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Media optimization for lipase from *Halomonas salina*, a moderately halophilic bactrium isolated from excreta of wild Ass.

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Abstract
Twenty-four moderate halophiles were isolated from excreta of wild Ass after enrichment in complete media broth and halophilic broth. All the isolates were Gram's positive and non-capsulated. Further, isolates were screened for extracellular lipase secretion on solid media containing tributyrin as carbon source. *Halomonas salina* was the potential lipase producer identified on the basis of 16s r-RNA sequencing and further optimized for lipase secretion in liquid media. Maximum lipase secretion was after 264 hours of incubation i.e. during stationary phase. Isolates were found to secrete maximum lipase at pH 5, 14% NaCl, 30- 40˚C temperature and 3% substrate concentration.

Key-Words: Lipases, Moderate halophiles, Rann of Kutch, *Halomonas salina*

Introduction
Little rann of Kutch is bland, brown and flat. The landscape is punctuated with saline dessert plains, stony and thorn scrubs, arid grasslands, lakes and marshes. The Wild Ass sanctuary is located in the little rann of Kutch. Wild Ass is a protected species of the Indian Wildlife Protection Act, 1972. Sanctuary covers an area of 4954 km². The sanctuary is named after a wild Ass (*Equus hemionus khur*), highly endangered species of mammal. Wild Ass contains moderately halophilic bacteria in their intestine due to presence of salt in local grass [1].

Extremophilic microorganisms are inhabitant of extreme environment of earth. These organisms are known to tolerate harsh conditions like higher and lower temperature, high pressure, high salinity, acidity, alkalinity etc. Halophiles are the group of extremophiles that could tolerate and grow in the presence of high salinity. Microbes that could tolerate presence and absence of salt, categorized as halotolerant microorganisms, while microbes that grow above 15% (w/v) salt are categorized as extreme halophiles [2].

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Lipases (triacylglycerol acylhydrolases- E.C.3.1.1.3) are the group of enzymes that catalyze hydrolysis of triacylglycerol to glycerol and fatty acids when absorbed to oil-water interface [3]. Microbial lipases are applied in detergent industries [4], food industries [5], paper and pulp industries [6], for organic synthesis [7], bioconversions in organic media [8], regioselective acylations [9] etc.

Material and methods
Collection of samples
Samples (Excreta of wild Ass) were collected from little rann of Kutch, from wild Ass sanctuary near Dhrangadhra, Gujarat, India [Latitude- 22°98'4.181"N and Longitude- 71°51'0.242"E].

Enrichment and isolation of halophiles
Halophiles were enriched in halophilic broth (Himedia) containing Casein acid hydrolysate-10 (g/l), Yeast extract- 10 (g/l), Protease peptone-5 (g/l), Trisodium citrate- 3 (g/l), Potassium chloride- 2 (g/l), Magnesium sulfate- 25 (g/l), Sodium chloride- 50-150 (g/l), pH- 7.0-7.4 as well as complete media broth containing Glucose- 10 (g/l), Potassium dihydrogen phosphate- 10 (g/l), Yeast extract- 5 (g/l), Peptone- 5 (g/l), Sodium chloride- 50-150 (g/l), pH- 7.0-7.4. From enriched 15% 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 24 isolates were obtained, designated as Mk-1 to Mk-24 and preserved on N-agar slant at 4˚C for further studies. *Halomonas salina* was

identified on the basis of 16s r-RNA sequence analysis and selected for further studies.

Growth and kinetics of lipase producers

Potential isolate *Halomonas salina* was selected on the basis of zone ration on solid media and grown in broth containing: Tributyrene-1 ml, Peptone-1gm, Yeast extract-0.5gm, Sodium chloride- 10 gm, Distilled water- 100 ml and pH- 7.2. Growth and enzyme production was monitored at an interval of 8 hours.

Enzyme Assay

Lipase activity was determined as described by Pignede *et al*., [10].The substrate emulsion was prepared with, 50 ml. olive oil. 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 37ºc 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.

Optimization of Medium for lipase production.

Optimization of salt

Optimization of salt for optimum enzyme production was carried out by varying NaCl concentration in liquid media. NaCl concentration was maintained 10%, 11%, 12%, 13%, 14%, and 15%. After incubation period, growth and enzyme production was monitored.

Optimization of pH

Optimization of pH for optimum enzyme production was carried out by varying pH (adjusted by adding 1N HCl or 20% Na_2Co_3) concentration in liquid media. Different pH i.e. 4, 5, 6, 7, 8, 9, 10 was optimized in liquid media.

Optimization of Temperature

Temperature was optimized by incubating inoculated media at different temperature 20˚C, 30˚C, 40˚C, 50˚C, 60˚C.

Optimization of Substrate concentration

Substrate concentration optimization was carried out by inoculating organisms into medium with different substrate (Tributyrin) concentration i.e. 1%, 2%, 3%, 4%, 5%, 6%. All the flasks were incubated in shaking condition followed by measurement of growth and enzyme activity.

Results and Discussion

After enrichment and pure culture isolation, twenty four moderate halophiles were obtained from excreta of wild Ass and designated as Mk-1 to Mk-24. All the isolates were screened on tributyrin agar media and on the basis of zone-ratio; *Halomonas salina* was selected for further studies.

Growth kinetics with reference to lipase production

Halomonas salina on liquid media containing tributyrin, shows very little enzyme production till 120 hours followed by steadily increase in lipase secretion. Maximum lipase secretion was obtained on 264 hours of incubation i.e. during stationary phase (Figure-1). Contrary, *B. thermoleovorans* ID-1, showed extracellular lipase activity at late exponential phase [11]. Further incubation period decrease lipase activity sharply.

Media optimization

Optimization of media is important at industrial scale to improve the efficiency of the process without increasing the cost [12]. Media optimization is necessary practices to use available resources maximally.

Effect of salt

Halomonas salina shows maximum biomass production at 10% NaCl and maximum lipase at 14% NaCl. Higher or lower salt affects extracellular lipase secretion (Figure-2). This optimum salt concentration for *Halomonas salina* is not compatible with optimum salt requirement by *Staphylococcus warneri* for lipase secretion [13].

Effect of pH

Halomonas salina secretes maximum lipase at pH 5 while maximum growth was observed at pH 7. Alkaline pH found to be unsuitable for growth and enzyme secretion (Figure-3). Contrary, *Penicillium wortmanii* produced maximum lipase (12.5 U/ml) after 7 days culture using olive oil (5% w/v) as the carbon source. Lipase production was maximum at pH 7 [14].

Temperature optimization

Among the range of temperature tested, *Halomonas salina* grows maximally at 40˚C temperature and secrete maximum lipase at 30-40˚C. 20˚C and 50˚C affects growth and enzyme secretion drastically (Figure-4). Above optimum temperature is lower as compare to *Bacillus* sp. RS-12, produced maximum lipase at 50˚C [15].

Optimization of tributyrin

Among 1-6% (v/v) tributyrin, *Halomonas salina* grows maximally at 4% and secretes maximum lipase at 3% substrate concentration (Figure-5). Tributyrin is an inducer for lipase production. In turn, extracellular lipase production by *Rhodotorula glutinis* was maximum in the medium containing palm oil at a concentration of 2% [16].

Extremophilic microorganisms have been widely explored industrially and biotechnologically for its valuable products. Halophilic microorganisms can secrete salt and thermo-tolerant enzymes viz. protease, lipase, amylase, cellulase, chitinase etc.

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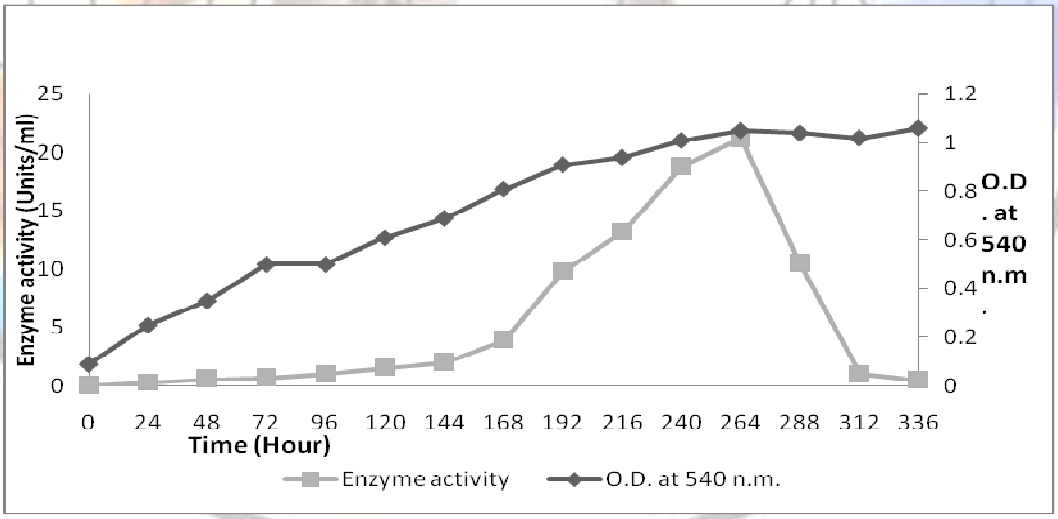

Fig. 1: Growth kinetics with reference to lipase production from *Halomonas salina*

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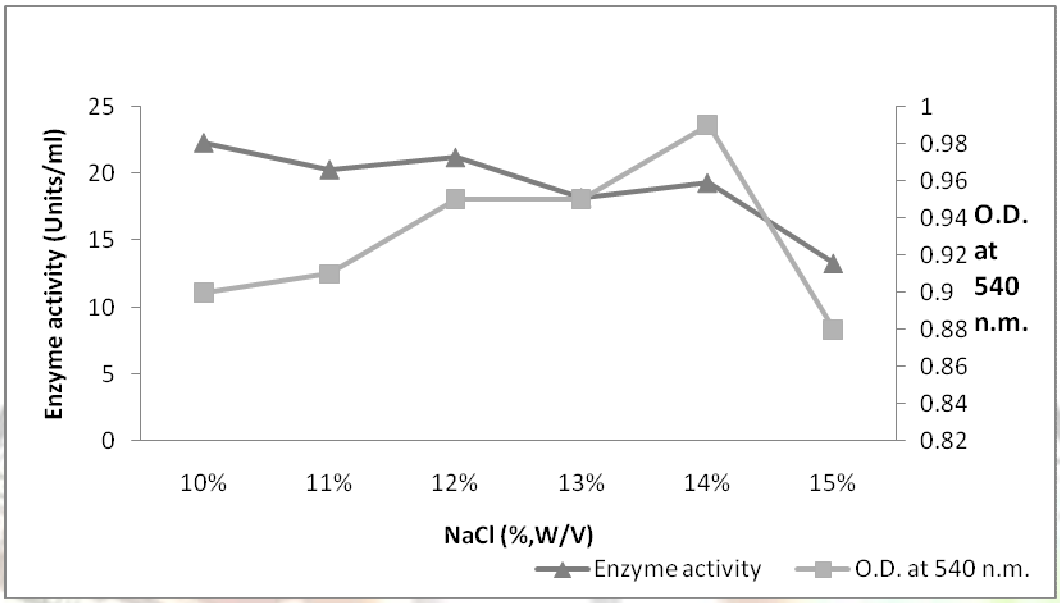

Fig.2: Effect of salt on growth and lipase secretion from *Halomonas salina*

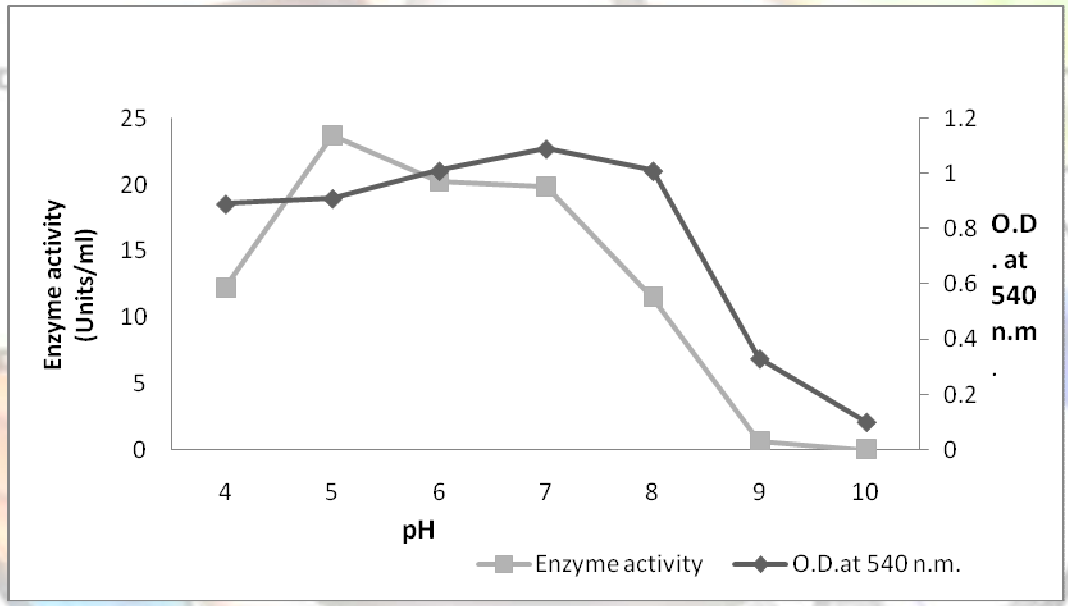

Fig.3: Effect of pH on growth and lipase secretion from *Halomonas salina*

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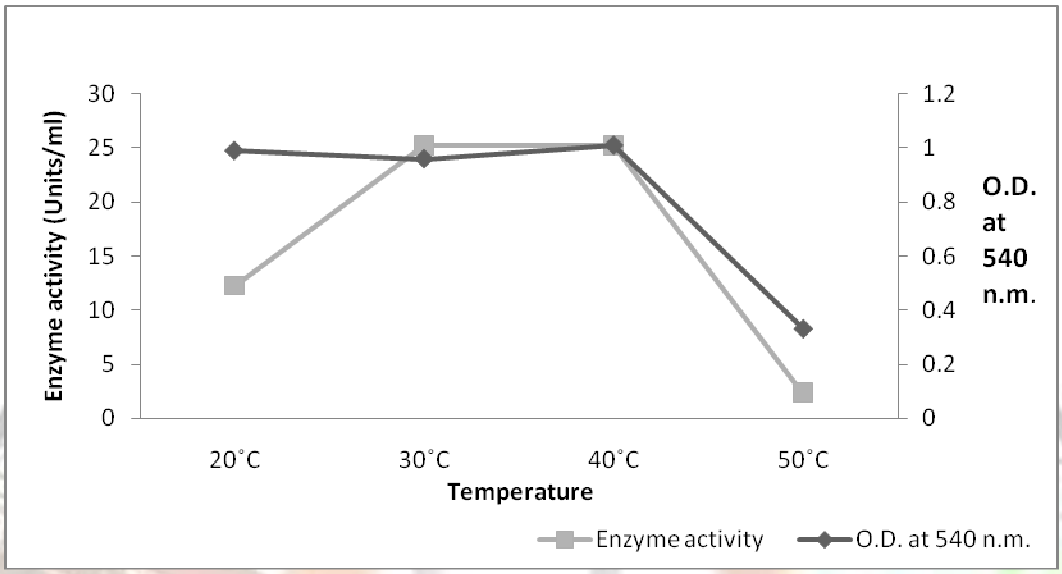

Fig. 4: Effect of temperature on growth and lipase secretion from *Halomonas salina*

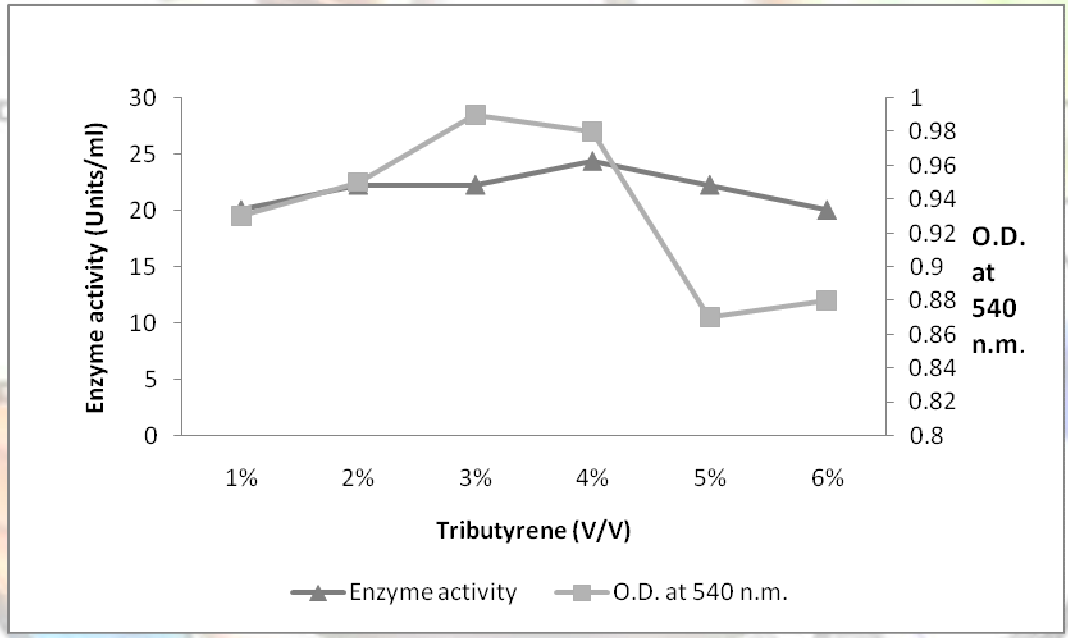

Fig. 5: Effect of substrate concentration on growth and lipase secretion from *Halomonas salina*