

Pediocin CP2GENE Localisation To Plasmid PCP289 Of *Pediococcus Acidilactici* MTCC 5101

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Citation

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Abstract

Pediocin producing *Pediococcus acidilactici* MTCC 5101 has been investigated for localization of pediocin gene. A plasmid CP289 was isolated using dl-threonine (10mg/ml) in MRS medium in conjunction with treatment, of overnight grown cell culture, with lysozyme and SDS. The molecular weight of pCP289 was estimated to be 8.9kb in comparison with *E. coli* V517, the standard molecular weight marker. Curing was carried out by exposing cell culture overnight to ethidium bromide, which resulted in linking of ped+ phenotype to pCP289. PCR based assay further confirmed pediocin PA-1 specific amplification of 711bp fragment containing pedA and pedB genes on pCP289. The pediocin CP2 produced by *Pediococcus acidilactici* MTCC 5101 is a thermostable variant of pediocin PA-1.

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INTRODUCTION

Food technologists started their pioneering work in 1970's to unravel the relationship of plasmid encoded food fermentation capabilities and bacteriocin production trait associated with some strains of *Pediococci*. Fleming and coworkers (1975) were the first to report the bacteriocin production by food associated *Pediococci*. Further experiments on plasmid curing revealed the association of small cryptic plasmids with genetic determinants encoding bacteriocin production trait in majority of the strains (Tagg et al, 1976). Graham & McKay (1985) reported the presence of a 10.5 Mdal plasmid in a number of bacteriocin producing *Pediococcus cerevisiae* FBB63 strains. Since the establishment of pediocin production as a plasmid linked trait in *Pediococci* (Gonzalez & Kunka, 1987; Hoover et al, 1988; Ray et al, 1989a) studies on cloning these plasmids have started. The plasmids may be transferred conjugally or by electroporation into a plasmidless homo- as well as heterologous hosts, and the expression of cloned genes encoded on them was studied (Ray et al, 1989b; Marugg et al, 1992; Bukhtiyarova et al, 1994; Buyong et al, 1998; Horn et al, 1998; Miller et al, 1998a, Miller et al, 1998b; Horn et al, 1999; Schoeman et al, 1999).

Pediocin PA-1 produced by *Pediococcus acidilactici* PAC1.0, has been extensively studied for its different characteristics. The original strain is NRRL B-5627 that excretes pediocin in the broth. *Bac*⁺ *Pediococci* have also been isolated from minimally processed vegetables (Gonzalez & Kunka, 1987; Bennik et al., 1997). Genetic determinants for the pediocin PA-1 production are located on a 9.3kb plasmid: pRSQ11. The pediocin PA-1 operon consists of four open reading frames: pedA, pedB, pedC & pedD (Fig.:1).

Figure 1

Figure 1: Organization of pediocin PA-1 gene cluster, structural genes are shown as bars, promoter as black arrowhead and rho-independent terminator as lollipop symbol (Ray et al., 2001)



Pediocin PA-1 structural gene i.e. pedA encodes a 62 amino acid precursor peptide which is posttranslationally modified (by removal of the 18 amino acid leader peptide) into an active 44 amino acid mature peptide. Precursor peptides are having a conserved processing site 'Gly-Gly' at positions -1 and -2 (Horn et al., 1998). PedB gene product (112 amino acid) is involved in the immunity of producer strain against its own secreted bacteriocin. PedC (174 amino acid) and

PedD (724 amino acid), both of these gene products are required for the translocation and secretion of the active molecules outside the cell (Marugg et al., 1992 and Venema et al., 1997).

PCR and plasmid profiles of the producer strains have already linked plasmid-encoded secretion of pediocin AcH (Bhunja et al, 1994). PCR based detection of pediocin biosynthesis genes in certain strains of *Pediococcus* sp., using a single set of primers has been reported in a number of studies (Bennik et al, 1997; Rodriguez et al, 1997; Kim et al, 2000).

Pediocin PA-1 and pediocin AcH producing *P. acidilactici* PAC 1.0 and *P. acidilactici* H strains respectively show a similar genetic organization of the pediocin operon. Mora et al (1997) devised a 16S rRNA and *ldhD* gene targeted multiplex PCR analysis using a couple of primer sets designed from sequences of D-lactate dehydrogenase from *P. acidilactici* and 16S rRNA from strains of *P. acidilactici* and *P. pentosaceus*. Multiplex PCR assay thus distinguished 6 strains of *P. acidilactici*, and 9 strains of *P. pentosaceus* individually, from other similar species viz. *P. parvulus*, *P. damnosus*, *Lactobacillus pentosus*, *Lb. casei*, *Lb. rhamnosus*, *Lb. delbrueckii*, *Lb. helveticus*, and *Streptococcus thermophilus*. Further development in multiplex PCR successfully discriminated among pediocin PA-1/pediocin AcH producer strains of *Pediococci*, when three set of primers targeted against *pedA*, *pedB*, *pedC*, *pedD*, and *ldhD* genes were designed (Mora et al, 1998).

A Bac⁺ *Pediococcus acidilactici* MTCC 5101 strain was isolated from chilly pickle by pourplating method (Dr. Bibek Ray, Personal Communication) and by spot-on-lawn assay (Sarkar & Banerjee, 1996). The strain was identified on the basis of its physiological growth parameters and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology – 9th edition and by comparing total cellular protein profiles using SDS-PAGE (Kerstens, 1985; Holt et al, 1994). The inhibitory activity of the strain MTCC 5101 was attributed to a low molecular weight, highly thermostable and anti-listerial pediocin CP2 of 3kDa. Antimicrobial range of the pediocin produced by isolate MTCC 5101 was tested against twenty-four strains including bacteria, molds and fungi (Kaur and Balgir, 2004). Isolation of plasmid DNA from MTCC 5101 isolate was carried out to determine its molecular weight. Plasmid curing experiments were conducted using MRS medium containing ethidium bromide to unravel the localization of pediocin operon on plasmid DNA in *P. acidilactici* MTCC 5101.

MATERIALS AND METHODS

The material for the present study comprised of a standard plasmid molecular weight marker *Escherichia coli* V517 (MTCC131) procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. MRS medium, LB medium and other chemicals required for plasmid DNA isolation, curing and agarose gel electrophoresis were procured from Hi-media.

Isolation of plasmid DNA was carried out using overnight grown broth cultures of *Pediococcus acidilactici* MTCC 5101 and *Escherichia coli* V517, from MRS and Luria broth respectively. dl-threonine at a concentration of 10µg/ml was added to MRS medium to culture *Pediococcus acidilactici* MTCC 5101, that induces protoplast formation which became sensitive to lysozyme and SDS induced cell lysis. The cells were harvested by centrifugation of 3ml culture broths in eppendorfs for 5 min at 5000 rpm. *E. coli* V517 pellets were subjected to plasmid isolation as per standard protocol of Birnboim and Doly (1979). For the isolation of plasmid DNA from *Pediococcus* isolate standard protocol of Anderson & McKay (1983) was followed with little modifications.

Plasmid curing was accomplished through a growth medium containing ethidium bromide. A 1% (v/v) inoculum of *P. acidilactici* MTCC 5101 (18 hour old culture) was added to sterile MRS broth containing 20µg/ml ethidium bromide. After incubation at 37°C for 24h, subculturing was carried out using 1% (v/v) inoculum in MRS broth lacking ethidium bromide and it was incubated for another 24h at 37°C. Then, the culture was serially diluted and plated onto MRS agar plates, which were incubated at 37°C for 24h (Gupta and Batish, 1992). Colonies were selected at random and tested for their Bac⁻ characteristics by performing Agar-spot assay (Sarkar & Banerjee, 1996). Cured MTCC 5101 derivatives were then grown in MRS broth and plasmid isolation was carried out as described above.

Agarose gel electrophoresis of plasmid preparations of both Bac⁺ *P. acidilactici* MTCC 5101, cured MTCC 5101 derivatives and *E. coli* V517 was carried out in 0.6% agarose gels using 1X TBE buffer system (10X TBE buffer-0.89M Tris base, 0.89M Boric acid, 0.02M Na₂-EDTA.2H₂O, pH-8.3). Electrophoresis was carried out at 50V for 1.5-2h. Plasmid profiles were stained with ethidium bromide, visualized by placing on a UV transilluminator and photographed.

PCR was carried out to characterize the thermostable and

anti-listerial pediocin CP2 produced by *P. acidilactici* MTCC 5101. Pediocin PA-1 specific primers (Rodriguez et al., 1997), designed from the single strand DNA sequence of the region of *P. acidilactici* PAC1.0 plasmid containing pediocin PA-1 genes were used in the experiment. The DNA sequences of the two primers i.e. coding strand primer (primer G1) was 5' – AAA ATA TCT AAC TAA TAC TTG – 3', while that of the complementary strand primer (primer A2) was 5' – TAA AAA GAT ATT TGA CCA AAA-3'. The 5' end of primer G1 is 91 nucleotides upstream from the start of *pedA* and the 5' end of primer A2 is 33 nucleotides downstream from the start of *pedC*. The primers used in PCR amplified a 711 bp DNA fragment containing *pedA* and *pedB* genes from plasmid DNA of the *Pediococcus acidilactici* PAC1.0. The primers G1 and A2 were synthesized and supplied by Bangalore Genei Pvt. Ltd., India, at a concentration of 66 and 70nM respectively. DNA concentration of the plasmid preparation so obtained was calculated as per standard equation.

PCR was carried out in 50µl reaction volumes in a sterile 200µl PCR tubes. DNA amplification core kit supplied by Bangalore Genei Pvt. Ltd. India, was used. The PCR reaction mixture comprised of 500ng plasmid DNA, 50 M of each dNTPs, primer G1 and A2 at 500pM concentrations, and 1.5 units of Taq DNA polymerase. Standard protocol of Rodriguez et al. (1997) with slight modifications was used to amplify *pedA*-*pedB* gene fragment from pCP289. It consisted of an initialization step (1 cycle) at 95°C for 3 min; PCR amplification (29 cycles) at 92°C for 2 min, 45°C for 2 min and 72°C for 2 min; and final extension (1 cycle) of the growing fragments at 92°C for 2 min, 45° C for 2min, and 72°C for 10 min respectively. Amplified ssDNA was allowed to form dsDNA by complementary base pairing by cooling at 4°C for 5 min.

Amplification was carried out in techne thermal cycler (programmed as described above) and visualized in 1% agarose gels. The gels were electrophoresed at 100v for 1h using 5 µl λx174 dna/hae iii digest as a standard molecular weight marker. On completion of the run, the gel was stained with ethidium bromide, and visualized.

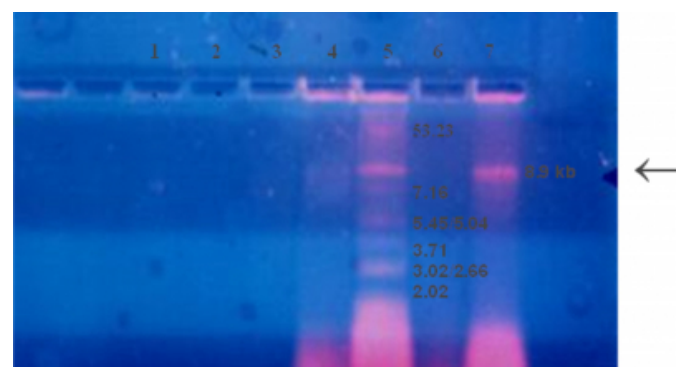
RESULTS AND DISCUSSION

Currently, pediocin pa-1 is one of the well-studied lab bacteriocins. After nisin, pediocin pa-1 will probably be the second lab bacteriocin to find practical applications as a biopreservative in the food industry, especially because of its attractive inhibitory spectrum. Pcr based molecular method

employed in the study may rapidly detect lab strains associated with different food substrates that has the potential to produce pediocin pa-1. The plasmid profiles of *p. Acidilactici* mtcc 5101 and a standard ccc mw marker *e. Coli* v517 are shown in fig.2. Isolate mtcc 5101 contained a single 8.9 kb plasmid and *bac*⁺ derivatives of the isolate that have lost their anti-listerial and anti-bacterial property were devoid of this plasmid designated as *pcp289*. Thus, curing experiments carried out with isolate mtcc 5101 successfully correlated the association of 8.9 kb plasmid with pediocin *cp2* activity.

Figure 2

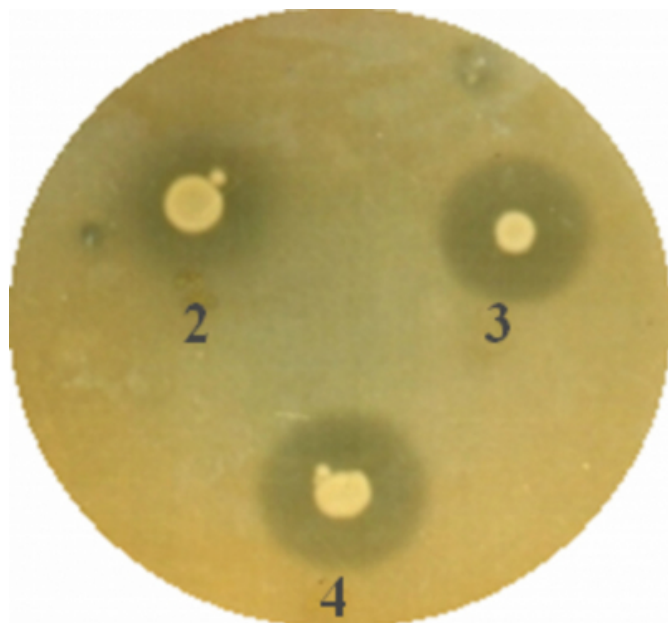
Figure 2: The plasmid profile of MTCC 5101 and its plasmid cured derivatives. The 8.9 kb plasmid is indicated by an arrow. Lane 1, 2, 3 and 6: *Bac*⁺ mutant strains of MTCC 5101; Lane 5: molecular size markers prepared from V517 containing 53.23, 7.16, 5.45, 5.04, 3.91, 3.02, 2.66, 2.02 Kb plasmids; Lane 4, and 7: Wild type strain of MTCC 5101.



Loss of selected phenotype i.e. *Bac*⁺ character was invariably reported in the test culture *Pediococcus acidilactici* MTCC 5101, after treating with ethidium bromide and subsequent sub-culturings in fresh growth medium lacking ethidium bromide. The loss of *Bac*⁺ phenotype is concomitant with the loss of specific plasmid. In fig.3 colonies having zone of inhibition lacking a clear-cut boundary represent the cured derivatives that had lost their *Bac*⁺ character. Curing experiments using ethidium bromide yielded approx. 13% cured derivatives of the isolate MTCC 5101.

Figure 3

Figure 3: Agar-spot assay demonstrating bacteriocin activity as seen by a clear zone of inhibition around spots 3, and 4. The two spots contained Bac MTCC 5101. The spot 2 is Bac variant of MTCC 5101, which was isolated after curing the culture with ethidium bromide.

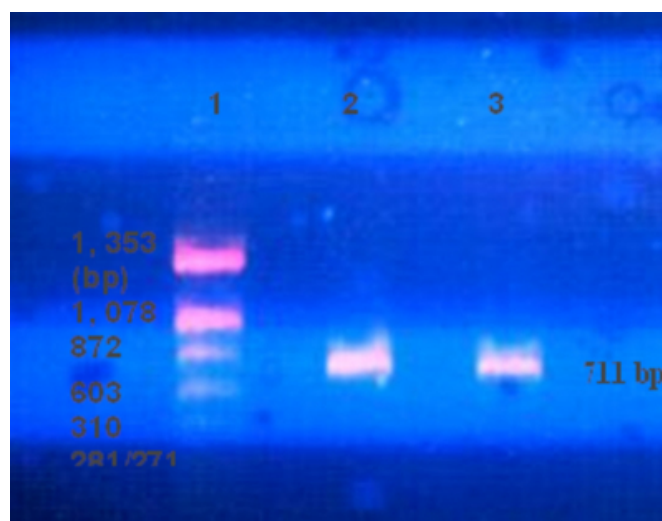


These observations are consistent with findings of several other workers who also reported loss of Bac⁺ activity after curing of selected plasmids in *Pedococcus*. Ray et al. (1989a and 1989b) indicated by plasmid curing experiments and plasmid profile analysis that a 7.4 Mdal plasmid is involved with bacteriocin activity in *P. acidilactici* strain H isolated from fermented sausages. Hoover et al. (1988) also conformed the association of pediocin PO2 production with a 5.5 MDa plasmid in *P. acidilactici* PO2.

Pediocin PA-1 specific primers used in the present study amplified 711 bp DNA containing ped A and ped B genes from plasmid DNA preparation of the ped⁺ *P. acidilactici* MTCC 5101. Size of the amplified fragment is detected by comparing it with standard MW marker λ x174/Hae III digest DNA. The amplified product appeared at a region between the band 3 (872 bp) and band 4 (603bp) of standard MW marker (fig.4). Thus, gene for pediocin PA-1 variant pediocin CP2 is localized on the pCP289 of *Pedococcus acidilactici* MTCC 5101.

Figure 4

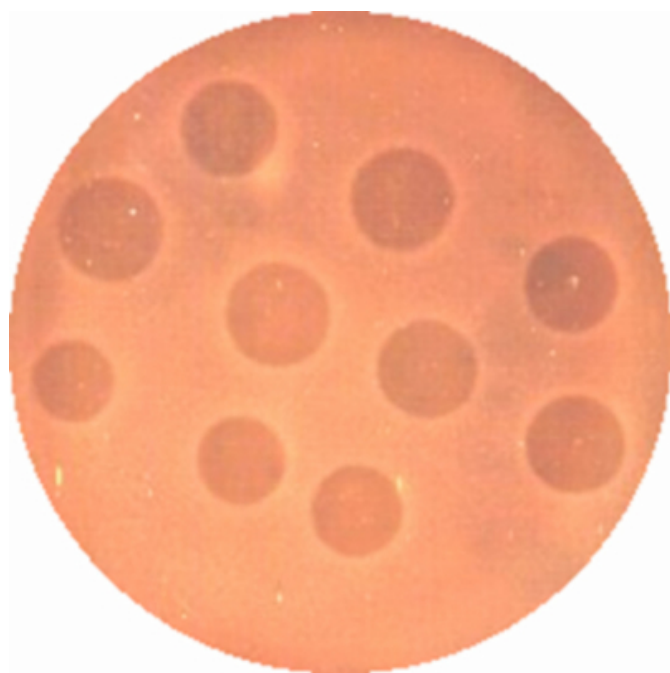
Figure 4: PCR based detection of Pediocin PA-1 production gene in plasmid preparations. Lane 2 and 3. MTCC 5101, and Lane 1. X 174/Hae III digest.



Pedococcus acidilactici MTCC 5101 isolate displayed activity against a number of spoilage, and opportunistic pathogens of interest in the food industry. It was found to inhibit food borne pathogens viz. *Listeria monocytogenes*, *Enterococcus faecalis*; opportunistic pathogens such as *Pseudomonas putida* and *Pseudomonas aeruginosa* (Fig.5); dental caries causing *Streptococcus mutans*; mucosal infections causing *Neisseria mucosa*; food spoilage bacteria including *Leuconostoc mesenteroides*, *Clostridium sporogenes*; apart from some LAB strains such as *Pedococcus acidilactici* LB42, *Pedococcus pentosaceus*, *Lactobacillus brevis* and a fungal strain of *Aspergillus flavus* (Kaur, and Balgir, 2004). The bactericidal activity of the isolate MTCC 5101 was attributable to a highly thermostable protein designated as pediocin CP2. Pediocin CP2 was relatively stable to treatment with organic solvents and retains its bactericidal activity at pH ranges between 2.5 to 9.0 as compared to pediocin PA-1 with pH stability range of 4.0 to 7.0 only. These properties indicate a wider spectrum of application for pediocin CP2 as compared to pediocin PA-1 due to its increased stability to temperature and pH. Development of application needs further investigation.

Figure 5

Figure 5: Spot-on-lawn assay to demonstrate inhibition of LB42 by pediocin CP2.



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References

- r-0. Anderson DG, and McKay LL. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Applied and Environmental Microbiology*. 1983; 46(3): 549-552.
- r-1. Bennik MHJ, Smid EJ, and Gorris LGM. Vegetable associated *Pediococcus parvulus* produces pediocin PA-1. *Applied and Environmental Microbiology*. 1997; 63: 2074-2076.
- r-2. Bhunia AK. Monoclonal antibody-based enzyme immunoassay for Pediocins of *Pediococcus acidilactici*. *Applied and Environmental Microbiology* 1994; 60: 2692-2696.
- r-3. Bhunia AK, and Johnson MC. Monoclonal antibody-colony immunoblot method specific for isolation of *Pediococcus acidilactici* from foods and correlation with Bacteriocin production. *Applied and Environmental Microbiology*. 1992; 58: 2315-2320.
- r-4. Birnboim HC, and Doly J. *Nucleic Acid Research*. 1979; 7: 1513.
- r-5. Bukhtiyarova M, Yang R, and Ray B. Analysis of Pediocin AcH gene cluster from plasmid pSMB 74 and its expression in a pediocin-negative *Pediococcus acidilactici* strain. *Applied and Environmental Microbiology*. 1994; 60: 3405 - 3408.
- r-6. Buyong N, Kok J, and Luchansky JB. Use of a genetically enhanced, pediocin-producing starter culture, *Lactococcus lactis* subsp. *lactis* MM217, to control *Listeria monocytogenes* in Cheddar Cheese. *Applied and Environmental Microbiology*. 1998; 64: 4842-4845.
- r-7. Fleming HP, Etchells JL, and Costilow RN. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Applied and Environmental Microbiology*. 1975; 30: 1040-1042.
- r-8. Gonzalez CF, and Kunka BS. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. *Applied and Environmental Microbiology*. 1987; 53: 2534 - 2538.
- r-9. Grahm DC, and McKay LL. Plasmid DNA in strains of *Pediococcus cerevisiae* and *Pediococcus pentosaceus*. *Applied and Environmental Microbiology* , 1985; 50: 532-534.
- r-10. Gupta R K, and Batish V K. Genetic evidence for plasmid-encoded lactococcin production in *Lactococcus lactis* subsp. *lactis* 484. *Current Microbiology*. 1992; 42: 231-238.
- r-11. Holt JG, Krieg NR, Sneath PHA, Staley JT, and Williams ST (eds.). *Bergey's Manual of Determinative Bacteriology* – 9 th edition. Williams and Willkins Publications, USA, 1994.
- r-12. Hoover DG, Walsh PM, Kolaetis KM, and Daly MM. A Bacteriocin produced by *Pediococcus* species associated with a 5.5-megadalton plasmid. *Journal of Food Protection*. 1988; 51: 29-31.
- r-13. Horn N, Martinez MI, Martinez JM, Hernandez PE, Gasson MJ, Rodriguez JM, and Dodd HM. Production of pediocin PA-1 by *Lactococcus lactis* using the lactococcin A Secretory Apparatus. *Applied and Environmental Microbiology*. 1998; 64: 818-823.
- r-14. Horn N, Martinez MI, Martinez JM, Hernandez PE, Gasson MJ, Rodriguez J M, and Dodd HM. Enhanced production of pediocin PA-1 and co-production of Nisin and pediocin PA-1 by *Lactococcus lactis*.. *Applied and Environmental Microbiology*. 1999; 65(10): 4443-4450.
- r-15. Kaur B, and Balgir PP. Biopreservative Potential of a Broad – Range Pediocin CP2 obtained from *Pediococcus acidilactici* MTCC 5101. *Asian Journal of Microbiology, Biotechnology, and Environmental Sciences* –in press.
- r-16. Kaur, B. Purification, characterisation and molecular genetics of bacteriocin obtained from an isolate of *Pediococcus* spp. Ph.D. Thesis, Department of Biotechnology, Punjabi University, Patiala, Punjab, India, 2002.
- r-17. Kersters K. In: M Goodfellow, D Jones, and FG Priest (eds.). *Numerical Methods in the Classification of Bacteria by Protein Electrophoresis, in Computer-Assisted Bacterial Systematics*. Academic Press, London, 1985; 337-365.
- r-18. Kim C H, Ji G E, and Ahn C. Purification and molecular characterization of a bacteriocin from *Pediococcus* sp. KCA 1303-10 isolated from fermented flat fish. *Food Science and Biotechnology*. 2000; 9(4): 270-276.
- r-19. Marugg JD, Gonzalez CF, Kunka BS, Ledeboer AM, Pucci MJ, Toonen MY, Walker SA, Zoetmulder LCM, and Vandenbergh PA. Cloning, expression and nucleotide sequence of genes involved in production of Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC 1.0. *Applied and Environmental Microbiology*. 1992; 58: 2360-2367.
- r-20. Miller KW, Schamber R, Chen Y, and Ray B. Production of active chimeric Pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus* Pap operon. *Applied and Environmental Microbiology*. 1998a; 64: 14-20.
- r-21. Miller KW, Schamber R, Osmanagaoglu O, and Ray B. Isolation and characterization of Pediocin AcH chimeric protein mutants with altered bactericidal activity. *Applied and Environmental Microbiology*. 1998b; 64: 1997-2005.

- r-22. Mora D, Fortina MG, Parini C, and Manachini. Identification of *Pediococcus acidilactici* and *P. pentosaceus* based on 16S rRNA and *ldh -D* gene targeted multiplex PCR analysis. *FEMS Microbiology Letters*. 1997; 151: 231-236.
- r-23. Mora D, Parini C, Fortina MG, and Manachini PL. Discrimination among pediocin AcH/PA-1 producer strains by comparison of *pedB* and *pedD* amplified genes and multiplex PCR assay. *Systematic and Applied Microbiology*. 1998; 21: 454-460.
- r-24. Ray B, Miller KW, and Jain MK. Bacteriocins of lactic acid bacteria : Current prospectives. *International Journal of Microbiology*. 2001; 41: 1-21.
- r-25. Ray SK, Johnson MC, and Ray B. Bacteriocin plasmids of *Pediococcus acidilactici*. *Journal of Industrial Microbiology* . 1989a ; 4(3): 163-171.
- r-26. Ray SK, Kim WJ, Johnson MC, and Ray B. Conjugal transfer of a plasmid encoding bacteriocin production and immunity in *Pediococcus acidilactici* H. *Journal of Applied Bacteriology*. 1989b; 66: 393-399.
- r-27. Rodriguez JM, Cintas LM, Casaus P, Martinez MI, Sourez A, and Hernandez PE. Detection of pediocin PA-1 producing *Pediococci* by rapid molecular biology techniques. *Food Microbiology*. 1997 ; 14: 363-371.
- r-28. Sarkar PK, and Banerjee S. Antibacterial activity of lactic acid bacterial isolates obtained from natural habitats. *Journal of Food Science and Technology*. 1996; 33 (3): 231-233.
- r-29. Schoeman H, Vivier MA, duToit M, Dicks LMT, and Pretorius IS. The development of bactericidal yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (*ped A*) in *Saccharomyces cerevisiae*. *Yeast*.. 1999; 15(8): 647-656.
- r-30. Tagg JR, Dajani AS, and Wannamaker LW. Bacteriocins of gram-positive bacteria. *Bacteriology Reviews*. 1976; 40: 722-756.
- r-31. Venema K, Chikindas ML, Seegers JFML, Haandrickman AJ, Leenhouts KJ, Venema G, and Kok J. Rapid and efficient purification method for small, hydrophobic cationic bacteriocins: Purification of Lactococcin B and Pediocin PA-1. *Applied and Environmental Microbiology*. 1997; 63: 305-309.

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