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Research Article

Screening of Pectinase Producing Gram Positive Bacteria: Isolation and Characterization

Fouzia Tabssum[1]* and Syed Shahid Ali[2]

[1]Microbial Biotechnology Lab, Department of Zoology University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan
[2]Institute of Molecular Biology & Biotechnology, The University of Lahore, Defense Road Campus, Lahore-54500, Pakistan.

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Authors' Contributions
FT perform experiments and draft initial draft, SSA design, supervise the experiments and improve the manuscript.

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Screening, Extracellular microbial enzymes, Pectin, Alkaline pectinase, Acidic pectinase.

Abstract | Microbial pectinases are enzymes of industrial importance; they are being used commercially in a variety of ways such as clarification of the juices in beverage industries, tea making, and wastewater treatment among others. The present investigation was undertaken to isolate pectinase-producing bacteria from local habitats. To enrich the diversity of isolated bacteria that produce pectinase, samples were collected from the soil, water, fruits and vegetables wastes. Initial screening of pectinase producing bacteria was carried out on pectin containing agar plates of pH 7 incubated at 37°C. Among isolated thirty-seven strains, a total of thirteen strains showed satisfactory pectinase productivity. Pectinase production of the thirteen strains was then determined qualitatively as well as quantitatively. Zones of hydrolysis produced by the bacterial strains were ranged from 0.9-2.6 mm^2 in diameter. Enzyme production units were 59.0, 57.0, 58.0and 58.0 µg/ml/min by the bacterial isolates designated as BCTL-SL-197, BCTL-FL-24, BCTL-VL-23 and BCTL-FL-27, respectively. Bacterial growth was checked at different pH and temperature conditions. Best growth was observed at pH 7 and at 37°C.

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Introduction

Enzymes are biological catalysts, which facilitate the chemical reactions under diverse sets of physicochemical conditions. Enzymes are specific in their "actions" and are usually protein in nature and some enzymes may have non-protein part known as prosthetic group. Enzymes were discovered in middle of nineteen century, and they were first introduced in industrial application by Dr. Jokichi Takamine (1894-1914). He basically introduced the fungal enzymes but after twenty years Boidin and Effront introduced the bacterial enzymes in the industry. As the energy demand is increasing with passage of time the natural resources are going to be exploited by man at very high rate, so there is an urgent need of new energy resources.

Most of the industrial processes and technologies employ different chemicals fundamentally, which are responsible for significant environmental pollution. Many extracellular microbial enzymes are environmental friendly and have industrial applications. Environmental pollution is no longer accepted as inevitable in technological societies. People are now more aware of the effects of pollutions and public pressure is increasing on both industries and government to use more environmental friendly technologies. So there is increasing demand to replace chemical based industries with biotechnological processes involving microorganisms and use of enzymes such as amylases, lipases, xylanases, cellulases, chitinases and pectinases *etc.* (Underkofler *et al.*, 1957; Bajpai, 1999; Bruhlmann *et al.*, 2000; Demirijan *et al.*, 2001).

Pectinases are the group of enzymes, which degrade pectin substances. Pectinases are basically hydrolases, lyas-

***Corresponding author:** Fouzia Tabssum
fouziatabassum66@gmail.com

es and estrases (Danielle *et al*., 2009). Microbial pectinases are secreted by fungi, bacteria and yeast. Pectins are the variety of substances, which are present in higher plants and are macromolecules of high molecular mass. They are basically the component of plant cell wall and middle lamella, and very thin extracellular layer, which joins the young, cells together. The function of the pectic substances is to give the structural integrity to the cells and make them cohesive. There are three major groups of pectic substances, all of these contains D-galacturonic acid as the common component which may be in lesser or greater extent depending upon the group. The three major groups of pectic substrates include: homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II).

Homogalacturonans are the linear polymers of pectins, which are formed by D-galacturonic acid monomers, which may be methyl esterified and/or acylated. This region of pectin is called smooth region. These molecules are further classified on the basis of esterification level or carboxylic groups. If 75% of carboxylic acids are methylated then it is called pectin, if less than 75% of carboxylic acids are methylated the substances are called pectic acids and if there is no methyl esterification of carboxylic acid it is called polyglacturonic acid. Pectin is the generic name for all above groups (Alkorta *et al*., 1998; Jayani *et al*., 2005; Namasivayan *et al*., 2011).

Pectinases are the enzymes, which degrade the pectic substances. Microbial pectinases have great potential and have commercial applications to serve mankind. Pectinases may be alkaline and acidic. The acidic pectinases are employed in juices and beverages industries for purification and clarification of wines and juices; whereas the alkaline pectinases are applied in wastewater treatment of vegetables processing origin having the pectinicious materials. The present study was aimed at isolation, characterization and preservation of pectinase producing bacteria from local habitate. The reported isolates demand additional research inputs to streamline the economical provision of pectinases to food processing units.

Materials and Methods

Collection of samples, isolation and screening of bacteria

Soil and water, fruits and vegetables samples containing bacterial were collected from different localities of Lahore. Test samples were preserved in polythenes bags and sterilized glass tubes and stored at 4ºC. Bacteria were isolated by serial dilutions of water and soil samples followed by spreading on pectin containing agar plates. Whereas the fruits and vegetables' infected portions were saved aseptically and suspended and sterile water, which was then processed for serial dilution. The plates were incubated at 37ºC in the dark for 24 h. Colonies of bacteria with clear zones of hydrolysis were selected and sub cultured on nutrient agar medium in 9cm diameter of Petriplate for culture purification. The purified strains were preserved in refrigerator at 4ºC on nutrient medium.

Estimation of enzyme activity

Qualitative enzyme analysis

The enzyme was checked by making spot of a loopful of a 24 h grown bacterial culture on 2.5% pectin suphlented LB agar plates. The plates were incubated at 37ºC at dark for 24 h. The diameter of clear zone of hydrolysis around the spot after 24 and 48 h, followed by staining with Lugel, siodine solution.

Quantitative enzyme analysis

Pectinolytic activity of the all thirteen strains was determined by a modified (colori-metric method of Miller, 1859 modified by Aguillar and Huitron (1990)). Pectinase enzyme 100µl was taken in Eppendorff and 100µl of 1% pectin solution added to enzyme. Pectin solution of 1% was prepared by dissolving 1 g of pectin in 100 ml of sodium citrate buffer at pH 6.5. The enzyme substrate mixture was incubated at 40˚C for 15 min. Then 400µl of dintrosalicylic acid (DNS) was added to the mixture to terminate the reaction and it was kept in the boiling water bath for 15 min. Blank was prepared along with by adding the 100µl of distilled water in place of enzyme. Absorbance was recorded at 570nm wavelength in spectrophotometer in glass cuvettes against the blank Standard curve of glacturonic acid was prepared by reading its 1,2.....5 mg/ml concentrations as mentioned above. Concentrations of reducing sugars in the sample were then measured with the help of following equation:

Conc. of reducing sugars (mg/ml) = O.D. of sample x 0.4 x *dilution factor*

The amount of reducing sugars was then converted into the enzyme's units according to the method referred above.

Protein estimation

The protein was estimated by the method of Lowry *et al.* (1951). The copper reagent was prepared by adding 100ml of solution A (0.4g NaOH and 2g Na_2CO_3 in 100ml water) and 2ml of solution B (1g of sodium potassium tartarate in 100ml water) and 2ml of solution C (0.5 g of $CuSO_4$ in 100ml distilled water). The supernatant from centrifuged broth culture (0.5ml) was added to 5ml of the alkaline copper reagent and allowed to stand for 10 min followed by addition of 0.5ml of Folin-Ciocalteau phenol reagent. The solution was then allowed to stand for 30 min at room temperature and the absorbance was measured at 750 nm with spectrophotometer. Standard curve was prepared by processing 0.2, 0.4.....1mg/ml of bovine serum albumin. The protein content of samples was then estimated with the help of following formula:

Protein content (mg/ml) = O.D of sample x 0.32 x dilution factor

Characterization of bacteria

Identification of bacteria

Identification of pectin degrading organisms was performed in terms of different morphological physical and biochemical characteristics. The data was compared with standard description given in Burgey's Manual of Determinative Bacteriology.

Optimization of growth conditions

All the above pectinolytic strains were grown on different temperatures to evaluate their thermophilic behavior. For this purpose bacterial isolates were incubated at different temperature ranges from 30-50˚C. All isolates were further tested by culturing them at different pH to evaluate their best pH for growth. For this purpose isolates were grown at different pH ranges from 4-13.

Results and Discussion

Collection of samples, isolation, purification and preservation of bacterial strains

In addition to soil and waters samples the fruits tomato, orange, grape fruit and apple and the vegetables; lemon, carrot, potato and onion were also samples for isolation of bacteria. The fruits, vegetable and soil samples were collected from fruit and vegetable Market Kot Lakhpat, Lahore, Pakistan. The soil and water samples were collected from Punjab University hostels at New Campus, Lahore, Pakistan. Thirty-seven bacterial strains were isolated from soil, vegetables, water and fruits by serial dilution method followed by spreading of LB medium with pectin containing agar plates. All thirty-seven strains were purified and preserved in refrigerator at 4°C on nutrient agar slants.

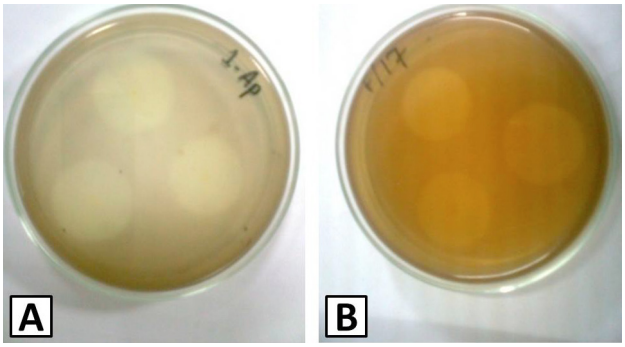

Figure 1: Zone of hydrolysis of BCTL-VL-23 after 24 h (A) and 48 h (B).

Screening of pectinolytic bacteria

All isolated thirty-seven strain were screened for pectinase production on LB agar plates at pH 7. Thirteen strains out of thirty-seven were pectinase producing because they showed clear zones of hydrolysis of different diameter around the colony of bacteria in substrate containing agar plates (Figures 1, 2 and Table I). Further these thirteen bacterial strains were checked for pectinase production shown in Table II. Maximum enzyme production was 59.0 µg/ml/min from the bacterial strain BCTL-SL-197.

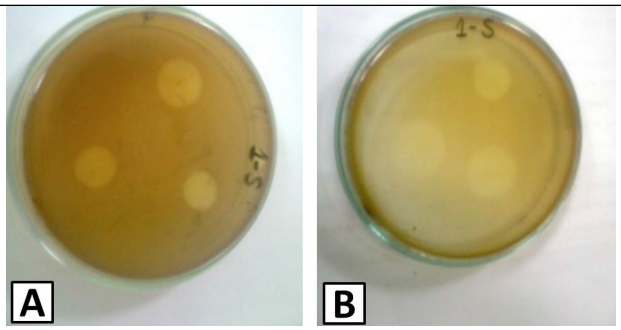

Figure 2: Zone of hydrolysis of BCTL- SL-197 after 24 h (A) and 48 h (B).

Table I: Qualitative analysis of all collected strains*.

Bacterial strain	Colony diameter (mm)	Diameter of Hydrolysis zone (mm)	Pectinase +ve or -ve
BCTL-AB-04	-	1.1	+ve
BCTL-AB-05	-	No zone	-ve
BCTL-AB-06	-	2.0	+ve
BCTL-AB-07	-	1.2	+ve
BCTL-SL-197	-	2.0	+ve
BCTL-SL-198	-	No zone	-ve
BCTL-SL-199	-	No zone	-ve
BCTL-FL-22	-	No zone	-ve
BCTL-VL-23	-	No zone	-ve
BCTL-FL-23	-	No zone	-ve
BCTL-FL-24	-	2.0	+ve
BCTL-VL-23	-	2.3	+ve
BCTL-VL-24	-	No zone	-ve
BCTL-VL-25	-	No zone	-ve
BCTL-FL-25	-	No zone	-ve
BCTL-FL-26	-	No zone	-ve
BCTL-FL-27	-	2.6	+ve
BCTL-FL-28	-	1.1	+ve
BCTL-FL-29	-	1.9	+ve
BCTL-VL-26	0.2	No zone	-ve
BCTL-VL-27	0.2	2.1	+ve
BCTL-VL-28	0.2	No zone	-ve
BCTL-VL-29	0.2	No zone	-ve
BCTL-VL-30	0.2	No zone	-ve
BCTL-FL-30	0.2	No zone	-ve
BCTL-FL-31	0.2	No zone	-ve
BCTL-FL-32	0.2	No zone	-ve
BCTL-SL-200	0.2	No zone	-ve
BCTL-SL-201	0.2	0.9	+ve
BCTL-SL-202	0.2	No zone	-ve
BCTL-SL-203	0.2	2.1	+ve
BCTL-SL-204	0.2	No zone	-ve
BCTL-SL-205	0.2	No zone	-ve
BCTL-SL-206	0.2	No zone	-ve
BCTL-SL-207	0.2	2.4	+ve
BCTL- SL-208	0.2	No zone	-ve
BCTL-WL-109	0.2	1.5	+ve

BCTL, Biochemistry and Toxicology Lab; VL, Vegetable Lahore; SL: Soil Lahore; FL: Fruit Lahore.

*colonies of all the isolates had 0.2 cm diameter following 24 hrs of incubation in LB medium

Table II: Morphometric and biochemical characterization of the select bacterial isolates.

Strain No.	Colony morphology (color)	Gram reactions (cell shape)	Spore staining	Catalase (oxidase)	Pectinase activity (ug/ml/min) n=3	Protein estimation (mg/ml/min) n=3
BCTL-SL-207	Irregular (White)	Positive (Bacilli)	Positive	-------	29.15 ± 0.01	0.798±0.02
BCTL-AB-O5	Regular (Orange)	Positive (Cocci)	Negative	+ve (+ve)	27.7 ±0.01	0.693±0.01
BCTL-SL-197	Irregular (White)	Positive (Cocci)	Negative	+ve (-ve)	59.69± 0.01	0.938±0.04
BCTL-FL-29	Regular (White)	Positive (Cocci)	Negative	+ve (+ve)	34.70± 0.01	0.850±0.01
BCTL-AB-03	Irregular (White)	Positive (Cocci)	Positive	----	24.99±0.01	0.843±0.01
BCTL-FL-27	Regular (White)	Positive (Bacilli)	Negative	+ve (+ve)	58.31± 0.01	0.951±0.02
BCTL-VL-27	Regular (Pink)	Positive (Cocci)	Positive	----	40.26±0.01	0.672±0.02
BCTL-VL-23	Regular (White)	Positive (Cocci)	Negative	+ve (+ve)	58.31±0.01	0.747±0.04
BCTL-FL-24	Regular (White)	Positive (Cocci)	Positive	----	56.92±0.01	0.693±0.03
BCTL-AB-06	Regular (Yellow)	Positive (Cocci)	Positive	----	30.54 ±1.6	0.636±0.03
BCTL-SL-201	Regular (White)	Positive (Cocci)	Positive	----	11.10±1.82	1.07±0.01
BCTL-FL-28	Regular (White)	Positive (Bacilli)	Positive	----	33.32±1.82	0.636±0.06
BCTL-SL-203	Regular (Yellow)	Positive (Cocci)	Positive	----	26.37±0.01	0.877±0.01

Rokade *et al.* (2015) have isolated and screened pectinase producing bacterial strain form Osmanabad soil and fruit samples.Among these, one strain was best enzyme producer identified as *Staphylococci* sp. Raju *et al.* (2013) reported 3 bacterial pectinase producing isolates from *B. licheniformis*, *B. cerus* and *S. aureus* from dump yards of Banglore. Kumar *et al.* (2012) isolated and screened *Bacillus* sp. MFW7 on cassava waste for pectinase production.

Characterizations of bacterial isolates

Identification of bacteria

Identification of pectin degrading bacteria was performed on the basis of different morphological, physical and biochemical characteristics shown in Table II. The data was compared with standard description given in the Burgey's Manual of Determinative Bacteriology (Buchanan an Gibbons, 1974). The data was compared with standard was shown in Table II. Gectha *et al.* (2012) isolated and characterized *Bacillus* sp. And *Pseudomonas* sp. on gram staining, spore formation on selective media plate and on biochemical reactions.

Growth of bacteria at different temperatures and pH

All the above pectinolytic bacteria were grown on different temperatures to check their thermophilic behavior by incubating them at different temperatures ranges from 30-50°C. All pectinolytic isolates showed maximum growth at 37°C. Moderate growth was also observed at 30°C but growth was reduced at 41°C and no growth was observed after 48°C. All pectinolytic strains were thermotolerent. All the pectinolytic strains were further tested for their alkalophilic or acidophiic behavior by growing them at different pH ranges from pH 4-13. They showed maximum growth at pH 7 but scanty growth was observed at pH 12 whereas on the other hand very little growth was observed at pH 5 and no growth was observed at pH 4. All above pectinolytic bacteria had 7 optimum pH.

Conclusion

In the present study thirteen bacterial strains, capable of producing extracellular pectinases, were isolated from local environment. In anagri-based economy, demand-supplies of such bacterial enzymes for indigenous sources provide economical solution to multiple sectors and strengthen the in-country research infrastructures. The bacteria reported in this communication have potential of pectinases production employing bio-wastes resources abundantly available in Pakistan for their cultivation.

Conflict of interest

Authors claim no conflicts of interest associated with this research work.

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