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Probiotic Potential of *Pediococcus pentosaceus* CRAG3: A New Isolate from Fermented Cucumber

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Abstract A novel strain of lactic acid bacterium isolated from fermented cucumber was selected due to its high glucansucrase activity. It was identified on the basis of 16S rRNA sequence analysis as *Pediococcus pentosaceus* CRAG3 (GenBank accession number JX679020). The isolate was round shaped, Gram positive, and catalase negative displaying typical features of lactic acid bacterium. It produced 145 ± 3.27 mg lactic acid per ml of cell-free supernatant. It showed ability to ferment carbohydrates such as sucrose, dextrose, and arabinose; showed resistance to antibiotics such as ciprofloxacin, kanamycin, vancomycin; displayed acid production in triple sugar iron agar test and non-motile nature. Interestingly, the isolate also displayed potential probiotic properties such as hydrophobicity, autoaggregation, coaggregation, and in vitro cell adhesion ability. It exhibited resistance against lysozyme and simulated gastric juice at pH 3.0 with 75 and 58 % survival, respectively. It also showed tolerance toward 0.3 %, w/v bile salts with 73 % survival and ability to deconjugate bile salts. The isolate exhibited antibacterial activity and ability to utilize prebiotics such as inulin and raffinose. These results indicate both probiotic property and glucansucrase-producing ability of *P. pentosaceus* CRAG3.

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Introduction

Lactic acid bacteria (LAB) comprise a group of Gram-positive, non-spore forming, catalase-negative cocci or rod shaped microorganisms having great importance in fermented food industries. Although there are reports available on the isolation of LAB from different sources, only few emphasized on the isolation from fruits and vegetables [1, 2], and the LAB from lettuce, cucumber, and cabbage are scarcely studied. Cucumbers serve as traditional fermented foods in some areas although it is cultivated throughout the world [3]. It has been reported that the fermentation in cucumbers occurs by lactic acid bacteria [4].

Pediococci are a group of homofermentative, coccus-shaped, Gram-positive lactic acid bacteria that are involved in the manufacturing of fermented foods [5]. These include *P. damnosus*, *P. parvulus*, *P. inopinatus*, *P. cellicola*, *P. ethanolidurans*, *P. claussenii*, *P. stilesii*, *P. acidilactici*, *P. pentosaceus,* and *P. dextrinicus* [6]. They have been isolated from various sources like soil [7], plants [8], sausages [9], pickles [10], wines [11], beverages [12], and cheese [13]. The members of the *Pediococcus* have great economic importance in fermented food industry [14] and as starter cultures in fermentation processes of milk, meat, vegetable products, and sausages [15]. These are also used in fermentations of sauerkraut [16], cucumber, green bean, soymilk, bread sourdoughs, and silage [17]. There are reports describing the probiotic properties of *Pediococcus* spp. [18, 19]. The glucansucrase- and glucan-producing ability of *Pediococcus* spp. has not been much explored [7].

Probiotics are live microorganisms which provide health benefit to host by producing inhibitory substance like acid against pathogenic strains and also by competing with them for essential nutrients and adhesion sites. In recent years, there has been tremendous increase in research on the potential health benefits of probiotics. They play important role in the treatment for various infectious diseases including viral or bacterial diarrhea and chronic inflammatory diseases such as ulcerative colitis and pouchitis [20]. *P. acidilactici* has GRAS (Generally Recognized as Safe) status and is used as probiotic culture and nutritional enhancer in silage [15]. For a microbe to behave as probiotic, it should fulfill certain criteria like adhesion to gastrointestinal tract, antibacterial activity, resistance to acid, bile salts, and lysozyme [21]. To the best of our knowledge, the isolation of probiotic *Pediococcus* spp. from fermented cucumber is not yet reported. This is the first report on the glucansucrase-producing probiotic *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber. The isolate was identified by 16S rRNA gene sequence analysis. A number of in vitro tests were performed to screen *P. pentosaceus* CRAG3 for its probiotic properties such as cell adhesion property, deconjugation of bile salts, and resistance to biological barriers.

Materials and Methods

Isolation and Culturing of Microorganism

The sample of freshly chopped cucumber soaked in 2.5 % (w/v) NaCl solution in 1-L air-tight bottle was subjected to fermentation at 25 °C for 5 days. 1 g paste of fermented cucumber was mixed homogeneously in 10 ml of saline (0.85 %, w/v) contained in test tubes and the serial dilutions up to 10^{-7} were made. 100 µl from each dilution from 10^{0} to 10^{-7} was taken and spread on 1.7 % (w/v) MRS agar plates [22]. Petri plates were incubated at 28 °C for 24 h. Forty-five isolated colonies were randomly isolated from plates of 10^{-5}–10^{-7} and were grown in enzyme production medium [23]. All the colonies were evaluated for their ability to produce glucansucrase. The enzyme assay was carried out in 1 ml reaction mixture containing 5 % (w/v) sucrose in 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell-free supernatant. The enzymatic reaction was performed at 30 °C for 15 min. 100 µl aliquot from the reaction mixture was taken for the estimation of the reducing sugar, which was analyzed using the method of Nelson [24] and Somogyi [25]. Based on the highest glucansucrase activity displayed by the isolate CRAG3 (abbreviated after the name of source from where it was isolated and the initials of two authors and colony number), it was selected for further characterization.

Morphological Characterization of the Isolate CRAG3

The morphology of the isolate grown in MRS medium overnight at 28 °C was observed directly and by light microscopy. The Gram reaction was performed using the KOH method of Gregesen [26]. The cell morphology of the selected isolate was examined by field emission scanning electron microscopy (Carl Zeiss, Model Sigma) operated at 10.0 kV. 1 ml sample from 12 h old culture was centrifuged at 8,000*g* for 10 min. The pellet containing cells was dissolved in 1 ml of sterile saline solution (0.85 %, w/v) and fixed with equal volume of gluteraldehyde (2.5 %, v/v) for 4–5 h. Gluteraldehyde was removed by centrifuging at 8,000*g* for 10 min and cells were dehydrated using different concentrations of acetone and finally dried in vacuum desiccator.

Molecular Identification of the Isolate CRAG3

The isolate CRAG3 was identified by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified by PCR using universal primers (8F and 1492R) with genomic DNA as template and its sequencing was obtained from Xcelris Labs Limited, Ahmedabad, India. Consensus sequence of 1,269 bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence of the isolate CRAG3 was used to carry out BLAST with the non-redundant database of NCBI GenBank database. First ten sequences, based on maximum identity score, were selected and aligned using ClustalW, the multiple alignment software program.

Biochemical Characterization of the Isolate

The isolate *P. pentosaceus* CRAG3 was analyzed for its ability to produce lactic acid. The concentration of lactic acid was measured by using the method described by Mirdamadi et al. [27] with certain modifications. The isolate was grown in 5 ml MRS medium [22] at 25 °C and 180 rpm for 24 h. The cells were removed by centrifugation at 10,000*g* and 4 °C for 10 min and the cell-free supernatant was used for the determination of lactic acid concentration. To 0.5 ml of cell-free supernatant, 3.0 ml conc. sulfuric acid (18 M) was mixed and heated in boiling water bath for 10 min. The mixture was cooled to 25 °C and 50 µl of cupric sulfate (4 %, w/v in water) and 100 µl phenolphthalein (1.5 %, w/v in 95 % ethanol) were added. The absorbance at 570 nm was read against blank. The standard graph was plotted using lactic acid in the range 10-150 mg/ml.

The *P. pentosaceus* CRAG3 was tested for its ability to ferment 21 different carbohydrates using the method described earlier [28]. The MRS medium without glucose containing 1.8 % (w/v) agar and phenol red (100 µg/ml) was first poured into petri plate. The overnight grown culture (1 %) of the isolate was seeded in MRS-soft agar (0.8 %, w/v) containing phenol red (100 µg/ml) and overlaid in the petri plate having a bottom layer of 1.8 % (w/v) MRS agar. The plate was allowed to dry for 2–3 min. The carbohydrate discs were gently placed on to the surface of soft agar and the petri plates were incubated at 28 °C for 48 h to observe the color change from red to yellow and formation of zone around carbohydrate discs.

The sensitivity of *P. pentosaceus* CRAG3 against 42 antibiotics was evaluated using agar disc diffusion method [29]. The MRS medium containing 2 % (w/v) glucose and 1.8 % (w/v) agar was first poured into the petri plate and allowed to solidify. The overnight grown culture (1 %) of the isolate was seeded in MRS-soft agar (0.8 %, w/v) having 2 %, w/v glucose and overlaid in the petri plate having a bottom layer of 1.8 % (w/v) MRS agar. The plate was allowed to dry for 2–3 min. The octodiscs were gently placed on to the surface of soft agar and the petri plates were incubated at 28 °C for 24 h to observe the zone of inhibition around the discs.

Triple sugar iron (TSI) agar test was carried out using TSI agar slant (HiMedia) having sucrose, glucose and lactose as triple sugars for acid, H_2S, and/or gas production. A loopful of overnight grown culture of *P. pentosaceus* CRAG3 was streaked over the slant and then stabbed deep into the butt and incubated at 28 °C for 48 h. The acid production was inferred by changing color from red to yellow. The isolate was tested for its ability to display catalase activity using the method of Maehly and Chance [30]. To the 5 ml 12–16 h grown culture of the isolate CRAG3, few drops of 3 % (v/v) H_2O_2 were added and observed for bubble formation. *Escherichia coli* was taken as positive control for catalase test. The motility test of CRAG3 was carried out by stabbing a loopful of culture deep into the Motility Indole Lysine Agar Slant (HiMedia).

Probiotic Characteristics of *P. pentosaceus* CRAG3

Cell Surface Traits

Hydrophobicity Kiely and Olson [31] suggested the use of hexadecane to qualitatively determine the adhesion property of bacteria. The hydrophobic behavior of the *P. pentosaceus* CRAG3 was determined by the method described by Perez et al. [32]. The cells from the overnight grown culture in 5 ml MRS medium at 28 °C and 180 rpm were harvested by centrifugation at 12,000*g* for 5 min at 4 °C. The cell pellet was washed twice by 5 ml 50 mM K_2HPO_4 buffer (pH 6.5) and finally re-suspended in the same buffer. The absorbance (A_{600}) of cell suspension was taken at 600 nm and adjusted to the value of approximately 1.0 with the buffer. To 3 ml of the bacterial suspension, 0.6 ml of *n*-hexadecane was added and mixed well for 2 min. The two phases were allowed to separate at 37 °C for 20 min from which the aqueous phase was removed cautiously and the absorbance (A_{600}) at 600 nm was taken. The cell surface hydrophobicity (H %) was measured by observing the drop in the absorbance of the aqueous phase using the formula

$$H\% = [(A_0 - A)/A_0] \times 100$$

where A_0 and A are the absorbance before and after extraction with *n*-hexadecane, respectively.

Autoaggregation and Coaggregation Assay The autoaggregation assay was performed by the method described by Del Re et al. [33] with the following modifications; the incubation time for autoaggregation was 5 h instead of 2 h. The culture (4 ml) of *P. pentosaceus* CRAG3 containing approximately 4×10^8 cells was centrifuged at 12,000*g* and 4 °C for 10 min. The cell pellet was re-suspended in 4 ml 150 mM phosphate-buffered saline (PBS, pH 7.0) and allowed to aggregate by further incubating at 37 °C for 5 h. Aliquots of 100 µl were withdrawn at regular interval of 1 h from the upper suspension and mixed in 3.9 ml 150 mM PBS (pH 7.0) and absorbance (A_{600}) at 600 nm was measured. The autoaggregation percentage (A_a%) was calculated using the following equation [33]

$$A_a\% = 1 - (A_t/A_0) \times 100$$

where A_t = absorbance at time $t = 1$, 2, 3, 4, or 5 h and A_0 = absorbance at $t = 0$ h.

The autoaggregation percentage was expressed as a function of time until it was constant.

The coaggregation assay was performed using the method described by Kos et al. [34]. The samples for coaggregation assay were prepared same as autoaggregation. The cultures (4 ml) of *P. pentosaceus* CRAG3 and *E. coli* containing approximately 2×10^8 CFU each were centrifuged at 12,000*g* and 4 °C for 10 min. The cell pellet of both cultures was re-suspended separately in 2 ml 0.1 M sodium phosphate buffer (pH 7.0). Equal volumes (2 ml) of both cell suspensions were mixed and allowed to aggregate by incubating at 37 °C for 5 h. The tubes containing 4 ml of each bacterial suspension on its own were taken as control. Aliquots of 100 µl were withdrawn at regular interval of 1 h from upper part of suspension and mixed with 3.9 ml same buffer and absorbance at 600 nm (A_{600}) was measured. The percentage of coaggregation (C_a%) was calculated using the equation given below:

$$C_a\% = \frac{(Ax + Ay)/2 - (Ax + Ay)}{(Ax + Ay)/2} \times 100$$

where Ax and Ay represent absorbance of each of the two strains in the control tubes at 600 nm and $A(x + y)$ the absorbance of the mixture.

In Vitro Cell Adhesion Assay The adhesive capacity of *P. pentosaceus* CRAG3 was studied using cervical cancer (HeLa) cell line and murine monocyte-macrophage (J774A.1) line as described earlier [35]. The cell lines were grown in Dulbecco's Modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 10 % (v/v) fetal bovine serum (Sigma, St Louis, MO, USA), 50 µg/ml streptomycin, and 50 IU/ml penicillin (Himedia India Pvt. Ltd., India) at 37 °C in 5 % CO_2 atmosphere. The monolayers were prepared on glass coverslips in six-well tissue culture plates. Both HeLa and murine J774A.1 cells were seeded at a concentration of 4×10^4 cells/cm^2 and were incubated at 37 °C in a 5 % CO_2 incubator. The cells of *P. pentosaceus* CRAG3 from overnight grown culture in MRS medium at 28 °C and 180 rpm were counted and $\sim 10^8$ cells were added over the monolayer of each cell line at post-confluence. The plates were incubated at 37 °C under 5 % CO_2 atmosphere for 90 min. The unbound bacteria were removed from monolayers by washing with PBS (pH 7.0) twice, followed by fixing with methanol and finally stained with Giemsa solution. The adherent bacteria were counted under microscope using oil immersion at 20 random microscopic fields per glass coverslip monolayer. The adhesive capacity of the bacterial strains was calculated based on the number of adherent bacterial cells; the bacterial strains were put under non-adhesive (<5 bacterial cells adhered to 100 cells), adhesive (6–40 bacterial cells adhered to 100 cells), and strongly adhesive (>40 bacterial cells adhering to 100 cells) [33].

Resistance to Biological Barriers

Lysozyme Resistance The tolerance of *P. pentosaceus* CRAG3 against lysozyme was determined using the method of Zago et al. [36]. The culture was grown overnight in 10 ml MRS medium at 25 °C and 180 rpm. The cells were centrifuged at 12,000*g* and 4 °C for 10 min. The cell pellet was washed with 5 ml 0.1 M potassium phosphate buffer (pH 7.0) two times and re-suspended in 2 ml of Ringer solution (Sigma Aldrich, USA). 0.5 ml suspension of *P. pentosaceus* CRAG3 from the above solution was inoculated in 5 ml sterile electrolyte solution (SES; in g/l, 0.22 $CaCl_2$, 6.2 NaCl, 2.2 KCl and 1.2 $NaHCO_3$) containing 100 mg/l of lysozyme (Sigma Aldrich, USA) and incubated at 37 °C for 2 h. The bacterial suspension in SES without lysozyme was taken as control. The aliquot (10 µl) from each sample was spread on MRS agar plates and incubated at 25 °C for 48 h. The microbial colonies grown on plates were counted and the survival rate was calculated as percentage of CFU/ml (colony forming unit per ml) obtained from lysozyme treatment with respect to the control (without lysozyme treatment).

Tolerance to Simulated Gastric Juice The resistance of *P. pentosaceus* CRAG3 against simulated gastric juice was determined by the method of Charteris et al. [37]. The simulated gastric juice was prepared by mixing pepsin (1,000 U/mg, 3 mg/ml) and NaCl (0.5 % w/v) in water and pH was adjusted to 2 and 3. 30 ml overnight grown culture of the isolate was centrifuged at 6,000*g* and 4 °C for 20 min. After removing the supernatant, the cells were washed twice with 10 ml 50 mM K_2HPO_4 (pH 6.5) and re-suspended in 3 ml of the same solution. 1 ml of cell suspension was added to 9 ml each of gastric solution with pH 2 and 3 and incubated at 37 °C for 3 h. The total viable cells (CFU/ml) were counted, before and after incubation period, and expressed as the difference in colony counts.

Bile Salts Resistance The ability of *P. pentosaceus* CRAG3 to tolerate different concentrations bile was evaluated by the method of Walker and Gilliland [38] with following modifications; instead of MRS-THIO medium (containing 0.2 % sodium thioglycollate), MRS medium was used. The overnight grown culture of *P. pentosaceus* CRAG3 (2 %, v/v) was inoculated into 10 ml MRS medium (pH 6.4) containing 0.3, 0.5, or 1 % (w/v) of bile salts (Sigma Chemical Co., St. Louis, MO, USA). The cultures were incubated at 25 °C and after 24 h, absorbance was measured at 600 nm and compared to a control culture (without bile salts). The results were expressed as the percentage of growth (A_{600} nm) in the presence of bile salts with respect to the control.

Bile Salts Hydrolase Activity

Bile salts hydrolase (BSH) activity of the *P. pentosaceus* CRAG3 was determined according to the method of Taranto et al. [39]. The MRS medium (pH 6.4) plates were made supplemented with 1.7 % (w/v) agar and 0.5 % (w/v) sodium salt of taurodeoxycholic acid (Sigma Chemicals Co., USA). The medium was autoclaved (121 °C, 15 min, 15 psi) and immediately used. 12 h old culture of *P. pentosaceus* CRAG3 was streaked on the petri plate and was incubated anaerobically in air-tight gas jar containing the gas-pak sachet at 37 °C for 72 h. The presence of precipitated bile acid around colonies (opaque halo) confirmed the hydrolysis of bile salts.

Antibacterial Assay

The ability of CRAG3 to exhibit antibacterial activity was determined by the method of Yuksekdag and Aslim [18]. Test microorganisms (*E. coli*, *Bacillus subtilis,* and *Staphylococcus aureus*) were grown in 10 ml nutrient broth at 37 °C for 18–24 h and *P. pentosaceus* CRAG3 was grown in 10 ml MRS medium [22] at 25 °C for 18–24 h. *P. pentosaceus* CRAG3 cells were removed by centrifugation at 8,000*g* and 4 °C for 10 min and the cell-free supernatant was sterilized by filtering through 0.22 µm membrane. The petri dishes containing nutrient agar, previously inoculated with 1 % each of test microorganisms, were prepared. The wells (6 mm) were made in each petri dish, and 100 µl cell-free filtrate was added to each well. In another set of experiment, the cell-free filtrate was neutralized by 2 N NaOH and then 100 µl added to each well. The plates were incubated at 37 °C for 24 h and the zone of inhibition was measured.

Utilization of Prebiotics

The isolate *P. pentosaceus* CRAG3 was explored for its ability to utilize prebiotics such as raffinose and inulin (Himedia Pvt. Ltd., India) as described earlier [36]. The MRS medium (20 ml) containing raffinose or inulin (2 %, w/v) was prepared separately. MRS medium that contains 2 % (w/v) glucose was taken as positive control. The overnight grown culture of *P. pentosaceus* CRAG3 (1 %) was inoculated in each of 20 ml MRS media and incubated at 37 °C and 180 rpm for 24 h. The percentage growth of *P. pentosaceus* CRAG3 in the presence of each prebiotic was calculated as follows [36],

$$\left[\left(\left(\mathrm{MRS}_p - \mathrm{MRS}_b\right) \times 100\right)/\left(\mathrm{MRS}_g - \mathrm{MRS}_b\right)\right]$$

where MRS_p, MRS_b, and MRS_g are absorbance (A_{600}) at 600 nm of culture grown in MRS medium with prebiotic, MRS medium without carbon source (negative control), and MRS medium with glucose (positive control), respectively.

Results and Discussion

Isolation and Morphological Characterization of Isolate

Out of the selected 45 strains, the isolate named as "CRAG3" showed the maximum enzyme activity (2.7 U/ml) and hence selected for further characterization. The purple color of the cells after Gram's staining reaction displayed Gram-positive nature of CRAG3. The colonies of isolate on MRS agar medium supplemented with sucrose were translucent, circular and produced slimy layer due to the formation of glucan polymer. The scanning electron microscopic analysis showed that the isolate was phenotypically homogeneous with round cells in pairs or chains with a diameter of 0.7–0.9 µm (Fig. 1). The catalase negative, Gram positive, and coccus shape indicated that the isolate is a lactic acid bacterium as also reported earlier [40].

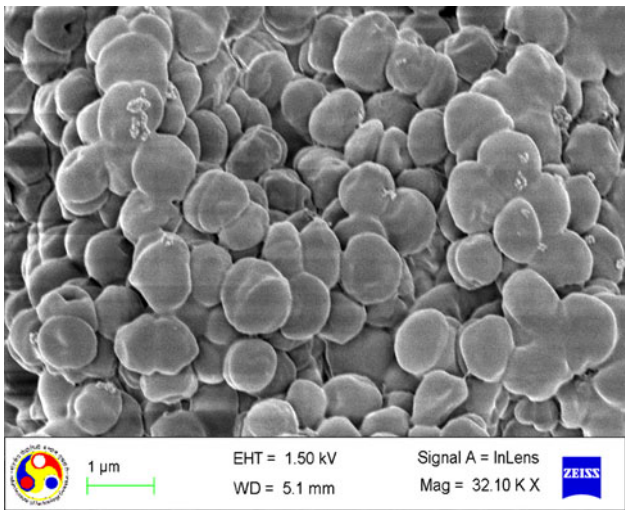

Fig. 1 Scanning electron micrograph of the isolate *P. pentosaceus* CRAG3

Molecular Identification of the Isolate CRAG3

The identification of CRAG3 was done on the basis of 16S rRNA sequence analysis. The new isolate clustered with *P. pentosaceus* strain ULAG75 (GenBank Accession Number: JN944736.1) (Fig. 2) which confirmed the isolate to be *P. pentosaceus*. The isolate was identified as *P. pentosaceus* CRAG3 and assigned the Genbank accession number JX679020.

Biochemical Characterization of Isolate

Pediococcus pentosaceus CRAG3 produced 145 ± 3.27 mg lactic acid per ml of cell-free supernatant. Similar results were reported for *Pediococcus* spp. GS4 which produced 130 mg/ml lactic acid [19]. The ability of the *P. pentosaceus* CRAG3 to ferment carbohydrates was evaluated by observing the change in color of phenol red to yellow due to production of lactic acid during fermentation. *P. pentosaceus* CRAG3 displayed yellow zone around cellobiose, dextrose, arabinose, rhamnose, sucrose, mannitol, fructose, maltose, trehalose, mannose, selicin and galactose showing its ability to ferment these sugars (Table 1). However, it failed to utilize raffinose, lactose, adonitol, sorbitol, inositol, inulin, mellibiose, xylose and dulsitol, since no zone was observed around these sugars. These results of *P. pentosaceus* CRAG3

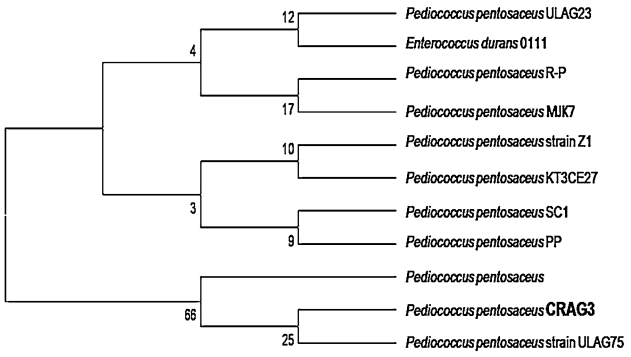

Fig. 2 Phylogenetic Tree made in MEGA 3.1 software using neighbor-joining method demonstrating the relationship of isolate CRAG3 to other *Pediococcus* species from NCBI

Table 1 Fermentation of different carbohydrates by *P. pentosaceus* CRAG3

S. No.	Carbohydrate	Isolate CRAG3	*P. pentosaceus* SPA ([7])
1	Cellobiose	+++	+++
2	Dextrose	+++	+++
3	Arabinose	+++	nd
4	Rhamnose	++	++
5	Sucrose	+++	+++
6	Mannitol	++	−
7	Fructose	++	+++
8	Maltose	+++	+++
9	Trehalose	+++	+++
10	Mannose	+++	nd
11	Selicin	+++	nd
12	Galactose	+++	+++
13	Raffinose	−	−
14	Lactose	−	++
15	Adonitol	−	nd
16	Sorbitol	−	nd
17	Inositol	−	nd
18	Inulin	−	nd
19	Mellibiose	−	+++
20	Xylose	−	+
21	Dulsitol	−	nd

(+++) Strongly positive; (++) fairly positive; (+) weakly positive; (−) negative

nd not determined

corroborated with *P. pentosaceus* SPA [7] with some variations (Table 1). Unlike *P. pentosaceus* SPA which weakly fermented lactose and unable to utilize mannitol, the isolate *P. pentosaceus* CRAG3 was able to ferment mannitol but unable to utilize lactose (Table 1).

The antibiotic sensitivity of isolate CRAG3 was evaluated by measuring the diameter of zone of inhibition around the discs and comparing with those given in standard chart. Depending upon the diameter of zone, the isolate was categorized as resistant, moderate or susceptible. The isolate CRAG3 was resistant to cefixime, ciprofloxacin, co-trimoxazole, kanamycin, nalidixic acid, sulphamethoxazole, tobramycin, trimethoprim, vancomycin, and gentamicin (Table 2). However, it displayed susceptibility to amoxycillin, amoxyclav, cefoperazone, cephaclor, cephalexin, cephalothin, clindamycin, erythromycin, imipenam, lincomycin, nitrofurantoin, novobiocin, oleandomycin, oxytetracycline, piperacilin, tetracycline, ticarcillin, carbenicillin, cephotaxime, chloramphenicol, co-trimazine and oxacillin. The similar results with exceptions were observed in the previously reported strain of lactic acid bacteria such as *P. pentosaceus* SPA [7] as listed in Table 2 and *Leuconostoc* spp [41]. Unlike *P. pentosaceus* SPA, which was resistant to amikacin, ampicillin, gentamicin and norfloxacin, *P. pentosaceus* CRAG3 was moderately sensitive to these antibiotics. The resistance of *Pediococcus*, *Leuconostoc* and *Lactobacillus* spp. to vancomycin is a common trait among these lactic acid bacteria [42]. Triple sugar agar test also confirmed that CRAG3 was able to ferment sucrose and glucose since the color of slant changed to yellow due to the formation of acid. It was catalase negative since no bubble formation was observed on the addition of H_2O_2. The motility agar test served the non-motile nature of CRAG3.

Probiotic Potential of *P. pentosaceus* CRAG3

Cell Surface Traits and Adhesion Capacity

The hydrophobicity shown by *P. pentosaceus* CRAG3 was 33 % after 20 min, which was similar to other reported probiotic bacteria such as *Lactococcus acidophilus* (38.1 %), *Lactococcus casei* (24.1 %), *Lactococcus lactis* (31.3 %) [43]. The autoaggregation (%) of *P. pentosaceus* CRAG3 increased with increasing time showing 43 % autoaggregation after 5 h displaying its ability to form clumps (Fig. 3). *P. pentosaceus* CRAG3 showed 10.65 ± 0.84 % coaggregation with *E. coli* after 5 h (Fig. 3) similar to probiotic strain *Lactobacillus acidophilus* M92 which showed 4.36 and 15.11 % coaggregation with *Lactobacillus plantarum* L4 and *E. coli* 3014, respectively [34]. Hydrophobicity and auto-aggregation of microorganism are phenotypically related to its adhesion capacity [32, 33]. It is the hydrophobic nature of outer layer of microbe which helps in attaching to the mammalian cell surface [36, 44]. The adhesion property of a probiotic microbe also supports in competing with other microorganisms in gastrointestinal tract [45]. The adhesion of *P. pentosaceus* CRAG3 on HeLa and J774A.1 cell lines is shown in Fig. 4. The microscopic examination of adhesion between mammalian cell lines and

Table 2 Antibiogram of *P. pentosaceus* CRAG3 using antibiotic octodiscs on MRS agar

S. No.	Antibiotic	Concentration (μg)	*P. pentosaceus* CRAG3	*P. pentosaceus* SPA[a]
1	Amikacin	10	M	R
2	Ampicillin	25	M	R
3	Bacitracin	10 U	M	M
4	Ceftazidime	30	M	nd
5	Cephaloridine	30	M	R
6	Cloxacillin	01	M	R
7	Gentamicin	10	M	R
8	Methicillin	05	M	S
9	Norfloxacin	10	M	R
10	Penicillin-G P	10 U	M	S
11	Cefixime	05	R	nd
12	Ciprofloxacin	10	R	R
13	Co-trimoxazole	25	R	nd
14	Kanamycin	30	R	R
15	Nalidixic acid	30	R	nd
16	Sulphamethoxazole	50	R	nd
17	Tobramycin	10	R	R
18	Trimethoprim	2.5	R	nd
19	Vancomycin	30	R	R
20	Gentamicin	10	R	nd
21	Amoxycillin	10	S	S
22	Amoxyclav	10	S	M
23	Cefoperazone	75	S	nd
24	Cephaclor	30	S	nd
25	Cephalexin	10	S	M
26	Cephalothin	30	S	M
27	Clindamycin	02	S	M
28	Erythromycin	15	S	S
29	Imipenam	10	S	nd
30	Lincomycin	02	S	S
31	Nitrofurantoin	50	S	nd
32	Novobiocin	30	S	M
33	Oleandomycin	15	S	S
34	Oxytetracycline	30	S	S
35	Piperacilin	100	S	S
36	Tetracycline	100	S	nd
37	Ticarcillin	75	S	nd
38	Carbenicillin	100	S	S
39	Cephotaxime	30	S	M
40	Chloramphenicol	30	S	S
41	Co-trimazine	25	S	R
42	Oxacillin	05	S	M

Zone of inhibition of growth of microorganisms: 0–2 mm (*R* resistant); 3–6 mm (*M* moderate); 7–13 mm (*S* sensitive). *nd* not determined

[a] [7]

the isolate showed good adhesion property of the isolate with both cell lines (Fig. 4a–d). The cells of *P. pentosaceus* CRAG3 adhered to HeLa cell line (Fig. 4b) and also with J774A.1 cell line (Fig. 4d). As shown in Fig. 4b–c, d more than 40 cells adhered to cell lines indicating good adhesion property of the isolate. The adhesion capacity is a key property of probiotic through which it colonizes in intestine and competing with pathogenic strains. The characterization of many probiotic bacteria has been done on the basis of their adhesion capacity [33]. It has been reported that the bacteria having high autoaggregation capacity also show good adhesion capacity [33].

Resistance to Biological Barriers

Resistance to Lysozyme The overall resistance of *P. pentosaceus* CRAG3 to lysozyme, bile salts and simulated gastric juice was expressed in terms of percent survival. The isolate showed resistance to 100 µg/ml of lysozyme

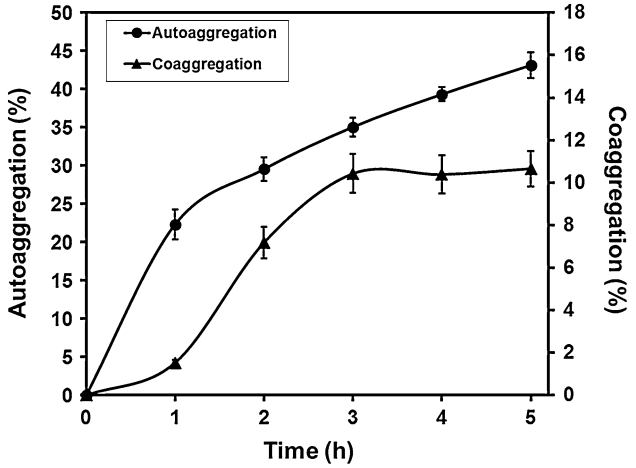

Fig. 3 Autoaggregation and coaggregation (with *E. coli*) property shown by *P. pentosaceus* CRAG3 with time (h)

with 75 % (6.0 x 10^5 CFU/ml) survival after 2 h as compared to control having 7.95 × 10^5 CFU/ml (Fig. 5). Similar results were observed in other probiotic bacteria such as *L. plantarum* Lp751 (72.52 %), *L. plantarum* Lp803 (72.64) and *L. plantarum* Lp813 (74.04 %) [36]. The swallowed probiotic microorganisms generally encounter the first biological barrier of lysozyme of saliva in the mouth. The next barrier is gastric juice in stomach where the pH is between 1.5 and 3.0 and the upper part of small intestine, which contains bile [46].

Resistance to Gastric Juice The resistance of CRAG3 toward gastric juice is shown in Fig. 5. The number of CFU/ml before incubation with gastric juice of pH 2 and 3 was 4 × 10^8 and 12.6 × 10^8, respectively. It showed resistance to simulated gastric juice of pH 3.0 with 57.68 ± 2.4 % survival (7.2 × 10^8 CFU/ml) but no survival was observed in pH 2.0. A lactic acid bacterium strain, *Leuconostoc mesenteroides* isolated from the intestine of snakehead fish (*Channa striatus*) showing growth at pH 3–7 has also been considered as probiotic [47]. According to Erkkilä and Petäjä [48], the strains of *Pediococcus acidilactici* (P2), *Lactobacillus curvatus* (RM10)

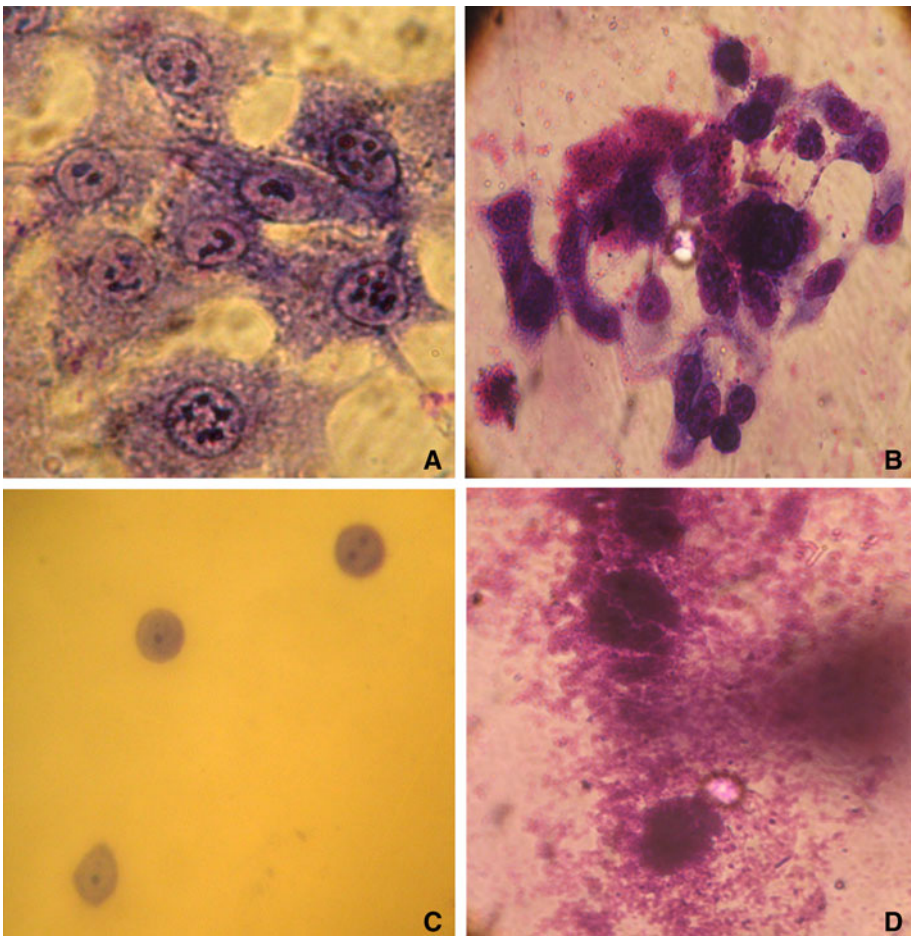

Fig. 4 Determination of adhesion capacity of *P. pentosaceus* CRAG3 with HeLa and J774A.1 cell lines. **a** Untreated HeLa cells (control); **b** Adherence of *P. pentosaceus* CRAG3 with HeLa cells; **c** Untreated J774A.1 cells (control); **d** Adherence of *P. pentosaceus* CRAG3 with J774A.1 cells

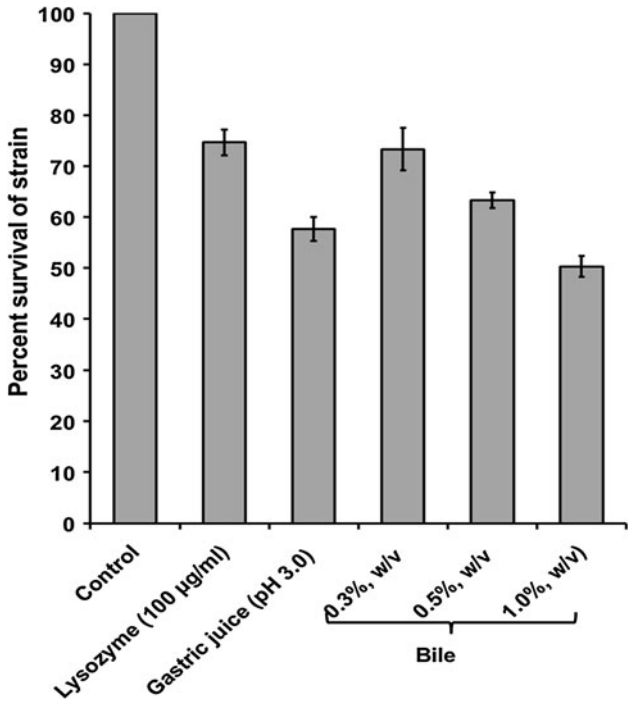

Fig. 5 Resistance of *P. pentosaceus* CRAG3 against biological barriers. Percent growth of the strain in presence of 100 µg/ml lysozyme after 2 h, simulated gastric juice (pH 3.0) and bile salts at 0.3, 0.5 and 1.0 % (w/v) concentration

and *P. pentosaceus* (FF) showed tolerance to acid (pH 3.0). The survival of *P. pentosaceus* CRAG3 at pH 3.0 indicated that it could transit through the stomach.

Resistance to Bile Salts *Pediococcus pentosaceus* CRAG3 showed 73.33 ± 4.2 % survival in the presence of 0.3 % bile salts, 63.3 ± 1.5 % survival in the presence of 0.5 % bile salts and 50.0 ± 2.1 % survival in the presence of 1.0 % bile salts (Fig. 5). *P. pentosaceus* CRAG3 showed 8 % higher survival than *P. pentosaceus* Z13P, which showed 65 % viable cells at 0.3 % bile salt concentration as reported by Yuksekdag and Aslim [18]. The human body contains bile salts in large intestine and the relevant physiological concentrations of human bile range from 0.3 to 0.5 % [49]. Thus, it is generally considered necessary to evaluate the ability of potentially probiotic bacteria to resist the effects of bile acids [50].

Bile Salts Hydrolase Activity

The appearance of halos (nearly 6 mm) around colonies after growth in MRS-TDCA medium validated the ability of strain to hydrolyze sodium salt of taurodeoxycholate (data not shown). The inhibition of common intestinal bacteria had been related to the presence of free (deconjugated) bile acids rather than conjugated ones [51, 52]. The ability to hydrolyze bile salts could help the microorganism in sustaining the balance of the gut microflora [39, 44]. It had also been suggested that bile salt hydrolase (BSH) enzyme might be a detergent shock protein that enables LAB to survive the intestinal bile stress [51].

Antibacterial Assay

Pediococcus pentosaceus CRAG3 displayed antibacterial activity against all three test microorganisms. The zone of inhibition observed around *E. coli*, *B. subtilis* and *S. aureus* was 12 ± 0.2 mm, 7 ± 0.1 mm and 15 ± 0.2 mm, respectively. Similar results were observed in *Pediococcus* spp. isolated from Turkish type fermented sausages which showed antibacterial activity against *Listeria monocytogenes*, *E. coli,* and *Micrococcus flavus* [18]. No zone of inhibition was observed around the wells containing neutralized cell-free filtrate of *P. pentosaceus* CRAG3. The antibacterial activity exhibited by *P. pentosaceus* CRAG3 might be due to the production of lactic acid, hydrogen peroxide or other acids. Lactic acid bacteria have the ability to produce antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins to inhibit the growth of pathogenic and spoilage microorganisms [53].

Utilization of Prebiotics

The growth profile of *P. pentosaceus* CRAG3 in the presence of different prebiotics is shown in Fig. 6. *P. pentosaceus* CRAG3 utilized inulin with 37.5 % relative cell growth as compared to glucose. It proved to be better than

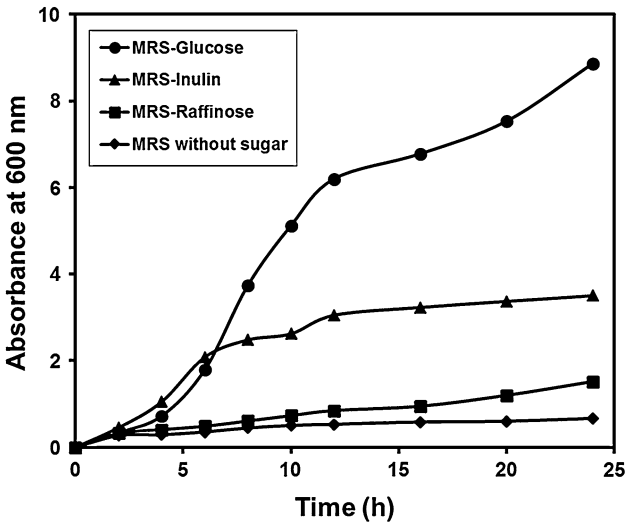

Fig. 6 Growth profile of *P. pentosaceus* CRAG3 in MRS medium containing inulin, raffinose or glucose as carbon source at 37 °C. The MRS medium without any carbon source was taken as negative control

previously reported probiotic strains of *L. plantarum* which were not capable of using inulin within 24 h [36]. However, it showed only 6 % relative cell growth in the presence of raffinose as carbon source (Fig. 6).

Conclusions

The lactic acid bacterium CRAG3 was isolated from fermented cucumber on the basis of glucansucrase activity (2.7 U/ml) and was evaluated for probiotic properties. It was a Gram-positive, catalase-negative and round-shaped bacteria indicating the common properties of lactic acid bacteria. It showed acid formation in triple sugar iron agar test and non-motile nature. The isolate CRAG3 was identified as *P. pentosaceus* CRAG3 (GenBank accession number JX679020) on the basis of 16S rRNA gene sequence analysis. It produced 145 ± 3.27 mg lactic acid per ml of cell-free supernatant. It showed utilization of carbohydrates and susceptibility to antibiotics. However, it displayed its ability to ferment mannitol and inability to utilize lactose. *P. pentosaceus* CRAG3 was moderately sensitive to amikacin, ampicillin, gentamicin and norfloxacin and resistant to vancomycin showing common feature of LAB. Interestingly, *P. pentosaceus* CRAG3 displayed probiotic properties such as 33 % hydrophobicity, 43 % autoaggregation, 10 % coaggregation and in vitro cell adhesion ability. It exhibited resistance against lysozyme and simulated gastric juice at pH 3.0 with 75 and 57 % survival, respectively. The isolate showed tolerance toward bile salts with 73 % survival and bile salts hydrolase activity. It exhibited antibacterial activity against *E. coli*, *S. aureus* and *B. subtilis* and ability to utilize prebiotics such as inulin and raffinose with 37.5 and 6 % relative cell growth, respectively. Based on the above observations, it could be concluded that *P. pentosaceus* CRAG3 can serve as potential probiotic bacterium and the glucansucrase and glucan producing ability could play a potential role for functional food applications.

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Conflict of Interest The authors declare that they have no conflict of interest.

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