JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2008. Vol. **2**(2), p. 457-461

Isolation and Characterisation of Enzyme Invertase from Halotolerant Yeast

Rajesh C. Patil[1], Ujjwala Jadhav, Abhishek Mule[1], Eruch Gorwalla[1], Amit M. Parekh[2] and Varsha M. Jadhav[2]

[1]Department of Microbiology, Bhavan's College, Andheri (W.), Mumbai - 400 058, India.
Department of Life Sciences, University of Mumbai, Santacruz(E), Mumbai - 400 098, India.
[2]Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai - 400 614, India.

(Received: 06 April 2008; accepted: 18 July 2008)

Sixty-seven pure cultures of halophilic bacteria and yeasts were isolated from Thane creek water, soil, and other materials. High salinities (15% NaCl) were maintained during all phases of the isolation procedure to avoid possible destruction of obligate halophiles. Four of the cultures, all yeasts, proved to be obligate halophiles. Three are species of *Candida* and one of *Torulopsis*. They grew in the range of 10% to 20 % salinity and the optimum salinity was 15 %. They died rapidly when exposed to lower saline conditions between 0-5%.

Key words: Invertase, halophilic bacteria, halotolerant yeast.

The environment has a profound influence on the microbial population of an aquatic medium. Past work has shown that, in hypersaline media, the bacterial communities are particularly rich in halophilic and hyperhalophilic bacteria.

Compared to the extensive literature on the physiology, biochemistry, and ecology of the aerobic red halophilic archaea (family Halobacteriaceae), the aerobic halophilic bacteria, and halophilic yeasts have been relatively little studied. Research on the halotolerant yeast often seems to be less glamorous than the study of the halophilic and halotolerant bacteria, with their unique adaptations, including a highly saline cytoplasm, specialized salt-requiring proteins, and the unique light-driven proton and chloride pumps bacteriorhodopsin and halorhodopsin. (Aitken *et al.*, & Abrahm *et al.,*) Hypersaline environments are generally thought to be characterized by low species diversity, probably because eukaryotic organisms are largely or entirely absent. The number and diversity of halophilic bacterial strains and species (i) that exist in nature and (ii) that coexist in local hypersaline environments are unknown. It can be hypothesized that a relatively large diversity of strains, species, or both exists within the bacterial floras, a condition that requires experimental scrutiny rather than the examination of gross morphology used for identifying unicellular and multicellular eukaryotes.

MATERIAL AND METHODS

Isolation of halophilic bacteria

Extreme halophilic bacteria have been isolated from a broad diversity of hypersaline environments, especially those resulting from the evaporation of seawater (Larsen, H. 1962).

* To whom all correspondence should be addressed.

Although it is likely that these organisms could be dispersed throughout the world via ocean currents, little is known about this subject. On the other hand, the extremely halophilic bacilli are considered to be unable to survive in media with less than 10% NaCl (at this concentration the cell envelopes are dissolved), and although the cocci do not lyse below this limit, they do not show detectable growth at these concentrations.

The present study was designed to determine whether extreme halophilic bacteria could be isolated systematically from seawater and to study their invertase activity . Surface seawater samples were collected in plastic containers disinfected with alcohol. Samples were collected from the surface at a depth of 0 to 1 m and 5 km from the Thane Creek (North East to Mumbai). They were taken from sites separated by about 5 km. Three samples of 1 liter were taken from each point. All were collected in October. The 1-liter samples were filtered through membrane filters (Millipore filters 50 mm Millex, Polypropylene 0.2, µm pore size, 47-mm diameter) at ambient temperatures under strict aseptic conditions. The filters were then placed on petri plates containing either Eimhjellen or Sehgal and Gibbons media for extreme halophiles (Gibbons 1969). The petri plates were placed in plastic bags which were sealed hermetically and incubated at 38°C. After 5 to 7 days of incubation, each filter had between 10 and 25 non pigmented colonies. Both gram-positive bacilli and yeast were represented. Upon further incubation (15 to 20 days), 2 to 35 colourless colonies also formed on each filter. The colonies were made up of yeast. The yeast from the nonpigmented colonies were able to grow in artificial seawater medium with (Gonzalez, 1978) supplemented with 1% yeast extract, but the bacteria from the pigmented colonies were able to grow only if the media contained at least 2 M NaCl. Thus, the latter were considered to be extreme halophiles. Extreme halophiles were also isolated from seawater by the addition of 1,000 IU of penicillin per ml (final concentration) to the Eimhjellen medium. Since the extreme halophiles lack peptidoglycan in their cell wall (Larsen, H. 1962), they could grow in the presence of penicillin. Under these cultural conditions, only pigmented colonies were observed, and all were gram-negative cocci. One of these isolates, hereafter referred to as Y 1, was selected for further study. It was compared with Halococcus sp. NCMB 757. The ability of Y 1and NCMB 757 to grow in various salt concentrations was determined. The media were composed of inorganic salts corresponding in proportions to the artificial seawater solution (Rafaeli-Eshkol 1968). Keeping these proportions, the concentration of all salts (except $CaCl_2$ and $NaHCO_3$, which were maintained at seawater concentrations) was increased over a range of concentrations. Yeast extract and antifoam were added to media to final concentrations of 1 and 0.5%, respectively. Media (100 ml) were dispensed into 500-ml Erlenmeyer flasks. The flasks were inoculated with 1-ml samples of cultures grown in media with 25% salt concentration that had attained an absorbance of 0.3 at 520 nm. The flasks were incubated at 38°C, and the media were continuously agitated via a magnetic stirrer. Finally, we estimated the ability of the two strains for surviving in seawater by harvesting an exponentially growing population (same medium and incubation conditions as before) by centrifugation and suspending in sterile seawater at the original cell density. The suspension was kept at room temperature with shaking. Samples were taken at intervals and plated on Eimhjellen medium.

Both organisms were maintained on YEPD medium and for the growth of the organisms for the extraction of intracellular and extracellular invertases, cells were routinely grown in 20 liters of the salts-vitamin-1.0% glucose medium of Gibbons (Gibbons, N. E. 1969.), supplemented with 0.5% Casamino Acids (Difco) (CAA-salts), for 18 h at 25°C.

Organisms were checked for enzyme activity Enzyme assays. Invertase activity was measured as total reducing sugar production upon incubation with sucrose by the method described by Ross *et al* 1981. Conditions for the assay, including optimal temperature and pH, as well as substrate concentrations based on the Km for sucrose, were determined for the GS5invertase previously . A 0.1-ml portion of enzyme sample was combined with 0.1 ml of 1.0 M potassium phosphate buffer (KPB), pH 7.0, 0.2 ml of a 20%solution of sucrose, and 0.02% sodium azide to a final volume of 1.0 ml. Mixtures were

incubated for 30 min at 37°C, and 2 volumes of 0.04 N NaOH was then added to stop the reaction. A 1.0-ml amount of Somogyi reagent was added, and the tubes were heated at 100°C for 20 min. After the tubes had cooled, 1.0 ml of Nelson's reagent was added, and the tube contents were thoroughly mixed and allowed to stand at room temperature for 15 min prior to recording the absorbance at 560 nm. One international unit of enzyme activity (IU) was defined as the amount of enzyme required to hydrolyze 1.0 tmol of sucrose per min under standard assay conditions. 2.2 Crude enzyme preparation.

Cultures were centrifuged at 10,000 x g for 15 min. The culture fluids were brought to 60% saturation with ammonium sulfate and allowed to stand for 24 h in the cold. The precipitates were collected and dissolved in 0.01 M potassium phosphate buffer (pH 6.5). This was dialyzed against the same buffer and then concentrated with the aid of a collodion bag. The concentrated fraction was employed as the starting material for further purification.

Purification of intracellular invertase.

The concentrated fraction was washed with 3 N NaCl three times to remove cell-associated glucosyltransferase activity, once with 0.01 M KPB, and finally with saline- 0.02% sodium azide. Cells, 0.75 mg (wet weight), were suspended in 7.0 ml of saline-sodium azide containing 1.0 mM phenylmethylsulfonyl fluoride (Sigma) as a general inhibitor of protease activity (McCabe 1973). This suspension was added to Glass beads (5.0 g) and shaken in a Mickel disintegrator (H. Mickle, Gomshall- Surrey, England) for 10 to 15 min. Broken-cell suspensions were centrifuged once at 13,000 x g for 10 min in a Eltech centrifuge at 4°C to remove cell debris and then at 73,000 x g for 60 min to separate the particulate fraction from the soluble fraction.

Chromatography on Sepharose 6B

The crude enzyme preparations were applied to a Sepharose 6B column (2 by 130 cm) that was previously equilibratedwith 0.01 M potassium phosphate buffer (pH 6.5). Enzymes were eluted with the same buffer. Enzyme activity was detected in eluates of fractions 13 to 27 . The two fractions of tubes 13 to 19 and tubes 20 to 27 were collected separately. Ammonium sulfate was added to the fractions (60% saturation). The precipitates were taken up in 0.01 M phosphate buffer (pH 6.5) and dialyzed overnight against the same buffer**.** The first fraction (tubes 13 to 19) synthesized water-insoluble polysaccharides from sucrose. Ethanol was added, up to 50%, to the supernatant fluid to remove the polysaccharides. No precipitates appeared. The second fraction (tubes 20 to 27) produced soluble polysaccharides. Which were precipitated by addition of ethanol to 50%. A preliminary experiment showed that the acid hydrolysate of the insoluble polysaccharides contained glucose. It was suggested that the first fraction might contain the glucosyltransferase responsible for the formation of the insoluble glucan.

Chromatography on DEAE-cellulose

The second fraction (i.e., eluate from fractions 20 to 27) was applied to a column (1 by 25 cm) of DEAE-cellulose equilibrated with 0.01 M potassium phosphate buffer (pH 6.5). Elution was performed with a gradient of sodium chloride inthe same buffer (Fig. 2). Enzyme activity appeared as two separable components. The enzyme in the first component produced neither insoluble nor soluble polysaccharide, but released both free glucose and fructose from sucrose. On the other hand, the enzyme in the second component never synthesized insoluble polysaccharide, but only soluble polysaccharide which was precipitated by the addition of equal volume of 99% ethanol to the reaction mixtures. The results suggest that the early-appearing and the late-appearing enzymes may be invertase and dextransucrase, respectively.

Determination of kinetic parameters

In order to determine maximum velocity of the reaction (vmax) and the Michaelis–Menten constant (Km) for each purified enzyme, activity assay was applied for different concentrations of sucrose (invertase assay)

Determination of optimum temperature

Optimum temperatures for immobilized invertase was determined by changing incubation temperature between 10,20,30,40,50,60 °C, respectively, while keeping substrate concentration constant.

Determination of optimum pH

Optimum pH for immobilized invertase was determined by changing incubation pH between pH values 3,5,7,9 respectively, while

keeping substrate concentration and temperature constant.

Immobilization of enzyme

Enzyme immobilization was done by calcium alginate method in which 9 g of sodium alginate was dissolved in 300 ml of growth medium it was stirred until all sodium alginate is completely dissolved. The final solution contains 3% alginate by weight Thoroughly Y 1 wet cells were suspend 250 g of wet cells in the alginate solution prepared in the previous step air bubbles allowed to escape The yeast-alginate mixture was dripped from a height of 20 cm into 1000 ml of cross-linking solution. (The cross-linking solution was prepared by adding an additional 0.05M of $CaCl_2$ to the growth media). The calcium cross-linking solution is agitated on a magnetic stirrer. Gel formation can be achieved at room temperature as soon as the sodium alginate drops come in direct contact with the calcium solution. Relatively small alginate beads are preferred to minimize the mass transfer resistance. A diameter of 0.5-2 mm can be readily achieved with a syringe and a needle. The beads should fully harden in 1-2 hours. Note that the concentration of the $CaCl_2$ is about one fourth of the strength used for enzyme immobilization. The beads were washed with a fresh calcium cross-linking solution.

RESULTS AND DISCUSSION

Survival ability of isolate in saline condition

Y 1 strain was isolated and compared with standard strain *Halococcus* sp. NCMB 757 their growth response in original environment that is in Eimhjellen medium was studied for growth response; both cultures showed the ability to grow for 3 days and could survive for 15 days. (Fig. 1)

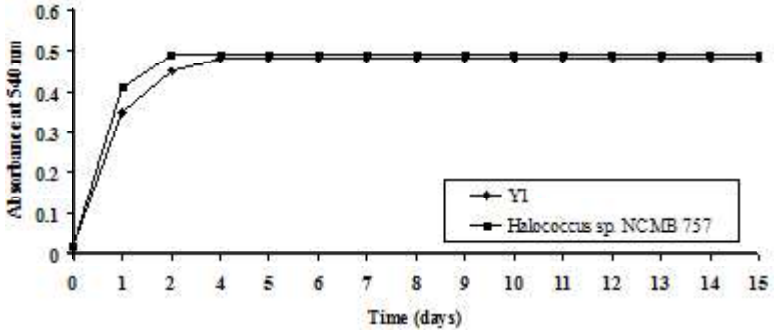

Fig. 1: Ability of the isolate to survive in saline condition

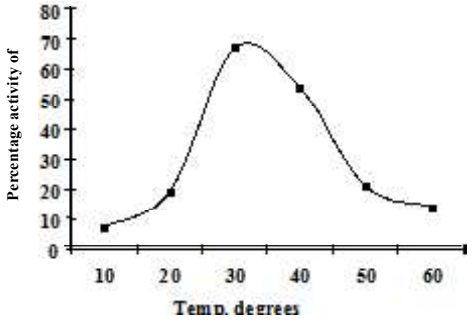

Fig. 2: Determination of optimum temperature

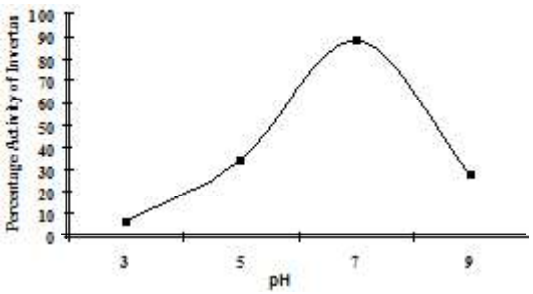

Fig. 3: Determination of optimum pH

Enzyme purification

The Km value was determined at sucrose concentrations between 10 and 125 mM at pH 6.0, and was 20 mM as obtained from a Lineweaver- Burk plot.

Optimum temperature

Temperature range of 10-60°C was tried, a typical bell shaped graph was formed, temperature range of 30-40°C was found to be optimum (Fig. 2).

Optimum pH

The pH range of 3-9 was tried the, optimum pH was found to be at 7 (Fig. 3).

Isolation of invertase producing enzyme isolates was done from Thane creek. The activity of the invertase is strongly dependent on temperature, with the optimum temperature being observed between 30 and 40 °C. As the temperature increases after is further increased above the optimum temperature, the structure of the enzyme becomes altered and consequently, its catalytic properties are reduced, and eventually destroyed. The effect of temperature on the enzyme was investigated (Fig. 2). The maximum activity was found at 30°C for the enzyme

The maximum reaction rate, Vmax, and Michealis-Menten constant, Km were obtained from Lineweaver-Burk plot. The Km value was determined at sucrose -concentrations between 0.6 and 10 mM at pH 6.0. Its value obtained from a Lineweaver-Burk plot was 2.0 mM.

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