Antibacterial activity of aqueous extract of pomegranate peel against *Pseudomonas stutzeri* isolated from poultry meat

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Abstract In this study antibacterial activity of pomegranate peel (PPE) was evaluated against bacteria isolated from poultry meat. The bacteria were identified using 16S rRNA gene and DNA sequencing. Results of molecular characterization showed that the bacteria isolated were having 100% homology with the *Pseudomonas stutzeri* strain CTSP36 and further analysis showed that sample sequence clustered with the *P. stutzeri* strain CTSP36. Antibacterial activity of PPE was demonstrated by clear zone of inhibition in plates inoculated with extract. The diameter of inhibition zones were significantly ($p < 0.05$) higher in PPE as compared to standard antibiotic discs used (tetracycline, vancomycin and streptomycin). Results of broth dilution assay also revealed that PPE at 1%, 5% and 10% were effective in inhibiting bacterial growth in test plates. Further, a decrease in the growth of bacterial cells and a gradual decline in protein content of bacterial cells were also observed when bacterial culture was grown with different concentration of PPE along with a control. These results showed the potential application of pomegranate peel extract as antibacterial agent against *P. stutzeri*.

Keywords Poultry meat · *Pseudomonas stutzeri* · DNA sequencing · Pomegranate peel · Broth dilution assay · Antibacterial activity

Introduction

Major bacterial contamination of poultry products occurs during processing and supply chain. Pathogenic bacteria (*E. coli*, *Salmonella*, *Campylobacter jejuni*, *Staphylococcus aureus* and *Listeria monocytogenes*) and spoilage bacteria (*Pseudomonas*, *Alcaligenes*, *Loactococcus* spp. etc.) are the major factors influencing the safety and shelf-life of poultry products. Several antimicrobial treatments have been used in the meat industry to decontaminate/inhibit disease-causing pathogens and extend shelf-life. These treatments are either synthetic chemicals or antibiotics. Increased awareness about antibiotic resistance and adverse effects of synthetic preservative has revived the search for natural sources of antimicrobials as alternative preservatives in meat products. Previously studied natural antimicrobials in meat products include bacteriocins, lactoferrin, lysozyme, spices, essential oils and variety plant extracts. Antibacterial activity of rosemary extract (Del Campo et al. 2000), garlic extract (Tsao and Yin 2001), grape fruits extract in fish (Cho et al. 1990), capsicum extract in raw beef (Monica et al. 2003), oregano essential oils in sheep meat (Govaris et al. 2010) have been reported. The antimicrobial activity of many plant extracts, traditional spices and essential oils is well documented (Ahmad and Beg 2001; Naz et al. 2007; Shan et al. 2007; Burt 2004).

Pomegranate (*Punica granatum*) peel is an inedible part obtained during processing of pomegranate juice. Pomegranate peel is a rich source of tannins, flavonoids and other phenolic compounds (Li et al. 2006). Antioxidant and antibacterial properties of pomegranate peel in in-vitro model systems have been reported (Negi and Jayaprakasha 2003; Reddy et al. 2007; Opara et al. 2009; Alzoreky 2009). However, pomegranate peel has received less attention as natural preservatives in meat. Except two recent studies (Naveena et al. 2008; Devatkal et al. 2010)

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no efforts have been made to develop a natural preservative from pomegranate peel. The objective of present study was to evaluate the antibacterial activity of aqueous extract of pomegranate peel against *Pseudomonas stutzeri* isolated from poultry meat.

Materials and methods

Materials Poultry meat samples were obtained from a local retail meat processing unit and chilled at 4 °C before use. Fresh pomegranate (*Punica granatum*) fruits were collected from retail fruit market. Microbiological media, standard antibiotic discs and other reagents were obtained from Himedia (Himedia, New Delhi, India). All glassware and bacteriological growth media used in microbiological works were either sterilized using a hot air oven (185 °C for one hour) or an autoclave (121 °C and 15 lb pressure and 15 min).

Preparation of pomegranate peel extracts (PPE) Fresh pomegranate fruits were peeled manually and collected peels were then rinsed with distilled water. Peels were further cut into small pieces and dried in an air circulatory tray drier (Narang Scientifics, New Delhi, India) at 60 °C for 48 h. Dried pieces were powdered in a heavy duty kitchen grinder and sieved using a sieve, ASTM No.10 (1.651 mm). The powder (10 g) was extracted with 25 ml boiled distilled water at room temperature for 1 h. The clear extract obtained was centrifuged (5,000 rpm for 10 min at 5 °C) and filter sterilized (0.45 µm) or autoclaved. This filtrate was used for determination of antibacterial activity.

Isolation and identification of bacteria from poultry meat sample About 250 g minced poultry meat was stored aerobically at 30±1 °C for 24 h. One g of this meat sample was transferred aseptically into 10 ml of sterile nutrient broth and incubated at 35±1 °C for 48 hours. Suitable ten fold dilution of the broth supplemented with spoiled meat was made and was transferred to sterile nutrient agar plates. A prominent bacterial colony was selected and streaked on sterile nutrient agar plates and subcultured in sterile nutrient broth for further experimentation. The viable bacterial stock culture was maintained at 4±1 °C on nutrient hard agar (peptone 1 g, meat extract 0.5 g, and sodium chloride 0.25 g and agar 1 g/100 ml sterile water) slants and subcultured periodically. The above isolated bacterial culture was used for molecular characterization. The isolates were maintained on nutrient agar slants at 4±1 °C and sub-cultured every month.

DNA isolation DNA isolation from the bacterial culture was carried out as per the standard protocol (Sambrook 2001). Briefly, bacteria cell pellets were resuspended in TE

buffer and incubated at 37 °C for 2 h with 0.5% SDS and 100 µl/ml of proteinase K. Then, 200 µl of 5 M NaCl and 160 µl of 10% CTAB/NaCl solution were added and incubated at 65 °C for 10 min followed by extraction with equal volume of phenol:chloroform: iso-amyl alcohol (25:24:1) twice. DNA was precipitated with 3 M sodium acetate and ethanol followed by washing with 70% ethanol. DNA pellet dried and resuspended in TE buffer.

PCR amplification PCR was performed using the universal primers 8F and 1429R for 16S rDNA gene (8F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3') (Danilo et al. 2007). The PCR reaction was carried out in a 25 µl reaction mixture containing 10 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 2.5 µl of MgCl₂, 200 µM of dNTP mix, 10 p mol each of the primers and 1 unit of Taq DNA polymerase (MBI Fermentas) with initial denaturation temperature of 95 °C for 5 min, followed by 30 cycles of denaturation for 1 min, annealing temperature of 55 °C for 1 min and extension of 72 °C for 2 min. Final extension was carried out at 72 °C for 5 min. The amplified product was analyzed by agarose gel electrophoresis and visualized under UV gel documentation system.

Sequencing and analysis Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using Cycle sequencing kit on ABI 3730xl Genetic Analyzer at a custom sequencing facility (Xcelaris Labs, India). Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using Seqman programme of Lasergene software (DNASTAR Inc., USA). The 16S rDNA gene sequence was used to carry out BLAST against NCBI database. Based on maximum identity score several sequences were selected and aligned, sequence distances were generated and the phylogenetic tree was constructed using Lasergene software (DNASTAR Inc., USA) version 2.7.

Determination of antibacterial effect of PPE Antibacterial activity of PPE was tested using agar diffusion method (Mothershaw and Jaffer 2004; Jaiswal et al. 2005), broth dilution assay (Negi and Jayaprakasha 2003) and growth inhibition test (Toda et al. 1989).

Agar Diffusion method Stock cultures of bacteria isolated from poultry meat were cultured in nutrient broth for 24 h at 37±1 °C. After the growth conditions were observed, 5 µl of the inoculums containing approximately 10⁷ cfu/ml of bacteria was added to 10 ml soft nutrient agar and mixed thoroughly. The soft nutrient agar was then added onto a petri dish containing 15 ml hard agar. Sterile filter paper disc (5 mm) were impregnated with PPE (0.02 g) for 10–

Fig. 1 Identification of the bacterial culture. PCR amplification of 16S rDNA gene of the sample bacterial culture. Lane 1. Amplified product of 16S rDNA; Lane 2. DNA ladder

15 min and allowed to dry completely for 20–25 min, then evenly placed on the surface of previously inoculated agar. Different antibiotic discs (Hi media, India) were positive control and sterile diluent (0.1% peptone water) was negative control for comparison of inhibition zone with sample. Plates were incubated at 35 ± 1 °C for 24–36 h, until visible growth of bacteria was evident in control plates. Clearly visible inhibition zones around discs were measured in three directions and averaged. The antibacterial activity was expressed as the diameter of inhibition zone produced by extract against test bacteria.

Broth Dilution assay Antibacterial activity of PPE against bacteria isolated from poultry meat was tested by the method of Negi and Jayaprakasha (2003) with minor modifications. Different concentrations of PPE (1%, 5% and 10%) were added to flask containing 20 ml melted molten agar. In case of control equal amount of sterile diluent was added. About 100 μ l of different dilution of exponentially grown bacterial culture ($\approx 10^5$ cfu/ml) was

inoculated into the flasks under aseptic conditions. The media was then poured into sterilized petri dishes in triplicate and incubated at 35 ± 1 °C for 24–36 h. The colonies developed after incubation were counted. The inhibitory effect was calculated by comparing the number of colonies in control plates and test plates.

Growth inhibition test The bacterial culture isolated from poultry meat was inoculated in a sterile nutrient broth medium (Hi media, India), containing varying concentrations (0.5%, 1% and 5%) of pomegranate peel extract. The growth of bacterial culture was monitored by change in turbidity and protein content measured using Lowry's method (Herbert et al. 1971).

Statistical analyses The experiment was replicated thrice and all parameters were measured in duplicate. Data obtained were analyzed using statistical software, Agristat (Indian Agriculture Statistical Research institute, New Delhi, India). Statistical significance was identified at the 95% confidence level ($P < 0.05$).

Results and discussion

Identification of the bacterial sample DNA isolated from the cultured bacteria and 16S rDNA gene of the bacterial sample was amplified using the universal 16S rDNA primers, which yielded a product of 1,500 bp size (Fig. 1). Amplified PCR product was sequenced at the custom sequencing facility and the sequence was assembled. 1,397 bp sequence of 16S rDNA of bacterial sample was subjected to BLAST

		Percent Identity																	
Divergence		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
	1		99.8	99.7	99.7	100.0	99.8	99.7	99.8	99.5	99.7	99.8	99.7	99.8	99.8	99.8	99.2	1	Sample
	2	0.1		99.9	99.9	99.8	100.0	99.9	100.0	99.7	99.9	100.0	99.9	99.9	99.8	99.8	99.4	2	<i>P. stutzeri</i> 20G AJ312158
	3	0.2	0.1		99.9	99.7	99.9	99.9	99.9	99.8	99.9	99.9	99.9	99.8	99.9	99.7	99.4	3	<i>P. stutzeri</i> 24a97 AJ312172
	4	0.1	0.0	0.0		99.7	99.9	99.9	99.9	99.7	99.9	99.9	99.9	99.8	99.8	99.7	99.4	4	<i>P. stutzeri</i> 24a112 AJ312174
	5	0.0	0.1	0.2	0.1		99.8	99.7	99.8	99.5	99.7	99.8	99.7	99.8	99.8	99.8	99.2	5	<i>P. stutzeri</i> CTSP36 EU855213
	6	0.1	0.0	0.1	0.0	0.1		99.9	100.0	99.7	99.9	100.0	99.9	99.9	99.8	99.8	99.4	6	<i>P. stutzeri</i> DSM 50227 PSU26415
	7	0.2	0.1	0.1	0.1	0.2	0.1		99.9	99.6	99.9	99.9	99.9	99.8	99.7	99.7	99.4	7	<i>P. stutzeri</i> LC2-8 FJ859914
	8	0.1	0.0	0.1	0.0	0.1	0.0	0.1		99.7	99.9	100.0	99.9	99.9	99.8	99.8	99.4	8	<i>P. stutzeri</i> aa-28 EU852047
	9	0.4	0.3	0.2	0.2	0.4	0.3	0.4	0.3		99.6	99.7	99.6	99.6	99.6	99.6	99.3	9	<i>P. stutzeri</i> DN7 EU873348
	10	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.3		99.9	99.9	99.8	99.7	99.7	99.4	10	<i>P. chloritidis</i> mutans AY017341
	11	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.3	0.0		99.9	99.9	99.8	99.8	99.4	11	<i>P. hyss58</i> FJ613311
	12	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.4	0.0	0.1		99.8	99.7	99.7	99.4	12	<i>P. knackmussii</i> B13 HM007154
	13	0.3	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.4	0.1	0.1	0.2		99.6	99.6	99.3	13	<i>P. sp.</i> 79 FJ613623
	14	0.4	0.2	0.1	0.1	0.4	0.2	0.3	0.2	0.4	0.2	0.2	0.3	0.4		99.6	99.2	14	<i>P. sp.</i> LE9 EF683122
	15	0.4	0.2	0.3	0.2	0.4	0.2	0.3	0.2	0.4	0.2	0.2	0.3	0.4	0.4		99.6	15	<i>P. sp.</i> ORNaP2a EF025737
	16	0.4	0.2	0.3	0.2	0.4	0.2	0.3	0.2	0.4	0.2	0.2	0.3	0.4	0.4	0.0		16	<i>P. sp.</i> pah3 EU221617
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16			

Fig. 2 Nucleotide sequence similarities and divergences of the 16S rDNA gene

Fig. 3 Phylogenetic tree based on the nucleotide sequence of 16s rDNA gene. Branched distances correspond to sequence divergences

analysis against NCBI database as described in material and methods, which yielded several hits. Based on the maximum identity score, several of the sequences were selected and aligned with the sample sequence. Sequence analysis showed that the sample sequence has 100% homology with the *Pseudomonas stutzeri* strain CTSP36 (Fig. 2). Phylogenetic analysis also showed that sample sequence clustered with the *P. stutzeri* strain CTSP36 (Fig. 3). Among various molecular techniques, PCR-restriction fragment length polymorphism is the most definitive and accurate method of characterization by sequencing selected genes (Lalucat et al. 2006). The most extensively used genes include 16s rRNA gene, although other genes such as internal transcribed spacer genes, ITS1, *gyrB*, *rpoD*, *nosZ* and *catA* were also used (Cladera et al. 2004). In the present study, we have sequenced the 16s rRNA gene of *P. stutzeri* to characterize the bacteria isolated from the spoiled meat sample. BLAST analysis of the obtained 16s rRNA gene followed by sequence analysis using that of reported *Pseudomonas* strains indicated that the bacteria isolated from the spoiled meat is indeed *P. stutzeri* strain CTSP36. Other studies have also successfully used sequencing of 16s rRNA gene to characterize the various *Pseudomonas* species from various foods including meat (Franzetti and Scarpellini 2007). These data indicated that the bacterial sample was indeed the *P. stutzeri* strain CTSP36. It is a ubiquitous organism found in soil, water and foods with optimal growth temperature of 35 ± 2 °C. It has also been isolated as an opportunistic pathogen from humans in hospital environments (Noble and Overman

1994). It is a gram negative saprophytic microorganism, which was earlier almost discarded as a plate contaminant, but for its unusual features, it has now been given special attention in clinical specimens. Patients with *P. stutzeri* infections have generally been found to have serious underlying diseases or predisposing risk factors such as haemodialysis (Goetz 1983) and chronic obstructive pulmonary disease (Campos-Herrero 1997).

Antibacterial activity *P. stutzeri* isolated from spoiled poultry meat was tested for its sensitivity to PPE. The potency was initially determined by the agar diffusion method. Diameter of inhibition zones (clear zones around discs) exerted by the various PPE and standard antibiotic discs towards *P. stutzeri* is presented in Table 1. The marked inhibition was associated with PPE extracts and other antibiotics used. However, the diameter of inhibition zones were significantly ($p < 0.05$) higher in PPE1 (extract obtained from fresh peels) and PPE2 (extract obtained from fine powder) as compared to antibiotic discs containing tetracycline, vancomycin and streptomycin. Among two PPE tested, extracts obtained from powder (PPE2) was having significantly ($P < 0.05$) higher inhibition than extract obtained from fresh peels (PPE1). As expected, the sterile diluent (negative control) was inactive against tested bacteria. Antibacterial effect observed in this study is in accordance with other studies (Akiyama et al. 2001; Negi and Jayaprakasha 2003; Opara et al. 2009). The inhibitory activity of water-methanol extract of pomegranate peel

Table 1 Comparative Antibacterial activity of PPE and standard antibiotic discs using agar diffusion method

Test solution	PPE1	PPE2	PPE1 (Autoclaved)	PPE2 (Autoclaved)	Tetracycline (30mcg)	Vancomycin (30mcg)	Streptomycin (10mcg)	Sterile diluent
Diameter of inhibition zone (mm)	21.0 ^c	26.0 ^d	20.0 ^c	26.0 ^d	13.0 ^a	15.0 ^b	12.0 ^a	–
Mean standard error	3.2	2.5	1.8	1.5	0.98	1.3	0.78	–

Means within a row with different superscripts are significantly different. $p < 0.05$. $n = 6$

PPE1: Extract obtained from peels; PPE 2: Extract obtained from powder; - No inhibition

against different pathogenic bacteria was reported (Alzoreky 2009). The antibacterial activity of pomegranate peels may be indicative of presence of some metabolic toxins or broad spectrum antibiotic compounds (Voravuthikunchai et al. 2004). Hydrophilic extracts are reported to be better solvents for polyphenols and hydrophilic phytochemicals found in pomegranate peel (Negi and Jayaprakasha 2003; Alzoreky and Nakahara 2003). In pomegranate peels, tannins and other phenolics are the major bioactive compounds having antioxidant and antimicrobial activity (Voravuthikunchai et al. 2005; Reddy et al. 2007). These antimicrobial constituents like phenols and flavanoids might be responsible for inhibitory action of PPE against bacteria. Several other workers (Prashanth et al. 2001; Negi and Jayaprakasha 2003; Alzoreky 2009) reported comparatively weaker effect of water extract than acetone and/or methanol extract. The contrasting findings might be due to different methods of extraction and type bacterial species used in different studies. Further, the autoclaved extract of both peel and powder were found to be equally efficient as evident from the zone of inhibition indicating that the active compound responsible for the antimicrobial activity is heat stable. Similarly, Alzoreky (2009) reported that water methanol extract was thermo stable, since they retained activity after boiling or sterilization. McCarrell et al. (2008) also demonstrated antibacterial activity of autoclaved pomegranate peel extract against *Staphylococcus aureus* and *B.subtilis*.

The antibacterial effect of PPE was further tested by broth dilution assay. The plates containing different concentrations (1%, 5% and 10%) of PPE showed a significant ($P<0.05$) reduction in the bacterial count (cfu) as compared to control (nutrient agar) plates (Table 2). In order to study the effect of PPE on bacterial growth, the bacterial culture was grown with different concentrations (0.5%, 1% and 5%) of PPE along with a control. Results indicated that PPE significantly reduced the growth of bacterial cell right from the 4th hour of incubation, as evident from the reduction in turbidity (Fig. 4). Further, a gradual decline in protein content of

Fig. 4 Growth of bacterial cells in medium treated with different concentrations of PPE. Medium (Nutrient agar) without PPE serves as control. Vertical bars on each point represent $\pm$ SE. $n=6$

bacterial cells was also observed as compared to control (Fig. 5). At 28 h of incubation 98.5% decline in protein content was observed in the bacterial cells treated with 5% extract as compared to control. Similar to these findings, Alzoreky (2009) had also observed that pomegranate extracts interfered with bacterial protein secretion. Polyphenols play an important role in protein precipitation and enzyme inhibition of microorganisms (Naz et al. 2007; Fan et al. 2008). The mechanism responsible for phenolic toxicity to microorganisms was related to reaction with sulphhydryl groups of proteins and unavailability of substrates to microorganism (Machado et al. 2003; Naz et al. 2007).

Table 2 Antibacterial activity of PPE using broth dilution method

Dilution factor	Number of Colonies (cfu)			
	Control	1% PPE	5% PPE	10% PPE
10^{-1}	>300 ^a	36	—	—
10^{-2}	>300	b _—	—	—
10^{-3}	>300	—	—	—
10^{-4}	>300	—	—	—
10^{-5}	68	—	—	—
10^{-6}	4	—	—	—

>300^a: Innumerable colonies were counted as >300

b^b: Absence of any bacterial colonies. $n=6$

Fig. 5 Percentage decline in the protein content of bacterial cells grown in medium containing PPE as compared to control medium. (Nutrient agar without PPE). $n=6$

Conclusion

Pseudomonas stutzeri, an opportunistic bacteria isolated from poultry meat showed sensitivity in vitro to aqueous extract of pomegranate peel. The autoclaved extract also showed antibacterial activity indicating that active compounds are heat stable. Further research is required to isolate and characterize the active antimicrobial molecules in pomegranate peel in order to develop natural food preservatives from pomegranate by-products.

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