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Full Length Research Paper

Molecular characterization of polygalacturonase producing *Klebsiella* and *Staphylococcus* species by 16S rRNA sequencing collected from rotten fruits and vegetables

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Pectinases are of commercial importance due to their application in manufacturing fruit juices (apple/ citrus), paper, textile and jute retting for its up gradation. The main thrust idea of the present study was concerned with the isolation of bacteria from different habitats for the production of the enzyme polygalacturonase. 16 bacterial colonies selected from preliminary screening by well plate method were subjected for morphological and biochemical characterization. Bacterial isolates, ZS-03 and ZS-17 which showed maximum zone sizes of 27 and 30 mm in well plate assay were characterized as *Klebsiella* species, whereas ZS-10 exhibiting zone size 25 mm was characterized as *Staphylococcus* sp. Quantification of polygalacturonase in the cell free supernatant was carried out by dinitrosalicylic acid (DNS) method and detected upto 2.8 U/µl in cell the free supernatants. Molecular characterization by 16S rRNA sequences identified ZS-03 as *Klebsiella pneumoniae,* ZS-10 as *Klebsiella veriicola* and ZS-17 *Staphylococcus guallinarium*.

Key words: DNS method, *Klebsiella pneumonia*, pectinases, polygalacturonase, *Staphylococcus guallinarium*.

INTRODUCTION

The living cells exclusively produce enzymes or biocatalysts and no life in any form could exist without enzymes. Most of the enzymes are protein in nature and are very specific in their action. Enzymes are considered as friends of many industries. Enzymes are among the most important products obtained for human needs through microbial sources. Microorganisms such as bacteria (Reda et al., 2008), yeast (Chaudhri,and Suneetha, 2012) and filamentous fungi (Hannan et al., 2009; Akhtar et al., 2011) are capable of producing pectin degrading enzymes. Pectinases are a group of enzymes produced by variety of microorganisms that catalyze the breakdown of glycosidic bonds of the long chains of galacturonic acid residues of pectic substances. Pectic substances are widely distributed in vegetables and fruits (Lowe, 2002), hence they form important natural substrate for pectinases. Major pectinases are polygalacturonases (PGases) and pectin lyases (PLases) which split the molecular chain of respective polymers (Kashyap et al., 2001). Pectin methyl esterases (PME) hydrolyze the methyl ester of galacturonide chain and liberate methanol. Many microorganisms have been found to produce only one type of each pectinase; however, some appear to produce multiple isozymes that differ in isoelectric point, molecular weight and often in their regulation (Keon et al. 1987). Several microorganisms produce different types of pectinases to degrade pectin that are classified by their substrates and mode of action on the pectin polymer (Pe´rombelon and Kelman, 1980). Microbial rotting is an ancient process dating to the civilization. The traditional rotting uses mixed microbial populations and has major disadvantage such as the bad odor that develops in the rotting tanks during handling, and in the discharge of the effluent; its uncontrolled nature and its slowness. Enzymatic rotting is

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faster than traditional retting, is readily controlled and produces fewer odors, but further development is required to make it commercially competitive with the traditional methods. Current developments in biotechnology are yielding new applications for enzymes. The ability to synthesize the pectinases is wide spread among all microbial groups but moulds (*Aspergillus niger, Trichoderma harzianum, Rhizopus*) are preferred because as much as 90% of the enzyme can be extracted into culture medium (Blandino et al., 2001). Only the limiting factor for fungi is the slow growth of microorganisms. Bacteria are fast growing and can be used for the production of polygalacturonase to fulfill the local demand by different industries. It is dreadful needed for the isolation of local microorganisms for commercial production of polygalacturonases. In the present study, we report the bacterial isolates from local sources (rotten fruits and vegetables) having pectinolytic activity.

MATERIALS AND METHODS

Collection of samples

Samples of rotten fruits and vegetables from STC Shopping Centre, University of the Punjab were collected and stored at 4°C in airtight bags.

Isolation and purification of bacteria

Ten fold dilutions of each 10% sample were prepared in autoclaved distilled water and 100 µl from each dilution was spread on Nutrient agar plates. The plates were incubated overnight at 37°C. Different bacterial colonies were picked from each plate and streaked on the same medium. The plates were incubated under the same conditions as before to get pure single colonies.

Preliminary screening of pectinase producing bacterial isolates by well plate method

Fifteen (15) purified bacterial colonies were preliminary screened for pectinase activity by well plate method (Zeroual et al., 2001; Hannan et al., 2009). Supernatant from overnight bacterial cultures (1.0 optical density at 600 nm) were used in all experiments to determine the pectinase activity. Petriplates containing autoclaved modified MS medium containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 02% NH_4Cl, 0.5% NaCl, 0.01% $MgSO_4$. $7H_2O$, 1.5% agar supplemented with 0.2 % pectin were prepared. After solidification of the medium, four wells of 5 mm in diameter were made in the agar with the help of cork borer and filled with 50 µl of cell supernatant. After incubation for 24 h at 37°C, plates were flooded with iodine solution containing 0.25% iodine, 0.5% potassium iodide and 31 ml of 20% ethanol (Cappuccino and Sherman, 2002). The pectinase activity was observed by a clear zone around the well. The result was observed by measuring the diameter of the clear zone.

Morphological and biochemical characterization of polygalacturonase producing bacterial isolates

Bacterial strains which hydrolyzed pectin in well plate assay were selected for morphological and biochemically tests such as gram staining, gelatin liquefaction, oxidase, catalase, starch hydrolysis, triple sugar iron (TSI), motility indole urease (MIU), hydrogen sulfide (H_2S) production, methyl red (MR), Voges Proskauer (VP) and citrate (Cappuccino and Sherman, 2002).

Quantitative analysis for polygalacturonase production

Dinitrosalicylic acid (DNS) method was used to determine the reducing sugar released by the enzyme action (Miller 1959; Hannan et al., 2009). A standard curve of galacturonic acid was prepared for the estimation of polygalacturonic acid activities in terms of mg/ml and then converted in units (U). One unit is the amount of enzyme which catalyzes the formation of 1 µmol of galacturonic acid per minute at 40°C from pectin at pH 5.0.

Molecular characterization of bacterial isolates by 16S rRNA sequencing

Bacterial isolates (ZS-03, ZS-10 and ZS-17) selected on the basis of enzyme activity (zone size) and maximum enzyme productions were molecularly characterized through 16S rRNA sequencing. DNA isolation was done by CTAB method (Weising et al., 1991c; Ausubel et al., 2002) with the modification of 4% CTAB in the lysis solution. Amplification of 16S rRNA gene by polymerase chain reaction (PCR) was performed successfully by using universal primers including forward primer RS-1: 5′-AAACTCAAATGAATTGACGG-3′ and reverse primer RS-3: 5′-ACGGGCGGTGTGAC-3′ (Hasnain and Thomas, 1996). PCR products were sent to Centre for Advanced Molecular Biology (CAMB), Ministry of Science and Technology, Pakistan for sequencing. The data (sequences) obtained from CAMB Sequencing facility were BLAST (NCBI database; http://www.ncbi.nlm.nih.gov/BLAST) and submitted for accession numbers. The sequences were aligned with closely related matches. A dendrogram based on homologous sequences was constructed with Neighbor-Joing on the TN93 distance with Bootstraping value 1000 (No. of data sets).

RESULTS

Preliminary screening of pectin degrading bacterial isolates

Fifteen (15) bacterial isolates purified from rotten fruits and vegetables were preliminary screened out on the basis of zone size, ranging from 8 to 30 mm after flooding the well plate with iodine solution. Bacterial isolates, ZS-03, ZS-10 and ZS-17 were gave maximum zone size of 27, 30, and 25 mm respectively (Figure 1) as compared to others.

Morphological and biochemical characterization of selected bacterial isolates

Three bacterial isolates selected in well plate assay on the basis of maximum zone size subjected for morphological (Table 1) and biochemical (Table 2) characterization were identified as *Klebsiella* and *Staphylococcus* species.

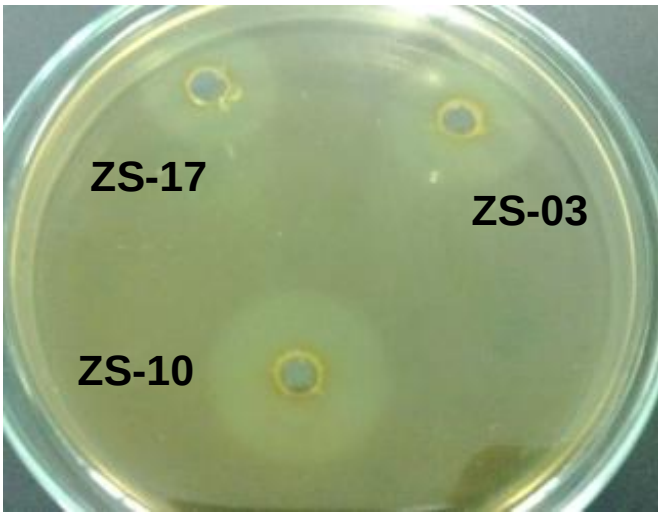

Figure 1. Zone formation by bacterial isolates indicating pectin hydrolysis on MS medium supplemented with 0.2% pectin flooded with iodine solution.

Table 1. Morphological characteristics of selected bacterial isolates.

Bacterial strain	Colony characteristic (form, elevation, margin, surface, opacity, chromogenesis, consistency, odor)
ZS-03,ZS-17	4.3 mm, white, mucoid, raised, regular margin, rough, translucent and tenacious.
ZS-10	3.5 mm, yellow, round, entire smooth, raised, opaque

Table 2. Biochemical characteristics of selected bacterial isolates.

Strain	Source	Gram/spore staining	GL	Oxid	Cata	SH	TSI	MIU	H_2S	MR	VP	Citr
ZS-03	Mango	- Rods/ spore	-	-	+	-	YR	--+	-	-	-	+
ZS-10	Apple	- Rods/ spore	-	+	+	-	YR	--+	-	-	+	-
ZS-17	Tomato	+ Cocci/ spore	+	-	+	+	YR	---	-	+	-	-

Y, Yellow color; R, red color; GL, gelatin liquefaction; Oxid, Oxidase; Cata, Catalase; SH, starch hydrolysis; TSI, triple sugar iron; MIU, motility indole ureasE; MR, methyl red; VP, Voges Proskauer; Citr, Citrate.

Quantification of polygalacturonsase

The amount of polygalacturonase estimated (DNS method) in Figure 2 shows that the maximum enzyme released by *Staphylococcus* sp. (ZS-10) was 2.8 U/µl as it showed maximum zone in plate assay (Figure 1) whereas other two strains of *Klebsiella sp*.(ZS-03 and ZS-17) showed 1.6 and 2.0 U/µl polygalacturonase production.

Molecular characterization of selected bacterial strains by 16S rRNA sequencing

Three bacterial strains characterized as *Klebsiella* sp. (ZS-03, ZS-17) and *Staphylococcus* sp. (ZS-10) by biochemical tests were further molecularly characterized by 16S rRNA sequencing. The blast query revealed that ZS-03 16SrRNA gene was 99% homologous to the already reported gene of *Klebsiella pneumoniae* (accession no.JN848784) and *Klebsiella* sp. (accession no. HM037179). Bacterial strain ZS-10 showed 100% homology with *Staphylococcus guallinarium (*accession no. JQ279067) and *Staphylococcus* sp. (accession no. JN660060). ZS-17 characterized as *Klebsiella veriicola* in blast query was 87% similar to *K. veriicola* (JQ659780, HQ259961) and *Klebsiella* sp. Accession nos. JN660056 and GU458293. Dendrogram based on 16S rRNA gene sequence comparison showed the relationship between members of *Klebsiella* and *Staphylococcus* (Figure 3).

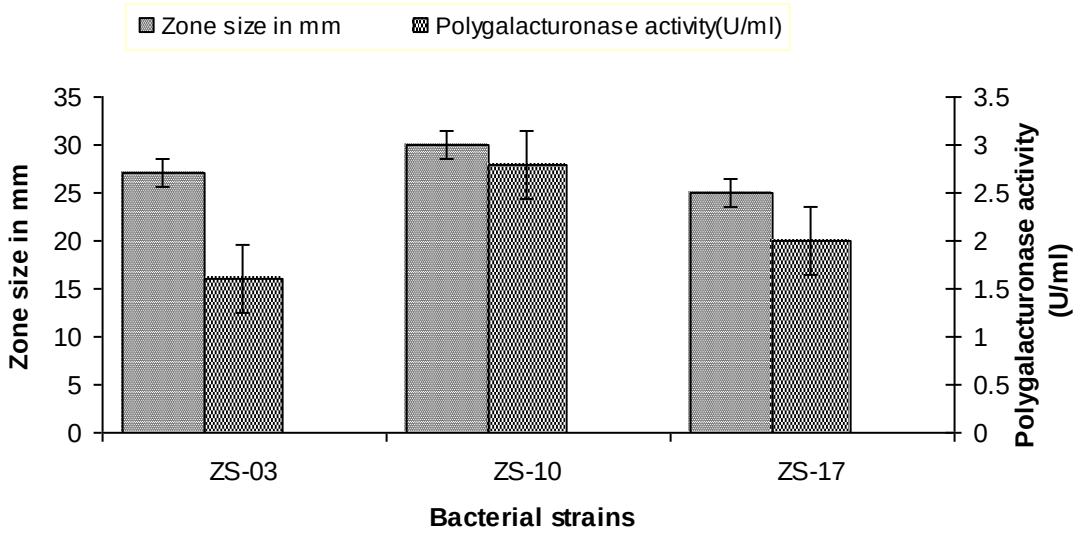

Figure 2. Zone size (mm) and polygalacturonase production (U/µl) by pectinolytic bacterial strains identified biochemically as *Klebsiella* and *Staphylococcus* species.

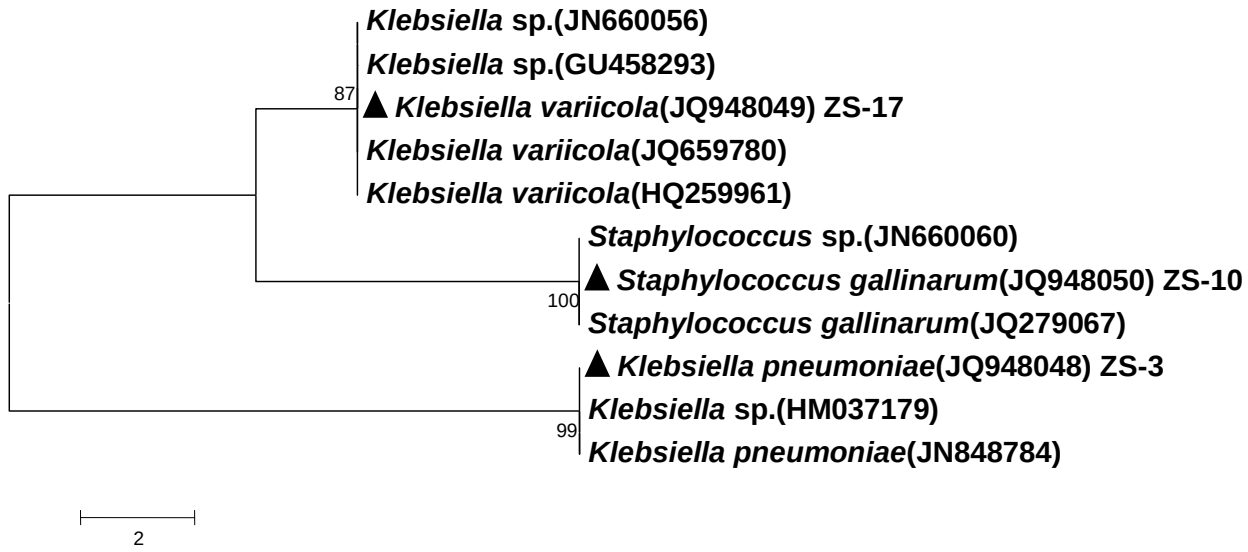

Figure 3. Dendrogram showing the similarity with closely related members of *Klebsiella* and *Staphylococcus* species.

DISCUSSION

Pectin naturally occurs in organic material present in plants, fruits and vegetables in large quantity. It is a complex polysaccharide with various types and variable molecular weight ranging from 25 to 360 kDa. All of them contain galacturonic acid in lesser or greater amount. Pectin is degraded into simple carbohydrates by pectinase group of enzymes and reduces the rigidity of plant tissues and intracellular adhesive properties (Pires and Filho, 2005). Pectinases have a significant role in food, textile (Phugare et al., 2011), paper and pulp industry, rotting of plant fiber, coffee and tea fermentation, production of animal feed, purification of plant viruses, oil extraction, and improvement of chromaticity and stability of red wines and other industries. Pectinolytic enzymes are naturally produced by microorganisms. *Aspergillus niger* is the most commonly used fungus for industrial production of pectinolytic enzymes. *A. niger* is generally recognized as a safe microorganism but is a slow growing fungus. This mould secretes other enzymes which are less desirable

for the fruit industry and can cause turbidity but are biotechnologically important for other industries. Commercial application of enzyme depends upon desirable physical and biochemical properties of enzyme but cost effectiveness is most important. Pectinases isolated from different microbial sources not only have different physical, biochemical properties but their mode of action is also different. Among the polygalacturonases obtained from yeast and fungal sources, most have the optimal pH 3.5 to 5.5 and temperature ranges from 30°C to 50°C. Thermostable pectinases are more suitable industrially due to treatment of fruit juices at higher temperature upto 70°C (Jayani et al., 2005; El-Sheekh, 2008). Exo-polygalacturonases produced mostly by bacterial species are alkaline in nature, easy to harvest, stable and active at high temperature (Jayani et al., 2005). Among bacteria, species of *Bacillus*, *Clostridium* and *Pseudomonas* are reported for pectinases production (Bhardwaj and Garg, 2010). Pakistan spends lot of valuable foreign exchange to import pectinases according to the national demand. In order to save the money and fulfill the demand of the industries, it is needed to search out new and novel microorganisms to enhance safe pectinases production. These efforts will greatly increase the production of pectinases, strengthen the economy and industry by adapting new biotechnological approaches to overcome these problems. The bacterial isolates isolated from rotten fruits and vegetables showing hydrolysis of pectin were initially biochemically characterized and then were characterized by 16S rRNA sequence. In this study, *S. gallinarum* (JQ948050) produced significant amount of exo-polygalacturonases (2.8 U/µl) whereas *K. pneumoniae* (JQ948048) and *K. veriicola* (JQ948049) produced 1.6 U/µl and 2.0 U/µl exo-polygalacturonases respectively. These values are comparable with already reported fungal and bacterial species (Jayani et al., 2005) which produce different levels of pectinases. This is the first report for the production of exo-polygalacturonases from *Klebsiella* and *Staphylococcus* species isolated from rotten fruits and vegetables. These plants associated novel strains especially *S. gallinarum* (JQ948050) can be used to produce polygalacturonase at pilot and industrial scale.

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