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## Evaluation of Soil Origin *Pseudomonas* sp. for Production of Bioactive Compounds

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**Abstract:** Nowadays, bioactive compounds produced by bacteria consider a source of new remedy to eliminate antibiotic-resistant bacteria. Therefore, the main purpose of this study was isolation and identification of soil origin Gram-negative bacteria and their evaluation for production of bioactive compounds. In general, three bioactive compounds producing bacteria were isolated from hundred and twenty five soil samples. These strains identified as *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Comamonas testosteroni* based on phenotypic and 16 SrRNA Gene sequencing methods. The bioactive compounds produced by isolated strains were partially purified and their activities evaluated at different pHs and temperatures. Then, their antimicrobial spectrums assessed against different pathogenic microorganisms. The results obtained indicated that the best temperature and pH for activity of the bioactive compounds were 40-50°C and 6.5-7.5, respectively. In addition, bioactive compounds produced by *P. aeruginosa* and *P. putida* showed an antimicrobial property against *Staphylococcus aureus*, *Bacillus cereus*, *Corynebacterium* sp. *Listeria monocytogens*, *Klebsiella pneumonia*, *Shigella dysenteriae* and *Proteus mirabilis*; however, *E. coli* was resistant to them. Although all the bacteria were resistant to the bioactive compound produced by *C. testosteroni*, *Aspergillus niger* and *Alternaria* sp. were sensitive. Overall bioactive compounds produced by *Pseudomonas* sp. showed activity against some pathogenic bacteria as well as fungi and might be considered an alternative therapeutics not only in pharmaceutical but also as a preservative in food industries.

**Key words:** Bioactive compound, *Pseudomonas*, antimicrobial effect

### INTRODUCTION

Bioactive compounds are peptides produced by various bacteria with antimicrobial property against different microorganisms (Motta and Brandelli, 2008). These compounds with use of hydrophobic/amphiphilic properties penetrate into the cell and consequently the bacterial compositions release from the cell (Oscariz and Pisabarro, 2001). However, production of bioactive compounds such as bacteriocins is similar to antibiotics but several advantages could be considered in compared to the antibiotics. For example, toxicity of bacteriocins on animal tissues is relatively nil. In addition, proteinaceous property of these compounds resulted in their degradation by protease enzymes; hence could not accumulate in the human body (Hechard and Sahl, 2002). In general, Bacteriocins are divided into four groups based on their structures. Class I bacteriocins are small peptides (<5 kDa) with unusual amino acids. Class II are thermo stable peptides with size of <10 kDa. Class III are heat-sensitive with high molecular weight (<30 kDa) and

Class IV are relatively complex with glycoprotein, lipoprotein and non-protein moiety (Ennahar *et al.*, 2000). It is confirmed that non-protein moieties of bacteriocins require for stability and antimicrobial activity (Garneau *et al.*, 2002).

Nowadays, application of bacteriocins considers as a way to reduce the frequency of occurrence of antibiotic-resistant bacteria. For examples, alternative drugs as well as safe food preservatives could eliminate antibiotic-resistant strains of bacteria and increase shelf life of foods (Settanni and Corsetti, 2008). Therefore, based on foregoing evidence present study was conducted to isolate and identify bacteriocin-producing bacteria from soil and assessed their antimicrobial effect against some pathogenic bacteria.

### MATERIALS AND METHODS

#### Isolation of gram negative bacteria from soil samples:

One hundred and twenty five soil samples were collected

from different areas of kazeroun city, Iran. Isolation of potent bioactive producing bacteria was carried out by serial dilution of soil sample ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-6}$ ) using sterile distilled water. Then, 0.1 mL of each dilution was transferred onto MacConkey agar and the plates were incubated at 35°C for 24 h. After this period the growth colony was picked up and subjected for assay the bioactive compound production.

**Screening of bioactive compound producing bacteria:** The bacterial colonies obtained from soil samples separately were inoculated into hundred milliliters of trypticase soy broth in the conical flask (250 mL) and incubated at 35°C, 200 rpm for 24 h. Then, 5 mL of each culture broth was centrifuged at 12000 rpm for 30 min and the supernatant was assessed for antimicrobial property against bioassay strains viz., *E. coli* PTCC 1330, *Staphylococcus aureus*, PTCC 1337 *Bacillus cereus* PTCC 1137, *Pseudomonas aeruginosa* PTCC1556 and *Candida albicans* PTCC 5027. Screening of bioactive compound producing strains in this study was done by the Well Diffusion Agar (WDA) method. To perform the test, bioassay strain was cultivated on Mueller Hinton agar and wells (5 mm in diameter) were made in plate agar using sterile sharp borer. Then, 100  $\mu$ L of each supernatant was added into each well and the plates were incubated at 35°C. After 24 h, observation of a clear zone of growth inhibition considered bioactive compound activity (Ten Brink *et al.*, 1994).

**Phenotypic identification of bioactive compound producing strains:** Promising bacteria for production of bioactive compounds were presumptive identified by Gram stain, growing on MacConkey agar, catalase and oxidase tests and then identification was confirmed using O/F, Nitrate, TSI, Urease, Lysin decarboxylase and Phenylalanine tests.

**Authentication of antimicrobial producing strains:** Verification of presumptive identification was carried out by Gene sequencing of 16SrRNA.

To perform the test DNA was extracted from the isolated strains using High Pure Template DNA PCR kit (Roche-Germany) according to the supplier's instructions. The purity of the extracted DNA was assessed based on absorbance of the extracted DNA at 260 and 280 nm wavelengths by Biophotometer (Eppendorf-Germany) and then the purity was calculated based on absorbance ratio 260/280 nm. The extracted DNA with ratio (260/280 nm)  $1.9 \leq$  corresponding to 121  $\mu$ g DNA mL<sup>-1</sup> was used for amplification of 16S rRNA by PCR. Amplification of 16S rRNA was performed using universal primers produced by TAG K openhagen (Denmark). The

sequence of Forward and Reverse primers were 5'-AGGAGGTGATCCAACCGCA-3' and 5'-AACTGGAGGAAGGTGGGGA-3', respectively. Each reaction was carried out in a total volume of 25.5  $\mu$ L contained 14.5  $\mu$ L of molecular biology-grade water (Sigma Aldrich Company Ltd.), 2.5  $\mu$ L of 10 $\times$ PCR buffer (Cinagen-Iran), 1  $\mu$ L of each forward and reverse PCR primers, 1  $\mu$ L of a 10 mM dNTPs (Cinagen-Iran), 0.5  $\mu$ L of Smar taq polymerase (cinagen-Iran), 1  $\mu$ L of 50 mM MgCl<sub>2</sub> (cinagen-Iran) and 5  $\mu$ L of DNA template.

PCR amplification conditions on an Eppendrof thermocycler were as follows: 95°C for 4 min, followed by 35 cycles of 95°C for 40 sec, 56°C for 30 sec and 72°C for 40 sec, with a final extension at 72°C for 5 min and storage at 4°C. All PCR products obtained were run on a 1.5% (w/v) agarose gel with a 100 bp DNA ladder (Fermntas-Russia). PCR products were electrophoresed at 75V for 20 min; DNA was visualized using ethidium bromide and photographed by Uvidoc (England). After visualization of pure DNA bands, the PCR products have been sent to Macrogen in South Korea (<http://www.macrogen.com/>) for DNA sequencing. The 16S rRNA sequenced data for bioinformatic applications were subjected to BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) in order to identify each respective 16S rRNA gene amplicon.

**Assay of bioactive compound production during bacterial growth phases:** To assess antimicrobial property of the metabolite during the bacterial growth phases, 5 mL of each bacterial culture was withdrawn at time intervals (5, 10, 15, 20...45 h) and the cells counted by optical density at 600 nm. Then the bacterial culture centrifuged at 12000 rpm for 30 min. Afterward, the antimicrobial property of the supernatant was assessed against bioassay strains (Van Reenen *et al.*, 1998).

**Partial purification of the bioactive compounds:** The bacterial cultures containing bioactive compound (as described above) were centrifuged at 12000 rpm, 30 min, 4°C. Then, the pellets were removed and ammonium sulfate was added into the supernatants (up to 40%) with stirring. After 12 h mixtures were centrifuged at 12000 rpm, 30 min, 4°C and surface pellicle from each suspension was recovered. The pellicle containing bacteriocin was resuspended in 50 mM sodium phosphate buffer (pH 6.5). Then 25 mL of mixture was treated with chloroform-methanol (2:1, v/v) for 1 h and centrifuged at 12000 rpm, 30 min, 4°C. The precipitate was air dried and dissolved in 10 mL of 10% 2-propanol/0.1% tri fluoroacetic acid (TFA).

**Effect of different pHs on activity of bioactive compounds:** One milliliter of each partial purified bioactive compound was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 by diluted NaOH and HCl for 1 h and readjusted to pH 7.0. To assess bacteriocin activity, 100 µL of each compound was added into the well on Mueller Hinton agar seeded by bioassay strain (Sharma *et al.*, 2009). After 24 h, observation of a zone of inhibition was considered bioactive compound activity.

**Effect of different temperatures on activity of bioactive compounds:** Stability of partial purified bioactive compounds at different temperatures was tested by overlaid of 10 mL of aliquot compounds using paraffin oil. The tubes were incubated at 40, 50, 60 and 70°C for 30 min then, 100 µL of each bioactive compound was added into the well of Mueller Hinton agar that previously seeded by bioassay strain (Sharma *et al.*, 2009). After 24 h, observation of a zone of inhibition was considered bioactive compound activity.

**Antimicrobial spectrum of the bioactive compounds:** Antimicrobial activity of the compounds against various microorganisms viz., *Listeria monocytogens* PTCC 1163, *Klebsiella pneumonia* PTCC 1053, *Salmonella typhi* PTCC 1609, *Shigella dysenteriae* PTCC 1183 and *Proteus mirabilis* PTCC 1076, *Aspergillus niger* PTCC 5011 and *Alternaria* PTCC 5224 was assessed by Well Diffusion agar method. To perform the test, each bioassay strain was full cultivated on Mueller Hinton agar and three wells were made in plate agar using sterile sharp borer. Then, 100 µL of purified bacteriocin of BC10, Bd9 and BSI were separately added into each well and the plate was incubated at 35°C. After 24 h, clear zone of growth inhibition was measured and considered antimicrobial activity. All experiments were carried out in three replicates.

## RESULTS

**Isolation of bioactive compound producing strains from soil samples:** The results obtained from screening of bioactive compound producing bacteria from soil samples indicated that three strains of Gram-negative bacteria could produce bioactive compounds. Two bioactive compounds (BC10 and Bd9) showed an antimicrobial property against *Bacillus cereus* and *staphylococcus aureus* with zone of inhibition of 22 and 20 mm; however, *Escherichia coli* was resistant to them. *Pseudomonas aeruginosa* was sensitive (with zone of inhibition of 15 mm) and resistant to BC10 and Bd9, respectively. Furthermore, of all antagonistic

microorganisms only *Candida albicans* was sensitive to bioactive compound BS1.

**Phenotypic and molecular identification of bacteriocin producing strains:** The results obtained from phenotypic identification of the isolates illustrated that bacteriocin producing bacteria were *Pseudomonas* sp. However, alignment analysis of 16SrRNA genes of bacteriocins BC10, Bd9 and BSI producing strains exhibited 100, 99 and 99% identical to *Pseudomonas aeruginosa* strain GDC10 with accession number gb|JN594664.1|, *Pseudomonas putida* strain CDd9 with accession number gb|GU248219.1| and *Comamonas testosteroni* with accession number gb|HQ200412.1, respectively (Fig. 1).

**Determination of the growth phase of bioactive compounds production:** The results obtained from production of bioactive compounds during the bacterial growth phases indicated that all bacteriocins were produced after 10 h and reach to maximum level at 20 h of bacterial growth. It means all the bioactive compounds were primary metabolites. Therefore, they must be noted as bacteriocins.

**Effect of various pHs and temperatures on activity of bioactive compounds:** As shown in Table 1 activity of three bioactive compounds maintains at pHs 6-7.5 however, at pHs 5, 5.5 and 8 was nil. In addition, BC10 and Bd9 showed weak and strong activity at pHs 6 and 6.5-7.5, respectively. The results obtained from activity of bioactive compounds at different temperatures indicated that the best temperatures for activity of the bioactive compounds were 40 and 50°C. In addition, BC10 and BS1 showed stable and unstable bioactive compounds against different pHs and temperatures, respectively.

**Antimicrobial spectrum of the bioactive compounds:** The result obtained from the antimicrobial spectrum of the bioactive compounds against some pathogenic microorganisms illustrated that the bioactive compounds BC10 and Bd9 exhibited an antimicrobial property to all bacteria tested with varied responses; however, their antimicrobial effects against fungi were nil. In addition,

**Table 1: Effect of different pHs and temperatures on bioactive compounds**

Bioactive compounds	pHs							Temperatures (°C)			
	5	5.5	6	6.5	7	7.5	8	40	50	60	70
BC10	-	-	+ <sub>w</sub>	+	+	+	-	+	+	+	+ <sub>w</sub>
Bd9	-	-	+ <sub>w</sub>	+	+	+	-	+	+	+	-
BS1	-	-	-	+	+	+	-	+	+	-	-

-: Negative in bacteriocin activity, +: Positive in bacteriocin activity and +<sub>w</sub>: Weak in bacteriocin activity

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gb|JN594664.1| Pseudomonas aeruginosa strain GDC10 16S ribosomal RNA gene, partial sequence Length=1500
Score = 693 bits (375), Expect = 0.0 Identities = 375/375 (100%), Gaps = 0/375 (0%) Strand=Plus/Plus

Query 11 TCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC 70
|||||
Sbjct 1123 TCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC 1182

Query 71 AAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCCGTACAAAGGG 130
|||||
Sbjct 1183 AAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCCGTACAAAGGG 1242

Query 131 TTGCCAAGCCGCGAGGTGGAGCTAATCCATAAAAACCGATCGTAGTCCGGATCGCAGTCT 190
|||||
Sbjct 1243 TTGCCAAGCCGCGAGGTGGAGCTAATCCATAAAAACCGATCGTAGTCCGGATCGCAGTCT 1302

Query 191 GCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAA 250
|||||
Sbjct 1303 GCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAA 1362

Query 251 TACGTTCCCGGCCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAG 310
|||||
Sbjct 1363 TACGTTCCCGGCCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAG 1422

Query 311 TAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGT 370
|||||
Sbjct 1423 TAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGT 1482

Query 371 CGTAACAAGGTAACC 385
|||||
Sbjct 1483 CGTAACAAGGTAACC 1497

gb|GU248219.1| Pseudomonas putida strain CDd-9 16S ribosomal RNA gene, partial sequence
Length=1504, Score = 676 bits (366), Expect = 0.0 Identities = 372/375 (99%), Gaps = 0/375 (0%)
Strand=Plus/Plus
Query 17 ATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA 76
|||||
Sbjct 1128 ATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA 1187
Query 77 AGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCCGTACAGAGGGT 136
|||||
Sbjct 1188 AGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCCGTACAGAGGGT 1247
Query 137 TGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTG 196
|||||
Sbjct 1248 TGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTG 1307
Query 197 CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAAT 256
|||||
Sbjct 1308 CAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAAT 1367
Query 257 ACGTTCCCGGGCCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCACAAGT 316
|||||
Sbjct 1368 ACGTTCCCGGGCCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCACAAGT 1427
Query 317 AGCTAGTCTAACCTTCGGGAGGAAGGTTACCAAGGTGTGATTCATGACTGGGGTGAAGTC 376
|||||
Sbjct 1428 AGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGATTCATGACTGGGGTGAAGTC 1487
Query 377 TTAACAAGGTAACCA 391
|||||
Sbjct 1488 GTAACAAGGTAACCA 1502

gb|HQ200412.1| Comamonas testosteroni strain SI 16S ribosomal RNA gene, partial sequence Length=1524
Score = 669 bits (362), Expect = 0.0, Identities = 367/369 (99%), Gaps = 2/369 (1%), Strand=Plus/Plus
Query 13 GTTGAGC-
CTCTAATGGGACTGCCGGTGAC-AACCGGAGGAAGGTGGGGATGACGTCAAG 70
|||||
Sbjct 1123 GTTGAGCACTCTAATGGGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAG 1182
Query 13 GTTGAGC-
CTCTAATGGGACTGCCGGTGAC-AACCGGAGGAAGGTGGGGATGACGTCAAG 70
|||||
Sbjct 1123 GTTGAGCACTCTAATGGGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAG 1182
```

Fig. 1: Continue

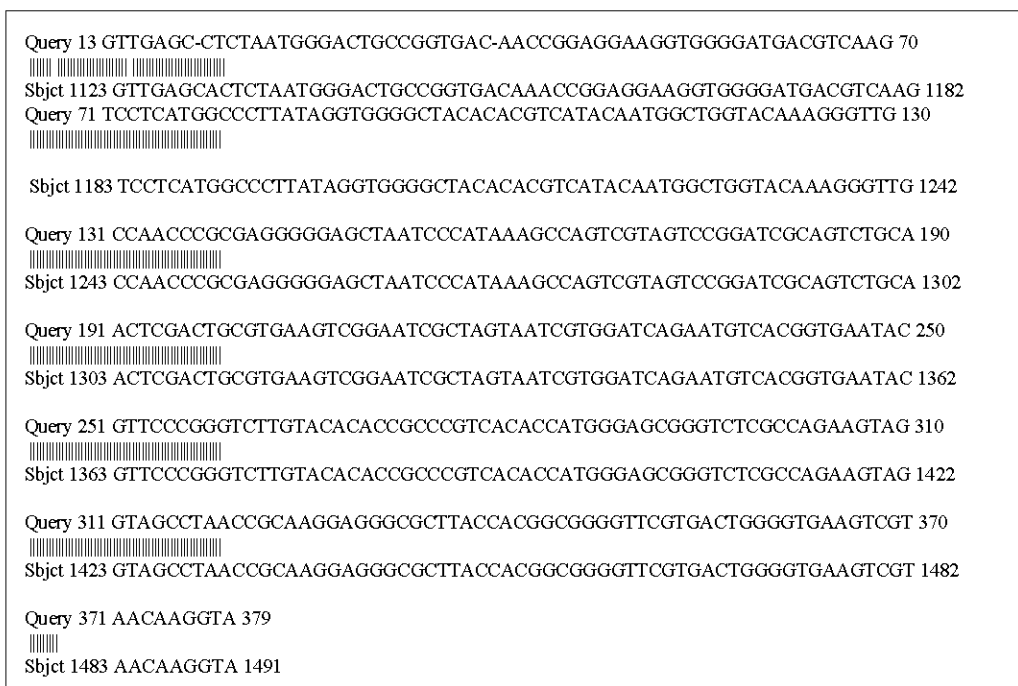


Fig. 1: Alignment analysis of 16SrRNA genes of bacteriocin producing strains

Table 2: Antimicrobial spectrum of bioactive compounds against pathogenic microorganisms

Bacteria	Inhibition zone diameter (mm)		
	BC10	Bd9	BS1
<i>Listeria monocytogens</i>	17	16	-
<i>Klebsiella pneumoniae</i>	18	11	-
<i>Salmonella typhi</i>	16	11	-
<i>Shigella dysenteriae</i>	17	12	-
<i>Proteus mirabilis</i>	15	10	-
<i>Corynebacterium sp.</i>	30	25	-
<i>Aspergillus niger</i>	-	-	18
<i>Alternaria sp.</i>	-	-	17

Numbers represent average of three replicate (mm), R: Resistance

*Corynebacterium sp.* and *Proteus mirabilis* exhibited high and less sensitive character. Bioactive compound BS1 showed antimicrobial activity against fungi (*Aspergillus niger* and *Alternaria sp.*) (Table 2).

### DISCUSSION

Several reports verified the antimicrobial effect of bioactive compounds against pathogenic microorganisms. For instance, bacteriocin as bioactive compound of *Paenibacillus polymyxa* strongly inhibited growth of *Xanthomonas campestris* strains CP-1-1 and M-5 and bacteriocin of *Bacillus subtilis* MTCC-8114 inhibited growth of *Microsporium fulvum* and *Trichophyton* species (Hammami *et al.*, 2009; Kumar *et al.*, 2009). Gupta and Garg (2009) reported that protection of urogenital treat of

human against pathogenic bacteria is possible by using two strains of bioactive producing bacteria viz., *Lactobacillus GG* and *Lactobacillus rhamnosus GR-1* and therefore, antimicrobial substances produced by these organisms can be considered new antimicrobial agents. Recently application of bacteriocins has focused on their antimicrobial effect because of specific target of these compounds and less frequency of developing bacterial resistance (Ketla *et al.*, 2003). In the present study bacteriocin producing bacteria were detected and identified from soil samples. These isolates were *Pseudomonas aeruginosa* and *Pseudomonas putida* and *Comamonas testosteroni*. *Pseudomonas aeruginosa* produced bacteriocin BC10, whereas *Pseudomonas putida* and *Comamonas testosteroni* produced bacteriocins Bd9 and BS1 respectively. The best pH for activity of bacteriocins was 6-7.5 however, the optimum temperature for their activity was 40-50°C. Although, bacteriocins BC10 and Bd9 showed antimicrobial effect against pathogenic bacteria, BS1 exhibited an antimicrobial effect against pathogenic fungi. Concerning to this result it must be noted that *Comamonas testosteroni* known as *Pseudomonas testosteroni* before its reclassification by molecular genetic techniques, metabolize testosterone (Gilligan *et al.*, 2003). Although, the pathogenic potential of *C. testosteroni* has not been well studied, it is usually considered a commensal. *C. testosteroni* has been

implicated in bacteremia-related septic shock, endocarditis and meningitis (Abraham and Simon, 2007). Our results showed antifungal activity of *Comamonas testosteroni* isolates. Parallel to our report El-Banna (2007) reported an antifungal activity of this bacterium. In addition, several reports confirmed capability of *Comamonas testosteroni* in degradation of steroids and non-steroid compounds (Arda *et al.*, 2003). In addition several reports affirmed capability of *Comamonas testosteroni* in degradation of steroids and non-steroid compounds.

### CONCLUSION

In general, natural habitat of bacteriocin producing bacteria is soil. In addition, bacteriocins produced by Gram negative bacteria have antimicrobial property against pathogenic microorganisms. Hence, in order to eliminate frequency of antibiotic-resistant bacteria and increase antimicrobial spectrum of the new drugs, production of bacteriocins from Gram-negative bacteria must be considered an especial area of investigation.

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