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Effect of Medium and Environmental Parameters on Lipase Production from Extreme Halophilic Isolate *Halomonas salina* ku-20

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Total 30 extreme halophilic isolates were obtained from salt, mud and water samples collected from little Rann of Kutch, Gujarat, India after enrichment. Morphologically all the isolates were Gram's positive or Gram's variable and non-capsulated. Primary screening of lipase producer was performed on tributyrene agar. On the basis of zone index, Ku-20 was found to be a potential lipase producer, selected for further study in liquid media. Organism was identified on the basis of 16s r-RNA sequencing as *Halomonas salina*. Organism was able to secrete maximum lipase after 264 hours of incubation, 10-15% NaCl, pH 7, 30°C temperature and 2% tributyrene concentration.

Key words: Extreme halophiles, *Halomonas salina*, Lipases, Rann of Kutch.

Little Rann of Kutch is situated within Gujarat, India along the border with Pakistan. The Little Rann of Kutch extends northeast from the Gulf of Kutch over 5,100 km^2. Little rann of kutch is a typical ecological system with saline desert climate having unique floral and faunal diversity.

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 extremophiles that could survive and grow in the presence of salt and hence widely distributed in natural habitats like sea, salt desert, salt Lake etc. Halotolerant microorganisms can grow both in the presence as well as absence of salt, while true halophiles can be further divided into slight halophiles, Moderate halophiles and extreme halophiles[1].

Hydrolytic enzymes like lipases, proteases, cellulases, and amylases have potential in different industrial processes[2,3]. Halophilic hydrolase usually produces by *Halomonadaceae*. Industrially useful enzymes such as cellulases, amylases, xylanases, proteases and lipases are useful hydrolase from *Halomonadaceae*[3-5].

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyze the hydrolysis of ester bond of triacylglycerol and convert it to glycerol and free fatty acid at oil-water interface and does not hydrolyze dissolved substrate in the bulk fluid[6]. Lipases are widely used in fat/oil processing, detergent formulation, paper-pulp industries, food industries, cosmetics and pharmaceuticals[7,8] and biodegradation of fatty acid containing waste[9].

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MATERIALS AND METHODS

Collection of samples

Total 10 wet samples were collected from sea 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.

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- 50-350 (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- 50-350 (g/l), pH- 7.0-7.4. From enriched 35% 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 30 isolates were obtained, designated as Ku-1 to Ku-30 and preserved on N-agar slant at 4°C for further studies.

Growth and enzyme production pattern

Halomonas salina Ku-20 was grown in broth containing: tributyrene-10 (ml/l), Peptone-10 (g/l), Yeast extract-5 (g/l), Sodium chloride-200 (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.* [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 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%, 15%, 20%, 25%, 30%, and 35%). 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%, 2%, 3%, 4%, 5% and 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

Halophiles were enriched with increasing salt concentration in halophilic broth and complete media broth. Thirty isolates were obtained in pure culture, designated as Ku-1 to Ku-30. All the isolates were able to grown on media containing 35% salt concentration indicated extreme halophilic nature of isolates. On the basis of zone index, Ku-20 was further selected for liquid media optimization for lipase production and identified as *Halomonas salina* on the basis of 16's r-RNA sequence.

Halomonas salina Ku-20 showed no lipase activity till 200 hour in liquid media. Maximum lipase activity was obtained after 264 hours. Enzyme secretion and growth pattern data indicated that maximum lipase secretion was obtained at initial stationary phase (Figure-1). Similar types of results were obtained by Werasit

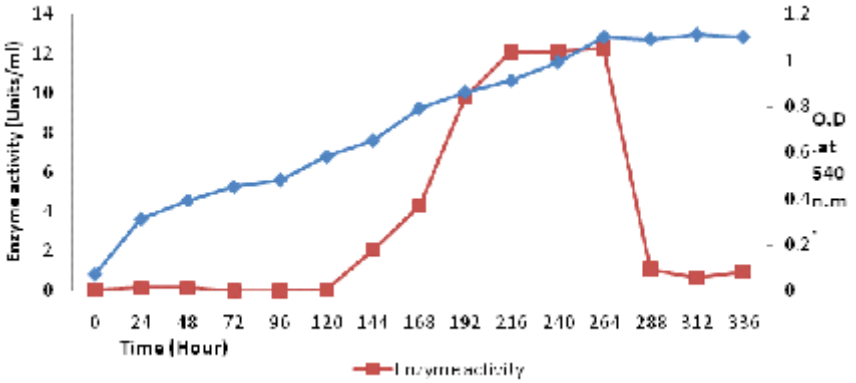

Fig. 1. Growth and enzyme production pattern from *Halomonas salina* Ku-20

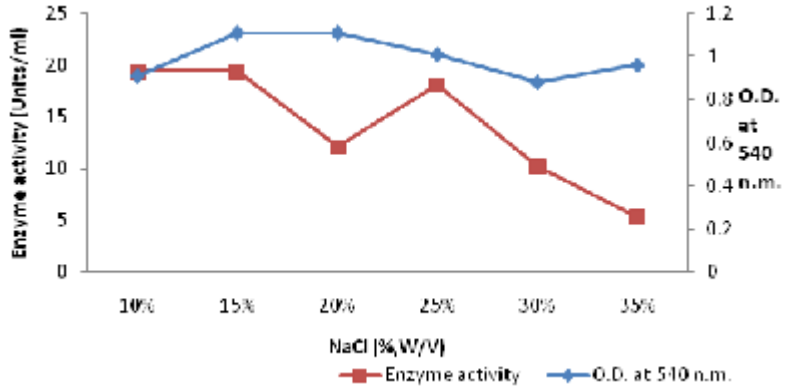

Fig. 2. Effect of salt on lipase production from *Halomonas salina* Ku-20

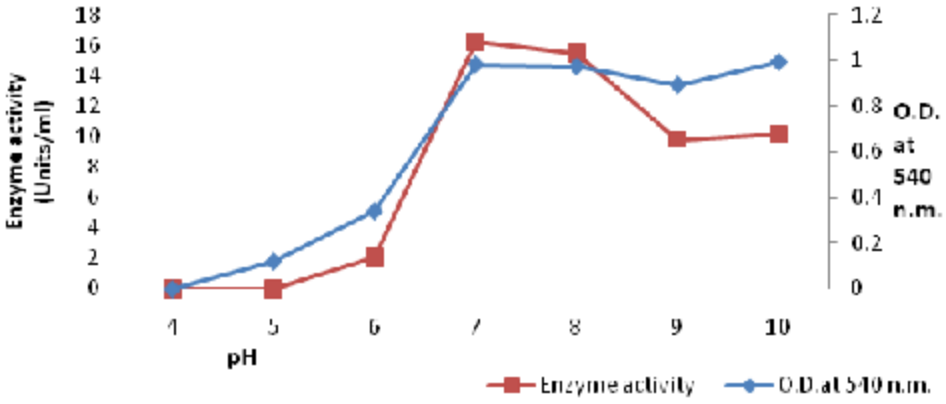

Fig. 3. Effect of pH on lipase production from *Halomonas salina* Ku-20

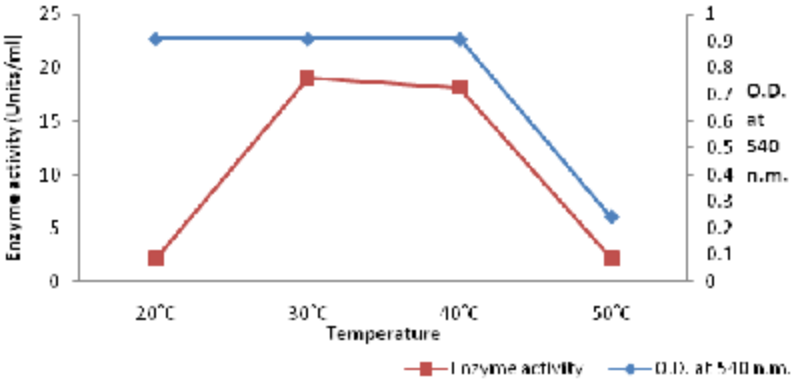

Fig. 4. Effect of temperature on lipase production from *Halomonas salina* Ku-20

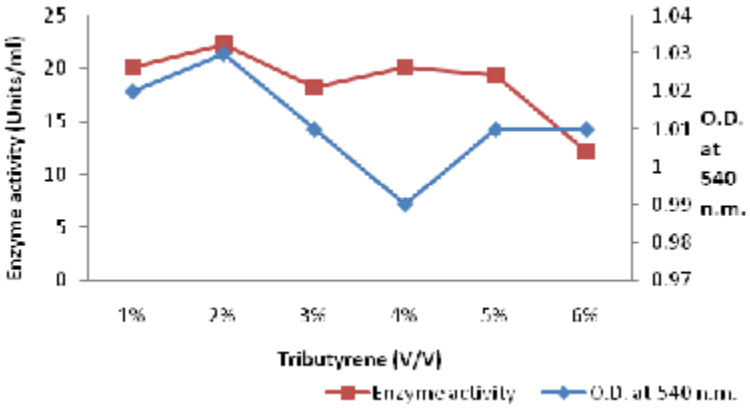

Fig. 5. Effect of substrate on lipase production from *Halomonas salina* Ku-20

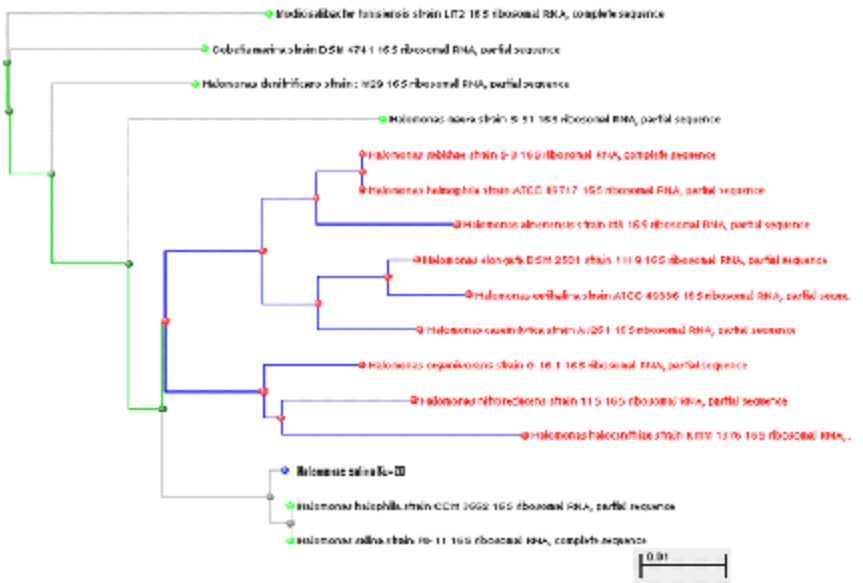

Fig. 6. Phylogenetic tree on the basis of 16's r-RNA sequences

and Anan[11] from halophilic *Staphylococcus warneri* PB233 at a stationary phase.

Media optimization

Halophiles are important at industrial scale for the production of salt and thermo-tolerant enzymes. Lipases are important in variety of industrial applications. Lipase production is affected by environmental factors and medium components. Optimization of media is important at industrial scale to improve the efficiency of the process without increasing the cost[12].

Halomonas salina Ku-20 produced maximum biomass and enzyme at 10% and 10-15% NaCl (w/v) respectively. Lipase secretion was adversely affected by 30-35% salt concentration (Figure-2). Optimum salt concentration for *Halomonas salina* is not compatible with optimum salt requirement by *Staphylococcus warneri* for lipase secretion[11].

Organism showed maximum growth and enzyme secretion at pH 7. Acidic pH was highly unsuitable for growth and enzyme while alkaline pH decreased enzyme activity (Figure-3). Similar results were also obtained from *Rhizopus oryzae*[13]. Among the range of temperature tested, *Halomonas salina* Ku-20 showed maximum growth at temperature 20-40°C while lipase secretion at 30-40°C i.e. in mesophilic temperature range. Growth and lipase secretion was adversely affected by higher temperature i.e. 50°C (Figure-4). Similar types of results were also obtained from other halophiles isolated from subterranean rock salt crystal[14] and *Rhizopus oryzae*[13].

Among 1-6% (v/v) tributyrene, *Halomonas salina* Ku-20 showed maximum growth and lipase production at 2% substrate concentration. Higher or lower concentration was found to decreased growth and enzyme secretion (Figure-5). Tributyrene is an inducer for lipase production. Sugihara *et al*,[15] reported lipase secretion from *Bacillus* sp. in medium containing 1% olive oil. No lipase production was observed in the absence of olive oil even after prolonged incubation.

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