

## ISOLATION AND SCREENING OF THERMOPHILIC BACTERIA AND ITS SUBSEQUENT EVALUATION FOR LIPASES PRODUCTION

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### Abstract

Thermophilic bacteria are always being attracted for their thermostable products including enzymes. In the present work, isolation and screening of thermophilic lipolytic bacterial strains were performed from hot spring environments of Azad Jammu, Kashmir, and Manghopir, Karachi. Forty-eight bacterial strains were isolated using serial dilution method. Phenol red agar medium was used to screen hyper producers for lipases production through agar well diffusion technique. Fifteen strains were found lipolytic positive. Six were selected as lipases hyper producers based on zone hydrolysis. Their compatibility was checked with all other strains for the formulation of bacterial consortium for maximum lipolytic activity. By fermentation process, the highest lipases production was observed by consortium of *Bacillus toyonensis* (L2AKh) and *Bacillus thuringiensis* (L2AKo), which was selected for subsequent lipases synthesis. Various physiological variables, including pH, incubation temperature and incubation time, were optimized for maximum lipases synthesis. Lipases produced by thermophilic bacteria had optimum pH of 6, incubation temperature of 60°C, and an optimum incubation period was found to be 24h. The thermostable lipases produced in the present study are suitable for exothermic reactions on an industrial scale.

**Key words:** Bacterial isolate, Fermentation, Lipases, Thermophilic and Screening.

### Introduction

Lipases are a class of hydrolytic enzymes that catalyze triacylglycerol acyl hydrolases into free fatty acids and glycerol. Lipases obtained from bacteria are more stable than plants, animals, and microorganisms (Bronsheuer, 2002). Bacterial lipases are easily cultivated and optimized, so these are extensively used in industry on a large scale (Hassan *et al.*, 2006). Currently, isolation and screening of new bacteria isolate increased owing to the presence of unique catalytic characteristics. Agro-industrial waste, vegetable oil, dairy plants, and soils tainted with oils are a reservoir for lipase-producing microorganisms (Sorhaug & Stepaniak, 1997; Sirisha *et al.*, 2010; Salihu *et al.*, 2012).

Thermophilic microbes are vital because they are not denatured at elevated temperatures (Lee *et al.*, 1999). Thermophilic microorganisms produce thermostable enzymes which can tolerate harsh industrial conditions and produce products at elevated temperatures (Demirjian *et al.*, 2001). Extremophiles are microbes that inhabit extreme environmental conditions, pH, temperature, and salinity. Microbes inhabiting extreme temperatures (45°C-80°C) are regarded as thermophiles and produce thermostable enzymes (thermophilic enzymes) (Fooladi & Sajjadian, 2012).

Microbial enzymes are applied in dairy, food, new polymeric materials, textile, biodiesel, pharmaceuticals, cosmetics, agrochemicals, and chemical production. Mostly microbial enzymes are produced by fermentation such as solid state and submerged technique (Lotrakul & Dharmsthiti, 1997). Submerged fermentation is superior to solid state protocol owing to the provision of proper oxygen, several kinds of microbes can be handled quickly, reduce cost and energy, design of fermentation vessel is simple, reduction in equipment and reduction downstream processing problems (Singhania *et al.*, 2010; Castilho *et al.*,

2000). In 1961, thermostable enzymes were reported from hot water springs, it was investigated that several bacteria not only tolerate but also exhibit high growth at elevated temperature (Brock, 2011). As thermophilic processes are stable, fast, lesser expensive, extensive product recovery, reactant activity, and capable of carrying biotechnology processes at raised temperatures have a lesser risk of contamination than common mesophiles (Haki, 2003). Thermophilic bacteria extensively affect the solubility and bioavailability of organic compounds, therefore ensuing greater bioremediation. Lipases from bacteria are affected by factors such as pH, temperature, carbon, nitrogen lipids, stirrer conditions, dissolved oxygen concentration, and inorganic salts (Rosenau & Jaeger, 2000). The present study was particularly designed to identify and examine the potential for the synthesis of lipases in lipolytic bacteria from hot water spring habitats using qualitative and quantitative methodologies.

### Material and Methods

**Collection of sample:** Samples of water were taken from the Tattapani hot spring of Kotli district of Azad Kashmir and Manghopir hot spring in Karachi in sterilized bottles. Sample bottles were labelled and stored in the refrigerator at 4°C.

**Isolation of thermophilic bacteria:** The bacterial species were isolated by serial dilution on nutrient agar. To make the dilutions, 1mL of inoculum was added to each test tube containing 9mL of distilled water. Each petri plate was prepared with nutrient broth agar medium and 100 µl of suspension from each test tube. By streak plate method, pure cultures were isolated and petri plates were incubated at 37°C for 24-48h to check the growth of bacteria on nutrient agar medium (Rhodes & Stanbury 1997; Adiguzel *et al.*, 2009).

### Screening of bacterial strains

**Qualitative screening:** The bacterial strains were initially grown for one day on an LB medium containing olive oil (1%). A sterile phenol red agar plate was utilized for the chromogenic assay with olive oil as a substrate. Sterile cup borer was aseptically perforated into sterile agar plates to produce a four millimeters diameter well. Separately, 100  $\mu$ l of enzyme were placed in each well and plates were incubated for one day in an incubator at 37°C. Colonies of yellow color appeared around the wells were measured in millimeters that showed lipases were being produced (Iqbal & Rehman, 2015; Femi-Ola *et al.*, 2018).

**Quantitative screening:** For quantitative screening of lipases, liquid and the solid medium were employed. The submerged production medium utilized in this investigation contained the following components: (MgSO<sub>4</sub>·7H<sub>2</sub>O 0.0.3%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5%, K<sub>2</sub>HPO<sub>4</sub> 0.05%, and d.H<sub>2</sub>O 100 mL). Microbial cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium at 37°C on a rotary shaker (200 rpm). After 48 hours' incubation, the culture was centrifuged (10,000 rpm) for 15 minutes at 4°C to extract extracellular enzymes from the cell-free culture supernatant (Sidra *et al.*, 2016). The solid production medium contained a solid substrate (rice bran). The solid medium, after inoculation, was incubated in the oven at 37°C for two days. Then, 100 ml of phosphate buffer was added, shaken on a shaker for 1 hour and the mixture was filtered to extract the enzyme (Iftikhar *et al.*, 2012). The titrimetric approach assayed lipases activity in the supernatant.

**Compatibility testing:** Higher zone showing bacterial isolates were used for compatibility testing (consortia formulation) for enhanced lipase production, and lipolytic activity was determined under standard assay conditions (Darma *et al.*, 2019).

**Morphological and biochemical characterization:** Nutrient agar was used to study the morphological and colony features. Using standardized procedures, the biochemical properties (Mannitol test, urease, indole, catalase, hydrogen sulfide generation, motility test, endospore development, and oxidase) were also detected (Mukesh Kumar *et al.*, 2012).

**Lipases enzyme assay:** For biosynthesis of lipases, the titrimetric assay was performed. For this purpose, an assay substrate was prepared to contain 2mL calcium chloride, 5ml phosphate buffer, and 10% olive oil gum acacia solution. Supernatant (1 mL), was added to the assay substrate and rotated on the shaker at 150-200rpm for 1 hr. After 1 hour, the reaction was stopped by adding 20 mL of alcohol acetone [1:1]. The mixture was titrated against 0.1N NaOH, whereas one drop of phenolphthalein was used as an indicator (Iftikhar *et al.*, 2008).

**Preparation of slants:** For the preparation of slants, NBA medium was prepared in a 250 ml flask and then poured into the test tubes. Then test tubes were sterilized by autoclaving for fifteen minutes at 121°C, 15lb/inch<sup>2</sup>. Then, the test tubes were kept in a slanting position inside laminar airflow and allowed to cool. When the media was solidified, the test tubes were inoculated with bacterial cultures and incubated at 70°C for 24 h in an oven. After 24 h, bacterial colonies appeared, the slants were shifted to the refrigerator at 4°C. 10 mL of distilled water that had been sterilized, combined on a vortex mixer with the slants to produce the inoculum. Statistical data analysis was done by Co-stat software (Snedecor & Cochran, 1989).

**Effect of incubation period:** The influence of different periods on the lipases activity was determined by measuring the enzyme activity at incubation period ranging from 24 to 120 hours with an interval of 24 hours under standard assay conditions (Akhtar *et al.*, 2022).

**Effect of incubation temperature:** The influence of various temperature for maximum lipases production was determined at incubation temperature range from 30-70°C under standard assay conditions (Akhtar *et al.*, 2022).

**Effect of initial pH of medium:** The impact of pH on the production of enzyme at various pH ranging from 6-9.2 with an interval of 0.8 pH was evaluated in a batch fermentation process containing a 100 ml production medium in Erlenmeyer's flask (Akhtar *et al.*, 2022).

### Result and Discussions

The isolation and systematic assessment of bacterial strains within hot spring ecosystems offer a valuable approach for the identification of thermophilic bacterial species proficient in the synthesis of thermophilic lipase enzymes. The lipases producing bacteria were isolated from Tattapani Azad Jammu and Kashmir and Manghopir Karachi hot springs. From the total of 48 isolates, the lipolytic negative strains were put aside by screening and the remaining isolates were (15 strains) were biochemically characterized Table 2. Based on morphological characteristics of bacteria as shown in in Table 1, seven bacterial isolates were identified as genus *Bacillus*. Tattapani and Manghopir Karachi hot springs' water physicochemical properties are illustrated in Table 3.

When bacterial isolates were checked on phenol red agar for lipolytic activity, all the isolates produced a zone of hydrolysis of varying sizes on phenol red agar. The pH of the medium was lowered because it contained chromogenic substrates, which causes the medium to become yellow rather than red when free fatty acids were released. Six of the bacterial cultures had the highest zone of clearance out of all the cultures tested (Fig. 1), while nine cultures had reduced zones. The zone formation demonstrated the presence of lipolytic-positive bacterial strains (Fig. 2). Similarly, Akhtar *et al.*, (2022) used phenol red agar well diffusion method for the screening of lipolytic activity by *Bacillus cereus* strains.

**Table 1. Morphological characteristics of bacterial colony.**

Isolate	Colony colour	Colony surface	Colony edge	Bacteria shape	Gram reaction	Lipase
L <sub>2</sub> AKc	Creamy	Dry, flat	Smooth	Cocci	Negative	Strong
L <sub>2</sub> AKn	Creamy	Moist, flat	Smooth	Bacillus	Positive	Strong
L <sub>2</sub> AKe	Creamy, pale	Dry, flat,	Rough	Cocci	Positive	Strong
LMPKf	Off-white	Dry, flat,	Smooth	Bacillus	Negative	Strong
LMPKo	Off-white	Moist, flat	Rough	Cocci	Positive	Strong
L <sub>2</sub> AKh	Off-white	Moist flat,	Smooth	Bacillus	Negative	Strong
LMPKe	Off-white	Moist, flat	Rough	Cocci	Positive	Strong
LMPKb	Creamy, pale	Dry, flat	Rough	Cocci	Negative	Strong
L <sub>2</sub> AKd	Off-white	Dry, flat	Rough	Bacillus	Negative	Strong
LMPKa	Creamy	Dry, flat	Smooth	Bacillus	Positive	Strong
L <sub>2</sub> AKg	Creamy, pale	Moist, flat	Rough	Cocci	Positive	Strong
L <sub>2</sub> AKo	Off-white	Moist, flat	Smooth	Bacillus	Negative	Strong
L <sub>2</sub> AKb	Off-white	Dry, flat	Smooth	Bacillus	Positive	Strong
L <sub>2</sub> AKj	Creamy, pale	Moist, flat	Smooth	Cocci	Positive	Strong
L <sub>2</sub> AKp	Creamy, pale	Dry, flat	Smooth	Cocci	Positive	Strong

**Table 2. Biochemical characterization of bacterial strains**

Bacterial strain	L <sub>2</sub> AKc	L <sub>2</sub> AKn	L <sub>2</sub> AKe	LMPKf	LMPKo	L <sub>2</sub> AKh	LMPKe	LMPKb	L <sub>2</sub> AKd	LMPKa	L <sub>2</sub> AKg	L <sub>2</sub> AKo	L <sub>2</sub> AKb	L <sub>2</sub> AKj	L <sub>2</sub> AKp
Motility test	-	+	+	-	+	+	-	-	-	-	-	+	-	+	+
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase test	+	-	-	+	-	+	-	+	+	-	+	+	-	+	-
Oxidase test	-	+	+	-	+	+	-	+	-	-	-	+	-	+	+
Mannitol test	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Endospore formulation	+	-	-	-	+	-	-	-	+	+	-	-	-	-	+
H <sub>2</sub> S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

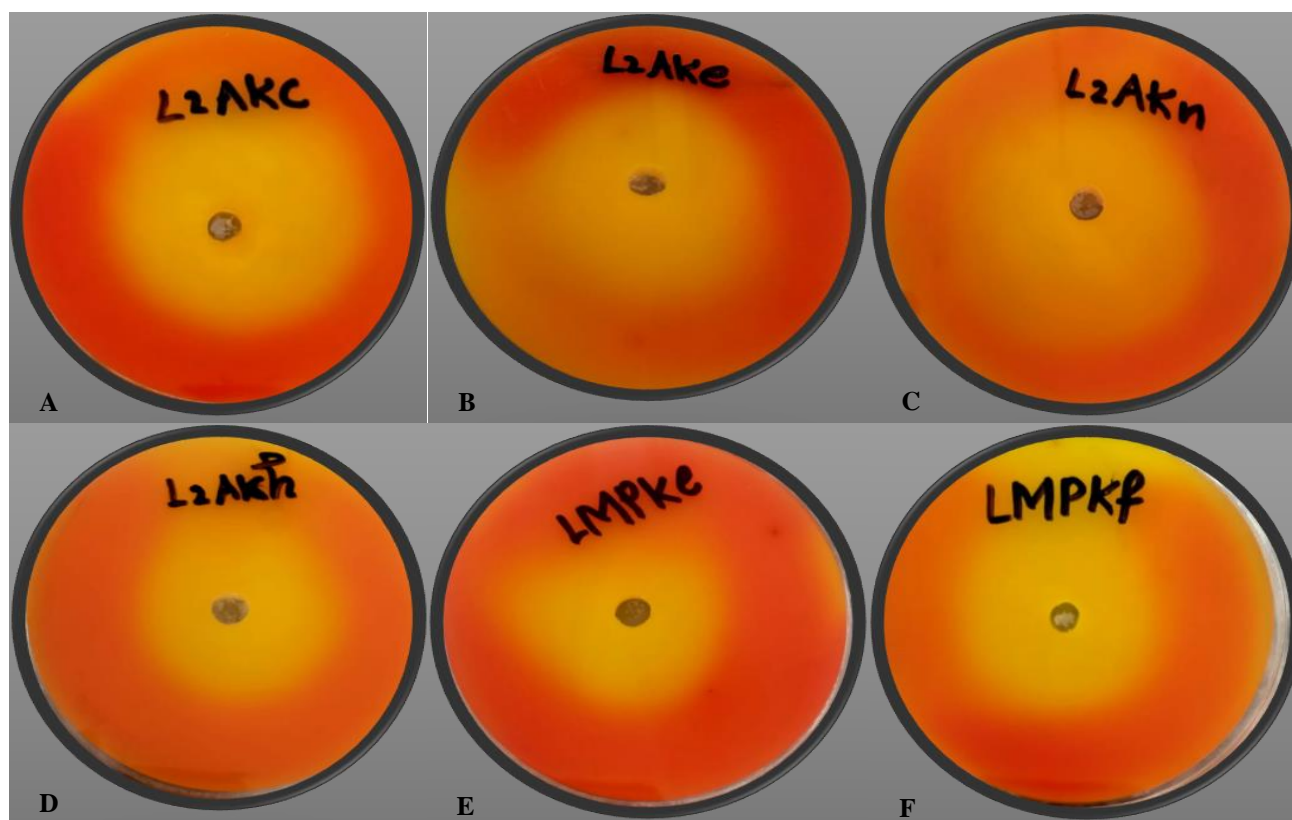


Fig. 1. Lipases hyper-producer bacterial strains showing yellow zone production (in mm) on phenol red agar plate (A) L<sub>2</sub>AKc showing highest zone of 25 mm diameter (B) L<sub>2</sub>AKe showing zone of 20 mm diameter (C) L<sub>2</sub>AKn showing zone of 24 mm diameter (D) L<sub>2</sub>AKh showing zone of 20 mm diameter (E) LMPKe showing zone of 20 mm diameter (F) LMPKf showing zone of 20 mm diameter.

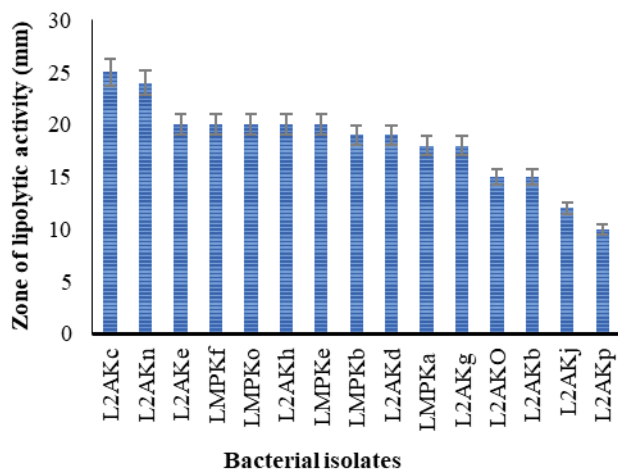


Fig. 2. The bacterial strains showing lipolytic activity by qualitative screening.

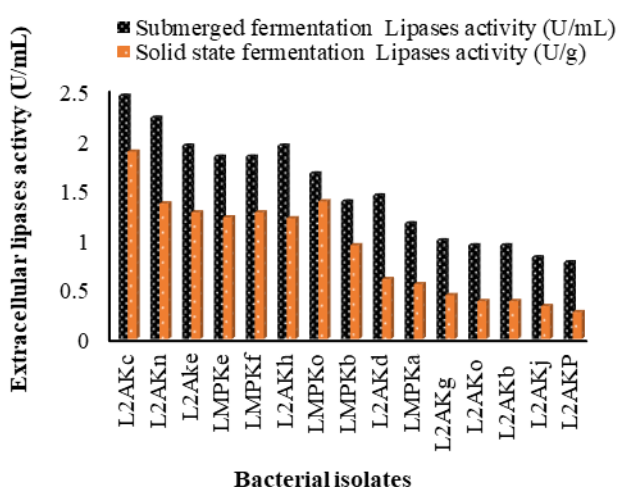


Fig. 3. Quantitative screening for lipases production by fermentation

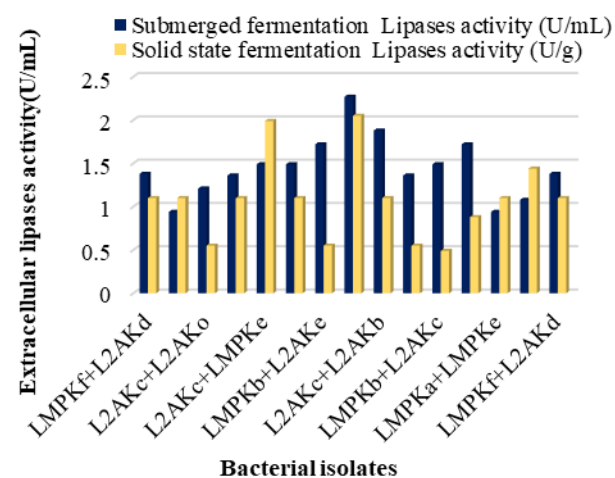


Fig. 4. Compatibility testing for production of lipases.

Bacterial isolates were quantitatively screened by using submerged and solid state fermentation protocol. The study results showed that all the isolates were lipolytic positive based on olive oil hydrolysis. But some bacterial strains showed highest lipases production

followed by other strains where the activity was reduced as illustrated (Fig. 3). Similar results were reported in previous study where quantitative estimation of lipases was conducted for lipolytic activity (Baloch *et al.*, 2021).

Table 3. Tattapani and Manghopir Karachi hot springs water physicochemical properties.

Parameters	Tattapani hot spring	Manghopir hot spring
pH	7.65-7.74	7.98
Electrical conductivity (mS)	1.08	2.32
Temperature	59.2 to 60.7°C	45-47.6°C
TDS (ppt)	0.54	1.15
Turbidity(ntu)	0.00	0.8
Smell	Nil	Nil
Colour	colorless	colorless
COD	0.00	0.00
BOD	0.00	0.00

Six hyper-producers were selected, and their compatibility (consortium formulation) was checked with all other strains. There were total 69 plates that were used in compatibility pairing among which 14 showed compatibility (data not shown). The highest lipases production was observed by consortia of L2AKh+ L2AKo ( $2.27 \pm 1.22a$  U/mL) under submerged fermentation technique and was selected for further studies (Fig. 4). Similarly, previous study by Darma *et al.*, 2019 used bacterial consortium for enhanced lipases production.

The incubation period significantly impacts the bacteria's ability to produce extracellular lipases. The enzyme in this study was active for a variety of incubation durations, ranging from 24 to 120 hours. The maximum activity was observed during a 24 h incubation period ( $2.91 \pm 0.21^a$  U/mL). However, lipases activity declined after 24 hours, and minimum production at 120 hours (Fig. 5a). Decreased lipases activity was obtained with longer incubation periods., which may have been caused by nutrient depletion, a change in medium pH, the buildup of hazardous byproducts, or a loss of moisture. Various researchers have reported different incubation times for the best synthesis of lipases. Our findings differ from those of Mazhar *et al.*, 2023, who conducted maximum lipases synthesis after 72 hours of incubation.

Incubation temperature is a very crucial factor for the production of lipases. An increase in temperature beyond the optimum level can decrease the enzyme activity because of a decrease in the activation energy. Temperature is one of the critical parameters to be optimized to study its effects on lipases production. Maximum enzyme activity ( $3.33 \pm 0.11^b$  U/mL) was observed at an optimal temperature of 60°C. However, significant amount of enzyme production occurred at a temperature ranging between 30°C to 70°C, and a decrease in activity was detected above 60°C (Fig. 5b). The results are consistent with Dominguez *et al.*, 2005 who obtained maximum lipolytic activity at a temperature of 60°C. However, these results disagree with the findings of Lokre & Kadam, 2014; Abol-Fotouh *et al.*, 2021), who reported 70°C optimal temperature for maximum lipolytic activity. Akhter *et al.*, 2022 study found that lipase was stable between temperature range 30–80°C whereas optimally active at 50°C using *Bacillus cereus* strain.



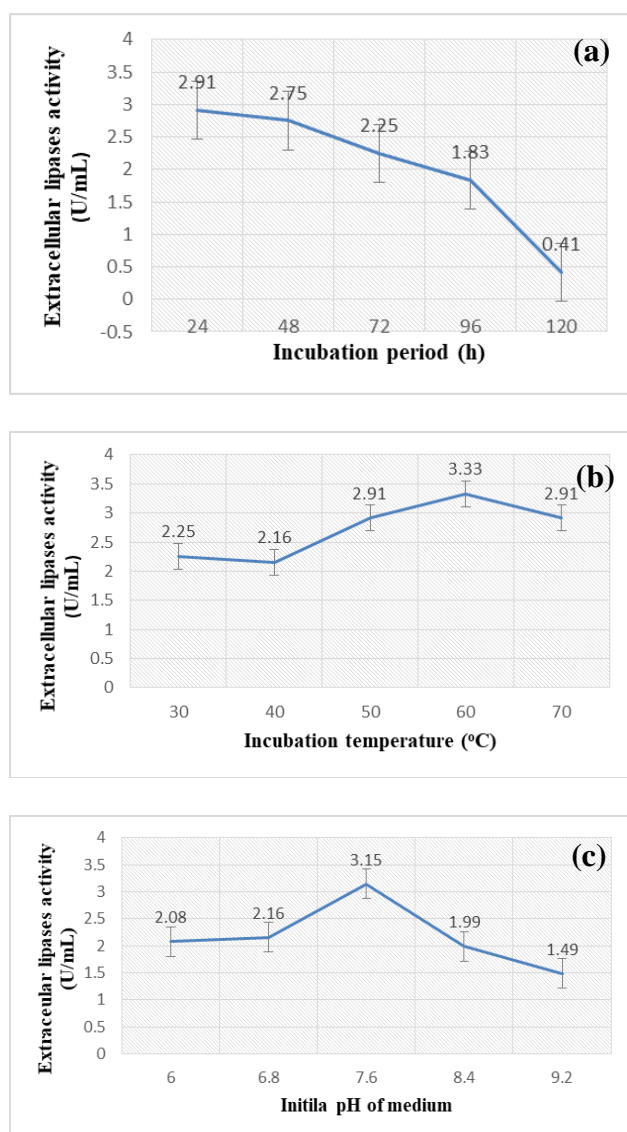


Fig. 5. Effects of (a) Incubation periods (b) Incubation temperature (c) Initial pH of medium on production of thermostable lipases.

The amount of lipases produced is affected by the pH of the culture medium. Current study revealed that the bacterium could produce lipases at pH levels ranging from 6.0 to 9.2 (Fig. 5c). Among all initial pH of fermentation mediums, the highest production of  $3.15 \pm 0.33^a$  U/mL was observed at pH 7.6. So, pH 7.6 was selected for further production of lipases. It might be because the microorganism opted for suitable environments, any increase or decrease in pH did not support the organism's growth, and development declined. Our findings are consistent with previous research (Celik *et al.*, 2014; Bharathi *et al.*, 2018), where the optimal pH for maximum lipolytic activity was pH 6. Khalil *et al.*, (2011), reported top growth above the pH range of 8.5. Mazhar *et al.*, 2023 described optimal activity at pH 7 by using *Bacillus amyloliquefaciens* strain. Maximum activity of lipases produced by using *Bacillus cereus* strain was observed at pH 8 as compared to our results (Akhtar *et al.*, 2022).

## Conclusion

The current study aimed to screen and isolate lipases producing bacteria from diverse water samples. Based on their growth in medium containing solely olive oil as a carbon source and zone of hydrolysis, 15 bacterial isolates were chosen. Two isolates were preferred for further study after a quantitative evaluation revealed them to be the highest lipases producers based on consortium formulation. The strain was recognized as belonging to *Bacillus toyonensis* and *Bacillus thuringiensis* after being morphologically and biochemically characterized. The physiological factors were optimized to increase lipases production. Thermophilic bacterial strain had excellent potential for maximum production of lipases and can be employed to produce biotechnological products industrially.

## Acknowledgements

I am cordially thankful to ORIC for funding and Department of Botany, Government College University, Lahore for motivation and inspiration for such a worthy task to my research work and providing me facilities and courage throughout the period of research.

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(Received for publication 22 September 2022)