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Media Optimization for Lipase from *Halomonas salina* Mk-23, Moderate Halophiles Isolated from Wild Ass Excreta

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Twenty-four moderate halophilic isolates were obtained 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 sole source of carbon and Mk-23 was potential lipase producer selected on the basis of zone index. Organism was identified as H*alomonas salina* on the basis of 16s r-RNA sequencing. Maximum lipase secretion was after 240 hours of incubation (During stationary phase). Organism was found to secrete maximum lipase at pH 6, 10% NaCl, 30°C temperature and 6% substrate concentration in liquid media.

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

The Rann of Kutch is an area of 18,000 km^2 situated within Gujarat along the border with Pakistan. The Little Rann of Kutch extends northeast from the Gulf of Kutch over 5,100 km^2. Once an extension of the Arabian Sea, the Rann has been closed off by centuries of silting. 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 km^2. 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].

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Extremophiles are the organisms that 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 while true halophiles can be further divided into slight halophiles, moderate halophiles and extreme halophiles[2].

Hydrolytic enzymes like lipases, proteases, cellulases, and amylases have potential in different industrial processes[3,4]. Halophilie plays an important role in producing salt and thermotolerant enzymes[5], like proteases[6], amylases[7], nucleases[8], lipases[9] etc.

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[10]. Lipases are widely used in fat/oil processing, detergent formulation, paper-pulp industries, food industries, cosmetics and pharmaceuticals[11], biodegradation of fatty acid containing waste[12] etc.

MATERIALS 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, a loopful suspension was 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.

Growth and enzyme production pattern

Halomonas salina Mk-23 was grown in broth containing: tributyrene-10 (ml/l), Peptone- 10 (g/l), Yeast extract-5 (g/l), Sodium chloride-100 (g/l) 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.* [13]. 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

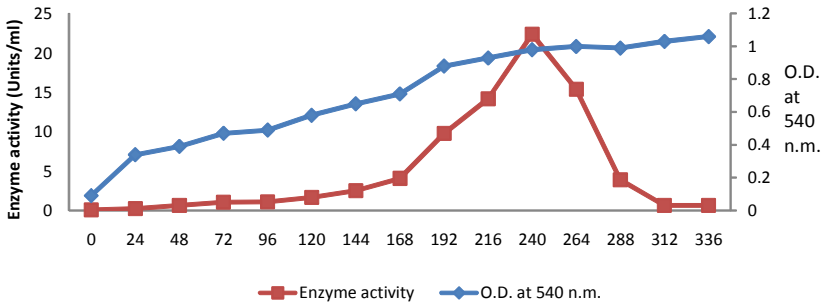

Fig. 1. Growth and enzyme production pattern from *Halomonas salina* Mk-23

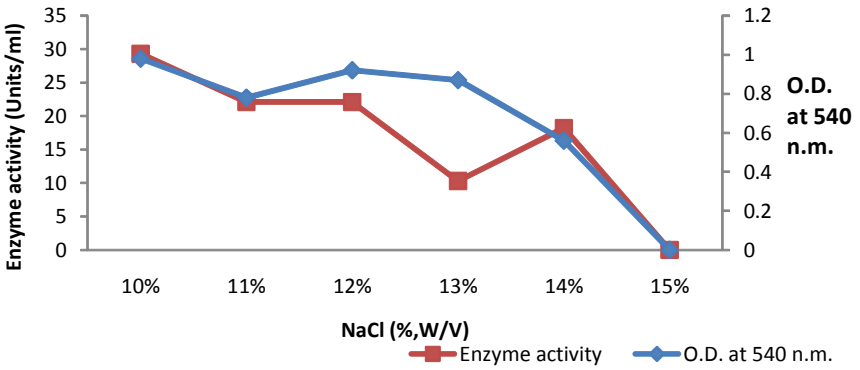

Fig. 2. Effect of salt on lipase production from *Halomonas salina* Mk-23

was determined by titration of the fatty acid released with 50mM sodium hydroxide. One international unit was defined as enzyme activity that produced 1 µM of fatty acid per min.

Optimization of Medium for lipase production

Optimization of salt

Salt optimization for maximum enzyme production was carried out by varying NaCl concentration in liquid media. Different NaCl concentration was optimized (10%, 11%, 12%, 13%, 14%, and 15%). After incubation period, growth and enzyme production was monitored.

Optimization of pH

pH optimization for 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 followed by measuring biomass and enzyme activity after incubation period.

Optimization of Substrate concentration

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

Phylogenetic analysis

Organism was phylogenetically analyzed on the basis of 16s r-RNA sequence and phylogenetic tree was prepared by bioinformatic tools.

RESULTS AND DISCUSSION

After enrichment of samples and pure culture isolation, twenty four halophilic bacterial isolates were obtained and designated as Mk-1 to

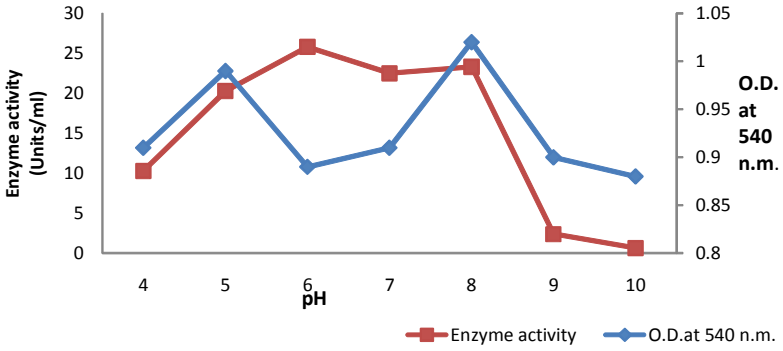

Fig. 3. Effect of pH on lipase production from *Halomonas salina* Mk-23

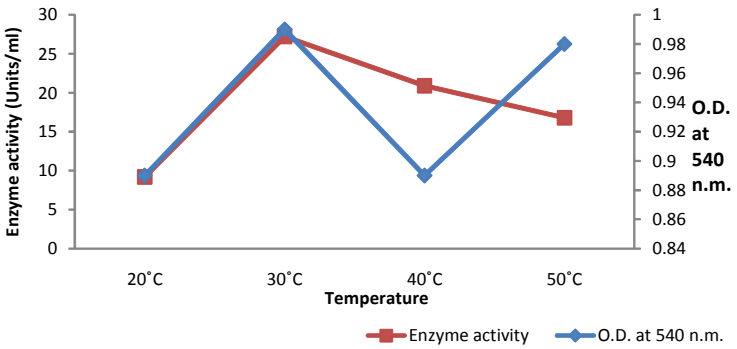

Fig. 4. Effect of temperature on lipase production from *Halomonas salina* Mk-23

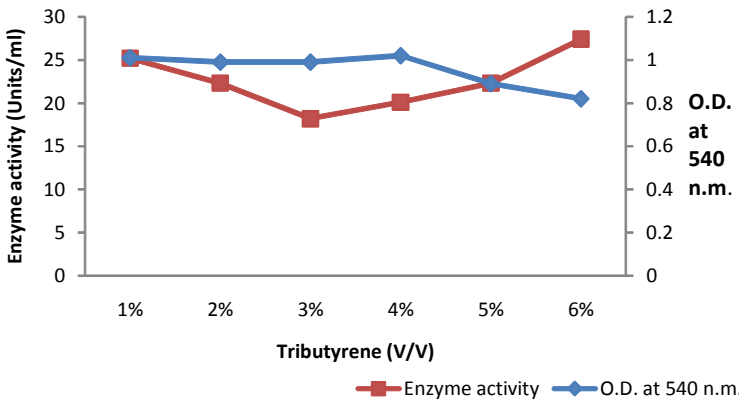

Fig. 5. Effect of substrate on lipase production from *Halomonas salina* Mk-23

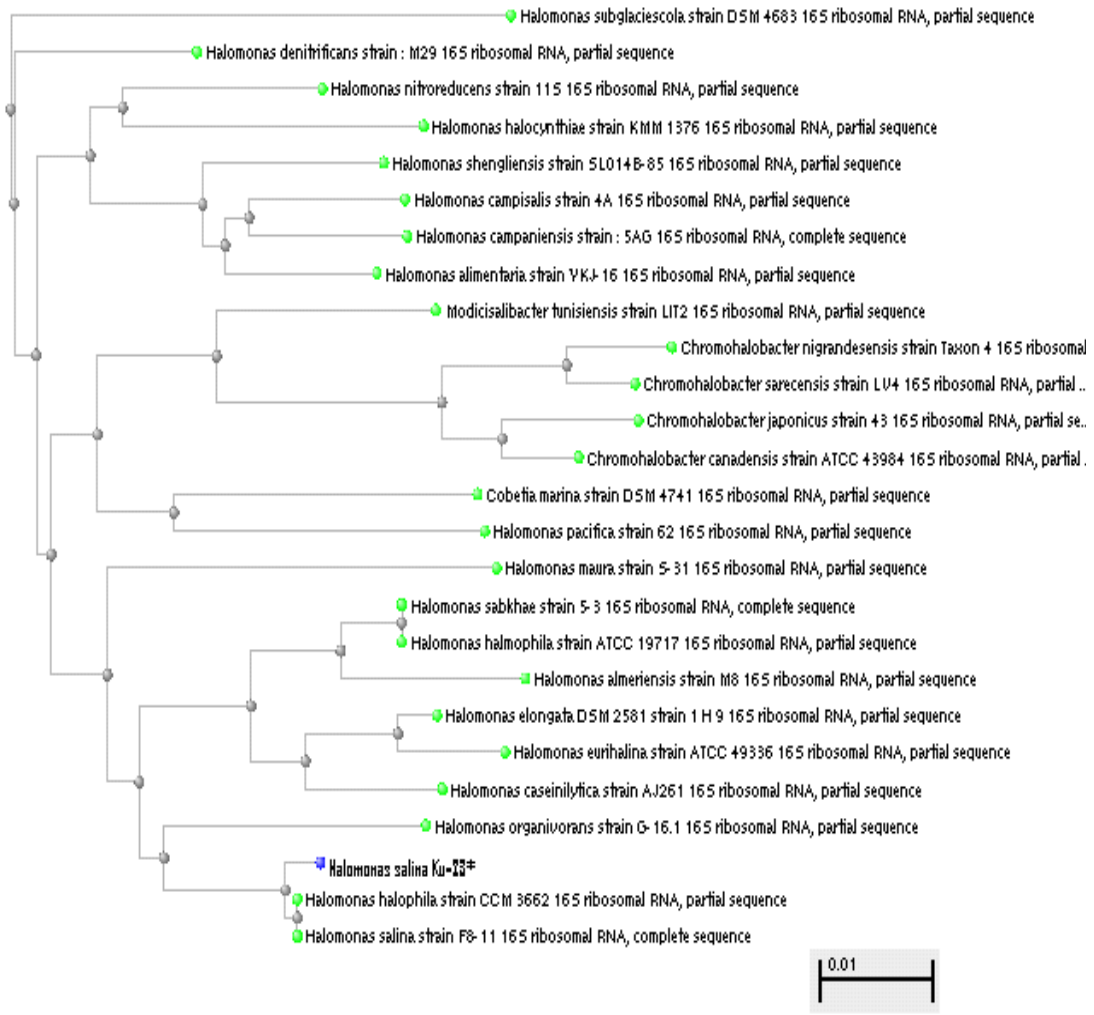

Fig. 6. Phylogenetic tree on the basis of 16's r-RNA sequences.

Mk-24. All the organisms have the ability to tolerate 2%-15% NaCl (W/V) indicated moderate halophilic nature of isolates. Isolates were preliminary screened on solid media containing tributyrene. On the basis of zone index, Mk-23 was further selected for liquid media optimization for lipase production. Organism was identified as *Halomonas salina* on the basis of 16's r-RNA sequence.

Halomonas salina Mk-23 showed little lipase activity in liquid media till 120 hours. Maximum lipase production was obtained on 240 hour of incubation. As shown in Fig.1, comparative data of growth and enzyme secretion indicates that maximum lipase secretion was during initial to mid stationary phase (Fig.1). Contrary, *B. thermoleovorans* ID-1 produced maximum lipase in exponential phase[14].

Media optimization

Halophilic bacteria are important industrially for the production of salt and thermotolerant lipases. Halophilic lipase used in variety of industrial applications because it can work under extremities. Lipase production is affected by environmental factors like temperature, pH etc. and medium components like carbon source, nitrogen source etc[15]. Optimization of media is important at industrial scale to improve the efficiency of the process without increasing the cost[16].

Halomonas salina Mk-23 showed maximum biomass and enzyme production at 10% NaCl (w/v). Lipase secretion was adversely affected by increasing salt concentration than optimum (Fig.2). Optimum salt concentration for *Halomonas salina* is not compatible with optimum salt requirement by *Staphylococcus warneri* for lipase secretion[9].

Halomonas salina Mk-23 produced maximum growth and lipase at pH 8 and pH 6 respectively. PH 4 and 10 were highly unsuitable for growth and enzyme (Fig.3). Similar types of results were obtained in from marine *salinivibrio* A2[17].

Among the range of temperature tested, *Halomonas salina* Mk-23 showed maximum growth and lipase secretion at 30°C. Growth and lipase secretion adversely affected by lower temperature while higher temperature i.e. 50°C affected only enzyme production (Fig.4). Similar types of results were also obtained from halophiles isolated from subterranean rock salt crystal[18].

Among 1-6% (v/v) tributyrene, *Halomonas salina* Mk-23 grown in entire range of substrate. In terms of lipase secretion 6% substrate (V/V) was optimum (Fig.5). Tributyrene is an inducer for lipase production. *Rhizopus oryzae* was found to produce three fold lipase in medium containing olive oil as compare to oil free media[19].

On the basis of 16'S r-RNA sequence, organism was identified as *Halomonas salina.* Phylogenetic tree shows evolutionary relationship of the organisms (Fig.6).

CONCLUSION

Extremophilic microorganisms have been widely explored industrially and biotechnologically for its valuable products. Halophilic microorganisms can secrete salt and thermo-tolerant enzymes viz. proteases, lipases, amylases, cellulases, chitinases etc. Lipases are important enzymes used in food, pharmaceutical, detergent and chemical industries.

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