Pectinase from an Extremely Haloalkaliphilic Archaeon, *Natronobacterium innermongoliae* SSBJUP-4 Isolated from Lonar Lake

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Abstract

An extremely haloalkaliphilic archaeon *Natronobacterium innermongoliae*.SSBJUP-4 showing potent pectinolytic activities was isolated from the saline alkaline Lonar Lake, India. Pectinase is active over the alkaline pH range from 8 to 10. Pectinase was stable over a broad temperature range of 40-80^{o}C than the protease but had the higher optimum (75^{o}C) for activity. The pectinase was stable over a broader salinity range from 12 to 18 % with an optimum salt concentration of 14-16%. Pectinase activity being markedly stimulated by Ca^{2+} and Mg^{2+} in the range of 0.4 to 0.7mM concentration but inhibited by Cu^{2+} and Hg^{2+}.

Keywords Haloalkaliphiles, Archaea, *Natronobacterium innermongoliae*.SSBJUP-4, Pectinase,

Introduction

Pectic substance is the generic name used for the compounds that are acted upon by the pectinolytic enzymes. They are high molecular weight, acidic, complex glycosidic macromlecules that are present in the plan kingdom. They are present as the major component of middle lamella between the cells in the form of calcium pectate and magnesium pectate (Rastogi, 1998). These materials have attracted considerable attention as an alternative feedstock and energy source, since they are abundantly available. Several microbes are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches. Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. Microbial pectinases are important in the phytopathologic process, in plant-microbe symbiosis and in the decomposition of dead plant material, contributing to the natural carbon cycle. Pectinases are abundantly

produced by saprophytic fungi, and decaying plant tissue represents the most common substrate for pectinase-producing microorganisms (Gummadi and Panda, 2003).

Pectinases are the single class of enzymes which play an important part in the metabolism of almost all organisms (Plants, Animals, Fingi, Bacteria and Viruses). Investigation of Pectinases is a central issue in enzymology due to their wide applications in Pharmaceutical, Food, Agricultural products and Bioremediation process. Pectinase (EC 3.2.1.15) belongs to the class hydrolase which are able to hydrolyse pectin more efficient than other Pectinases and their action are very specific, i.e., they acts only on pectin substrates, pectin is a major constituent of cereals, vegetables, fruits; fibers are complex, high molecular weight heterogeneous and acidic structural polysaccharide (Ceci and Loranzo, 2008).

Halophilic archaea, in contrast to halophilic bacteria which maintain a cytoplasm with low concentration of salt by production of compatible solute, use a high salt in strategy in order to survive osmotic challenges associated with life in hypersaline environments (Madigan and Oren, 1999). Thus, they have enzymes which are active at up to 5 M or higher concentration of NaCl or 4M KCl. These enzymes have catalytic function in the condition of low water activity, a situation common in the presence of organic solvents.

Despite advances in understanding the diversity and systematics of haloarchaea, studying their hydrolytic enzymes such as amylases, xylanases, cellulase pectinase and protease and their characterization has received less attention. However, some special haloarchaeal enzymes are characterized in detail (Ruiz and De Castro, 2007).

Characterization of pectinase from such extremely halophilic archaea for their high alkalinity and high salinity, therefore, assumes significance from the biotechnological point of view. But haloakaliphilic organism and in particular the archaea have received scant or no attention in this regard. Thus an attempt was made for the screening, isolation and characterization of pectinase producing archaea from soil samples of Lonar Lake.

Materials and Method

Bacterial isolation, media and culture conditions

Soil samples were obtained from the Lonar Lake situated in Buldhana District (Lat.$19^0$58', long. $76^0$34') of Maharashtra, India. Aliquots of the soil were enriched in Tindall medium (1980) containing (g/l): casamino acid, 7.5; yeast extract, 10; Tri-sodium citrate, 3; MgSO4.7H2O, 1; KCl, 2; FeSO4.7H2O, 0.05; NaCl, 200; Na_2CO_3, 18.5. The pH was self

adjusted. The sterilization of NaCl and Na_2CO_3 was carried out separately at 121^0C 15 min. Incubation was carried out at 40^0C under aerobic conditions for 15 days. Enriched samples were then streaked on the same medium for isolation and incubated at 40°C for up to 20 days. Pure isolates were obtained by successive cultivation on solid SH medium. Pure cultures were screened for extracellular pectinolytic activity. Storage of pure cultures was on slopes of the same medium at 4^0C.

Production of the pectinase was detected by conventional methods of flooding 1 % pectin agar with 0.3 % iodine after incubation at 40^o C for 15 days and observing for appearance of clear zones around the growth. After that diameter of zone and diameter of colony were measured to calculate S/R (Selection Ratio) value. The isolate showing higher S/R value was selected for further studies

Production, Partial Purification and assay of the enzymes

This was performed in SH medium supplemented with 1% pectin. After cultivation of the isolate at 40^0C for 15 days, cell-free supernatants were collected by centrifuging for 20 min at 10,000 rpm at 4 ^{0}C and the enzyme was precipitated with ammonium sulphate, stirring for 30' and refrigeration at 4^oC overnight. The precipitates were collected through centrifugation at 5000xg for 15' at 4^oC, dissolved in 50ml 50mM Tris-HCl buffer (pH 8.5) and dialysed against the same buffer at 4^oC overnight.

Pectinase produced was then assayed by the method described in De Siqueira et.al (1998) in which 0.5% pectin was incubated with pectinase in acetate buffer containing EDTA at 500c for 10 minutes and reducing sugars released were estimated by the dinitrosalicylate reduction method of Miller (1959). One unit of pectinase is defined as the quantity that releases 1 mille mole of reducing sugar from 0.5% pectin in one minute under the assay conditions.

Effect of temperature and pH on the activity of the enzymes

To determine the temperature optimum for the pectinase, assay was carried out at various temperatures from 0 to 100 ^{0}C. The effect of pH on pectinase activity was studied by incubating the reaction mixture at different pH values ranging from 3.0 to 11.0, in the following buffer systems: 0.1 M sodium acetate (pH 3.0–5.6); 0.1 M sodium phosphate (pH 6.0–7.0); 0.1 M Tris–HCl (pH 8.0–9.0); 0.1 M carbonate bicarbonate buffer (pH 9.0–11.0).

Effect of NaCl concentration, metal ions and substrate concentrations on the activity of the enzymes

Pectinase activity was assayed at optimum temperature and pH with different NaCl concentrations (0–26%) in the reaction mixture. To determine the effect of metal ions on pectinase activity, the assay was carried out in metal ions Ca^{2+}, Mg^{2+} Cu^{2+}, Na^{2+}, Hg^{2+}, and Zn^{2+}. The effect of substrate concentrations was studied by incubating the reaction mixture with different substrate pectin concentrations.

Results and discussion

Three haloalkaliphilic archaeal isolates were obtained from the soil samples on Tindall medium. Colonies on SH plate are reddish pink, circular, regular, convex and moist. In KOH method (Buck, 1982) the young culture showed Gram negative nature while the older culture was Gram positive. The isolate grew well in 20% salt concentration. Optimal bacterial growth was observed at pH 9, 37–42^{o}C, and 20% NaCl.

Only one isolate namely *Natronobacterium innermongoliae SSBJUP-4* producing pectinase as judged from the S/R value. This organism was cultivated in the suitable broth containing pectin to prod uce the enzyme for use in further study. The yield of enzyme obtained thus was 5.3 Units/ml with a specific activity of 11.77 Units/mg. The extracellular enzyme in the broth was separated from the cells by centrifugation, partially purified and used in characterization studies.

Effect of pH, Temperature and Salt (NaCl) Concentration on activity

The results of these studies are shown in Figures 1, 2 and 3 respectively. Effect of pH on enzyme activity showed that the enzyme is truly alkaline in nature showing maximum velocity at pH of 11.0.

The pectinase is more alkali stable with higher activity is in a narrower alkaline range from pH 8 to 10 and an optimum of 9. Beyond which activity goes on decreasing (Fig. 1).

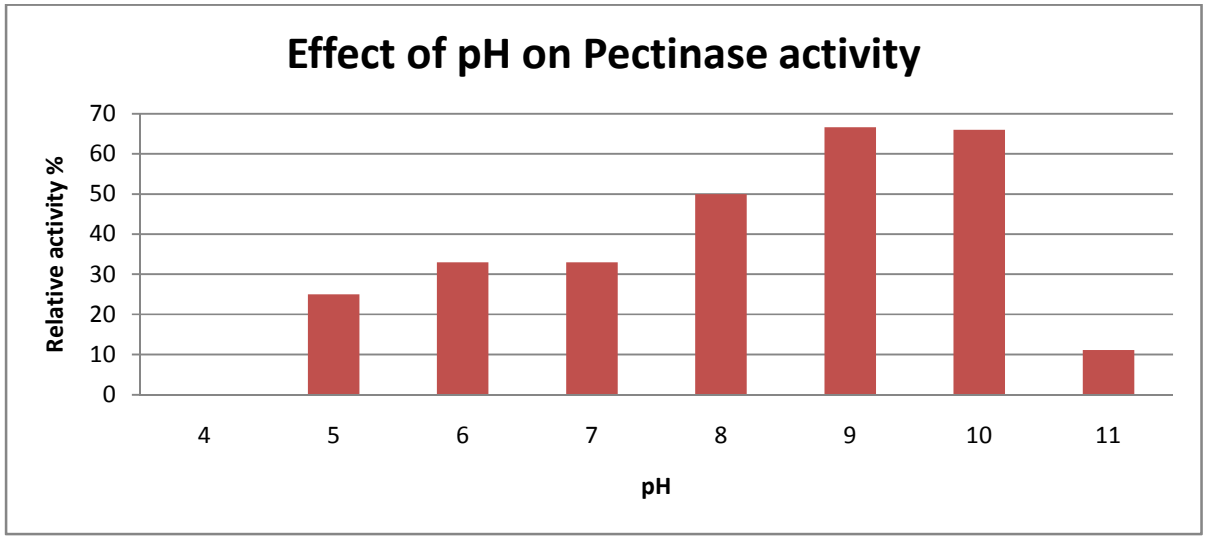


Fig.1: Effect of *p*H on the activity of pectinase from *Natronobacterium innermongoliae SSBJUP-4*. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments.

Effect of temperature on pectinase activity indicates that the enzyme is stable and functional over a wide range of temperature up to 75^{o}C beyond which it loses activity almost instantaneously. This nevertheless means that the enzyme is fairly thermo tolerant (Fig. 2).

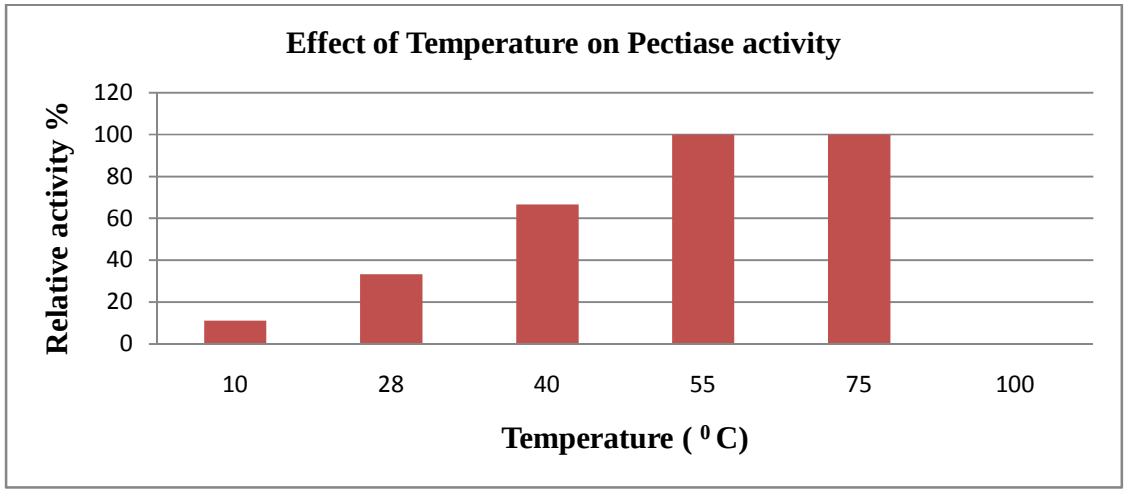


Fig. 2: Effect of temperature (^{o}C) on the activity of pectinase from *Natronobacterium innermongoliae SSBJUP-4*. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments

Again true to expectations that pectinase showed high activity at high salt concentration from 12% and above. Its maximum activity was at 14-16% salt concentration, falling rapidly at concentration higher than at 22% (Fig. 3).

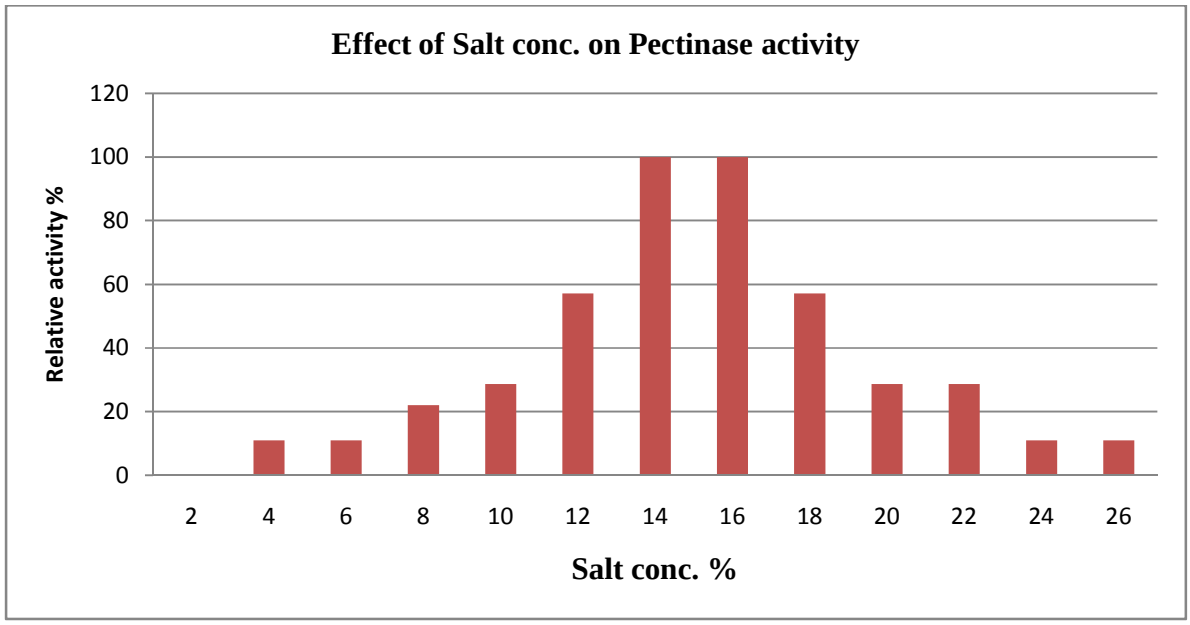
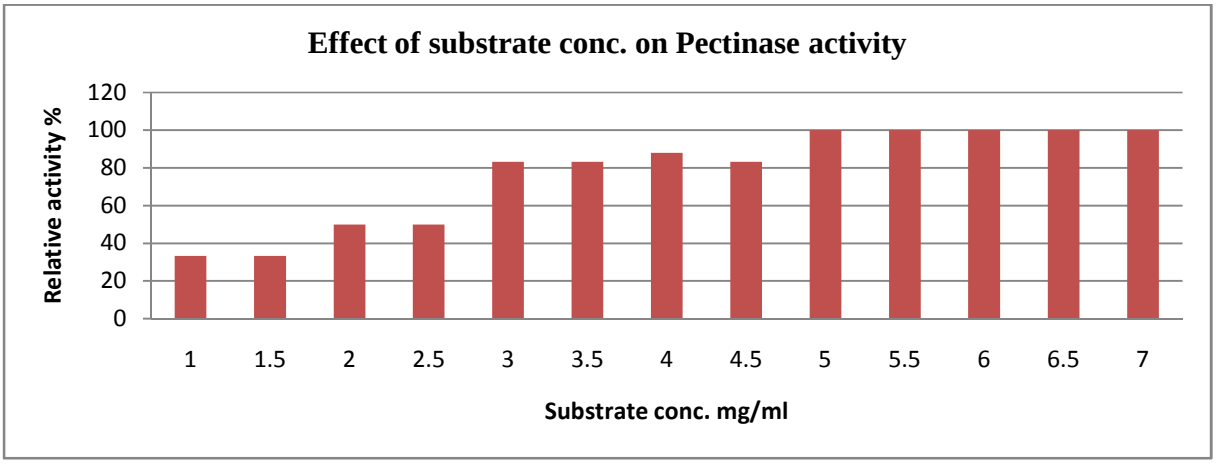


Fig.3: Effect of salt (NaCl) concentration (%) on the activity of amylase and protease from *Natronobacterium innermongoliae SSBJUP-4*. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments.

Effect of substrate concentration on pectinase activity is an important parameter commonly used to define the mathematical model for enzyme kinetics known as the Michalies-Menten relationship. The graphical presentation is usually in the form of a rectangular hyperbola known as the Michalies-Menten curve. The relationship of pectinase with its substrate pectin indicated in resemblance of the M-M curve with the velocity of the enzyme plateau from 5 mg substrate concentration to 7 mg (Fig. 4).



(Fig. 4) Effect of substrate concentration on pectinase activity showed in above graph. Relative activity (%) is enzyme activity detected at individual value in relation to the maximum enzyme activity taken as 100%. Values are averages of three independent experiments.
Pectinase activity being markedly stimulated by Ca^{2+} and Mg^{2+} in the range of 0.4 to 0.7mM concentration but inhibited by Cu^{2+} and Hg^{2+}.

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Conflict of Interest Statement

Authors have no conflict of interest

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