Probiotics & Antimicro. Prot. (2014) 6:47–58
DOI 10.1007/s12602-013-9153-z

Bacteriocin Production and Different Strategies for Their Recovery and Purification

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Published online: 5 December 2013
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Abstract Bacteriocins from lactic acid bacteria (LAB) are a diverse group of antimicrobial proteins/peptides, offering potential as biopreservatives, and exhibit a broad spectrum of antimicrobial activity at low concentrations along with thermal as well as pH stability in foods. High bacteriocin production usually occurs in complex media. However, such media are expensive for an economical production process. For effective use of bacteriocins as food biopreservatives, there is a need to have heat-stable wide spectrum bacteriocins produced with high-specific activity in food-grade medium. The main hurdles concerning the application of bacteriocins as food biopreservatives is their low yield in food-grade medium and time-consuming, expensive purification processes, which are suitable at laboratory scale but not at industrial scale. So, the present review focuses on the bacteriocins production using complex and food-grade media, which mainly emphasizes on the bacteriocin producer strains, media used, different production systems used and effect of different fermentation conditions on the bacteriocin production. In addition, this review emphasizes the purification processes designed for efficient recovery of bacteriocins at small and large scale.

Keywords Bacteriocin · By-products · Food-grade media · Cell immobilization · Purification

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Introduction

The preservation of foods is still considered as an important issue all over the world, irrespective of the developed and developing countries. The ribosomally produced peptides with antimicrobial activity from lactic acid bacteria (LAB) [25, 26] have attracted considerable attention to be used as natural food biopreservatives due to their bactericidal effect against many food spoilage and pathogenic bacteria [29, 62], without any toxic effect to human beings [22]. In addition, they are heat tolerant, active at acidic pH and offer no flavour or textural changes when used as biopreservatives in different food systems. According to Muriana [79], to inhibit pathogenic or spoilage microorganisms, bacteriocinogenic strains or partially purified bacteriocins can be added to foods. However, the effectiveness of bacteriocins may be reduced by different factors [57, 79]. First, the minimum inhibitory concentration differs widely among bacteriocins and sensitive strains [79]. Secondly, the activity spectrum of bacteriocins produced by Gram-positive bacteria is usually limited. These are not active against Gram-negative bacteria. As far as we know, only nisin and pediocins are the unique bacteriocins approved as food additives [30, 49] and are the most studied, not only because they exhibit a broad spectrum of activity, but also because they are bactericidal at low concentrations and exhibit thermal and pH stability in foods [29, 86]. Nisin, is FDA approved, used in more than 48 countries as natural food preservatives [22, 30]; however, pediocins, a group of class IIa bacteriocins produced by *Pediococcus* strains, have gained great attention in recent years and are extensively studied as well as well characterized [25, 59, 60]. Pediocin as potential food biopreservative have a wide inhibitory spectrum of activity against Gram-positive bacteria, including both spoilage and pathogenic organisms, such as *Listeria monocytogenes*, *Enterococcus*

faecalis, *Staphylococcus aureus* and *Clostridium perfringens* [31, 68, 100]. Pediocin-like bacteriocins (36–48 residues) share 40–60 % amino acid sequence similarity when the corresponding sequences are aligned. They contain two structural regions, a highly conserved N-terminal region and a less conserved C-terminal region (residues 18 and on). The N-terminal region of all pediocin-like bacteriocins are currently identified containing two cysteines, joined by a disulphide bond, in a motif known as the "pediocin box": $YGNGVX_1CX_2K/NX_3X_4C$, with X_{1-4} representing polar uncharged or charged residues [61, 89]. Among these bacteriocins, pediocin PA-1/AcH was shown to have an extra C-terminal disulphide bond that has been attributed to improve its potency at elevated temperatures and to widen its antibacterial spectrum [36]. Recently, two pediocins produced by *Pediococcus acidilactici* NCDC 252 and *Pediococcus pentosaceus* NCDC 273 (GenBank Acc No. FJ825757.1) were characterized and found identical to pediocin PA-1 at nucleotide sequence level [74, 75].

Bacteriocins like pediocin PA-1/AcH production have been extensively studied using various fermentation strategies with both free and immobilized cells in complex media [80], which promote abundant growth and relatively high bacteriocin levels; nevertheless, it seems more economical to use some of the by-product of food industry as the raw material as culture media [2, 21, 49, 51, 78, 88]. One of the main problems concerning the application of bacteriocins as food biopreservatives is their low yields and high cost of production and purification. Another is bacteriocins produced using complex media like de Man Rogosa Sharpe (MRS) medium is not food grade, so an approach need to be developed using food-grade media like by-products of dairy and food industry to produce food-grade bacteriocins. Low yields and high costs of production and purification are major bottlenecks for the commercial production of bacteriocins. Thus, for its economical use in foods, bacteriocins need to be produced in large amounts. Whey and whey permeate powders may serve as the basis of food-grade inexpensive fermentation media formulations and require minimum nutritional supplementation for the production of bacteriocins. Whey, which is a by-product of the dairy industry, provides an excellent growth medium for LAB bacteria as it has a high biological oxygen demand. It has been widely used for the production of various compounds including organic acids, single-cell protein, enzymes, ethanol [44] and bacteriocins [24, 45, 52–54, 68].

In addition, for developing bacteriocin for food biopreservation, it is necessary to produce it in purified form on a large scale. The purification at the industrial level is the main bottleneck for application of bacteriocins as biopreservative. This is due to the purification protocols which work well at laboratory-scale volumes but are not suitable at industrial scale due to expensive purification processes. In this review, we summarized and discussed all the available information regarding the bacteriocins production in complex and food-grade media and the techniques utilized for their recovery and purification.

Bacteriocin Production Using Complex and Food-Grade Media

Reports on lactic acid bacteria indicate the essential influence of temperature, pH and media composition on bacteriocin production [27, 96, 101]. Several studies have compared bacteriocin batch production by LAB strains on different complex media and have found that MRS and Elliker broths were the best media for the growth of LAB [27, 53, 82], which promote abundant growth and relatively high bacteriocin levels. However, such media are not suitable from financial point of view. In addition, bacteriocins produced using MRS media is not food-grade and some medium components (e.g. large amounts of proteins) may interfere with the subsequent bacteriocin purification. Necessity for reduction of pollutants in the environment and the need to maximize returns on raw materials have encouraged the search for new ways of using food industry and dairy industry waste as the basis of culture media. Possible alternatives include by-products such as milk whey and mussel-processing wastes [2, 51]. The most important feature of these substrates is their content of peptides that can act as inducers or precursors of the bacteriocin biosynthesis [27]. Moreover, these by-products are rich source of nutrient such as sugars and proteins; thus, it has been used as suitable culture medium for production of nisin and pediocin [45, 48], and lactocin 705 [95]. Whey was found to support the bacteriocin production by *P. acidilactici* NRRL B-5627, but the yield was lower than that obtained in MRS media [50]. By-products of food industry were effectively utilized for production of antimicrobial activity by *Bacillus* sp. P11 [16]. Jozala et al. [64] utilized supplemented powder milk whey as a culture medium for developing *Lactococcus lactis* cells and nisin production. These studies showed that biological processing of dairy and food industry by-product can be considered as one of the profitable utilization alternatives, generating high-value bioproducts and stimulates researches for its use.

Factors and Conditions for Bacteriocin Production

Bacteriocin Producer Strain

Different expression levels of bacteriocin genes in different strains along with the different activity of enzymes responsible for converting inactive bacteriocins into mature

Table 1 Bacteriocins production using free/immobilized producer strains in different production systems

Producer strain	Bacteriocin	Media	Production system	Bacteriocin production	Free/ immobilized cell	References
Pediococcus acidilactici PO2	Pediocin PO2	MRS broth	Continuous production in a packed-bed bioreactor	6,400 AU/ml	Immobilized cell	[18]
Leuconostoc mesenteroides subsp. *mesenteroides* UL5	Mesenterocin	Supplemented MRS, whey and whey permeate with YE (2 %), tween 80 (0.1 %), $MnSO_4$ (0.005 %), $MgSO_4$ (0.01 %)	Batch culture	4,096 AU/ml(MRS), 2,048 AU/ml(whey), 2048 AU/ml(whey permeate)	Free cell	[24]
Lactococcus lactis subsp. *lactis* and *P. acidilactici* UL5	Nisin Z and pediocin	Whey permeate (6 %) with YE (2 %) and tween 80 (0.1 %) [Glucose (0.5 %) added to SWPM]	Mixed-strain batch culture	Nisin and pediocin after 18 or 16 h incubation 3,000 and 730 AU/ml or 1,060 and 1,360 AU/ml, respectively	Free cell	[45]
L. lactis UL 719	Nisin Z	Whey permeate Powder (6 %) supplemented with 0.2 M KCL	Continuous fermentation	Maximum Nisin production during continuous free cell and immobilized cell with aeration is 2,560 and 2,430 IU/ml, respectively	Free cell and immobilized cell	[33]
L. lactis UL719	Nisin Z	Whey permeate (6 %) supplemented with 0.2 M KCL	Repeated-cycle batch cultures	8,200 IU/ml	Immobilized cell	[6]
L. lactis UL 719	Nisin Z	Whey permeate powder (6 %) with aeration supplemented with YE (1 %) and tween 80 (0.1–0.4 %)	Batch fermentation	8,200 AU/ml (without aeration), 41,000 AU/ml (with aeration)	Free cell	[2]
P. acidilactici UL 5	Pediocin PA-1	MRS broth supplemented. (1 % glucose) and whey permeate (SWP) medium	Repeated-cycle batch cultures (RCB)	By free cell, 187 and 342 AU/ml/h in SPM and MRS resp. By immobilized cells, 5,461 and 2,048 AU/ml/h, respectively	Free and immobilized	[80]
L. lactis subsp. *lactis* CECT 539 and *P. acidilactici* NRRL B-5627	Nisin and pediocin	Diluted whey (DW) and concentrated whey (CW)	Batch culture	Bacteriocin production from two strains were slightly higher in DW than in CW and production is lower as comparison to MRS medium	Free cell	[48]
L. lactis subsp. *lactis* CECT 539 and *P. acidilactici* NRRL B-5627	Nisin and pediocin	Influence of pH drop on bacteriocin production in non-buffered whey and buffered whey	Batch culture	Nisin and pediocin titres in whey 6.2 and 9.7 times lower than In MRS broth, respectively	Free cell	[53]
L. lactis subsp. *lactis* CECT 539 and *P. acidilactici* NRRL B-5627	Nisin and Pediocin	Whey supplemented with lactose and 4 nitrogen sources (YE, casitone, NH_4Cl and glycine)	Batch culture	YE and casitone increase pediocin titre from 55 BU/ml to 195 and 185 BU/ml, respectively, and nisin from 21 BU/ml to 74 and 59 BU/ml, respectively	Free cell	[48]
L. lactis subsp. *lactis* ATCC11454	Nisin	Whey permeate supplemented with YE or casein hydrolysate	Continuous fermentation using a packed-bed bioreactor	Maximum nisin titre 5.1×10^4 AU/ml	Immobilized cells	[70]

Table 1 continued

Producer strain	Bacteriocin	Media	Production system	Bacteriocin production	Free/ immobilized cell	References
P. acidilactici C20	Pediocin C20	Whey permeate supplemented with 2 % YE	Batch culture	150×10^3 AU/ml	Free cell	[56]
Bacillus licheniformis P40	Bacteriocin	Cheese whey powder supplemented with YE (1 %)	Batch culture	3,200 AU/ml	Free cell	[21]

bacteriocins are responsible for different levels of production of nisin and leucocin Lcm1 as compared to pediocin AcH [98]. A "ceiling" for bacteriocin production has been observed. Kim et al. [65] observed "ceiling or threshold" for nisin and De Vuyst et al. [28] for amylovoryn L471 production. Kim et al. [65] demonstrated the "ceiling or threshold" for nisin production is affected by both nutrient availability and nisin inhibition. Majority of pediocin-producing strains are unable to hydrolyse milk sugar lactose as a carbon source present in the medium [93, 94]. Most strains ferment glucose, ribose, galactose and fructose to DL-lactate. A few examples indicating Pediococci having β-galactosidase (β-gal) activity are reported [7], which may be able to utilize lactose present in whey and grow efficiently with effective bacteriocin production. Halami and Chandrashekar [56] found that a strain of *P. acidilactici* C20 had an ability to produce quantifiable amounts of pediocin C20 on whey permeate. The molecular basis for the presence of a β-gal like gene was shown by DNA dot-blot technique followed by β-gal assay on native polyacrylamide gel as experimental evidence for lactose hydrolysis. Production of pediocin C20 was found to be onefold to 1.5-fold excess in lactose-based medium as compared to medium with glucose. Optimized whey permeate supplemented with 2 % yeast extract gave cell growth of 3.5, OD_{600} and pediocin C20 activity of 150×10^3 AU ml^{-1}, equivalent to that obtained by growth in commercial MRS broth. Bacteriocins production using free/immobilized producer strains in different production systems is shown in Table 1.

Media

Studies on complex media and food-grade media demonstrated that bacteriocin production depends on the medium composition (mainly those of C and N sources) [9, 24, 34, 82, 83, 99] and greatly influenced by nutritional parameters, temperature, pH (initial and final) and aeration levels. Biswas et al. [9] showed that glucose, followed by sucrose, xylose and galactose are the best carbon sources for the production of pediocin AcH in an unbuffered medium. Li et al. [67] evaluated the effect of medium components on nisin production and cell growth, to search for the optimal medium composition for a higher nisin yield, which resulted in double yield as compared to that in CM medium. Among the different by-products (feather meal, grape bagasse, an industrial fibrous soybean residue and cheese whey) tested, cheese whey served as the best medium for maximum bacteriocin production and further increasing whey concentration resulted in increase of bacteriocin production [21]. Daba et al. [24] investigated the effects of various parameters (temperature, pH, nutrients and surfactants) on production and activity of mesenterocin 5, produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* UL5. This experiment proved that the large quantities of bacteriocin can be produced in whey and whey permeate medium supplemented with yeast extract in the presence of the surfactant (0.1 %). Tween 80 was a major factor in increasing mesenterocin 5 production and specific production might be due to the effect of the surfactant on cell membrane permeability, with acceleration in diffusion of the bacteriocin. Whey supported the growth of *Lactococcus lactis* subsp. *lactis* CECT 539 and *P. acidilactici* NRRL B-5627 and bacteriocin production by the two strains, but biomass and bacteriocin productions were lower than those obtained on MRS broth. However, supplementation of the whey with lactose and four different nitrogen sources further increased bacteriocins production by the two strains [48].

Effect of Fermentation Conditions

Krier et al. [66] showed that temperature and pH had a strong influence on the production of two bacteriocins by *L. mesenteroides*. Cladera-Olivera et al. [21] tested the effect of three variables (temperature, initial pH and whey concentration) on bacteriocin production by *Bacillus licheniformis* P40 on cheese whey medium and showed that, in the range studied, the three variables have a significant effect on bacteriocin production with maximum bacteriocin production at initial pH between 6.5 and 7.5 and temperature between 26 and 37 °C when the cheese whey concentration was 70 g l^{-1}. Conclusively, increasing whey concentration resulted in increased bacteriocin production, with an activity

at the maximum whey concentration tested. Aeration during the continuous fermentation by *L. lactis* UL719 in supplemented whey permeate (SWP) resulted in increased nisin Z production [33]. Goulhen et al. [45] studied the conditions for high production of nisin Z and pediocin during pH-controlled, mixed-strain batch cultures in SWP medium with *L. lactis* subsp. *lactis* biovar. *diacetylactis* UL719, a nisin Z producer strain, and variant T5 of *P. acidilactici* UL5, a pediocin-producing strain resistant to high concentrations of nisin. This study demonstrated that the high productions of both nisin Z and pediocin were obtained after 18 or 16 h incubation during mixed cultures, with titres of 3,000 and 730 AU ml^{-1}, or 1,060 and 1,360 AU ml^{-1}, respectively, corresponding to approximately 75 and 55, or 25 and 100 mg l^{-1} of pure nisin Z and pediocin, respectively. In pure cultures, nisin Z and pediocin productions were higher than in mixed cultures, and maximum activities were obtained after 10 h incubation, with approximately 10,000 AU ml^{-1} (250 mg l^{-1} pure nisin Z) and 2,500 AU ml^{-1} (190 mg l^{-1} pure pediocin). Amiali et al. [2] found that aeration have a large stimulatory effect on nisin Z production by *L. lactis* UL719 in a yeast extract/tween 80-SWP during batch fermentation.

Production Systems

Bacteriocin production was mostly studied in batch culture with synthetic media (MRS broth). Guerra et al. [47] reported increased cell growth and pediocin production by re-alkalized fed-batch fermentation by *P. acidilactici* NRRL B-5627 on whey compared with batch fermentation on MRS broth. The re-alkalized fed-batch culture was characterized by higher biomass (6.57 g/l) and pediocin [517.6 BU (bacteriocin activity units)/ml] concentrations compared with the batch processes on MRS broth (1.76 g/l and 493.2 BU/ml), DW (0.17 g/l and 57.7 BU/ml), DWG (0.14 g/l and 53.6 BU/ml), DWYE (1.43 g/l and 187.6 BU/ml) and DWGYE (1.28 g/l and 167.3 BU/ml) media. Guerra et al. [46] compared cell growth and pediocin production by *P. acidilactici* NRRL B-5627(on MRS broth and a culture medium from mussel-processing wastes (MPW)) using batch and two fed-batch fermentations on MPW with re-alkalization cycles. Mathematical models were developed to describe fed-batch production of biomass and pediocin by *P. acidilactici*. While cell growth was dependent on pH change, nitrogen and phosphorous availability and product inhibition (lactic acid, ethanol and butane-2, 3-diol), pediocin production was dependent on both growth and the final pH reached in each re-alkalization period. Cho et al. [18] developed a method for the continuous production of pediocin PO2 using immobilized *P. acidilactici* PO2 in a packed-bed bioreactor. Conditions for the optimum production of pediocin PO2 by the immobilized cells were also investigated [60, 72]. The authors obtained the maximum bacteriocin activity of 6,400 AU/ml when the medium was fermented with dilution rates of at least 1.19 day^{-1} and pH controlled at 4.5. This bacteriocin is a potent inhibitor of *Listeria monocytogenes*, a widespread food-transmitted pathogen [59, 60, 68]. Bertrand et al. [6] studied high nisin Z production during repeated-cycle pH-controlled batch cultures using *L. lactis* subsp. *Lactis* biovar. *diacetylactis* UL719 immobilized in k-carrageenan/locust bean gum gel beads in SWP. The RCB culture process developed in this study was stable and resulted in high nisin Z production (8,200 IU ml^{-1}) and volumetric productivity (5,730 IU ml^{-1} h^{-1}) at the end of 1-h incubation cycle. Naghmouchi et al. [80] studied the production of pediocin PA-1 by *P. acidilactici* UL5 cells immobilized in k-carrageenan/locust bean gum gel beads during repeated-cycle batch (RCB) culture with pH control in MRS broth supplemented with 1 % glucose and whey permeate medium. The maximum pediocin PA-1 activity obtained during RCB fermentation in SWP medium was 4,096 AU ml^{-1}. Liu et al. [70] investigated the continuous production of nisin in laboratory media and whey permeate using a packed-bed bioreactor. *Lactococcus lactis* subsp. *lactis* ATCC 11,454 was immobilized by natural attachment to fibre surfaces and entrapment in the void volume within spiral wound fibrous matrix. The authors observed optimal nisin activity at pH 5.5, 31 °C, 0.2–0.3/h dilution rate, and 30 g/l lactose in M17. The maximum nisin titre was 2.6×10^4 AU/ml. The bioreactor was fed whey permeate, supplemented with casein hydrolysate, and growth of *L. lactis* and associated nisin production were monitored. Optimal conditions for continuous nisin production in whey permeate were pH 5.5, 31 °C, 10–20 g/l casein hydrolysate and 0.2/h dilution rate. Under these conditions, a maximum nisin titre of 5.1×10^4 AU/ml was observed. Bhugaloo-Vial et al. [8] investigated the production of divercin, a bacteriocin active against Listeria, by whole cells of *Carnobacterium divergens* V41 by three means: continuous cultivation with free cells; with cells immobilized in calcium-alginate beads packed in a plug-flow bioreactor and with a membrane bioreactor. The productivity and bacteriocin concentrations of the systems were compared. Immobilized cells presented the best performances with $>10^5$ AU l^{-1} h^{-1}, which can be compared to the 2.8×10^3 AU l^{-1} h^{-1} of the batch system.

Strategies for Recovery and Purification of Bacteriocins

To develop bacteriocins for food biopreservation, it is necessary to produce these in purified form on a large scale. As crude form of bacteriocins may contain the components of media, which are undesirable when bacteriocins are to be used for biopreservation. Because bacteriocins are secreted

Table 2 Different strategies used for the bacteriocin purification with their recovery and purification fold

Bacteriocin	Media used	Steps of purification	Yield/ recovery	Purification fold	References
Pediocin PA-1	MRS broth	Cation-exchange chromatography	85 %	ND	[92]
		Reverse-phase HPLC (RP-HPLC)	110 %		
Enterocin EJ97	CM broth (101)	Cation-exchange chromatography	59.56 %	8.46	[71]
		Reverse-phase HPLC (RP-HPLC)	48.85 %	30.8	
Carnocin KZ213	MRS broth (101)	Hydrophobic interaction chromatography	0.58 mg/l	911	[87]
		Cation-exchange chromatography		34,000	
Enterocin AS-48	CM broth (251)	Cation-exchange chromatography	95.99 %	11.87	[1]
		Reverse-phase HPLC (RP-HPLC)	74.95 %	24.3	
Nisin Z	MRS broth (51)	Expanded-bed ion-exchange chromatography	90 %	31	[17]
Mesenterocin Y105	MRS broth	Cation-exchange chromatography	120 μg/l	60	[55]
		Hydrophobic interaction chromatography			
		HPLC			
AMP by *L. sakei*	MRS broth (81)	Acid extraction	3.33 %	2.9	[14]
		Cation-exchange chromatography	3.2 %	55.2	
Enterocin E-760	Brucella broth (6.51)	Cation-exchange chromatography	ND	ND	[69]
		Hydrophobic interaction chromatography			
Leucocin C	MRS broth	Cation-exchange chromatography	ND	ND	[37]
		Reverse-phase HPLC (RP-HPLC)			
Pediocin PA-1	MRS broth	Centrifugation	73 %	ND	[5]
		Cation-exchange chromatography			
		Hydrophobic interaction chromatography			
Plantaricin ST31	MRS broth	Centrifugation	100 %	1	[86]
		Cation-exchange chromatography	5.94 %	110	
Pediocin from *P. acidilactici* MM33	MRS broth	Centrifugation	100 %	1	[72]
		Cation-exchange chromatography	50.7 %	725	
		Rotavapor	40 %	5,725	
		Freeze drying	50.7 %	36,500	
Macedocin	Skim milk with yeast extract	Centrifugation	ND	ND	[42]
		Ammonium sulphate precipitation			
		Reverse-phase HPLC			
Acidocin CH5	MRS broth	Centrifugation	100	1	[19]
		Solid-phase extraction	3.9	0.2	
		Cation-exchange chromatography	4.0	66	
		Hydrophobic interaction chromatography	2.7	49	
		Reverse-phase HPLC (RP-HPLC)	ND	ND	
AMP from *L. helveticus*	Whey	Centrifugation	120 AU/ml	1	[10]
		Ultrafiltration		1.3	
		Precipitation		2.3	
		Gel-filtration chromatography		10.3	
		Ion-exchange chromatography		27.3	

Table 2 continued

Bacteriocin	Media used	Steps of purification	Yield/ recovery	Purification fold	References
Bacteriocin by *E. faecium* MMT21	MRS broth	Cation-exchange chromatography	ND	ND	[43]
		Hydrophobic interaction chromatography			
		Reverse-phase HPLC (RP-HPLC)			
Bacteriocin by *Leuconostoc mesenteroides* E131	MRS broth	Centrifugation	ND	1	[90]
		Ammonium sulphate precipitation		5.5	
		Resource S		4.6	
		Ammonium sulphate precipitation		0.6	
		Reverse-phase HPLC (RP-HPLC)-I		24.7	
		Reverse-phase HPLC (RP-HPLC)-II		9.3	
Sakacin P	MRS broth	Macroporous monolith column	87 %	156	[33]
Bacteriocin from *Carnobacterium divergens*	MRS broth	Centrifugation	100 %	1	[76]
		Triton X-114 phase partitioning	0.1 %	ND	
		Cation-exchange chromatography	0.04 %	13,000	
Sakacin A	MRS broth	Centrifugation	100 %	ND	[58]
		Ammonium sulphate precipitation	96 %		
		Cation-exchange chromatography	14 %		
		Hydrophobic interaction chromatography	51 %		
		FPLC	83		
Pediocin SA-1	MRS broth	Centrifugation	ND	ND	[3]
		Tricin SDS-PAGE			
Pediocin PD-1	MRS broth	Centrifugation	100 %	1	[4]
		Precipitation	86 %	8	
		Dialysis			
		Lyophilization	55 %	6	
		Methanol–chloroform extraction	47 %	11	
		Cation-exchange chromatography	34 %	1,700	

into the culture medium, most strategies start with a step to concentrate bacteriocins from the culture supernatant, such as either pH-dependent adsorption of bacteriocins on producer cells [81] or heat-killed producer bacteria [13], diatomite calcium silicate (Micro-Cel) [23], rice hull ash or precipitation with silicic acid [63], ammonium sulphate [13] or ethanol [95]. Although these procedures are used principally to reduce the working volume, these typically do not provide a high degree of purity. Therefore, subsequent steps of preparative isoelectric focusing [94] and/or multiple chromatographic separations, including gel-filtration [38, 73], cation-exchange [69, 77, 92], hydrophobic interaction [5, 20, 69, 87], and reverse-phase liquid chromatography [1, 35, 77, 92], are necessary to achieve significant purification of the bacteriocins. The different strategies used for the bacteriocin purification with their recovery and purification fold are shown in Table 2.

Majorly, three major strategies or methods for the purification of bacteriocins to homogeneity can be distinguished: conventional multi-step method, simple three-step method and single-step bed adsorption. Usually, but not always, the protein yields are low in case of conventional methods. This is probably due to the extra number of steps in the protocol, leading to low yield which is one of the problems for the purification of bacteriocins at the industrial level. This is due to the purification protocols which work well at laboratory-scale volumes but are not suitable at industrial scale. Purification of bacteriocins using conventional methods based on laborious series of subsequent steps of ammonium sulphate precipitation, ion-exchange, hydrophobic interaction, gel-filtration, and reversed-phase high-pressure liquid chromatography. Piva et al. [85] achieved purification of pediocin A from the culture of *P. pentosaceus* FBB61 by dialysing the cell-free culture supernatant against PEG 20000, followed by butanol extraction and electroendosmotic preparative electrophoresis, with a yield of 3.9 % and 7,834-fold purification. Cintas et al. [20] purified pediocin L50 from culture of *P. acidilactici* L50 by subjecting the precipitates obtained from the ammonium sulphate precipitation of cell-free culture supernatant to the cation-exchange chromatography, followed by

hydrophobic interaction chromatography and reverse-phase HPLC, resulting in 80 % yield. Casadei et al. [15] collected culture supernatant of *P. pentosaceus* FBB61, filtered through 0.45-µm pore-size filters, concentrated by polyethylene glycol dialysis and then concentrated solution was partially purified using ion-exchange chromatography. This procedure led to the recovery of 35 % of the activity present in the culture supernatant, indicating that the ion-exchange chromatography is an efficient purification method. Because of the number of laborious and time-consuming steps along with the low yield, there was a need to develop efficient protocols which require less time with efficient recovery.

Many simple three-step protocols have been developed, including (1) ammonium sulphate precipitation, (2) chloroform/methanol extraction/precipitation, and (3) cation-exchange/hydrophobic interaction/reverse-phase high-pressure liquid chromatography for the purification of bacteriocins from complex media on large scale. Ghrairi et al. [43] purified bacteriocins from culture supernatant of *E. faecium* MMT21 to homogeneity using a three-step procedure consisting of cation-exchange chromatography, C18 Sep-pack chromatography and C18 reverse-phase high-performance liquid chromatography. Recently, a simple and rapid protocol was developed in our laboratory which is suitable for small-scale purification and may prove suitable for large-scale purification of pediocin, which involved ammonium sulphate precipitation (80 % saturation) of cell-free culture supernatant at isoelectric point of pediocin PA-1 (pH 8.8), followed by cation-exchange chromatography using SP-Sephadex [97]. Similarly, pediocin PA-1 produced by *P. acidilactici* was purified by subjecting cell-free culture supernatant to the cation-exchange chromatography, followed by hydrophobic interaction chromatography, resulting in the yield of 73 % [5]. All these protocols involve the use of centrifugation for obtaining the cell-free culture supernatant, which is processed further for purification. At the industrial scale, centrifugation is considered to be a major bottleneck. Many protocols for the bacteriocin purification from complex culture media have been developed on large scale, skipping centrifugation step and exploiting the cationic and hydrophobic nature of bacteriocins. Uteng et al. [92] developed a rapid two-step procedure suitable for large-scale purification of pediocin-like bacteriocins and other cationic antimicrobial from complex culture medium of *P. acidilactici* LMG 2351 in which the bacterial culture was applied directly on a cation-exchange column, and then on a low-pressure reverse-phase column. The final bacteriocin preparation was more than 90 % pure as judged by analytical reverse-phase chromatography and capillary electrophoresis. Guyonnet et al. [55] developed the method for the rapid purification of mesenterocin Y105, by applying the overnight culture supernatant of *L. mesenteroides* Y105 to the carboxy-methyl cellulose column, followed by C18-cartridge and C8 Kromasil analytical HPLC column with a yield (60 %) and appeared to be at least 95 % pure. Fimland et al. [37] developed a rapid two-step procedure for the purification of leucocin C by applying an overnight grown culture of *L. mesenteroides* 6 directly on SP Sepharose Fast Flow cation-exchange column, followed by low-pressure reverse-phase column chromatography. Abriouel et al. [1] recovered enterocin AS-48 from *Enterococcus faecalis* subsp. *liquefaciens* A-48-32 by adding Carboxymethyl Sephadex CM-25 gel slurry to cultured broths followed by loading of active fractions on a reversed-phase high-performance liquid chromatography (RP-HPLC) column. By using a combination of cation-exchange and reversed-phase chromatography, ca. 75 % of the total activity in the cultured broths could be recovered. Lopez et al. [71] recovered enterocin EJ97 from cultured broth by direct mixing with the cation exchanger Carboxymethyl Sephadex CM-25 without previous separation of cells by centrifugation. The yield of this purification step was 59.46 %. Elute was further subjected to reverse-phase chromatography to obtain purified bacteriocin. The yield of this step was very high, and the specific activity of the bacteriocin was similar to the reported specific activity of 1.60 AU/g of protein for purified enterocin EJ97. This procedure is time saving and allows easier processing of large culture volumes. Line et al. [69] purified the enterocin E-760 by cation-exchange chromatography followed by hydrophobic interaction chromatography. Saint-Hubert et al. [87] developed a protocol for large-scale purification of carnocin KZ 213 from *Carnobacterium piscicola* 213 by loading the entire batch on the butyl Sepharose 4 Fast Flow column for hydrophobic interaction chromatography and then the eluted fraction was applied to the cation-exchange column. This protocol leads to a complete recovery of carnocin KZ 213 with 95 % purity and to a concentration factor of 83. From 10 l culture supernatant, 5.8 mg carnocin KZ 213 could be produced with a specific activity of 8,500 UA g^{-1}. The protocol is easy to implement for larger volumes. Skipping the centrifugation step resulted in the efficacy of purification and also reduced the time required for purification. However, Millette et al. [77] purified the bacteriocin produced by *P. acidilactici* MM33 using a modified version of the procedure described by Uteng et al. [92], in which culture of *P. acidilactici* in MRS broth was centrifuged at 8,000×*g* and 4 °C, and the supernatant was collected and vacuum filtered through a 0.20-µm pore-size nylon filter, which was then loaded directly on a SP Fast Flow cation-exchange column. About 50 % of total pediocin activity was recovered with a specific activity 725-fold higher than that of the cell-free supernatant.

Third, bacteriocins can be isolated through a unique unit operation, i.e. expanded-bed adsorption, using a hydrophobic interaction gel, after maximizing the bioavailable bacteriocin titre through pH adjustment of the crude fermentation medium

[11, 39, 84, 91]. Cheigh et al. [17] purified nisin Z by applying unclarified culture broth of *L. lactis* A164 on an expanded-bed ion-exchange chromatography and the fraction was eluted with 0.15 M NaCl. This simple one-step purification process resulted in 31-fold purification with a yield of 90 %. The advantages of expanded-bed ion-exchange chromatography includes reduced number of purification steps, shortened total processing time, increased productivity, and operation conditions such as high processing volume and high flow rate, which allow it to be used in large-scale process. This method may, therefore, provide a cost-effective alternative process for scale-up purification of nisin Z over other multi-step processes. Deraz et al. [32] captured bacteriocin directly from the non-clarified fermentation broth of *Lactobacillus sakei* CCUG 42687 using macroporous octyl- and phenyl-monolith columns and its screening demonstrated that at pH 6.2, about 80 % of the bacteriocin activity could be recovered with a purification factor of 150–160 in the cell-free eluate. It presents a promising approach for rapid analytical isolation of bacteriocins from numerous samples. Following the latter two methods, which are more rapid than the first conventional method and yet successful, several bacteriocins with interesting industrial potential have been purified, such as amylovorin L, enterocins, pediocins, nisin and macedocin [12, 40–42].

Conclusions

Bacteriocins can offer a promising role in the field of food biopreservation, but there are many hurdles to overcome to commercialize them on a large scale like production cost, lengthy and costly purification techniques involved. These hurdles can be overcome by using the food-grade media, which are available as a by-product of food and dairy industries like fish meal, grape waste, an industrial fibrous soya bean residue, soya bean meal and cheese whey. The costly production can be counteracted by suitable bioprocessing strategies designed for increasing yields and purity. The purification protocols can be simplified by reducing the number of steps required for the purification to the minimum steps so that the protocol can be scaled up to the large scale and at the same time remains cost-effective. Further genetic engineering of the bacteriocin producer strains may result in the enhanced expression of the bacteriocin, resulting in the high titre.

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

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