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FULL PAPER

Isolation and characterization of pediococcus Sp. with antimicrobial activity from phyllosphere of fruities trees

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The phyllosphere is a microbiology term which so-called to the plants portions that microorganisms adapted. Since fresh fruits and vegetables are not heated for administration, they could be the attractive source for isolation and identification of probiotic microbes. The isolation of probiotics from fermented foods including yogurt, cheese and native's dairy products are reported in vast studies but in this research 8 strains of bacteria with probiotic potentiality were isolated from phyllosphere of fruit trees. The bacteria were isolated from phyllosphere of fruit trees via culture in MRS agar at 35 °C for 48h. Tolerance of the favor strains were studied for there to acid [pH3] and bile salt [0.3%]. The isolates were characterized to the antibiotic resistance and antimicrobial activity. Two of isolates with high acid and bile tolerance, antibiotic sensitivity and antimicrobial activity were clearified by 16 s rDNA sequencing. The isolate bacteria were 100% similar to *Pedicoccus acidulates* and *P.cerevisae.* These bacteria displayed antimicrobial activity against both gram-negative and Gram-positive pathogenic bacteria. This study showed that phyllosphere of fruit trees could be an attractive source for isolation of human indigenous friendly microbe to application in antimicrobial products.

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KEYWORDS

Acid and bile resistant; antimicrobial activity; probiotics; *Pedicoccus sp*; 16s rDNA sequencing.

Introduction

Research into the identification of bacteria as both pathogenic or friendly in the phyllosphere is significant to the public health, which can help to understand the survival of human probiotic with health potentiality including antimicrobial activity and pestside detoxification[1]. It is also of great importance in discovering new methods to control food poisoning which connected with fruit and vegetables with pathogenic bacteria [2-4]. In addition, recently the use of probiotics has gained a growing interest in treatments of modern disease including cancer [5-11], diabetes [12], obesity [13], fatty liver disease [NAFLD] recovery [14], kidney disease [15], cardiovascular disease [16] and pathogenic biofilms [17].

Probiotics are eatable microorganisms with positive effects on intestinal microbiota adjusting[18], fortify antagonistic effect on the germination of adverse bacteria[19] and improve human immune system [20,21]. The most common types of probiotics is lactic acid bacteria with long–lasting survival in fermented products [16,22]. These bacteria are the gram-positive, non-sporeforming,

rarely motile rod-shape or spherical bacteria. They exist enormously in dairy products [23]. The main food sources of probiotics are yogurt, cheese and other fermented milk products [24,25]. Lactic acid bacteria in foods administration and dietary supplements have been common from ancient times and many strains are have been vastly carried out in fermented food production. Further, a large number of studies have been conducted in biomedical fields.

Currently, human-friendly lactic acid bacteria are extracted from natural sea salt [26], plants [27], abomasums driven rennet [28] [29], and traditional fermented foods [14,30]. Numerous studies have emphasized that the isolation of lactic acid bacteria should be done from natives [31]. Most of research isolated the probiotics in the genera *Lactobacillus* and *Streptococcus* from fermented foods but, in this study we isolated the other genera of probiotics with new source called phyllosphere. Then, the main goal of this research was to introduce the new source of probiotics and genus *Pedicoccus* with probiotic potential.

Material and methods

Sample preparation and isolation of bacteria

Samples of leaves, fruits, flowers and bourgeons were aseptically collected from fruit trees [grape, cherry, apple, pear, plum, mulberry, and peach]. One gram of each sample was crushed and then was suspended in 10 mL sterile sodium citrate buffer. After that, 1 mL was added to MRS broth and incubated in microaerophilic media for 48 h at 35 °C. Then, 0.1 mL of enriched culture was propagated onto MRS agar plates and incubated for 48 h at 35 °C. Some distinct colonies were evaluated morphologically and physiologically, i.e. cell morphology, gram staining, catalase production and etc. The isolates were sub cultured in MRS broth and conserved with addition of glycerol [25%] at minus 25 °C.

Acid bile resistance assay

Monitoring tolerance of acidophilic bacteria were performed in 1 mL of any enriched culture suspension which inoculated in 10 mL phosphate buffered saline buffer pH 2,5 and incubated for 3 h [32]. Cultures were incubated in MRS broth containing 0,3% [w/v] ox gall [Sigma, USA] overnight and then treated for 4 h at 35 °C. Some dilutions contained acid and bile resistant cultures, then 0,01 mL of 5-10 dilution were spread into MRS-agar medium and treated for 24 h at 35 °C. The number of living cell was defined on the basis of growing colony.

Molecular identification

Total chromosomal DNA was obtained from overnight broth cultures of the strains for molecular study of probiotic bacteria according to the known method [33]. 16s rDNA expression was studied by using the primer pair: 16lacF S´-AGAGTTTGATCMTGGCTCAG-3' 16lacR 5' -TACCTTGTTAGGACTTCACC–3' [34]. PSR expression was carried out using master mix, 0,5 mikM primer, 50 ng DNA and the final volume was reached to 25 mikl. The next step was denaturation of 94 °C for 4 min, 32 cycles of 94 °C for 50 s, 59 °C for 50 s and 72 °C for 90 s and final extension at 72 °C for 10 min and it was kept at 4 °C. PCR outputs were sent to a commercial sequencing facility [Macrogene, Korea]. The sequences were blasted to those reported in Gene Bank, using BLAST algorithm. The isolates were studied by similarity with standard strains in Gene Bank.

Antimicrobial activity

The antimicrobial activity of isolated strains was detected by the disc diffusion method [35]. The isolated strains were cultivated in MRS broth at 35 °C for 24 h, then were centrifuged at 20000 rpm 10 min. The empty discs of 6 mm diameter were immersed in bacterial free cultural fluid [supernatant], and then the discs were placed on inoculated

solid medium with-CFU/mL of the test culture, which is used for harmful gram-positive: Bacillus subtilis BDU 50 [food poisoning bacteria], *Staphylococcus aureus* BDU-23 [human pathogen] and gram-negative: *Acinetobacter baumanni* BDU-32 [opportunistic pathogen], *Esherichia coli* [conditionally pathogen], *Klebsiella pneumonia* BDU-44 [opportunistic pathogen], *Pseudomonas aeruginosa* BDU-39 [opportunistic pathogen] from culture collection of Microbiology Department of Baku State University. Positive results were recognized as clear zones around the discs after incubation at 35 °C for 24 h.

Results

Isolated bacteria morphology and characterization

By using MRS medium from surface of fruit trees, 8 spherical bacterial strains were isolated, which were gram - positive and catalase negative (Figure 1).

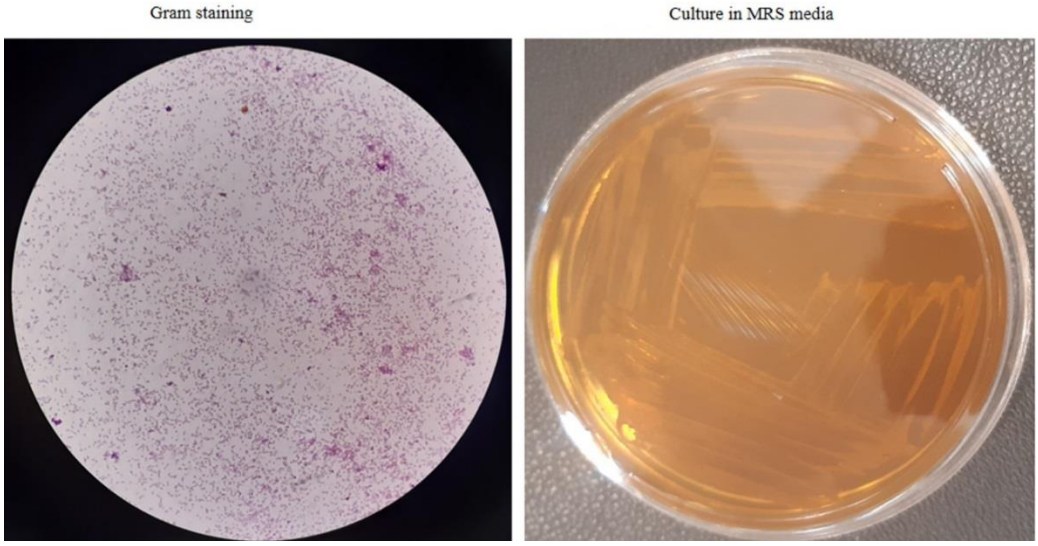

FIGURE 1 The morphology and gram staining of isolated bacteria

Resistance of isolated strains to simulated condition of human gastrointestinal system was studied [pH3 and 0,3% bile salt]. Received data are presented in Table 1. All strains showed tolerance to acid condition in disparate degree. The highest degree of acid resistance was observed in strains BDU-G2, bdu-Pl 14 and BDU-P6. As to bile tolerance, the high resistance was observed in strains BDU-G15, BDU-G2 and BDU-Pl14, low resistance in strains BDU-M12 and BDU – G3, no-resistance - in strains BDU-C8, BDU-P6 and BDU-A11. Two strains of BDU-G2 and BDU-Pl14 showed considerable tolerance to both and bile, and these bacterial strains were used for further investigation.

TABLE 1 Resistance of bacterial strains to acid and bile

Bacterial Strains	Number of living Cells Towards Control, % Resistance to Acid [pH=3]	Resistance to Bile [0.3%]
BDU – A11	32	0,0
BDU – C8	23	0,0
BDU – G2	62	38,0
BDU –G3	48	12,2
BDU –G15	42	40,0
BDU –M12	12	13,6
BDU –P6	52	0,0
BDU –Pl14	60	42,0

Antibacterial resistancy

Both identified bacterial strains displayed antimicrobial activity alongside both gram-negative and gram-positive bacteria study. High antimicrobial activity of *Pedicoccus acidilactis* BDU-G2 was observed against *Acinetobacter baumanni*, *Esherichia coli* and *Staphylococcus aureus*. But high antimicrobial activity of *P.cerevisiae* BDU-Pl14 was observed against *E.coli* and *S.aureus*. The antimicrobial activity of *P.acidilactis* BDU-62 was 1.2-1.4 times more than that of *P.cerevisiae* BDU-Pl14 (Table 2).

TABLE 2 The antimicrobial activity of *Pedicoccus acidilactis* BDU-G2 and *P.cerevisiae* BDU-Pl14

Test Cultures	Diameter of Inhibitory [pure] zone, mm	
	***P.acidilactis* BDU-G2**	***P.cerevisiae* BDU-Pl14**
Gram – positive:		
1. *Bacillus subtilis* BDU-50	16±0,4	12±0,5
2. *Staphylococcus aureus* BDU-23	20±0,6	17±0,4
Gram-negative:		
3. *Acinobacter baumanni* BDU-32	25±2,0	15±0,3
4. *Esherichia coli* BDU-12	23±1,2	18±0,7
5. *Klebsiella pneumonia* bdu-44	17±0,6	14±0,5
6. *Pseudomonas aeruginosa* BDU-39	15±0,6	14±0,3

Molecular identification

The 16s rDNA PCR product of the isolates BDU-G2 and BDU-Pl14 with high probiotic potential were electrophoresed [Figure 2]. After that sequencing reports were identified using BLAST [http://blast.nebi.nlm.nich.gov/Blast.cgi] and blasted with the sequences stored in NCBI GenBank for various species. The isolates have similarity with *Pedicoccus acidilactis and P.cerevisiae* (Figure 3).

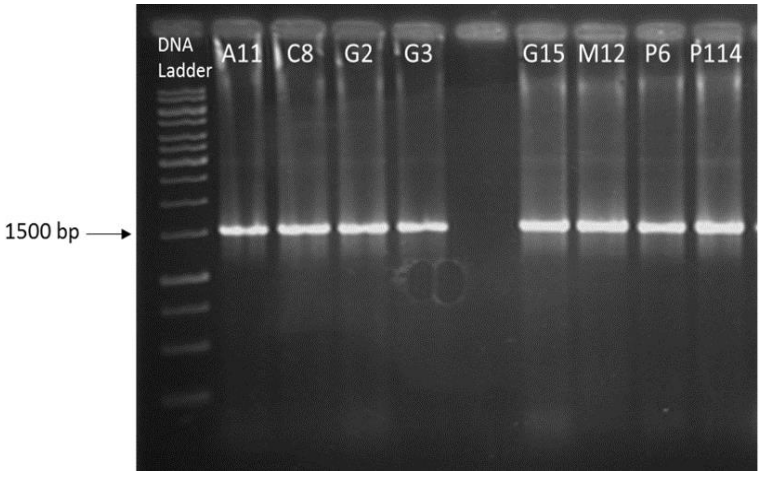

FIGURE 2 PCR electrophoresis of 16s rDNA PCR product. The bands were obtained in 1500 bp

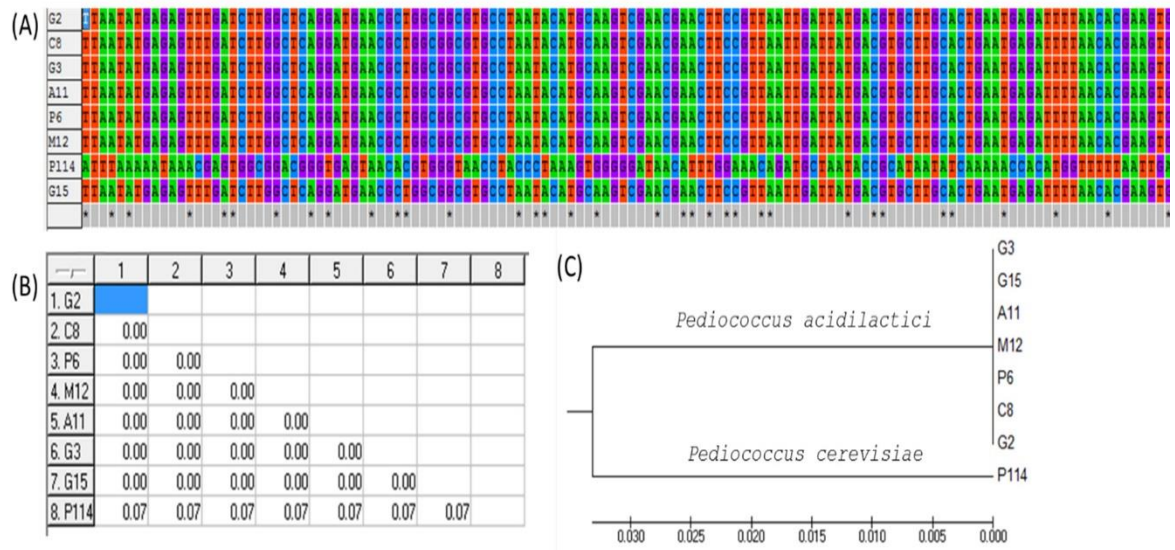

FIGURE 3 Aignment of 16 s ribosomal gene sequence and clustering of isolated bacteria. [A]; W alignment of sequencing prepared by Mega 4 software, [B]; A distance matrix of the obtained sequences, [C]; the clustering of isolated strains belong two distinct species including *P. acidilactici* and *p.cerevisiae*.

Discussion

The acid and bile resistant bacterial strains from fruit trees of Azerbaijan Republic were identified by 16s rDNA as *P. acidilactis* and *P.cerevisiae.* These bacterial strains showed high antimicrobial activity against both gram-negative and gram-positive unsafe bacteria and could be used as probiotics. Our study showed that human friendly probiotic bacteria can be isolated such as plants. In this study we found out that fruit trees could be a significant source to acquire the probiotic administrations in industrial dairy products to save the natives health and inhibit modern diseases as a result of industrial lifestyles. Another feature of these bacteria according to WHO guideline is its inhibitory effect on the growth of pathogenic gram-positive and gram-negative bacteria.

Conflict of Interest

The authors declare no conflict of interest.

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