academicJournals

Vol. 7(47), pp. 5338-5342, 28 November, 2013
DOI: 10.5897/AJMR12.2004
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Use of combination of bacteriocins from *Lactobacillus plantarum* MTCC 1407 and *Bacillus coagulans* MTCC 492

Garcha S* and Sharma N

Department of Biotechnology, Punjabi University, Patiala 147002, India.

Accepted 6 November, 2013

Bacteriocins are antimicrobials produced mainly by lactic acid bacteria as well other genera, a property which can be exploited in food biopreservation. However, narrow spectrum of activity of these bacteriocins is a limitation for their use in different food systems. Various approaches are being pursued to increase their antimicrobial efficacy like use of increased dosage, use of highly purified form in combination with other preservative techniques such as HPP, ultrasonic waves, use of a combination of bacteriocins, etc. Bacteriocins from two producers namely *Lactobacillus plantarum* MTCC 1407 and *Bacillus coagulans* MTCC 492 were used in the present study. Partially purified form of bacteriocin produced by them was tested individually as well as in combination with antimicrobial activity in liquid medium against food spoilage agents, Gram positive organisms such as *Streptococcus thermophilus, Leuconostoc mesenteroides, Micrococcus flavus* and also Gram negative organisms such as *Escherichia coli and Pseudomonas aeruginosa.* They were all susceptible to antimicrobial action of these bacteriocins, 26 to 72% inhibition was recorded with turbidometric method of assessment of bacteriocin activity. However, bacteriocins when used in combination of 1:1 (v/v) did not result in increased inhibition.

Key words: Bacteriocins, synergy, *Lactobacillus* sp., *Bacillus* sp., food spoilage agents.

INTRODUCTION

Despite modern advances in technology, the preservation of food is still a debated issue in developing as well as industrialized countries. Alleviation of economic losses due to food spoilage, lowering the food processing costs and avoiding transmission of microbial pathogens through the food chain while satisfying the growing consumers demands for food that are ready-to-eat, fresh tasting, nutrient and vitamin rich and minimally processed and preserved are the major challenges of the current food industry (Mangaraj and Singh, 2008). Consumers are concerned with possible adverse health effects of the presence of chemical additives in their foods (Soomro et al., 2002).

Reduction or inhibition of unwanted food based microorganisms by biological means is thus gaining importance. Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and their antibacterial products (Ross et al., 2002). Lactic acid bacteria have a major potential for use in biopreservation because they are safe to consume and during storage they naturally dominate the microflora of many foods. Lactic acid bacteria have been granted generally regarded as safe (GRAS) status (Stiles, 1996). Bacteriocins is their antimicrobial metabolite which has

*Corresponding author. E-mail: sgarcha@pbi.ac.in or seemagarcha@gmail.com. Tel: 91-0175-304-626306262. Fax: 91-0175-2283073.

potential to control the growth of spoilage and pathogenic bacteria in foods (O`Sullivan et al., 2002).

The term 'bacteriocins' was coined in 1953 to define colicin produced by *Escherichia coli*. They are ribosomally synthesized, extra-cellularly released low molecular mass proteins which have bactericidal or bacteriostatic effect on other micro-organisms (Klaenhammer, 1988; Tagg et al., 1976) either of the same species or other genera (Cotter et al., 2005). The first bacteriocin was discovered in 1925 by Gratia (Garneau et al., 2002). Majority of bacteriocin producers are *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp. and *Leuconostoc* spp. (De Vuyst and Vandamme, 1994) but those produced by *Lactococcus*, *Streptococcus*, *Clostridium* and *Carnobacterium* were also described.

Applications of bacteriocins for the control of some pathogens and food spoilage organisms has been approved in a number of countries (Cleveland et al., 2001; O'Sullivan et al., 2002; Chen and Hoover, 2003; Cotter et al., 2005; Fimland et al., 2005; Deegan et al., 2006; Drider et al., 2006). Advances in bacteriocins research and combination treatment for food preservation will benefit both the producer and consumer. The only bacteriocin given GRAS status is nisin (Federal Register, 1988). It is commercially used in food systems (Settani and Corsetti, 2008).

Bacteriocins are suitable for food preservation and recent studies conducted suggest that their use offers a lot of advantages such as a) extend shelf life, b) provide protection especially during times of temperature abuse, c) decrease the risk of transmission of food borne pathogens, d) decrease the losses due to food spoilage, e) reduce the application of chemical preservatives, f) permit the application of less severe heat treatment without compromising food safety (Hurdle Concept). Bacteriocins are non-toxic to eukaryotic cells and hence pose no threat to human intestinal cells. Being proteinaceous in nature they are readily degraded by protelytic enzymes in human gastro-intestinal tract. Moreover, they do not have any therapeutic application and are not known to cause allergies. Being of LAB origin they are probiotic in nature and also help in restoring the normal gut microflora (Thomas et al., 2000).

Lactobacillus spp. is an important bacteriocin producer. Bacteriocins produced by them exhibit bactericidal mode of action (Klaenhammer, 1988). They are effective against *E. coli* (Lade et al., 2006; Torodov and Dicks, 2004; Caridi, 2002), *Acinetobacter baumanii* (Torodov and Dicks, 2005); *Aeromonas hydrophila* (Messi et al., 2001); *Listeria monocytogenes, Staphylococcus aureus, Enterococcus faecalis, E. coli, Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (Miteva et al., 1998; Ennahar et al., 1996).

Bacillus spp. is comparatively a new entry to the list of bacteriocin producers. Their bacteriocins are less worked on as compared to bacteriocins from lactic acid bacteria. Most of them are lantibiotics (Abriouel et al., 2011). Production of bacteriocins has been detected in *Bacillus subtilis*, *Bacillus cereus*, *Bacillus stearothermophilus* and other Bacilli (Bizani and Brandelli, 2002). The bacteriocins produced by them have a broad spectrum, being effective against food borne pathogens such as *Streptococcus pyogenes* (Cherif et al., 2001). Lichenin produced by *Bacillus licheniformis* and megacin produced by *Bacillus megaterium* have been well characterized (Lisoba et al., 2006).

As stated earlier, bacteriocins have a narrow spectrum of activity. They are generally effective against closely related species (Cotter et al., 2005). They are effective against Gram negative organisms as well but only when used in high concentrations (Deegan et al., 2006). Also, most of them are ineffective or effective to a very less degree against yeast and molds which limits their use in food systems (Dalie et al., 2010). Efficacy of bacteriocins can be enhanced by using them in combination with other chemicals and other preservative techniques (Cleveland et al., 2001). Use of a combination of bacteriocins can broaden the antimicrobial spectrum and also prevent the emergence of bacteriocin resistant strains. The present investigation was carried out to elucidate the *in vitro* antimicrobial spectrum of bacteriocins produced by *Lactobacillus plantarum* MTCC 1407 and *Bacillus coagulans* MTCC 492 and investigate whether the use of a combination of them can be of advantage.

MATERIALS AND METHODS

L. plantarum MTCC 1407 and *B. coagulans* MTCC 492 were used as bacteriocin producers in the present study. The indicator organisms chosen were *Streptococcus thermophilus* MTCC 1928, *Leuconostoc mesenteroides* MTCC 107, *Micrococcus flavus* ATCC 10240, *E. coli* MTCC 1650 and *Pseudomonas aeruginosa* ATCC 10662. They were purchased from Microbial Type Collection Collection at Institute of Microbial Technology, India and maintained on their respective recommended media (Table 1).

The growth kinetics of the producer organisms was plotted spectrometrically at 600 nm and various growth phases identified. Bacteriocin production is related to growth phases of the producer. It was purified from broth culture at various stages of growth. Bacteriocin was purified by the method of Allende et al. (2007). Broth cultures were centrifuged at 5000 rpm for 10 min at 5°C using a cooling centrifuge (REMI C30). The supernatant was neutralized using 2N NaOH and then filter sterilized using membrane filters of pore size 0.22 µ. Subsequently it was heated at 80°C for 3 min. Lactic acid and other organic acids were neutralized by NaOH. H_2O_2 is degraded by heating to minimize the chances of getting a false positive result. This partially purified bacteriocin preparation was kept at -10°C throughout the time period of this study.

Bacteriocin assay was performed by turbidometric method of Turcotte et al. (2004) also known as percentage inhibition method with minor modifications. Suspension of metabolically active cells of the indicator organisms was prepared. It was calibrated to contain a population corresponding to McFarland Standard 0.5 (approximate cell density of 1.5×10^8 cells/ml). Bacteriocin preparation and indicator suspension were mixed in ratio 1:1 (v/v). Sterile broth was used as diluting medium to maintain the total volume of reaction mixture in the control run. The reaction mixtures were incubated for

Table 1. Details of microbial cultures used in the present investigation.

Organism	Recommended medium	Optimum temperature/pH	Incubation time (h)
Lactobacillus plantarum MTCC 1407	MRS	30°C/6.5±0.2	48
Bacillus coagulans MTCC 492	Nutrient Agar	37°C/7±0.2	24
Streptococcus thermophilus MTCC 1928	Brain Heart Infusion	37°C/7.0±0.2	48
Leuconostoc mesenteroides MTCC 107	MRS Agar	25°C/7.0±0.2	48
Micrococcus flavus ATCC 10240	Nutrient Agar	30°C/7.0±0.2	48
Escherichia coli MTCC 1650	Nutrient Agar	37°C/7.0±0.2	24
Pseudomonas aeruginosa MTCC 10662	Nutrient Agar	25°C/7.0±0.2	48

6 h (bacteriocin from *B. coagulans*) and 12 h (bacteriocin from *L. plantarum*). Taking A_m to be the absorbance of the sample recorded at 700 nm and A_o as absorbance of the control, percentage inhibition was calculated as per the formula:

Inhibition (%) = $1- A_m/A_o \times 100$

Use of combination of bacteriocins

Bacteriocin preparations from both organisms were mixed in equal amounts and assay performed as explained above against food spoilage organisms.

RESULTS AND DISCUSSION

The results presented are an average of multiple trials. The percentage inhibition mentioned is an average of at least five consistent recordings. As stated earlier, bacteriocin preparation prepared from one batch was used throughout the study. Initially, the various growth phases of the producer strains were indentified (data not shown). Bacteriocin was partially purified from various growth phases and assay was performed. Bacteriocin preparation exhibiting maximum inhibition was used for antimicrobial spectrum assays. *B. coagulans* MTCC 492 produced maximum amount of bacteriocin at 12 h of incubation. *L. plantarum* MTCC 1407 produced maximum amount of bacteriocin at 20 h of incubation.

Bacteriocin producers *L. plantarum* MTCC 1407 and *B. coagulans* MTCC 492 used in the present study exhibited secondary metabolite kinetics. Generally, bacteriocin production by bacteria is a growth associated process (Leory and De, 2002). It displays secondary metabolite kinetics (Ogunbanwo et al., 2003). Bacteriocin production in the early stationary phase is a characteristic feature of lactic acid bacteria (Tiwari and Srivastava, 2008). Like the lactic acid bacteria (LAB) some representatives of *Bacillus* spp., such as *B. subtilis* and *B. licheniformis*, also produce maximum bacteriocin in stationary phase (Sharp et al., 1992).

The antimicrobial spectrum of the bacteriocins from these two organisms was determined by percentage inhibition method (Table 2). It was chosen over well-diffusion assay which has some disadvantages of medium composition, poor diffusion by bacteriocins, concentration of bacteriocin in the extract, etc. which may result in false negatives. Partially purified bacteriocin of both producers, *L. plantarum* and *B. coagulans* were effective to varying degree against *S. thermophilus* MTCC 1928*, L. mesenteroides* MTCC 107 and *M. flavus* ATCC 10240*.* They also inhibited the growth of Gram negatives such as *E. coli* MTCC 1650 and *P. aeruginosa* ATCC 10602*.*

By and large, *L. plantarum*`s bacteriocin exhibited greater efficacy than bacteriocin produced by *B. coagulans*. Generally, the antibacterial activity of most bacteriocins is directed against species that are closely related to the producer and also against a number of other less closely related bacteria including spoilage bacteria (Schillinger et al., 1991; Ennahar et al., 1996; Rekhif et al., 1995).

A number of studies are being conducted to enhance the antimicrobial spectrum of the bacteriocins for greater application in food systems. Use of more than one bacteriocin is one such approach. The two bacteriocin preparations prepared in the present study were mixed in equal volume and assay performed (Table 2). Combination of these bacteriocins in equal ratio did not result in any appreciable increase in antibacterial activity in this investigation. However, no trend could be established.

Mainly synergistic effects have been reported between pairs of bacteriocins from lactic acid bacteria. Mulet-Powel et al. (1998) reported synergism and were the first ones to report antagonism with combination of bacteriocins. Their study dealt with different bacteriocins (nisin produced by *Lactococcus lactis*, pediocin AcH produced by *Pediococcus acidilactici*, lacticin 481 produced by *L. lactis*, lactacin F produced by *Lactobacillus johnsonii* and lactacin B produced by *Lactobacillus acidophilus*) against 10 different indicator strains. They could not assign any reason for antagonism of different pairs of bacteriocins. Increased antibacterial activity of combination than when used alone has been reported by Hanlin et al. (1993). Bacteriocins were obtained from four producers- *L. lactis*,

Table 2. Percentage inhibition of indicator organisms upon use of bacteriocins of *L. plantarum* MTCC 1407 and *B. coagulans* MTCC 492 independently and in combination.

Indicator organism	Inhibition by bacteriocins of *Lactobacillus plantarum* MTCC 1407 (%)	INHIBITION by bacteriocins of *Bacillus coagulans* MTCC 492 (%)	Inhibition using a combination of bacteriocins (%) (1:1 v/v)
Streptococcus thermophilus MTCC 1928	50	39	53
Micrococcus flavus ATCC 10240	36	32	39
Leuconostoc mesenteroides MTCC 107	65	32	67
Pseudomonas aeruginosa ATCC 10662	63	26	66
Escherichia coli MTCC 1650	72	51	74

P. acidilactici, *Lactobacillus sake* and *Leuconostoc carnosum.* Indicators strains used were *Lactobacillus plantarum* NCDO 955, *L. mesenteroides* Ly, *P. acidilactici* LB-42, *E. faecalis* MB1 and *L. monocytogenes* strains CA and Scott A. Synergism has also been observed by Vignolo et al. (2000). The antilisterial efficiency of three bacteriocins from lactic acid bacteria, lactocin 705 (produced by *L. casei* CRL705), enterocin CRL35 (produced by *E. faecium* CRL35), and nisin, was tested in the broth, individually and in combination against *L. monocytogenes* and *Listeria innocua*.

Antimicrobial action of bacteriocins occurs in steps-adsorption of the bacteriocin on cell wall, its transport across the cell membrane and finally its action within the cytoplasm. Bacteriocins are cationic proteins and their primary receptors are anionic lipids (O`Sullivan et al., 2002). Presence of receptors on cell surface plays a role in bacteriocin specificity (Drider et al., 2006). Synergistic effect occurs when receptor for one bacteriocin is not present but receptor for another bacteriocin is available for antibacterial action. Antagonism can occur when the bacteriocin producers compete for the same receptors on indicator cell surface.

Bacteriocins which belong to different categories and with different mode of actions are likely to exhibit synergistic effect (Vignolo et al., 2000). They suggested the use of combination of bacteriocins belonging to different classes to obtain enhanced activity. Both the bacteriocins used in this study belong to same class. As per the classification proposed by Abriouel et al. (2011), bacteriocins from *Bacillus coagulans* have been classified as belonging to class IIa which are non-modified pediocin like bacteriocins with antimicrobial activity against *Leuconostoc, Oenococcus, Listeria, Pediococcus* and *Enterococcus.* Bacteriocins from *L. plantarum* are generally classified as class IIb. They are small, ≤10 KDa, heat stable, non-lanthinone containing peptides (Chen and Hoover, 2003).

Further work on understanding of mechanism of interaction of bacteriocins is in progress. Use of combination of other bacteriocins from same class and different classes is being pursued. Amino acid sequencing of highly purified form of the bacteriocins can give a greater insight into the nature of interaction with each other.

ACKNOWLEDGEMENT

Laboratory facilities provided by the Department of Biotechnology, Punjabi University, Patiala is duly acknowledged.

REFERENCES

Abriouel H, Franz CMAP, Omar NB, Galvez A (2011). Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol. Rev. 35:201-232.

Allende A, Martinez B, Selma V, Gil MI, Suarez JE, Rodriguez A (2007). Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes in vitro* and in fresh-cut lettuce. Food Microbiol. 24:759–766.

Bizani D, Brandelli A (2002). Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Strain 8A. J. Appl. Microbiol. 93:512-519.

Caridi A (2002). Selection of *Escherichia coli* inhibiting strains of *Lactobacillus paracasei* subsp. *paracasei.* J. Ind. Microbiol. Biotechnol. 29:303-308.

Chen H, Hoover DG (2003). Bacteriocins and their food applications. Comprehensive Rev. Food Sci. Food Saf. 2: 82-100.

Cherif A, Ouzari H, Daffonchio D, Cherif H, Ben Slama K, Hassen A, Jaoua S, Boudabous A (2001). Thuricin 7: A novel bacteriocin produced *by Bacillus thuringiensis* BMG 1.7, a new strain isolated from soil. Lett. Appl. Microbiol. 32:2432-2247.

Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001). Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71:1-20.

Cotter PD, Hill C, Ross RP (2005). Batceriocins: Developing innate immunity for food. Nat. Rev. Microbiol. 3:777-788.
Dalie DKD, Deschamps AM, Richard-Forget F (2010). Lactic acid bacteria- Potential for control of mould growth and mycotoxins: a review. Food Control 21:370-380.
De Vuyst L, Vandamme EJ (1994). Bacteriocins of lactic acid bacteria: Microbiology, Genetics and Applications, Blackie Academic and Professional, London, UK.
Deegan LH, Cotter PD, Hill C, Ross P (2006). Bacteriocins: biological tools for biopreservation and shelf life extension. Int. Dairy J. 16:1058-1071.
Drider D, Fimland G, Hechard Y, McMullen LM, Prevost H (2006). The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. 70:564-582.
Ennahar S, Aoude-Warner D, Sorokine O, Van Dorsselaer A, Bringel F, Hubert JC, Hasselmann C (1996). Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. Appl. Environ. Microbiol. 62:4381-4387.
Federal Register (1988). Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Federal Register 54:11247-11251.
Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J (2005). Pediocin like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure and mode of action. J. Peptide Sci. 11:688-696.
Garneau S, Martin NI, Vederas JC (2002). Two peptide bacteriocins produced by lactic acid bacteria. Biochimie 84:577-592.
Hanlin MB, Kalchayanand N, Ray P, Ray B (1993). Bacteriocins of Lactic acid bacteria in combination have greater activity. J. Food Prot. 56:252-255.
Klaenhammer TR (1988). Bacterocins of lactic acid bacteria. Biochimie 70:337-349.
Lade HS, Chitanand MP, Gyananath G, Kadam TA (2006). Studies on some properties of bacteriocins produced by *Lactobacillus* species isolated from agro-based waste. Internet J. Microbiol. 2:1.
Leory F, De VL (2002). Bacteriocin production by *Enterococcus faecium* RZSC5. Intl. J. Food Microbiol. 70:155-164.
Lisoba MP, Bonatto D, Bizani D, Henriques JA, Brandelli A (2006). Characterization of a bacteriocin like substance produced by *Bacillus amyloliquifaciens* isolated from the Brazilian Atlantic forest. Int. Microbiol. 9:111-118.
Mangaraj S, Singh R (2008). Status of food processing industry in India and its future outlook. Indian Food Ind. 27:35-42.
Messi P, Bondi M, Sabia C, Battini R, Manicardi G (2001). Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. Int. J. Food Microbiol. 64:193-198.
Miteva V, Stefanova T, Budakov I, Ivanova I, Mitev V, Gancheva A, Ljubenov M (1998). Characterization of bacteriocins produced by strains from traditional Bulgarian dairy products. Syst. Appl. Microbiol. 21:151-161.
Mulet-Powel N, Lactoste-Armynot AM, Vinas M, Simeon de Buochberg M (1998). Interactions between pairs of bacteriocins from lactic acid bacteria. J. Food Prot. 61:1210-1212.
O`Sullivan L, Ross RP, Hill C (2002). Potential of bacteriocin producing Lactic acid bacteria for improvements in food safety and quality. Biochimie 84:593-604.
Ogunbanwo ST, Sanni AI, Onilude AA (2003). Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1. Afr. J. Biotechnol. 27:179-184.
Rekhif N, Atrith A, Lefebvre G (1995). Activity of plantaricin SA6, a bacteriocin produced by *Lactobacillus plantarum* SA6 isolated from fermented sausage. J. Appl. Bacteriol. 78:349-358.
Ross RP, Morgan S, Hill C (2002). Preservation and fermentation: past, present and future. Int. J. Food Microbiol. 79:3-16.
Schillinger U, Kaya M, Lucke FK (1991). Behavior of *Listeria monocytogenes* in meat and its control by a bacteriocin producing strain of *Lactobacillus sake*. J. Appl. Bacteriol. 70:473-478.
Settani L, Corsetti A (2008). Rev: Application of bacteriocins in vegetable food biopreservation Int. J. Food Microbiol. 121:123-138.
Sharp R, O`Donnell AG, Gilbert HG, Hazlewood GP (1992). Growth and survival of genetically manipulated *Lactobacillus plantarum* in silage. Appl. Environ. Microbiol. 58:2517-2522.
Soomro AH, Masud T, Anwaar K (2002). Role of Lactic acid bacteria in preservation and human health- a review. Pak. J. Nutr.1:20-24.
Stiles ME (1996). Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek 70:331-345.
Tagg JR, Dajani AS, Wannamaker LW (1976). Bacteriocins of Gram-positive bacteria Bacteriol. Rev. 40:722-756.
Thomas LV, Clarkson MR, Delves-Broughton J (2000). Nisin. In: Naidu, AS (Ed), Natural Food antimicrobials system. CRC Press, Boca-Raton, FL. pp. 463-524.
Tiwari SK, Srivastava S (2008). Characterization of bacteriocin from *Lactobacillus plantarum* strain LR/14. Food Biotechnol. 22:247-261.
Torodov SD, Dicks LMT (2004). Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. World J. Microbiol. Biotechnol. 20:643-650.
Torodov SD, Dicks LMT (2005). *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram negative bacteria. Enzyme Microbiol. Technol. 36:318-326.
Turcotte C, Lacroix C, Kheadr E, Grignon L, Fliss I (2004). A rapid turbidometric microplate bioassay for accurate quantifications of lactic acid bacteria bacteriocins. Intl. J. Food Microbiol. 90:283-293.
Vignolo G, Palacios J, Farias ME, Sesma F, Schillinger U, Holzapfel W, Oliver G (2000). Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. Curr. Microbiol. 41:410-416.