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Production and characterization of a haloalkaline pectinase from *Halomonas pantellerinsis* strain SSL8 isolated from Sambhar lake, Rajasthan

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Abstract

Haloalkaliphilic bacterium producing a pectinase was isolated from the Sambhar soda lake, Rajasthan, India. Chemical composition of water sample was analyzed. Pectinase production was studied in submerged fermentation, an appropriate medium for the growth and production was orange peel powder. The bacterium was gram negative and identified as *Halomonas pantellerinsis* strain SSL8 using biochemical tests and 16S rRNA sequencing. It was able to grow and produced pectinase that was stable and active at high pH, temperature and high NaCl concentration. Maximum pectinase production from isolate was observed after 120hr of incubation (0.70U/mL). The maximum pectinase activity was found at 9 pH (0.79U/mL), 40°C Temperature (0.70U/mL) and 10% NaCl concentration (0.85U/mL). Partially purified pectinase enzyme was used for the fruit juice extraction and clarification.

Keywords: Haloalkaliphilic, *Halomonas pantellerinsis,* Sambhar lake, orange peel powder, Pectinase

Introduction

Pectinase constitute a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls. Pectinases are commercially used in many processes and nearly 25% of the global enzymes sales are attributed to pectinases (1, 2).

In the industrial sector, acidic pectinases are used in the production and clarification of fruit juices, in maceration and solubilization of fruit pulps whereas alkaline pectinases are finding immense use in the degumming of ramie fibers retting of flax, textile processing, coffee and tea fermentations, paper and pulp industry, and in oil extraction (3). Pectinases are produced by many organisms such as bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. A quarter of the global food enzymes sale is met with microbial pectinases. Although the major sources of acidic pectinases are fungi, alkaline pectinases are produced from alkaliphilic bacteria (4)

The present paper describes isolation and identification of a haloalkaliphilic *Halomonas pantellerinsis* strain SSL8 from the hypersaline Sambhar Lake and production of extracellular haloalkaliphilic pectinase from such fruits and vegetable waste to minimize the cost by the selected isolate.

Material and Methods

Site description and sample collection: The Sambhar Lake is the largest inland saline lake located in Thar Desert of Rajasthan, India (26° 52'- 27° 2' N, 74° 53'- 75° 13'E) (Fig. 1). It is an elliptical and shallow lake, with the maximum length of 22.5 km. The width of the lake ranges from 3.2 km to 11.2 km. The total catchments area of the lake is 7560 km^2, most of which lies to

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the north and northeast. The lake has occupied an area of about approximately 225 Sq. Km and average depth of water is about 1 m whereas the maximum depth is about 3m (5, 6, 7). The surface (SU) and Sediment (SD) water samples were collected from four sampling stations located in main lake and salt pans towards Sambhar Lake city. Samples were collected in presterilized bottles in post-monsoon, season. The samples collected from each station were average of ten samples spanning the whole sampling point.

Abiotic characterization of water: The parameters like Temperature, pH were measured at the time of sampling by using Digital Thermometer and Digital pen pH meter respectively. Samples were transported to laboratory in cold box. The samples were filtered and stored in refrigerator during investigation (8).

Various physicochemical parameters were determined for both the samples individually. TS, TDS, TSS were analyzed according to procedures described in APHA. The salinity was measured by using Refractometer (Erma, Tokyo). The dissolved oxygen content (DO) was determined by azide modification method, Biological oxygen demand (BOD) and chemical oxygen demand (COD) were determined by potassium dichromate oxidation method. Chloride was determined by argentometric and sulphate by gravimetric methods. Sodium and potassium were measured directly using the flame photometer (Model Elico CL 361). Carbonates and bicarbonates were measured titrimetrically. Calcium and magnesium were determined by EDTA titrimetric method. Metal ions like Fe, Mn, Zn, As, Cr, Pb, Cu, and Cd were directly analyzed by atomic absorption spectrophotometer. (Model S2 Thermo- USA) (9, 10).

Enrichment, Isolation and cultivations of haloalkaliphiles: The 5ml water and Brine sample was inoculated into nine different media such as Alkaliphilic media at pH- 10.0,[A], Marine agar pH- 10.5 [MA], Nutrient broth at pH- 10.5 [ANA] with 30 % sodium chloride, Halophilic medium [H], modified Horikoshi II medium [H II], Synthetic Sea water medium[S], Alkaline peptone water [AP], Alkaline Bacillus medium (AB) and Tindal's medium [T].. Inoculated media flasks were incubated in shaking incubator for 8 days at 30°C temperature and 150 rpm speed. Incubated samples were further inoculated on respective agar plates. Inoculated agar plates were incubated for 15-20 days at 30°C temperature (11-14).

Screening of isolates for efficient pectinase producer: Isolates were tested qualitatively by growing the culture on modified alkaliphilic pectin agar medium followed by observing zone of hydrolysis around colonies. After incubation the plates were flooded with Iodine-potassium iodide solution (0.3 % iodine, 0.6 % potassium iodide solution) to enhance the clarity of zone. The isolate showing largest zone was selected for further production (2, 15, 16)

Identification of selected strain of the bacterium: Selected isolate was subjected to morphological and Biochemical observation. Sugar utilization pattern of selected isolate was determined using glucose, galactose, mannose, arabinose, fructose, ribose and lactose. Enzyme utilization profile was determined using starch, casein, gelatin, pectin substrates (17).

DNA extraction, amplification and 16S rRNA sequencing: 16S rRNA analysis was performed by extracting DNA of isolates. For DNA extraction isolates were suspended in an extraction buffer (10 mM Tris HCL, pH 8.0; 1 mM EDTA, pH 8.0). Proteinase K solution was added to a final concentration of 100 ug/ml and incubated at 55°C for 2 h with continuous shaking. 0.5 M NaCl was added and incubated at 72°C for 30 min. DNA was extracted by phenol–chloroform extraction. DNA was washed with 70% ethanol and dissolved in Tris–EDTA buffer (pH 8.0). Extracted DNA was analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining (18)

The amplification of 16S rRNA fragments were performed by using (PCR) thermocycler, (Eppendorf) with 530F (52 GTGCCAGC

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AGCCGCGG 32) and 1392R (52 ACGGG CGGTG T GTAC 32) primer pair. The PCR reaction mixture contained 1.5 mM $MgCl_2$, 200 uM dNTP mixture and 0.3 μM of each primer and 1 U of Taq DNA polymerase with a reaction mixture supplied by the manufacturer in a total volume of 100 μl. Reaction mixture was first denatured at 94°C for 3 min, followed denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min. Amplification was completed by a final extension step at 72°C for 7 min reaction was carried out for 30 cycles. PCR products were run on a 1% agarose gel. PCR products were purified by the PEG/NaCl method (19) and directly sequenced using Applied Biosystem model 3730 DNA analyzer (Foster, California, USA). The 16S rRNA sequences were initially analyzed using BLAST program (www.ncbi.nlm.nih.gov/blast/blast.cgi). Multiple sequence alignments of approximately 800 base pair sequences were performed using CLUSTALW2 program version 2.1. Phylogenetic tree was constructed using the neighbor joining method (20). Tree files were generated by PHYLIP and viewed by TREE VIEW program. Bootstrap analysis was applied.

***Pectinase production using synthetic and crude media**:* Pectinase production was carried out submerged fermentation using synthetic and crude media. Presterilized medium containing yeast extract, 1; pectin, 5; KH_2PO_4, 4; NaCl, 200; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 2 grams per liter was inoculated and incubated at 30°C temperature and 150 rpm speed at pH 9.

Presterilized crude medium contain 10g orange peel powder mixed with mineral salt solution contain 1% KH_2PO_4, 15% NaCl, 0.1% $MgSO_4.7H_2O$, 0.1% $CaCl_2$ was also inoculated and incubated at same conditions (21, 22).

Assay of pectinase activity: Polygalacturonase activity was determined by quantifying the amount of reducing groups expressed as galacturonic acid units, liberated during the incubation of 1 ml of 1% (w/v) citrus pectin, prepared in 0.2 M phosphate buffer (pH 8.2) with 500 ìl of the enzyme at 37°C for 30 min, by DNSA method. One unit of polygalacturonase activity was defined as the amount of enzyme required to release 1 ìmol of galacturonic acid per minute under standard assay conditions and expressed as units per litre (U/l). Specific activity was defined as the amount of enzyme required to release 1 μmol of galacturonic acid per minute per milligram of total enzyme protein and expressed as units per milligram (U/mg) (16, 21, 22).

Partial purification of pectinase: The crude pectinase enzyme supernatant was partially purified using chilled acetone and ammonium sulphate precipitation method. The precipitate was dissolved in 10 ml of 0.2 M phosphate buffer (pH 8.5) and desalting was carried out by dialysis (2).

Determination of protein content: The protein contents of the crude and purified pectinases were determined by the method specified by Lowry *et al.*, using bovine serum albumin as the standard (23).

Effect of pH, temperature, salt and reaction times on pectinase activity : The optimum pH and temperature of the pectinase activity was studied over pH range of 5 to 12 and temperature range from 20 to 50 °C respectively. The effect of various salt concentrations and reaction times on pectinase activity was measured over range of 5 to 30% and 10, 20, 30, 40, and 50 min respectively (2, 24, 25).

Application of pectinase in fruit juice extraction and fruit juice clarification: Fruits (apples) were obtained from a local market Nanded. For the extraction of juice from apple, the apples were chopped into small cubes (3-5mm in size). Ten grams of material were incubated with 1 ml of crude enzymatic extract for 1 h in a shaking water bath with a shaking rate of 100 rpm, at 40 °C. Later the samples were incubated in a boiling water bath for 5 min to inactivate the enzyme. After cooling to room temperature, the juice was filtered by vacuum through filter paper and the volume of juice obtained was measured

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DOI: 10.5530/ctbp.2020.3.33

by using 100 mL graduated cylinders. Inactivated enzyme was used as a control and for study the fruit juice clarification, 10 mL portion of juice was taken and centrifuged at 3000 rpm for 10 minutes. The clarity of fruit juice was determined by measuring at 450nm using UV-VIS spectrophotometer (9, 28).

Result and Discussion:

Abiotic characterization of water: Abiotic characterization of Sambhar lake water samples collected in post-monsoon season has yielded diverse results. The colour of post-monsoon water sample was pale green at the time of collection. The typical rotten egg like smell was experienced in the lake atmosphere. The average pH recorded was 10 for water sample.

The Total Solids (TS) and Total dissolved Solids (TDS) were recorded as 131050 mg/l and 88263 mg/l respectively. The Total Solids (TS) and Total dissolved Solids (TDS) recorded in present investigation were higher as compared to the very well studied African soda lake and Kenyan Soda Lake.[22]. Some anionic and cationic concentrations of water were recorded, among all dominating cations and anions were sodium (9930 mg/l) and chloride (7356 mg/l) and the divalent cations Ca^{2+} (1550 mg/l) and Mg^{2+}(1870 mg/l). Carbonates (396 mg/l) and sulphate (9152 mg/l)) anions also recorded in considerable amount.

Also in the water sample the metal concentrations were recorded. The Trace amount of chromium (0.01 mg/l) and arsenic (0.01mg/l) were recorded. Also lead (0.05mg/l), Zinc (0.36 mg/l) and cadmium (0.7mg/l) were present in considerable amount in water sample (27, 28).

Screening of bacterial isolates for Haloalkaliphilic pectinase production: Out of nine broth media used during enrichment the agar media have supported highest diversity and faster growths of haloalkaliphiles were used in further investigation. Out of total 10 isolates which have shown zone of clearance on pectin agar plates, and showing distinct colony characters were selected from alkaliphilc medium (A), synthetic Sea agar (SS), alkaline nutrient agar (ANA) and Marine agar (MA) plates. Small colonies were appeared after incubation of 10 days, further incubation of 10 days have yielded large colonies. Non pigmented and pigmented colonies were observed. Pigmented colonies showed cream, yellow, pink, and red colour pigment. Out of ten morphologically distinct isolates SSL8 rapidly growing extreme haloalkaliphilic strain was selected for further investigation which showing maximum zone of clearance on pectin agar plates. It is motile, cream pigmented, Gram negative, rod shaped bacterium growing at optimum 9 pH, 10% salt concentration and 40°C temperature. It is Catalase and oxidase positive, produces H_2S. The sugars Glucose, Fructose, Mannose, Ribose, Arabinose, Galactose and Lactose are not utilized It is negative for hydrolysis of starch, Casein and Gelatin (14, 28) (Table 1).

Based on morphological, physiological, biochemical characteristic and Phylogenetic analysis of its 16S rRNA gene sequence it was identified as *Halomonas pantellerinsis* strain SSL8 (Fig. 1). The 16S rRNA gene sequence was submitted to NCBI Genbank with accession number KC 434456

Alkaline pectinase production was recorded with two substrates orange peel powder and pectin. Maximum production was observed in growth medium containing orange peel as source of substrate (Fig. 2). Maximum pectinase production from *Halomonas pantellerinsis* strain SSL8 was observed after 120hr or 5 days of incubation (0.70U/mL). A gradual increase in the enzyme level was detected till the 120hr of the fermentation process, whereas, there was a steep decline in the pectinase activity after the 120hr of incubation, as shown in Fig 2. Beyond this period the enzyme production drastically reduced, probably due to the depletion of essential nutrients in the medium and/or accumulation of toxic secondary metabolites.

Effect of temperature: The effect temperature on the alkaline pectinase enzyme production was studied using alkaliphilic medium by conducting

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DOI: 10.5530/ctbp.2020.3.33

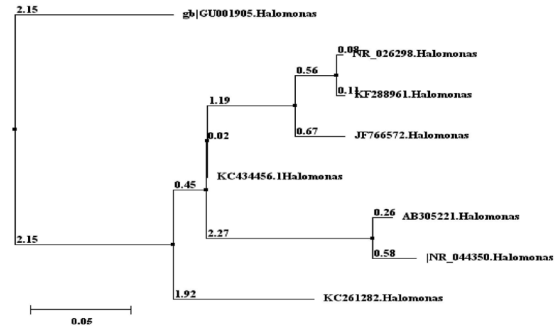

Fig. 1. Phylogenetic tree of isolate SSL8 to other Halomonas.Each number on a branch indicates the bootstrap values. The scale bar indicates 0.05 substitutions per nucleotide position

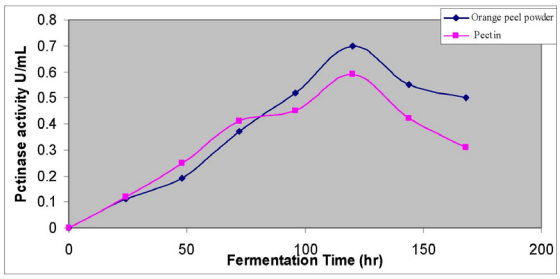

Fig. 2. Effect of fermentation time on pectinase production

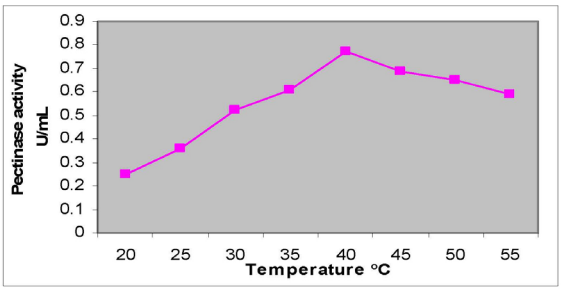

Fig. 3. Effect of Temperature on pectinase Activity

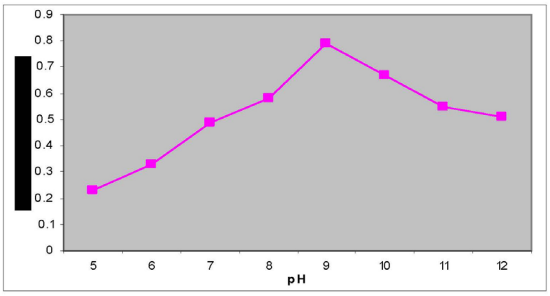

Fig. 4. Effect of pH on pectinase Activity

Table 1. Morphological and Biochemical characterization of Strain SSL8

Characters	SSL8
Morphology	Rod
Gram nature	-
Size (mm)	0.5-2
Colony pigmentation	Cream
Motility	Motile
Oxidase	+
Catalase	+
pH range	08-Nov
Optimum pH	9
Salt range	5-15%
Optimum Salt Concentration	10%
Temperature range (°C)	20-40
Urease	+
Nitrate reduction	+
H2S production	+
Hydrolysis of:	
Casein	-
Gelatin	-
Starch	-
Utilization of:	
Arabinose	-
Fructose	-
D-Glucose	-
D-Galactose	-
Mannose	-
Ribose	-
Lactose	-

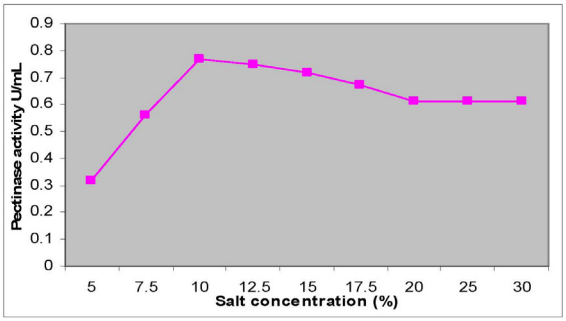

Fig. 5. Effect of Salt concentration on pectinase Activity

Vol. 14 (3) 319-326, July 2020, ISSN 0973-8916 (Print), 2230-7303 (Online)
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experiments at different temperatures, keeping all other conditions constant for the fermentation. As a temperature increase the pectinase enzyme activity was found to increase and maximum pectinase activity of 0.77 U/ml was found at 40°C (Fig. 3). Further increase in temperature beyond 40°C decreased the pectinase activity till the end of fermentation. Hence optimum temperature was 40°C and was used for further studies. The decrease in enzyme activity at higher temperature may be due to enzyme denaturation.

Effect of pH: The effect of pH on the pectinase production was studied by conducting experiments at different pH (pH range 5, 6, 7, 8, 9, 10, 11, 12) and by keeping temperature at 40 °C. As initial pH was increased from pH 5 to pH 9, the pectinase activity was found to increase. Further increase in initial pH beyond pH 9, the pectinase activity was found to decrease. The decrease in enzyme activity at higher pH may be due to growth and metabolism of organism. A maximum pectinase activity of 0.79U/ml was observed at a fermentation period of 6 days at temperature 40°C and at pH value of 9. Hence optimum pH value was selected as pH 9 (Fig. 4).

***Effect of salt concentration*:** The effect Salt concentration on the alkaline pectinase enzyme production was studied using alkaliphilic medium by varying Salt concentrations (5, 10, 15, 20, 25 and 30% w/v) keeping all other conditions constant. The maximum pectinase activity of 0.85U/ml was found at 10% Salt concentration (Fig. 5).

Pectinase for fruit juice extraction and clarification : The fruit juice extraction by using the pectinase enzyme as well as mixture of other enzymes (cellulose) with pectinase was showed significant results of fruit juice extraction. 31 mL of fruit juice was extracted when apple without peel was treated with pure pectinase and 24 mL juice was extracted when treated with crud pectinase. 17 mL juice was extracted when crude pectinase treatment was given to apple with peel. Whenever the treatment of cellulase on the fruit without peel was given, 26 mL juice was extracted and 19 mL was extracted from apple with peel. (1) 26.5 mL juice was extracted when treatment of crude pectinase and crude cellulose given to the fruit without peel and 19.7 mL was extracted from the same treatment on the fruits with peel. And the apple juice which was extracted by the treatment of crude pectinase has the more clarity as compare to other enzymatic treatment

Conclusions

A haloalkaliphilic bacterial strain isolated from the Sambhar salt lake of India was identified as *Halomonas pantellerinsis* strain SSL8. It produced halo alkaline pectinase that was stable and active at high pH, temperature and high salt concentration. It is showing optimum activity at pH 9, temperature 40 °C and at 10 % salt concentration. The *Halomonas pantellerinsis* strain SSL8 pectinase gives maximum production in fruit juice extraction and also showed good result in fruit juice clarification. Similar study was carried out by Kashyap et al. in 2000 (1).

Considering the high activity and stability in high alkaline pH and temperature, the *Halomonas pantellerinsis* strain SSL8 pectinase may find potential application in the degumming of ramie fibers, retting of flax, textile processing, coffee and tea fermentations, paper and pulp industry, and in oil extraction (25).

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