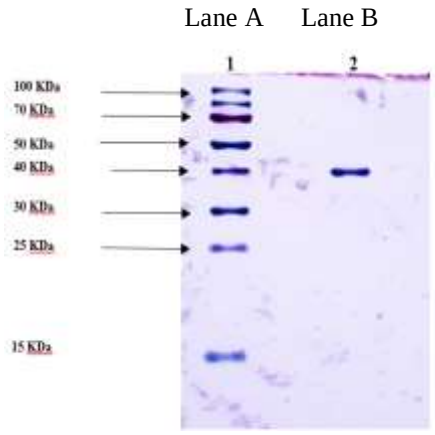

Fig. 1: Lane A: protein marker, Lane B: purified lipase

Thermo-stability of bacterial lipase was also studied by pre-incubating the purified enzyme for 4,8,12 and 24 hours at different temperatures. The enzymes retained its 99.32 % activity at 60ºC (pre-incubation temperature) for 8 h which reduced to 97.67% after pre incubation period of 24 h for purified lipase. However, 97.48% enzyme activity were observed for purified lipase, respectively after exposure at 70ºC (pre-incubation temperature) for 8h and reduced to 90.78% for pre-incubation period of 24 h (fig. 3). The enzyme activity either declines or is rendered inactive at extremely high temperatures. This could be the result of denaturation of the enzyme, which alters the enzyme's active sites by destroying the protein's three-dimensional structure and breaking the links that hold its secondary and tertiary structures together (Khalaf, 2012; Hassan and Al-Jobory, 2016). Our study is not in agreement with previous study (Zouaoui & Bouziane, 2011) where maximal stability of lipase at 25 to 35ºC temperature was analyzed. After 1

hour, at 35ºC stability of enzyme was observed. The enzyme kept its initial activity of 81% after one hour at 30°C. The particular nature of the protein and its thermos-stability at high temperatures, indicates that lipase have industrial potential

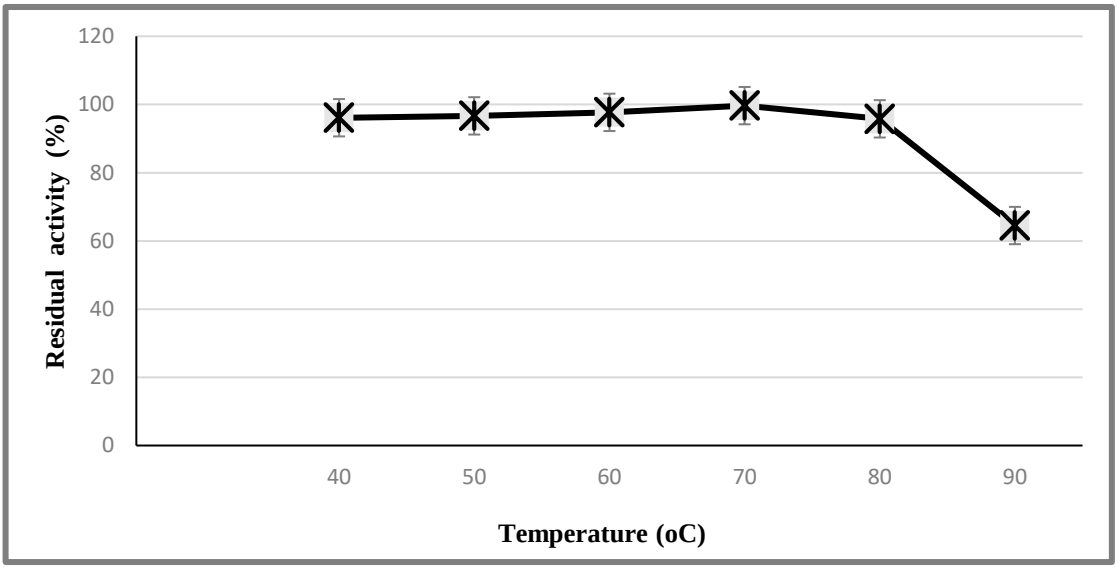

Fig. 2 Effect of temperature on purified lipases activity by *Bacillus* consortium

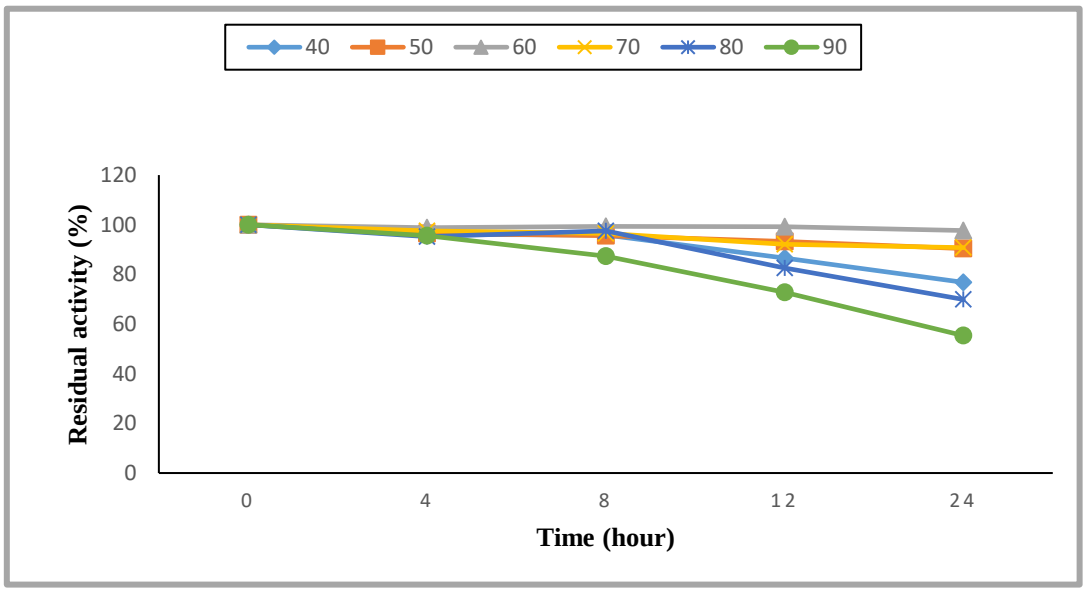

Fig. 3: Effect of temperature on thermostability

Protein structure is affected by change in pH which affects enzyme activity. Lipase exhibited the greatest activity at a pH of 7.8, although it remained active within a pH range of 3.4-9 following by decreased afterwards. So lipase produced by *Bacillus* consortium had highest

activity at pH 7.8 in presence of 100mM Tris HCl buffer (fig. 4). In earlier study, *Geobacillus thermodenitrificans* IBRL-nra sp. had peak lipolytic activity at pH 7 in presence of phosphate buffer followed by 6.5 and 7.5 pH (Balan *et al*., 2012). On the other hand, *Bacillus subtillis* had maximum lipolytic activity at pH 8 (Mazhar *et al*., 2016).

Purified lipase was pre-incubated for varying lengths of time, such as 4, 8, and 12 hours, at various pH levels (3.4–9) to determine their pH stability. The enzymes retained its 99.81% at pH 6.6 for 4 h which reduced to 98.74% after pre-incubation period of 24 h. However, 98.45% enzyme activity was observed after exposure at pH 7 for 4h and it reduced to 78.83% for pre-incubation period of 24 h (fig. 5). Correspondingly, Kojima and Schimizu, 2003, in their study, observed that the optimal pH stability range for bacterial lipase was between 6 and 8, and pH values above 8 make them unstable. However, 70 and 80% of the relative activity was maintained.

Lipase activity is influenced by reaction time. In current study, reaction time of 30min had maximum lipolytic activity followed by 60, 90, 120 and 150 mins (fig.6). The findings of this study are consistent with previous research on lipases produced by the *Bacillus* cereus (NC7401) strain, where optimum reaction time with the substrate was 30 minutes (Akhter *et al*., 2022). These findings are also in line with the lipases produced by *Bacillus methylotrophicus* PS3 strain (Sharma *et al*., 2017).

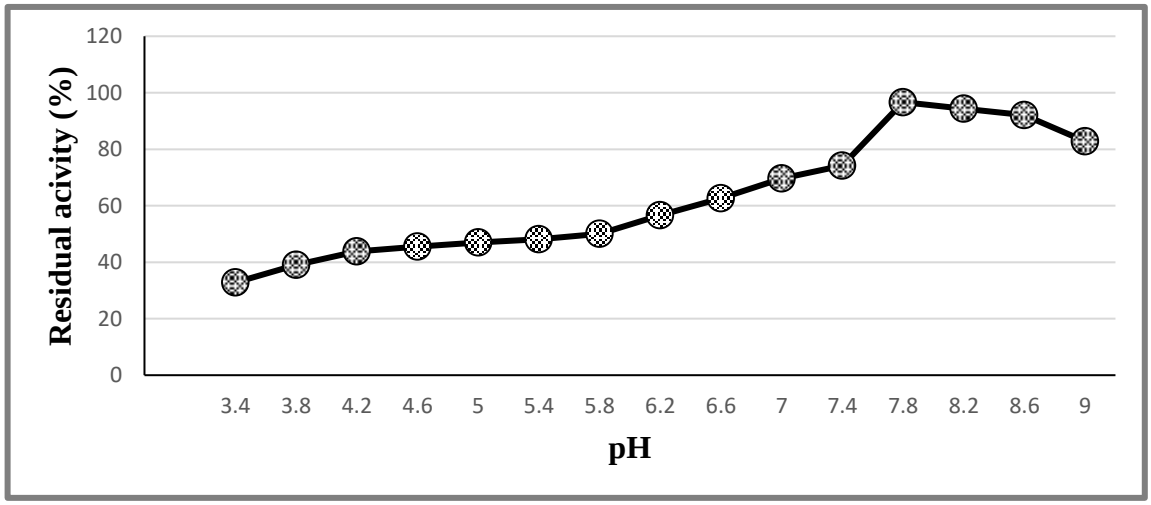

Fig 4 Effect of pH on purified lipase activity by *Bacillus* consortium

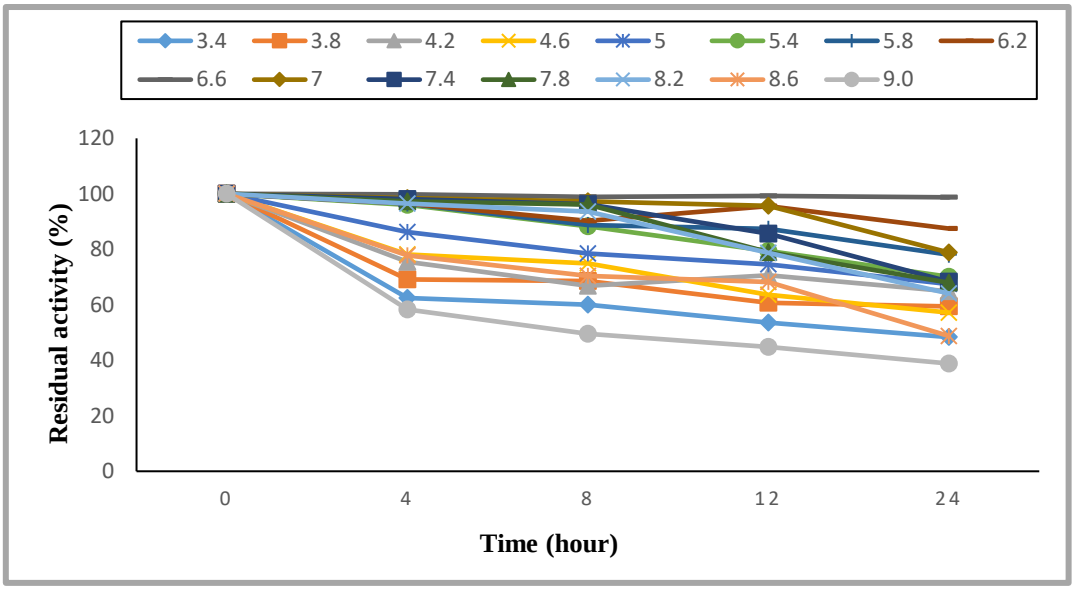

Fig. 5. Effect of pH on stability of lipase

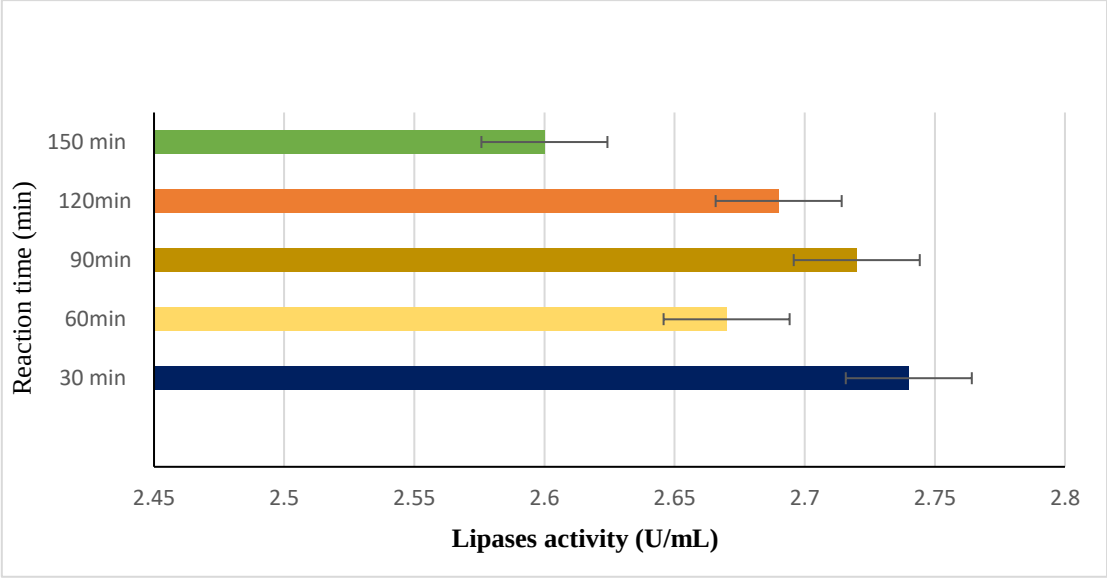

Fig. 6: Reaction time and lipase production

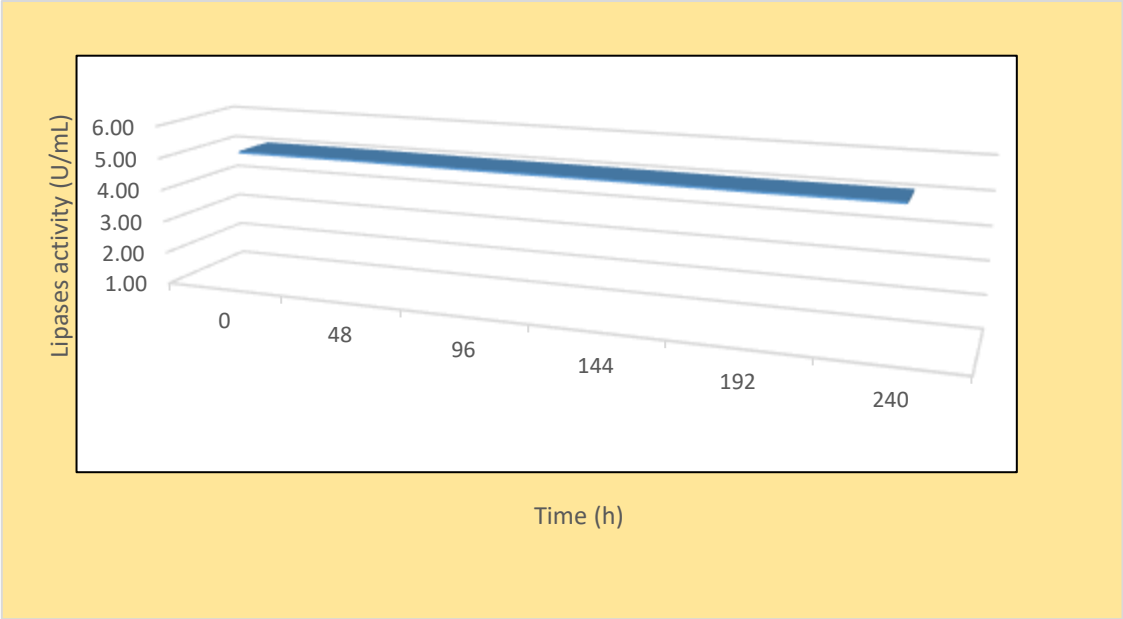

Fig. 7: Effect of shelf stability on lipase activity

Conclusion:

Thermophilic bacteria were isolated from hot springs. The thermophilic lipase purified and characterized from *Bacillus* consortium provides a stable biocatalyst for industrial processes. Due to its higher thermostability and pH stability it can be used in textile industry for sustainable and efficient alkaline processes and will be a good substitute of chemical processing.

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Conflict of interest

The authors have affirmed that they have no competing interests to report.

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