

PURIFICATION AND CHARACTERIZATION OF LIPASE FROM EXTREME HALOPHILES ISOLATED FROM LITTLE RANN OF KUTCH, GUJARAT, INDIA.

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ABSTRACT

Isolation of 30 extreme Halophiles was performed on the medium containing 35% NaCl (w/v) after enrichment in liquid media, designated as Ku-1 to Ku-30. Ku-10, Ku-19 and Ku-20 were selected as best lipase producer from solid media on the basis of zone ratio and were grown in liquid medium containing tributyrin as sole source of carbon. Lipase was partially purified by ammonium sulfate fractionation and highest lipase activity was obtained in 60% saturation fraction. Extracellular lipases from Ku-19 and Ku-20 were active maximally at pH 6 -8 and 40°C. Lipase from Ku-10 shows greater degree of temperature tolerance and shows highest activity at 60°C. Additionally, lipases from Ku-19 and Ku-20 denatured in presence of 8 M urea for 2 hours, while lipase from Ku-10 can able to tolerate its effect for 24 hours and retain little activity. Chemicals other than NaCl decrease enzyme activity and chemicals like EDTA and SDS affects activity drastically. After UV mutagenesis, no increase in lipase yield was observed among three halophiles.

Key words: Extreme halophiles, Little Rann of Kutch, Halophilic lipase, Enzyme characterization

INTRODUCTION

The Rann of Kutch is an area of 18,000 sq km situated within Gujarat along the border with Pakistan. The Little Rann of Kutch extends northeast from the Gulf of Kutch over 5,100 sq km. Once an extension of the Arabian Sea, the Rann has been closed off by centuries of silting. Little Rann of Kutch is a typical ecological system with saline desert climate having least floral diversity and unique faunal diversity.

Extremophiles are able to survive and grow in extreme environment and are widely distributed in natural habitats. Halophiles are the group of “salt loving” microorganisms present in saline habitats. Halophiles can be classified as halotolerant microorganisms that can grow both in the presence and absence of salt. True halophiles can be further divided into slight halophiles (Grow

optimally at 3-15% w/v salt), Moderate halophiles (Grow optimally at 3% w/v salt) and extreme halophiles (Grow optimally at 25% w/v salt) [Ventosa *et al.*, 1998]. Halophilic microorganisms have greater capacity to produce salt and thermo tolerant enzymes like cellulases, amylases, proteases, lipases and xylanases [Sánchez-Porro *et al.*, 2003; Govender *et al.*, 2009; Rohban *et al.*, 2009].

Lipases (E.C.3.1.1.3) is an important enzyme commercially, catalyze breakdown of triacylglycerol to glycerol and fatty acids when absorbed to oil-water interface [Martinelle *et al.*, 1995]. Lipases are widely used in fat/ oil processing, detergent formulation, paper-pulp industries, food industries, cosmetics and pharmaceuticals [Rubin and Dennis, 1997a; Rubin

and Dennis, 1997b], polyurethane [Masse *et al.*, 2001] and biodegradation of fatty acid containing waste [Takamoto *et al.*, 2001]. Lipase research is focused on kinetics studies, determination of 3D structure and genetic modification in lipase producing genes [Alberghina *et al.*, 1991].

MATERIALS AND METHODS

1. COLLECTION OF SAMPLES

Samples were collected from little Rann of Kutch from the back waters of sea. Total 10 wet samples were collected near Surajbari Bridge- Kutch, Gujarat, India (Latitude- 23°12'4.95"N and Longitude- 70°43'2.45"E) including 5 salt samples, 2 soil (mud) samples and 3 water samples from 2 km. area.

2. ENRICHMENT AND ISOLATION OF HALOPHILES

Halophiles were enriched in halophilic broth (Himedia) containing (gm/lit): Casein acid hydrolysate-10, Yeast extract- 10, Protease peptone-5, Trisodium citrate- 3, Potassium chloride- 2, Magnesium sulfate- 25, Sodium chloride- 50-350, pH- 7.0-7.4 as well as Complete media broth- CMB containing (gm/lit): Glucose- 10, Potassium dihydrogen phosphate- 10, Yeast extract- 5, Peptone- 5, Sodium chloride- 50-350, pH- 7.0-7.4. From enriched 35% NaCl (w/v) halophilic broth and complete media broth organisms were streaked on respective agar media by four sector method for the purpose of isolation into pure culture. Total 30 isolates were obtained and preserved on N-agar slant at 4°C for further studies.

3. ENZYME ASSAY

Lipase activity was determined as described by Pignede *et al.*, 2000. The substrate emulsion was prepared with olive oil, 50 ml. The reaction mixture contained 1 ml enzyme, 5 ml substrate and 2 ml of 50mM phosphate buffer, pH 6.8 and was incubated for 1 hour at 37°C with shaking. The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acid released with 50mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1μmole of fatty acid per min.

4. PARTIAL PURIFICATION OF LIPASE

Crude lipase preparation was fractioned with ammonium sulfate saturation at 4°C. 20%, 40%, 60%, 80% and 100% fractions were collected and dissolved in 50 mM phosphate buffer of pH 7. The fractions were dialyzed by dialysis membrane (Himedia) at 4°C against phosphate buffer. Protein content was determined by Folin's method.

5. CHARACTERIZATION OF LIPASE

5.1 Temperature optima of lipase

Temperature optimum was determined by incubating reaction mixture at 20°C to 70°C. Lipase activity was determined by the pignede method as described earlier.

5.2 Thermal stability

Thermal stability of lipase was determined by incubating enzyme at 60°C and 70°C for 30 min, 1 hour and 2 hour followed by rapid cooling at 4°C. Enzyme activity was measured and residual activity was calculated on the basis of available data.

5.3 Effect of pH on lipase

The effect of pH on lipase was determined by preparing the substrate emulsion in various buffers with varying pH. Buffers were Citrate phosphate buffer (pH 4-5); Phosphate buffer (pH 6-7); Tris-HCl buffer (pH 8-9); Glycine-NaOH buffer (pH10). After incubation of reaction mixture for 1 hour in shaking condition, lipase activity was determined.

5.4 Effect of inorganic salts on lipase

Effect of inorganic salts on lipase activity was determined by incubating enzyme at 30°C for 1 hour followed by determination of enzyme activity. Inorganic salts were NaCl (10 Mm), BaCl₂ (0.001 M), MgCl₂ (0.001 M), KCl (2 Mm), FeSO₄ (0.001 M), CaCl₂ (0.001 M), NaF (2 Mm), MnCl₂ (2 Mm), Ethylene diamine tetra acetic acid (0.5%), Sodium dodecyl sulphate (0.5%). To determine effect of inorganic salts on lipase activity, it was compared with control containing no inorganic salts.

5.5 Effect of urea on lipase denaturation

Urea was used as denaturant at 8 M. The partially purified lipase was incubated for 1 hour and 2 hour. Residual activity was calculated by comparison with control containing no urea.

5.6 UV mutagenesis

All the three isolates were exposed to UV radiation in order to improve lipase secretion. Mutated cultures were compared with non-mutated cultures and enzyme activities were compared.

RESULTS AND DISCUSSION

Extreme halophiles were isolated from sea samples collected from Little Rann of Kutch, Gujarat, India. Total 30 halophiles were isolated on solid

media after enrichment, designated as Ku-1 to Ku-30 and were preserved as pure culture. On the basis of zone ratio on solid media, Ku-10, Ku-19 and Ku-20 were grown on liquid media containing tributyrin as carbon source.

1. Purification of lipase

Table-1 explains purification fold and yield of partially purified lipase by ammonium sulfate fractionation. Highest purification fold was obtained from Ku-19. Much higher yield and purification folds were obtained from *Fusarium oxysporum* [Moataza *et al.*, 2004] as compare to Ku-19. Ku-10 and Ku-20 shows lower yield as compare to Ku-19.

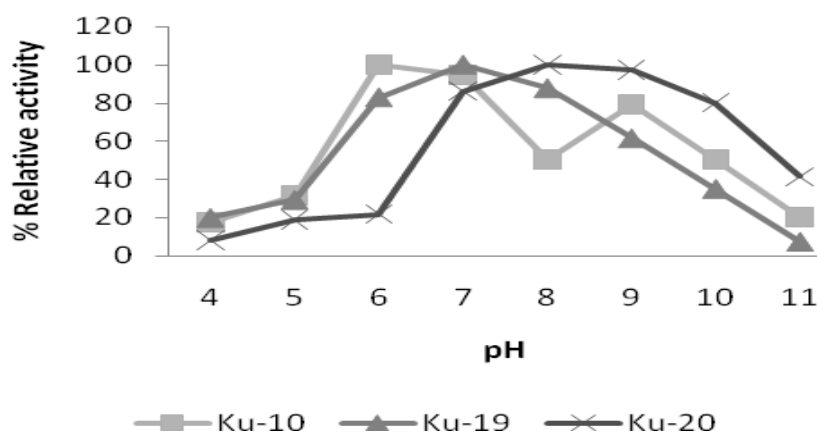
Table -1 Partial purification of lipase by ammonium sulfate fractionation.

Isolate	Crude/Partially purified	Specific Activity (Units/mg)	Purification fold	Yield (%)
Ku-10	Crude	10	-	100
	Partially purified	29.1	2.91	55
Ku-19	Crude	11.54	-	100
	Partially purified	57.76	5	54.15
Ku-20	Crude	10.2	-	100
	Partially purified	41	4	50.2

2. CHARACTERIZATION OF LIPASE

2.1 pH optima

Lipase from Ku-10, Ku-19 and Ku-20 was active maximally at pH 6, 7 and 8 respectively (Figure-1). The results are not compatible with lipase from *B.pumilus* LV01, gives maximum activity at pH 9 [Mariana *et al.*, 2008].

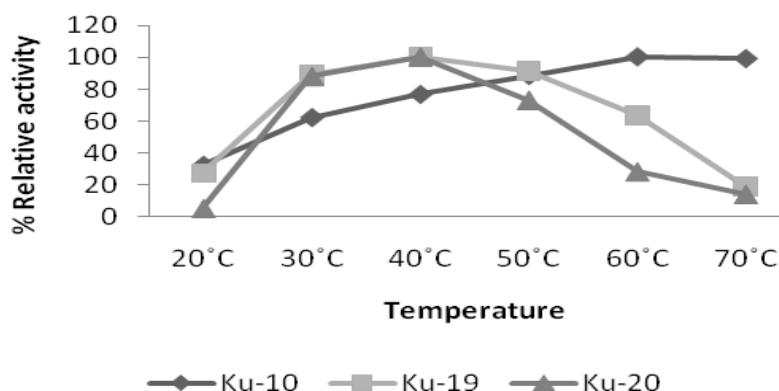


(Figure-1 pH optima of lipases)

2.2 Temperature optima

Ku-10 produces thermotolerant lipase active maximally at 60-70°C, the enzyme may have high industrial applications. Contrary, partially purified lipase from Ku-19 and Ku-20 less thermostable and can able to give maximum relative activity at

40°C (Figure-2). Similar to Ku-19 and Ku-20 but different temperature tolerant enzyme was obtained from halotolerant *Staphylococcus warneri* PB233 [Werasit and Anan, 2007], active maximally at 40°C.

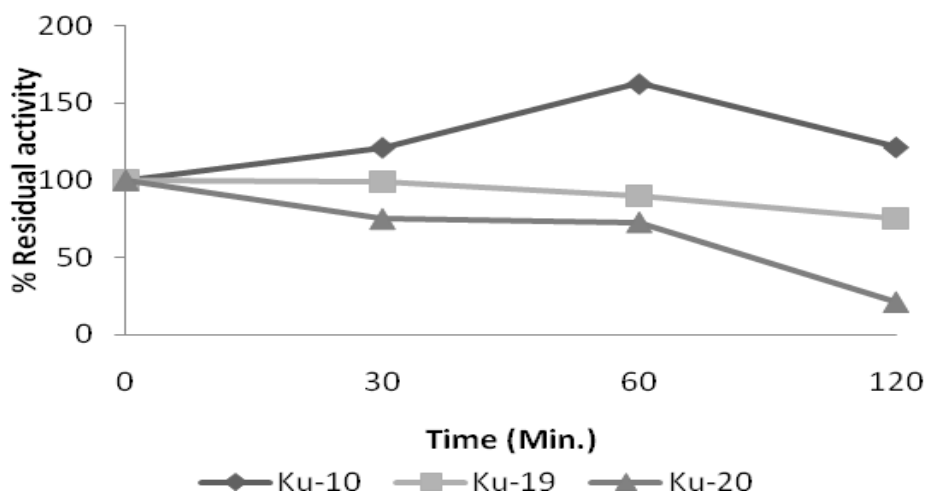


(Figure-2 Temperature optima of lipases)

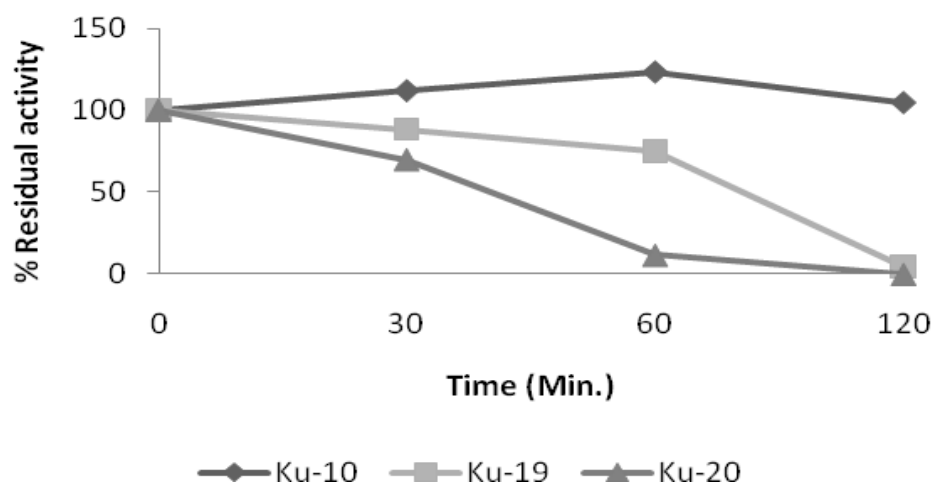
2.3 Thermal stability

Partially purified lipase from Ku-10 was showing high thermal stability with 163% and 123% relative activity at 60°C and 70°C respectively after 60 mins. (Figure-3, 4) Lipase with comparative less thermotolerant properties were obtained from

Salinivibrio sp. strain SA-2, which retain 90% of its activity at 80 °C for 30 min [Amoozegar *et al.*, 2008]. Lipase from Ku-19 and Ku-20 shows less thermostability and inactivated as temperature increases.



(Figure-3 Temperature stability of lipases at 60°C)

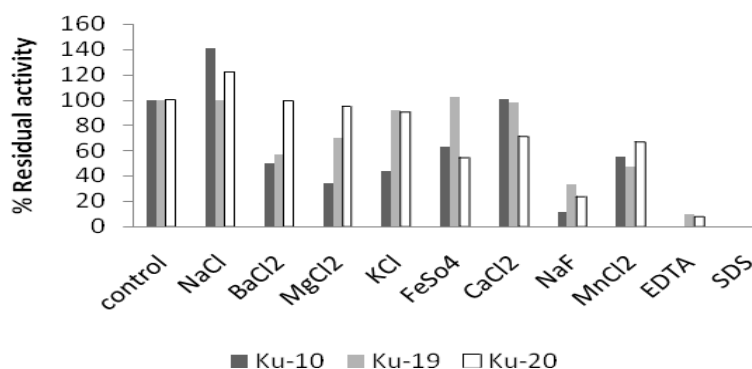


(Figure-4 Temperature stability of lipases at 70°C)

2.4 Effect of chemicals

Partially purified enzymes from extreme halophiles have high degree of halotolerance, chemicals other than NaCl decrease enzyme activity and chemicals

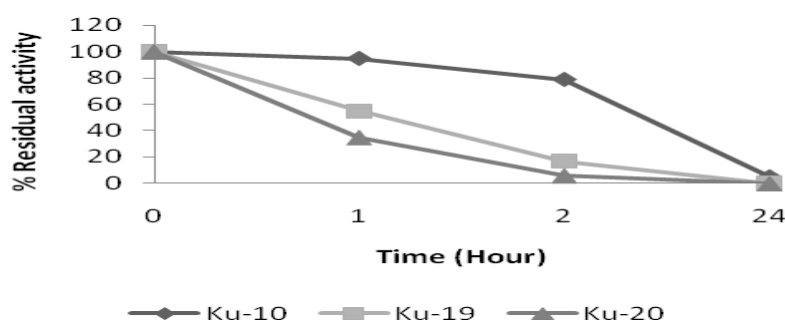
like EDTA, SDS affects activity drastically (Figure-5). Similar results were obtained in case of lipase Marine *Vibrio fischeri* [Ranjitha *et al.*, 2009].



(Figure-5 Effect of various chemicals on lipase activity)

2.5 Urea denaturation

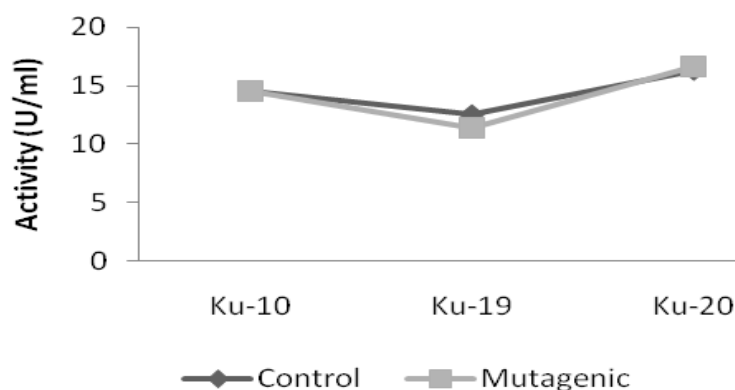
As shown in figure-6, partially purified enzyme from Ku-10 retains little activity after 24 hours in presence of Urea while lipase from Ku-19 and Ku-20 shows same activity after 2 hours.



(Figure-6 Effect of 8M Urea on lipase activity)

2.6 UV mutagenesis

As shown in figure-7, there was no considerable increase in lipase yield from extreme halophiles after exposure of physical mutagen UV radiation.



(Figure-7 UV mutagenesis for strain improvement)

CONCLUSION

Halophiles have capacity for secretion of salt and thermo-tolerant lipases important in food, detergent, pharmaceutical, paper industries. Media optimization, enzyme purification and

characterization, strain improvements are important practices to obtain good yield. Ku-10, an extreme halophile produces salt, thermotolerant lipase, may be explored at commercial point of view.

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