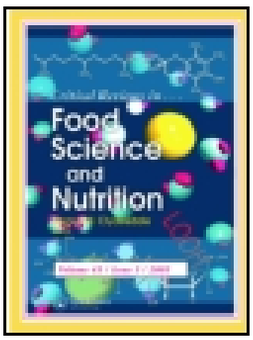

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Novel Approaches to Purifying Bacteriocin: A Review

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Abstract

Bacteriocin is a proteinaceous biomolecule produced by bacteria (both Gram-positive and Gram-negative) that exhibits antimicrobial activity against closely related species, and food-borne pathogens. It has recently gained importance and attracted the attention of several researchers looking to produce it from various substrates and bacterial strains. This ushers in a new era of food preservation where the use of bacteriocin in food products will be an alternative to chemical preservatives, and heat treatment which are understood to cause unwanted side effects, and reduce sensory and nutritional quality. However, this new market depends on the success of novel downstream separation schemes from various types of crude feedstocks which are both effective

and economic. This review focuses on the downstream separation of bacteriocin from various sources using both conventional and novel techniques. Finally, recommendations for future interesting areas of research that need to be pursued are highlighted.

Keywords: bacteriocin, food preservation, purification, conventional methods, alternative methods

ACCEPTED MANUSCRIPT

1. Introduction

Bacteriocins are antimicrobial peptides secreted by bacteria (both Gram-positive and Gram-negative) which show antibacterial activity against other bacterial species (Pingitore et al., 2007). However, they are not antibiotics despite being produced by bacteria; firstly, bacteriocins are ribosomally synthesized peptides, while antibiotics are synthesized by enzymes. Secondly, bacteriocins are targeted at a narrow spectrum of bacteria that are usually within or closely related to the species of the bacteriocin producer, while antibiotics are active against a wide range of bacteria. Another difference is that the working concentrations of bacteriocins are usually lower than antibiotics (Parada et al., 2007).

Klaenhammer (1993) comprehensively described four classes of bacteriocin. The first is termed lantibiotics which contain unusual amino acids (i.e., lanthionines and β-methyllanthionines). The second is bacteriocins which do not contain lanthionine residues, and this type is heat stable and membrane active. The third class is the large and heat labile bacteriocins, and finally the fourth class are bacteriocins that complex with other chemical moieties (carbohydrates and lipids). Discovery of other novel bacteriocins such as bacteriocin F1 from *Lactobacillus* subsp.*tolerans,* lactoxin MXJ 32 from *Lactobacillus coryniformis* MXJ 32, and bifidocin A from *Bifidobacterium animalis* BB04 have been reported (Liu et al., 2015; Lü et al., 2014; Miao et al., 2014), and these extensive findings further highlighted the importance and value of bacteriocins.

Bacteriocins from lactic acid bacteria (LAB) play an important role in the food industry, whether they are used as a starter culture, co-cultures, or bioprotective cultures that

give advantages in food quality and food safety (De Vuyst and Leroy, 2007). Many LAB bacteriocins have potential applications in the food industry, mainly by inhibiting the growth of foodborne bacterial pathogens such as *Listeria monocytogens, Bacillus cereus, Bacillus thuringiensis, Salmonella,* and *Staphylococcus aureus,* (Bizani et al., 2005; Corr et al., 2007; Martin-Visscher et al., 2008; Miao et al., 2014). Besides its role in the food industry, it is also important in healthcare applications: improving human health by fighting against gastrointestinal pathogenic bacteria (i.e. *Helicobacter pylori*, *Escherichia coli*, and *Salmonella)*(De Vuyst and Leroy, 2007), skin health dermatology (Bowe et al., 2006), and as an alternative to antibiotics (Joerger, 2003; Cotter et al., 2013). Therefore, pure bacteriocin is of great interest to the pharmaceutical industry (Bali et al., 2014).

It is important to highlight that the main bottleneck to the production of a commercial high quality bacteriocin is the high cost of its production, of which the two major contributors are: (1) the cost of synthetic medium and nutritional supplements used for the growth of bacteriocin-producing cells, for example, the Man, Rogosa, and Sharpe (MRS) complex medium, and, (2) the downstream separation of bacteriocin from the complex crude feedstock (Bali et al., 2014; Deraz et al., 2007; Jozala et al., 2008; Jozala et al., 2015). Bali et al. (2014) have extensively reviewed the first cost, and basically highlighted the current trend of bacteriocin production, i.e. use of agro-industrial byproducts such as whey, molasses, marine byproducts, and soy waste as low cost substrates. This approach is not only a cheaper alternative to the commercial

fermentation substrates, but also contributes to better waste management (Bali et al., 2014; Makkar et al., 2011).

The second cost is the downstream processing of bacteriocin where it is often synonymous with chromatography, a relatively efficient but expensive separation method in terms of capital and operational cost (Sadana, 1994). This review explores the conventional purification schemes of bacteriocin, as reported in the literature, such as ammonium sulfate precipitation, pH-mediated cell adsorption/desorption, membrane filtration, a variety of fixed-bed chromatography types, while also considering possible state-of-the-art alternatives that are more cost-effective, but with comparable performance to the chromatography-based methods; namely expanded bed adsorption (EBA), macroporous monoliths, liquid-liquid extraction-based aqueous two-phase systems (ATPS), and aqueous micellar two-phase systems (AMTPS).

2. Purification of Bacteriocin: Conventional vs Alternative Methods

In the food industry, bacteriocins have been widely used as a biopreservative agent in three different forms (i.e., viable cells, crude, and a purified form) in order to prevent food spoilage, and the growth of pathogenic bacteria such as *Listeria monocytogenes, Micrococcus luteus, Staphylococcus aureus,* and *Aeromonas hydrophila* (Pal and Ramana, 2010). Therefore, the development of a method to purify bacteriocin from a complex fermentation medium (broth) is important in order to lower its cost and enhance its use. Common fermentation medium for bacteriocin production such as MRS (Abbasiliasi et al., 2012; De Vuyst and Leroy, 2007; Elayaraja et al., 2014; Liu et al., 2015; Muriana and Klaenhammer, 1991) contains unwanted impurities for the final food

application; therefore, they should be removed in conjunction with other cell derived impurities. In this review, a summary of recently reported strategies for bacteriocin purification will be presented and discussed, along with their main advantages and disadvantages. A summary of conventional and alternative bacteriocin purification methods can be also found in Table 1 and Table 2, respectively.

2.1 Conventional methods

Conventional purification methods typically consist of ammonium sulfate precipitation, solvent-based precipitation, and chromatography-based techniques such as ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography (De Vuyst and Leroy, 2007). The shortcomings of these conventional methods have been reported by several researchers (Carolissen-Mackay et al., 1997; Guyonnet et al., 2000). In general, these conventional methods are laborious due to the fact that they consist of multistage operations that are expensive, time consuming, produce low yield, and are not efficient for industrial scale requirements. Furthermore, the low recovery levels of the conventional methods result in a significant loss of the target product (Carolissen-Mackay et al., 1997).

2.1.1. Ammonium sulfate precipitation

In a preliminary purification step, concentrating the bacteriocin from the culture supernatant is necessary for volume reduction, and aims at increasing the specific activity (Muriana and Klaenhammer, 1991). Commonly, the salting-out method with ammonium sulfate was used due to its high solubility and low costs (Pingitore et al., 2007). Ammonium sulfate precipitation works by compressing the DLVO layer and

reducing the repulsion of similar molecules which can then agglomerate, thereby separating proteins from a solution based on their solubility change in the presence of a high concentration of salt (Moore and Kery, 2009). The resulting bacteriocin concentrate resulted in an increase in the size of the inhibition zones against tested strains, indicating a stronger antagonistic effect (Sharma et al., 2011). In addition, Callewaert et al. (1999) found that this step could retain sufficient bacteriocin activity during elution, and many authors have used this technique to precipitate bacteriocin at different ammonium sulfate concentrations. Pediocin PD-1 from *Pediococcus damnosus* NCFB1832 has been purified with 70% saturated ammonium sulfate followed by cation exchange chromatography (Bauer et al., 2005). Lü et al. (2014) purified lactocin MXJ 32A produced by *L. coryniformis* MXJ 32 by ammonium sulfate precipitation (80%), dialysis, and ion exchange chromatography. Mørtvedt et al. (1991) used a 20% ammonium sulfate concentration, ion-exchange chromatography, hydrophobic interaction and reversed-phase chromatography, and gel filtration to purify lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. However, the concentration of ammonium sulfate needed depended on the type of bacteriocin (Pingitore et al., 2007). Several factors are considered in choosing the saturating concentrations - the initial volume of bacteriocin, volume that is desired after the procedure, molecular weight of the bacteriocin, absorptivity of bacteriocin, time requirement for concentration, and desalting of the bacteriocin solution (Pohl, 1990).

Nevertheless, Borzenkov et al. (2014) revealed that ammonium sulfate precipitation was not suitable for low molecular weight bacteriocin purification due to the poor

precipitation of the protein even at 75 - 80% saturation, and partial/full elimination of the protein when passing through dialysis sacs. In addition, an unsatisfactory result was obtained due to the varying compositions of the complex culture medium, and the floating of the protein pellet even after a centrifugation step which subsequently made it difficult to separate (Guyonnet et al., 2000). Other methods that have been reported previously to concentrate bacteriocin include ethanol precipitation (Chikindas et al., 1993), lyophilization (Mørtvedt et al., 1991), ultrafiltration (Muriana and Klaenhammer, 1991), and adsorption/desorption using Amberlite® resins XAD-16 (Martin-Visscher et al., 2008). As the required bacteriocin's purity was impossible to achieve using this single step, subsequent multistage chromatography then followed.

2.1.2. pH-mediated cell adsorption/desorption

Yang et al. (1992) developed an extraction method for bacteriocins i.e. sakacin A (*Lactobacillus sake*), pediocin AcH (*Pediococcus acidilactici*), nisin (*Lactococcus lactis*), and leuconocin Lcml (*Leuconostoc camosum*) based on the influence of pH on adsorption/desorption of bacteriocin onto the producer cell (Figure 1). The general method of extraction was: (1) pre-treatment of the cells by heating; (2) adsorption of bacteriocin at pH 5.5-6.5, and; (3) desorption under acidified conditions-pH 2, and high salinity-100 mM NaCl. The detailed procedure is illustrated in Figure 1.

It is important to highlight that for heat-stable bacteriocin, heating of the unclarified culture broth might have several benefits such as: (1) killing of the cells (Liu et al., 2015; Yang et al., 1992); (2) increasing the yield due to early proteolytic enzyme inactivation (Yang et al., 1992); (3) purification, where the step of sedimentation, i.e. centrifugation

of the denatured protein aggregate (impurities) can be performed in conjunction with the removal of cells hence improving the overall process economics (Buyel et al., 2014; Mehrnoush et al., 2011; Ng et al., 2006; Olichon et al., 2007). In addition, optimisation of the pH conditions for adsorption/desorption should also consider the stability range of the target bacteriocin. In fact, immediate buffer exchange of the desorbed bacteriocin solution was proposed to maintain its activity (Liu et al., 2015; Yang et al., 1992).

The bacteriocins recovered were then freeze-dried to obtain a dry preparation (Yang et al., 1992). In most bacteriocins, the amount lost in the supernatant after adsorption (unbound bacteriocin) was measured to be below than 7.7%, while less than 2.3% was lost with the cells after extraction (non-recovered). The totals obtained using freeze-drying were 106.7%, 93.3%, 44.3%, 96.2% for pediocin AcH, nisin, sakacin A, and leuconocin Lcml, respectively. Since then, many researchers have employed a similar method of purification, albeit with slight modifications, e.g. improvement in the mixing conditions (Dündar et al., 2014), use of methanol for desorption followed by a drying step using rotary evaporation (Wen et al., 2016), or further purification with additional chromatography steps (Liu et al., 2015; Wen et al., 2016; Zhang et al., 2009).

Although there was indications of ionic interactions during adsorption/desorption, the intrinsic mechanism of adsorption/desorption was not very clear. Several researchers (Manca De Nadra et al., 1998; Todorov and Dicks, 2006; Yildirim et al., 2002) have extensively investigated the adsorption of bacteriocin onto target strains; it was discovered that high adsorption was recorded for sensitive strains (to the specific bacteriocin) compared to their insensitive counterparts. Adsorptions of plantaricin 423

from *Lactobacillus plantarum* 423 onto *enterococcus* sp. HKLHS and *Lactobaciilus sakei* DSM 20017 were found to be influenced by environmental factors (pH and temperature), the presence of surfactants (i.e., sodium dodecyl sulfate), and inorganic and organic salts (Todorov and Dicks, 2006). Furthermore, differences in the adsorption conditions and the effect of additives (pre-treatment) were found to be species specific. Analysis of the plantaricin-treated cell morphology by atomic force microscopy indicated that it was deformed, and high concentrations of DNA and β-galactosidase were also detected (Todorov and Dicks, 2006). On the basis of this result, it was proposed that the mode of action of plantaricin 423 on the target cell was destabilising the membrane permeability. Nevertheless, it has not been confirmed whether the same adsorption mechanism was shared with the use of a producer cell (for the adsorption of bacteriocin).

Furthermore, it is important to note that if additives are going to be used in recovery, then those with food grade quality are desirable, for instance, salt, EDTA, and surfactant. However, if toxic chemicals are to be used, the carry-over issue needs to be addressed. Overall, provided that further insight can be gained, this method can be very economical as a 'natural' means of separating bacteriocin due to its relatively high yields, as well as being commercially attractive as an application for the biopreservation in food.

2.1.3. Chromatography

A series of chromatographic steps, which frequently includes ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid

chromatography (RP-HPLC), have been widely used for the further purification of bacteriocin. Cation exchange chromatography is usually applied since most of the bacteriocin is positively charged at a pH near neutrality (Pingitore et al., 2007). In addition, due to the amphiphilic properties of bacteriocin, hydrophobic chromatography has also been very useful to separate the highly hydrophobic bacteriocin (Borzenkov et al., 2014). Gel filtration chromatography can also be included in purifying the protein, and it is generally employed as the final polishing step possibly due to its lower protein loading capacity (Tan et al., 2015). Moreover, RP-HPLC was usually applied at the end of the purification scheme to eliminate any last contaminants (H-Kittikun et al., 2015; Hastings et al., 1991; Simha et al., 2012). In general, the application of these chromatographic methods were suitable for bacteriocin because of its stability to different organic solvents that are used as mobile phases, and the high pressure employed during the chromatographic procedures (Pingitore et al., 2007). Nevertheless, a low yield was also associated with these methods because of the large number of procedures which resulted in substantial loss in the bacteriocin's activity during the process (Parada et al., 2007). Although the chromatography-based purification schemes outlined by the previous researchers appeared to be almost identical, there was a marked difference in the purification degrees achieved; one example of this was highlighted by Hastings et al. (1991) and Mørtvedt et al. (1991) in Table 1 where the purification factor obtained by the latter was about 10 times higher than the former. Besides the variation in the original crude broth, other possible reasons behind the discrepancy in these results may be due to the innovations employed, e.g. addition of

ammonium sulfate to the sample to a final concentration of 6% (w/v) prior to its application to a hydrophobic interaction chromatography column (Mørtvedt et al., 1991). This result was supported by other researchers (Dayananda, 2007; Jozala et al., 2015), who stated that ammonium sulfate allowed for an increase in the concentration of the target proteins during binding which subsequently can be collected, and purified, hence, enhancing the degree of purification. On a separate note, Jozala et al. (2015) performed an almost identical single-step hydrophobic interaction chromatography (HIC) step to purify nisin produced by *Lactococcus lactis* cultivation in milk whey. Filter-sterilised cell-free-crude-supernatant (CFCS) was loaded directly onto the HIC without going through an ammonium sulfate precipitation step, but the salt was added to the sample at a concentration of 2 M instead just before chromatography. Ammonium sulfate was added to the sample (2 M) prior to HIC to enhance the hydrophobic interactions between the nisin, and the matrix. Impurities were eluted in the first elution step (2 M ammonium sulfate in water or PBS), while nisin was selectively eluted in the second step with only water or PBS. A high yield, and purification factor (284.88% and 774; 152.02% and 384) were recorded with water, and PBS elution, respectively. It is worth to note that the yield was more than 100% due to the much increment in specific activity.

In addition, affinity chromatography using bacteriocin-specific antibodies as ligands has also been employed previously in attempts to both detect and purify bacteriocins. This method is very selective, easy to carry out (as it allows for single-step chromatography), has a short operation time (i.e., few hours), and gives high productivity (Suarez et al., 1997). Purification of nisin A by immunoaffinity chromatography was first reported by

Suarez et al. (1997) using an immunoglobulin G1 isotype (produced by hybridoma cell line AD10) as a ligand, which was directly coupled to an N-hydroxysuccinimide-activated Sepharose; 72.7% activity recovery and 10 purification fold was reportedly achieved. However, some of the drawbacks of this method in relation to bacteriocin purification include the requirement for costly non-commercial antibody-conjugated resins, and lower yields compared to those obtained by other more lengthy chromatography schemes (Lohans and Vederaz, 2011), as well as the reusability limitations of the column used due to the harsh elution conditions employed (Rose et al., 2001).

Rose et al. (2001) explored the use of a crosslinking method where an enterocin B (partially purified) was conjugated to a carrier protein, BSA, in order to develop an immune response (as the small size of bacteriocins usually renders them poorly immunogenic). Immunoaffinity chromatography of the enterocin B on rabbit IgG-immobilised CNBr-activated Sepharose 4B was carried out, resulting in 25% activity recovery and a tenfold purification factor, and purity was subsequently confirmed through MALDI-TOF MS and SDS-PAGE.

Furthermore, immunopurification of enterocin P (EntP) from *E. faecium* P13 fermentation broth was also carried out using a 2 mL Immunopure rProtein A Plus Orientation column (where IgG was cross-linked to the matrix with disuccinimidyl suberate with a 15-100% coupling efficiency), and about 1.1-5.9 µg of EntP was recovered (Gutiérrez et al. 2004). Overall, there has not been much progress in the application of immunoaffinity-based techniques on bacteriocin purification primarily due

to the difficulties in producing bacteriocin-specific antibodies, and the development of economically viable antibody-conjugated resins.

2.1.4. Membrane filtration

In general, membrane filtration can be a highly selective and efficient separation method which does not require additives, can be performed at ambient (or below) temperature which allows for less energy consumption, is easy to scale up, not to mention the possibility of being able to be integrated into other separation or reaction processes (Bowen and Jenner, 1995; Saxena et al., 2009; Zacharof et al., 2013). Hence, this method has also attracted the attention of bacteriocin's researchers who have successfully employed it in bacteriocin purification, mainly because of its ability to separate proteins based on size and/or charge with no activity loss (Muriana and Klaenhammer, 1991; Zacharof et al., 2013).

Muriana and Klaenhammer (1991) discovered that lactacin F from the culture supernatant of *Lactobacillus acidophilus* was associated with a larger molecular complex through the result of two different purification methods. Firstly, crude lactacin F was filtered by ultrafiltration (TCF10 thin-channel filtration device) through membranes with sequentially decreasing pore sizes (i.e., 300, 100, 50, 30, and 10 kDa); it was determined that membranes in the size range of 100, and 300 kDa were capable of retaining almost 100% of lactacin F activity (i.e., 20 and 80% of yield, respectively), with total activity in the retentate increasing by 14-fold compared to the original supernatant. Next, lactacin F was subjected to a series of purification steps (ammonium sulfate precipitation, gel filtration, and HPLC) resulting in a 474-fold increase in specific activity.

In ammonium sulfate precipitation, the lactacin F from a culture supernatant was recovered as a floating pellicle, while from gel filtration, lactacin F was sized at approximately 180 kDa, and finally, from a column fraction of the HPLC, it was observed as a micelle-like globular particle under electron microscopy. After scrutinising these experimental results, it was postulated that lactacin F was associated with a larger molecular complex.

An attempt to perform a primary recovery of pediocin PA-1 from *Pediococcus acidilactici* 003 MRS broth using a combined microfiltration [polyvinylidene difluoride membrane (PVDF)]-diafiltration [polysulfone (PS)]-nanofiltration [polyamide (PA)] process has also been reported (Zhang et al., 2014). However, for further purification, rotary vacuum evaporation (20-fold concentration), and HPLC were employed because many peaks were still obtained, indicating the presence of impurities. Alternatively, the resulting permeate was also subjected to spray drying directly to obtain a pediocin-rich preservative. During ultrafiltration, a PS-10 membrane (MWCO = 10 kDa) was selected as it exhibited the highest permeability and activity recovery (63.3% as compared to 47.8%, and 62.6%, for PS-5, and PS-30, respectively), had the lowest permeate flux decline over permeate volume (based on flux-permeate volume profile), albeit only with average membrane anti-fouling performance (flux recovery ratio, FRR = 67.2% as compared to 54.5%, and 69.5%, for PS-30, and PS-5, respectively). After the nanofiltration (volume concentration factor = 4.5), the activity recovery was 71.6% with 4.5-fold purification.

2.1.5. Rapid purification methods

2.1.5.1. Three-step procedure

Methods for the rapid purification of bacteriocin were also reported by previous researchers using a short sequence of rather conventional unit operations. Callewaert et al. (1999) proposed a novel three-step method for the purification of amylovorin L471 from *L. amylovorus* DCE 471 which consisted of ammonium sulfate precipitation, chloroform/methanol extraction, and reversed-phase HPLC which resulted in about 64 000 AU/mL activity. Guyonnet et al. (2000) developed a three-step method for the purification of mesentericin Y105, a bacteriocin produced by *Leuconostoc mesenteroides* Y105 which yielded about 60% activity. The procedure consisted of cation exchange chromatography on a carboxy methyl cellulose-filled column (2.5 by 18 cm), C18 cartridge, and C8 Kromasil analytical HPLC column. The same method was also applied to four other anti-*Listeria* bacteriocins with purification yields varying from 10 to 66% (Table 1).

In addition, two bacteriocins from *Enterococcus durans* were successfully purified by cation exchange chromatography, reversed-phase column R1, and RP-HPLC (Batdorj et al., 2006). Two major peaks obtained during RP-HPLC represented enterocin A511A, and A511B, reaching purification factors of about 1650, and 2250-fold, and yields of 16% and 64%, respectively. The analysis of these two bacteriocins showed that they had slightly different molecular masses and amino acid composition (Batdorj et al., 2006). In addition, bifidin I produced by *B. infantis* BCRC 14602 was purified by subjecting its cell-free culture supernatant to a batch binding onto silicic acid (ADSA),

followed by cation exchange chromatography, and RP-HPLC, resulting in 25.6% activity yield, and 1390-fold purification (Ahmad et al., 2010). Interestingly, 100% adsorption was achieved between pH 5.0 and 7.0, indicating that complete isolation of bifidin I from the sample can be achieved at a wider range of pH values compared to the method performed by Cheikhyoussef et al. (2009) which used the producer cell itself as the adsorbent. 100% adsorption was recorded at a narrow pH range, i.e., between pH 6.0 and 7.0 (Cheikhyoussef et al., 2009).

2.1.5.2. Two-step procedure

Uteng et al. (2002) developed a rapid purification method for pediocin-like bacteriocin, and other cationic anti-microbial peptides from a complex culture medium by applying the bacterial culture directly onto a cation-exchange column followed by a low pressure reverse column. The results showed that greater than 90% purity can be achieved as determined by analytical reversed-phase chromatography, and capillary electrophoresis. This two-step method allowed purification to be quick (less than two hours), and resulted in a high yield of bacteriocin (80%) (Uteng et al., 2002). The short processing time was a marked improvement compared to the purification of pediocin from the same culture using ammonium sulfate precipitation, cation exchange chromatography, hydrophobic interaction chromatography, and reversed-phase chromatography which only gave about 10% activity yield. Moreover, this purification procedure took up to more than a week to complete (Uteng et al., 2002). Bacteriocin produced by *Bacillus subtilis* R75 which was isolated from fermented chunks of mung bean was partially purified by ammonium sulfate precipitation followed by single-step

gel exclusion column chromatography, resulting in 22.1% yield, and 22.3 purification fold (Sharma et al., 2011). Furthermore, Dündar et al. (2014) purified mesentericin W3 by direct adsorption onto a Micro-Cel matrix without any loss of activity, followed by desorption using SDS, and a cation exchange chromatography, resulting in a yield of 64%.

2.2. Alternative methods

The demand for alternative purification methods is mainly driven by the need to overcome problems associated with conventional strategies, as highlighted previously. In addition, alternative purification methods offer potential benefits such as high yield, low cost, short time to reach equilibrium, and a potential for up-scaling (Lappe et al., 2012).

Generally, at the beginning of a bacteriocin purification scheme a solid-liquid separation step is often needed, for example, the two centrifugation steps for the separation of cells from fermentation broth, and the collection of precipitate after ammonium sulfate precipitation (Simha et al., 2012). These two steps are time consuming, and laborious (Bizani et al., 2005; Garsa et al., 2014; H-Kittikun et al., 2015; Hastings et al., 1991; Liu et al., 2015), and hence researchers have been trying to develop alternative methods to eliminate them.

Simha et al. (2012) proposed the use of immobilised cells (*Pediococcus pentosaceous* NCDC 273) using a xanthan gum-alginate-chitosan combination for the production of pediocin PA-1 during fermentation, which removed the first centrifugation step for obtaining a cell-free crude extract; secondly, sedimentation of the bacteriocin precipitate

after ammonium sulfate precipitation was achieved using a high speed magnetic stirring technique (approx. 1200 rpm) rather than centrifugation. 134.4% activity yield, and 320 purification fold were achieved, which was much higher than the results reported by Guyonnet et al. (2000), i.e. 25%.

Furthermore, researchers have now shifted their attention towards bacteriocin purification schemes that are simpler in terms of equipment design and operation, cost and energy-saving, and shorter processing times compared to conventional chromatography-based methods, yet offering the best degree of purification. In the next section, several novel alternative bacteriocin purification strategies that have been reported, including expanded bed adsorption, a macroporous monolith, aqueous two-phase extraction, and an aqueous micellar two-phase system will be highlighted.

2.2.1. Expanded bed adsorption chromatography (EBA)

Clarification using centrifugation and filtration is a prerequisite before fixed bed chromatography during traditional downstream processing to overcome the clogging effect of the cells (Draeger and Chase, 1990). This lengthens the purification scheme, increases product loss, and thus reduces overall productivity (Draeger and Chase, 1990). With this in mind, Draeger and Chase (1990) created a stable fluidized (expanded) bed system that had similar chromatographic characteristics to packed bed mode, with plug flow in the column. A stable expanded bed is formed when adsorbents are freely suspended due to a balance between the particle sedimentation velocity, and the upward liquid flow velocity; voids generated between the adsorbent particles created by the upward flow during feedstock application permits the unhindered flow of

particulate materials such as cells, cell debris, particulates, and contaminants through the bed (and therefore removing them from the target protein), while the target protein is being simultaneously captured (Chase, 1994; Chow et al., 2007). Hence, EBA is an integrative unit operation that can achieve clarification, product concentration, and initial purification, and to date it has been widely used for the recovery of target proteins from unclarified bacterial broth (Hu et al., 2000; Tong et al., 2005), yeast broth (Heo et al., 2002; Chow et al., 2007), and mammalian cell cultures (Batt et al., 1995).

However, to our knowledge, there are only a few reports on the application of this technique to purify bacteriocins reported by De Vuyst et al. (1996), Callewaert and De Vuyst (1999), and Moreno et al. (2001). Low recovery (i.e. 30% of potential yields) was obtained using a strong cation exchanger-based EBA (i.e. Streamline SP^{TM}) to recover amylovorin L471 from a fermentation broth of *Lactobacillus amylovoros* DCE 471 (Callewaert and De Vuyst, 1999). Furthermore, a 47.6% yield with 140 purification fold was achieved via a hydrophobic interaction medium, Streamline^{TM} Phenyl, to recover the same bacteriocin (Moreno et al., 2001). After examining the results it was determined that the major loss occurred on the column (49.9%), indicating a low elution efficiency (Moreno et al., 2001). Although the difference in the yield for both cases was not significant, a 13 times higher purification was obtained through the later EBA variant (specific activity of 12900 as compared to 1000 AU.mg^{-1} as obtained by Streamline SP^{TM}). The optimum conditions determined for Streamline^{TM} Phenyl EBA were: equilibration-1.0 M ammonium sulfate at pH 4.0, and elution-50% of ethanol (v/v) at pH 6.0. Equilibration and elution steps were performed in packed bed mode at a flow rate of

2 $l.h^{-1}$, except for the loading stage where the degree of bed expansion (i.e. expanded bed height over settled bed height) recorded was close to 3.

The above optimum conditions for amylovorin recovery were also adopted for enterocin RZS C5 purification (Moreno et al., 2001), and resulted in a 47.6% yield, and 140 times purification. In comparison, only 1.6% yield, and 92 purification fold was obtained using conventional precipitation (ammonium sulfate-chloroform/methanol). In addition, the EBA system (Streamline SP^{TM}) was also tested for pediocin (from *Pediococcus acidilactici* ATCC 8042), and enterocin A (from *Enterococcus faecium* CTC 492) recoveries (Callewaert et al., 1999), and similarly, low recoveries were obtained, i.e. 26% and 15%, for pediocin, and enterocin, respectively. The authors attributed these low recoveries to: non-optimal loading/elution conditions, bacteriocin inactivation on the cationic resin, insolubilisation of bacteriocins, and activity reduction of the purified hydrophobic bacteriocin aggregates (Callewaert et al., 1999).

2.2.2. Macroporous monolith

Limitations in bead-packed column liquid chromatography such as the time-consuming packing process, and the slow diffusion of solutes within the pores have led to the introduction of monolithic columns for biomolecule purification (Luo et al., 2002). Basically, a monolith is composed of a single piece of highly porous organic or inorganic material with a well-defined pore size distribution that form highly interconnected channels (Mihelič et al., 2001). Monoliths eliminate diffusional limitations by transporting the solute to binding sites by convection rather than diffusion, which is a result of independent control of the silica skeleton size, and through pores (Etzel and Riordan,

2009; Zabka et al., 2008). Consequently, a faster volumetric throughput rate relative to bead-based columns is attainable (Etzel and Riordan, 2009). Hence, this method is similar to EBA in the sense that no clarification step is needed, and direct application of culture broth into the column can be carried out (Deraz et al., 2007).

Deraz et al. (2007) recovered sakacin P from *Lactobacillus sakei* CCUG 42687 MRS broth using macroporous polyacrylamide monoliths with functional epoxy groups (pAAm monoliths); the purification was performed in a typical chromatography mode, and a 96-well format. An octyl ligand-coupled monolith column (4 mL, d = 10 mm) was used while a phenyl ligand was coupled to each pAAm monolith (4 & 8 mL, d = 10 mm), and minicolumns for the 96-well format (volume 0.5 mL, d = 7 mm). The parameters investigated were the effect of pH (3.6, 4.6 and 6.2), salt (ammonium sulfate), and cell load (0.3–0.4, and 2.0-2.2 mg dry cell weight/mL column volume). Meanwhile, for minicolumns, the effect of sample volume i.e. 20-200 µL, on purification was investigated.

Under optimised conditions (using phenyl-pAAm monolith, pH 6.2, no ammonium sulfate, and 0.3-0.4 mg dry cell weight/mL column volume cell load), the cells were either found to be present in the breakthrough liquid (i.e. 92%), or non-recoverably bound onto the matrix; the recovery was about 80% of sakacin P in a cell-free eluate, with a purification fold of 150-160. In addition, cryogels in a 96-well format were shown to be effective for rapid pre-purification, concentration, and screening of sakacin P.

2.2.3. Aqueous two-phase systems (ATPS)

ATPS is considered to be a good purification technique for the separation, extraction and concentration of biomolecules because of its high productivity, simplicity, short processing time, cost effectiveness, scalability and versatility (Abbasiliasi et al., 2014; Li et al., 2001; Rito-Palomares, 2004). Two phases are formed when two incompatible polymers or polymer/inorganic salts are mixed in water above a certain critical concentration that is characterised by a high water content (80/90%), and low interfacial tension (Lappe et al., 2012). In general, there are two types of ATPS: a polymer-based, and a polymer-salt-based system, where the most popular examples are polyethylene glycol (PEG)/dextran, and PEG/potassium phosphate-based systems (Przybycien et al., 2004).

Lappe et al. (2012) purified cerein 8A using an ATPS that was composed of different combinations of components (PEG and ammonium sulfate; PEG, 1 M NaCl and ammonium sulfate; PEG, 0.1 M NaCl, and ammonium sulfate). The highest cerein 8A partition coefficient obtained (i.e., 9.4±4.9) was with PEG and ammonium sulfate, while the highest yield (i.e., 87.71%), and purification (i.e., 1.04) achieved was via the system with PEG, 1 M NaCl and ammonium sulfate. The purification expectedly resulted in a lower purification fold, but with a higher yield compared to the conventional method, i.e. a single gel filtration chromatography step with only 1.1% yield, and a 13 purification factor (Bizani et al., 2005; Lappe et al., 2012) (Table 1 and 2). Nevertheless, it seems that there is still further room for improvement as the authors only addressed the basic variables in the ATPS such as: the type of inorganic salt used (potassium phosphate,

sodium sulfate, sodium citrate and ammonium sulfate), effect of NaCl addition (0.1 M and 1 M). Optimisation of other important parameters (e.g., system composition, molecular weight of PEG, amount of crude load, and pH) that could influence the separation should be investigated.

In addition, Abbasiliasi et al. (2014) purified a bacteriocin-like inhibitory substance (BLIS) from a *Pediococcus acidilactici* Kp10 fermentation broth using an ATPS comprised of PEG and sodium citrate; the detailed procedure is illustrated in Figure 2. The purification recorded was 8.43 times with a yield of 81.18% which was achieved at 26.5% PEG (8000)/11% sodium citrate with a tie-line length (TLL) of 46.38% (w/w), volume ratio of top and bottom phases (V_R) of 1.8, and 1.8% crude load at pH 7 without the addition of NaCl. Hence, the potential of ATPS as a primary recovery method for bacteriocin from a complex fermentation broth is evident.

2.2.4. Aqueous micellar two-phase system (AMTPS)

AMTPS is a variant of the ATPS-based technique for separating a desired biomolecule using a non-ionic surfactant that results in the formation of binary phases (a micelle-rich phase, and micelle-poor phase) at certain surfactant concentrations, and temperature (Rangel-Yagui et al., 2004). The basis of separation is the difference in the physiochemical characteristics of both phases which drives the partitioning of the target protein (Liu et al., 1996). Jozala et al. (2008) investigated the feasibility of using AMTPS to extract nisin (commercial and biosynthesized), where a single non-ionic surfactant, Triton-X 114, was used. Firstly, in order to ensure that there was no *L. sakei* growth inhibition and nisin inactivation in the presence of the surfactant, they performed the

partitioning experiment at different Triton X-114 concentrations (i.e. 2, 4, 6 & 8%), and found that 2% Triton X-114 was optimal. Meanwhile, for commercial nisin, the total activity was enhanced by 10^3 AU in the presence of Triton X-114 (Jozala et al., 2008). The authors studied the effectiveness of AMTPS for nisin extraction based solely on the partition coefficient, K_{Nis} which is determined by Equation 1:

$$K_{Nis} = \frac{[Nis]_{\alpha}}{[Nis]_{\beta}} \quad \text{(Eq. 1)}$$

Where $[Nis]_{\alpha}$ and $[Nis]_{\beta}$ are the concentrations of nisin in the micelle-rich, and micelle-poor phase, respectively. Thus, the higher K_{Nis}, the higher the partitioning of the target bacteriocin into the micelle-rich phase. Nisin showed preferential partitioning into the micelle-rich phase, and the authors described this phenomenon as due to the effect of hydrophobic interactions, and the small size of the nisin molecule (3 kDa). However, besides the K_{Nis} value (i.e., in the range of 1-2), the authors did not examine the performance with regards to activity yield, and degree of purification.

Jozala et al. (2012) also extracted nisin using AMTPS composed of Triton X-114 and an electrolyte (Figure 3). In the presence of $MgSO_4$ and $(NH_4)_2SO_4$, the activity was 5.0 log AU/mL which was greater than the system without electrolyte. In spite of this, extraction of cerein 8A was also successful with 4% Triton X-114 which maintained the initial activity of the crude supernatant (3200 AU/mL), exploiting the affinity of the bacteriocin for the micelle-containing phase (Lappe et al., 2012). However, the authors did not mention either the yield or purification extent of the separation. In addition, when the concentration of Triton X-114 was 2%, and 6%, the partition coefficient (K_{Cer}) of cerein 8A was estimated to be lower than 1.5, while with 4% the K_{Cer} was greater than 2.5.

This implied that at a higher concentration of Trition X-114 the effect of excluded volume drove the partitioning of most proteins into the micelle-poor phase, resulting in the decline of K_{Cer} (Lappe et al., 2012).

In another example, Métivier et al. (2000) purified a divercin V41 from a complex culture medium of *Carnobacterium divergens* V41 by combining Triton X-114 phase partitioning, and cation exchange chromatography; a high specific activity (about 10^8 AU mg^{-1}) was obtained. From the study, they discovered that Triton X-114 phase partitioning can replace the preliminary step of cell separation by centrifugation. They also highlighted the fact that the detergent used can be easily removed by the first elution volume of the chromatographic step (Métivier et al., 2000). Triton X-114 (a non-ionic surfactant) was chosen because of its mildness, hence offering a non-denaturing environment (Liu et al., 1996; Jozala et al., 2008)

3. Other Aspects of Bacteriocin Purification

One of the unique features of bacteriocin observed during its purification is the increase in its specific activity (therefore purification factor), along with the increment in its purity following a purification step (Jozala et al., 2008). Moreno et al. (2001) attributed this to the removal of inhibitory compounds, and the dissociation of less active bacteriocin aggregates. For instance, Zhang et al. (2009) has recorded a 1,381.9-fold increase in specific activity of a purified pentocin 31-1 with a yield of 76.8% of the original activity. High purification factors (774, and 384 for water, and PBS elution, respectively) were also reportedly achieved by Jozala et al. (2015) after employing HIC to purify nisin produced by *Lactococcus lactis* fermentation in milk whey.

The other concern in the downstream purification of bacteriocin is the different levels of product purity required, and therefore the extent of the purification scheme. The level of purity required depends primarily on the subsequent bacteriocin application (Jozala et al., 2008; 2015; Yang et al., 1992), and the safety aspects of the purified product (Lappe et al., 2012). For bacteriocin characterisation studies (structure and function), a high product purity would be necessary, while in contrast, for biopreservation a high purity may not be required (Zhang et al., 2014). For example, Zhang et al. (2014) produced a pediocin PA-1-rich preservative after adding NaCl to the retentate of a nanofiltration step (with high content of impurities), followed by a final spray-drying step.

4. Conclusions

Various techniques consisting of conventional (long, and sequential chromatography-based), and alternative methods for the downstream purification of bacteriocin from its complex fermentation broth have been summarised from numerous studies in the literature. Varying results were obtained due to variations in the characteristic contents of the bacteriocin mother liquor, i.e., purity level. In addition, there were other factors which impacted on bacteriocin purification, such as the strain used, and the purification techniques themselves (Carolissen-Mackay et al., 1997). Alternative methods were highlighted (namely EBA, monolith, ATPS, and AMTPS) which exhibited superior performance compared to their conventional counterparts (in terms of activity yield) as a primary recovery method, but with a lower degree of purification. These methods also exploited the size, charge, and hydrophobic properties of the target bacteriocin.

The common notion about alternative methods addresses the ambition of ‘’anything but chromatography’’ that promises a lower cost, and a shorter processing time compared to conventional chromatography. In this context, the alternative methods presented here allowed for the handling of a non-clarified crude extract, thereby shortening the purification scheme, reducing product loss, and ultimately improving overall process economics.

In light of the above evidence, the liquid-liquid extraction-based methods: ATPS and AMTPS appear to be the most promising primary purification methods of bacteriocin. They have been well-established at a practical level, and have been widely used in biotechnological settings. In addition, they offer a relatively fast partial purification with a high yield, less energy consumption, and are scalable and economical. The major thrust in improving these methods has been towards developing a biocompatible method through the use of ‘’generally recognized as safe” (GRAS) biosurfactants like Tween and lecithin (from soy bean). Furthermore, the potential use of these surfactants to improve the bacteriocin’s stability and activity (as the result of a ‘’superactivity’’ effect) can be further explored. Concomitantly, intense scrutiny of the problem of surfactant carry-over can be set aside because of the non-necessity of complete surfactant removal, which in turn will reduce the overall downstream processing cost. On the contrary, if the phenomena of bacteriocin’s inactivation by these components, and the problems of interference in bacteriocin’s activity and protein assays are encountered, near complete removal of these components can be readily carried out. Besides, the

regeneration/recycling of the phase-forming components like salt and surfactant is crucial from an economic perspective.

On the other hand, it was shown that the classical method of pH-mediated cell adsorption/desorption continue to be a popular choice for bacteriocin recovery owing to its practicality, high-yielding process, ease of operation as well as being a 'natural' process which can be important for subsequent food application. More research on this method should be carried out to gain further insight into the mechanism of the target bacteriocin-cell interactions, which has not been well understood up until now. Also, a study of the use of food grade quality compounds like salt, EDTA, and surfactant as alternatives to toxic chemicals could be implemented in order to improve the recovery performance.

Moreover, simpler and more established methods like the precipitation-based methods (PBMs) (solvents, salts, polymers, polyelectrolytes, or surfactants) could also be contemplated for the use in the purification of bacteriocin owing to their enormous potential for large-scale application. PBMs are actually more cost-effective than chromatography techniques since only tanks and precipitants are required, instead of the expensive chromatographic columns, media, and eluents. In addition, the scale-up of PBMs is independent of the target protein concentration, and hence the dimensions and operating volume of the reactor used can be retained (Hammerschmidt et al., 2014). In addition, the feasibility of employing the more advanced PBMs such as the hybrid precipitation technology and centrifugal precipitation chromatography for bacteriocin recovery could also be examined (Przybycien et al., 2004). Furthermore,

future work on the application of other advanced methods such as integrated processes (i.e., incorporation of affinity ligands in an extraction system, three-phase partitioning), and the technique that exploits the distinctive properties of bacteriocin for simple purification, such as the application of thermal treatment for the recovery of heat-stable bacteriocin (i.e., class II bacteriocin - pediocin-like bacteriocins) should also be investigated.

Abbreviations

$(NH_4)_2SO_4$	Ammonium sulfate
AMTPS	Aqueous micellar two-phase system
ATPS	Aqueous two-phase system
AU/mL	Arbitrary units per millilitre
$AU.mg^{-1}$	Arbitrary units per milligram
BLIS	Bacteriocin like inhibitory substance
CFCS	Cell free crude supernatant
DLVO	Derjaguin Landau Verwey Overbeek
EDTA	Ethylenediaminetetraacetic
EBA	Expanded bed adsorption

FRR	Flux recovery ratio
HIC	Hydrophobic interaction chromatography
HPLC	High-pressure liquid chromatography
LAB	Lactic acid bacteria
$MgSO_4$	Magnesium sulfate
mL	Millilitre
mm	Millimetre
MRS	Man, Rogosa, and Sharpe
MWCO	Molecular weight cut off
NaCl	Sodium chloride
PBMs	Precipitation-based methods
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PVDF	Polyvinylidene difluoride membrane
PS	Polysulfone
PA	Polyamide

RP-HPLC	Reverse phase high-pressure liquid chromatography
SDS	Sodium dodecyl sulphate
TLL	Tie-line length
V_R	Volume ratio

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Table 1: Summary of the conventional methods used for the purification of bacteriocins, with their yield and purification fold.

No.	Method/Purification Scheme	Bacteriocin	Microbial Strain and Source	Activity Yield (%)	Purification Fold	Reference
1.	Ammonium sulfate or acid (pH 2.5) precipitation, hydrophobic interaction chromatography, gel filtration, RP-HPLC	Leucocin AUAL 187	*Leuconostoc gelidum* UAL 187 isolated from vacuum-packaged meat.	58	4 500	Hastings et al. (1991)
2.	Ammonium sulfate precipitation, ion exchange, hydrophobic interaction, RP-HPLC , gel filtration	Lactocin S	*Lactobacillus sake* L45 isolated from natural fermented sausage	3	40 000	Mørtvedt et al. (1991)
3.	Ammonium sulfate precipitation, 1-butanol extraction , ion-exchange chromatography	Cerein 8A	*B. cereus* 8A isolated from soil of native woodlands of south of Brazil	6.7	54.2	Bizani et al. (2005)
4.	Ammonium sulfate precipitation, cation exchange chromatography	Pediocin PD-1	*Pediococcus damnosus* NCFB1832 isolated from commercial malolactic starter culture	34	1700	Bauer et al. (2005)
5.	Ammonium sulfate precipitation, RP-HPLC	Salivacirin CRL1328	*Lactobacillus salivarius* CRL 1328 isolated from healthy woman vagina	7.3	-	Pingitore et al. (2007)
7.	Ammonium sulfate precipitation, gel exclusion column chromatography	Not specified	*Bacillus subtilis* R75 isolated from fermented chunks of mung bean	22.10	22.30	Sharma et al. (2011)
8.	Ammonium sulfate precipitation at isoelectric point, cation exchange chromatography, RP-HPLC	Pediocin PA-1	*Pediococcus pentosaceous* NCDC 273 from dairy products	134.4	320	Simha et al. (2012)
9.	Ammonium sulfate precipitation, cation exchange chromatography, hydrophobic interaction chromatography	Not specified	*L. mirunus* AU06 isolated from marine sediments	28.92	4.74	Elayaraja et al. (2014)
10.	Ammonium sulfate	Not specified	*Enterococcus faecalis*	4	48.10	H-Kittikun et al.

	precipitation, reverse phase cartridge, cation exchange chromatography, RP-HPLC		KT2W2G isolated from the mangrove forest in southern Thailand			(2015)
11.	pH-mediated cell adsorption/desorption	Sakacin A	*Lactobacillus Sake*	44.3	-	Yang et al. (1992)
		Pediocin AcH	*Pediococcus Acidilactici*	106.7	-	
		Nisin	*Lactococcus Lactis*	93.3	-	
		Leuconocin Lcml	*Leuconostoc comasum*	96.2	-	
12.	Adsorption–desorption of the bifidin I onto /from silicic acid, cation exchange chromatography, RP-HPLC	Bifidin I	*B. infantis* BCRC 14602 from stock cultures in MRS	25.6	1390	Ahmad et al. (2010)
13.	pH-mediated cell adsorption–desorption method, gel permeation chromatography, RP-HPLC	Not specified	*Weissella paramesenteroides* DFR-8 isolated from cucumber (*Cucumis sativus*)	4.42	177.94	Pal and Ramana (2010)
14.	Adsorption-desorption of the bacteriocin from culture supernatant onto Micro-Cel (diatomite calcium silicate), cation exchange chromatography	Mesentericin W3	*Leuconostoc mesenteroides* subsp. *cremoris* W3 isolated from Turkish white wine during malolactic fermentation	64	-	Dündar et al. (2014)
15.	pH mediated adsorption and desorption, SP-Sepharose Fast flow cation exchange column, GF Sephadex G10, RP-HPLC	Bifidocin A	*Bifidobacterium animals* isolated from centenarians' intestines	7	115	Liu et al. (2015)
16.	Cation-exchange chromatography, hydrophobic interaction chromatography, HPLC	Sakacin A	*L. sakei* 2675	10	-	Guyonnet et al. (2000)
		Sakacin P	*L. sakei* 2525	50	-	

		Enterocin A	*E. faecalis* 336	66	-	
		Pediocin PA-1	*P. acidilactici* 1521	25	-	
17.	Cation exchange chromatography – pressure reverse column	Pediocin PA-1	*Pediococcus acidilactici LMG2351*	80	-	Uteng et al. (2002)
18.	Cation exchange chromatography, reverse phase column R1, RP-HPLC	Enterocins A5-11A	*Enterococcus durans* isolated from Mongolian airag	16	1650	Batdorj et al. (2006)
		Enterocins A5-11B		64	2250	
19.	Hydrophobic interaction chromatography, elution of nisin with water or PBS	Nisin	*Lactococcus Lactis* from cultivation of milk whey	Water: 284.88 PBS: 152.02	Water: 724 PBS: 384	Jozala et al. (2015)
20.	Immunoaffinity chromatography	Nisin A	*Lactococcus lactis* BB24	72.7	10	Suarez et al. (1997)
21.	Immunoaffinity chromatography	Enterocin B	*Enterococcus faecium* BFE 900	25	10.7	Rose et al. (2001)
22.	Immunoaffinity chromatography	Enterocin P	*Enterococcus faecium* P13	1.1-5.9 µg	-	Gutiérrez et al. (2004)
23.	Ultrafiltration	Lactacin F	*Lactobacillus acidophilus*	100	-	Muriana and Klaenhammer. (1991)

24.	Microfiltration, diafiltration, nanofiltration	Pediocin PA-1	*Pediococcus acidilactici* 003	71.6	4.5	Zhang et al. (2014)

Table 2: Summary of alternative methods used for the purification of bacteriocins with their yield and purification fold.

No.	Method/Purification Scheme	Bacteriocin	Microbial Strain and Source	Activity Yield (%)	Purification Fold	Reference
1.	Cation exchanger-based EBA	Amylovorin L471	*Lactobacillus amylovoros* DCE 471	30	-	Callewaert and De Vuyst. (1999)
	EBA system (Streamline SP^{TM})	Pediocin Enterocin A	*Pediococcus acidilactici* ATCC 8042 *Enterococcus faecium* CTC 492	26 15	- -	
2.	Phenyl – pAAm monolith	Sakacin P	*Lactobacillus sakei* CCUG 42687	80	150 - 160	Deraz et al. (2007)
3.	ATPS by PEG, ammonium sulfate	Cerein 8A	*B. cereus* 8A was purified from the culture supernatant (Bizani et al., 2005)	81.7±27.5	0.96	Lappe et al. (2012)
	ATPS by PEG, ammonium sulfate + 1 M NaCl			65.3±38.5	0.81	
4.	Aqueous two-phase system (ATPS) consisting of PEG with sodium citrate	Bacteriocin-like inhibitory substance (BLIS)	*Pediococcus acidilactici* Kp10 from culture broth	81.18	8.43	Abbasiliasi et al. (2014)
5.	TX-114 partitioning, cation exchange chromatography	Divercin V41	*Carnobacterium divergens V41* isolated from fish viscera	0.04 (protein yield)	-	Métivier et al. (2000)

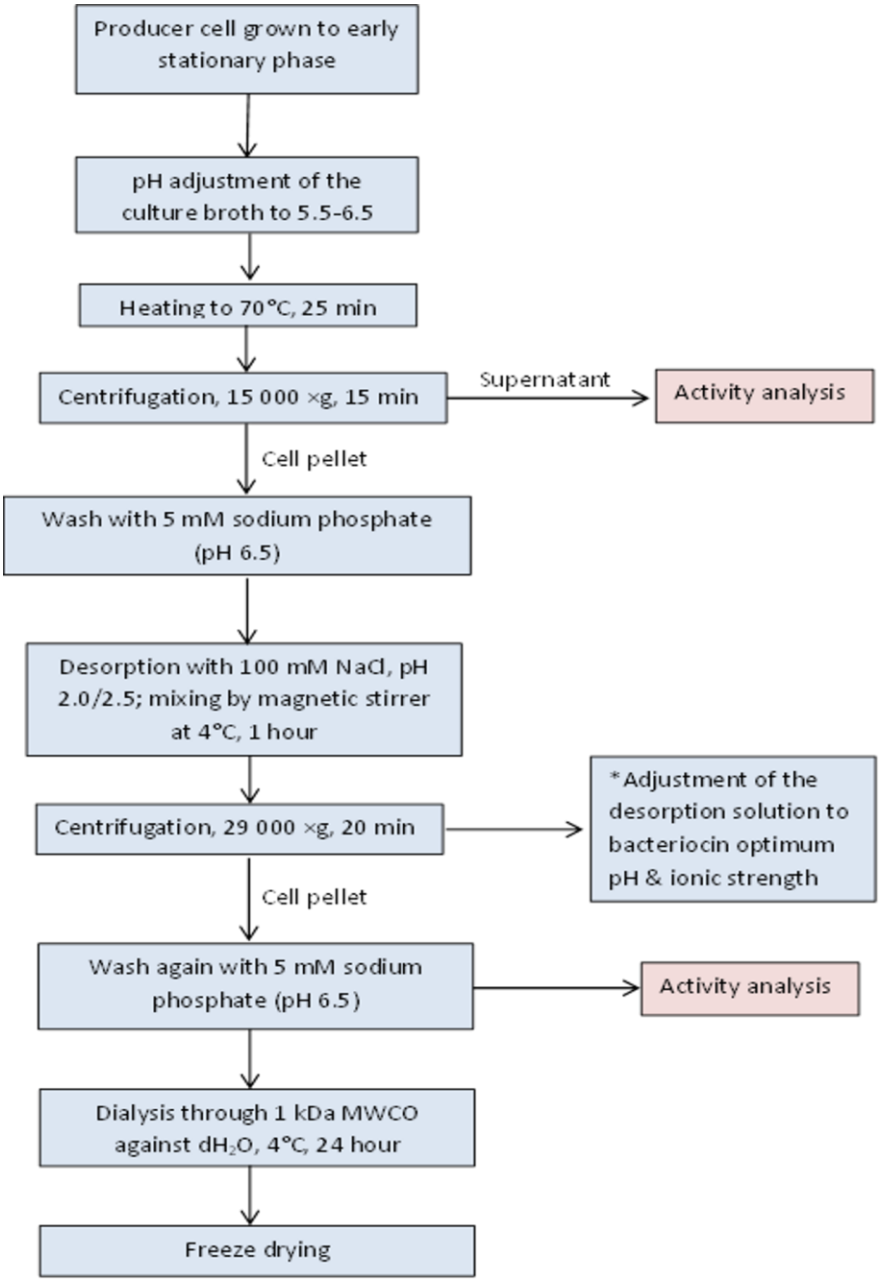

Figure 1: Schematic diagram of bacteriocin extraction method based on adsorption onto the producer cells (Yang et al., 1992; Miao et al., 2014). *Adjustment of pH was performed to preclude the influence of pH during bacteriocin activity determination, therefore ensuring the calculation accuracy of non-adsorbed fraction.

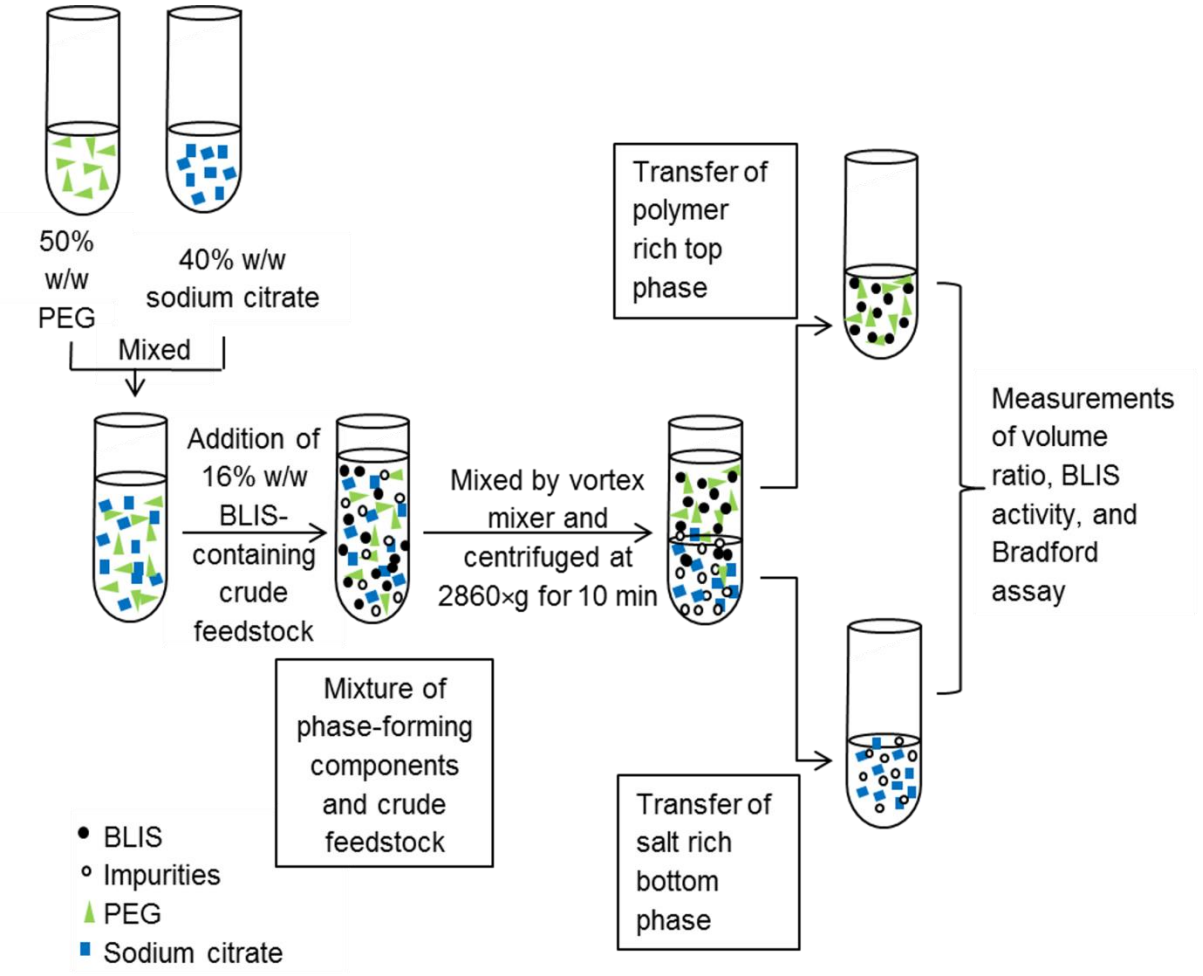

Figure 2: Schematic diagram of BLIS purification via ATPS performed by Abbasiliasi et al. (2014)

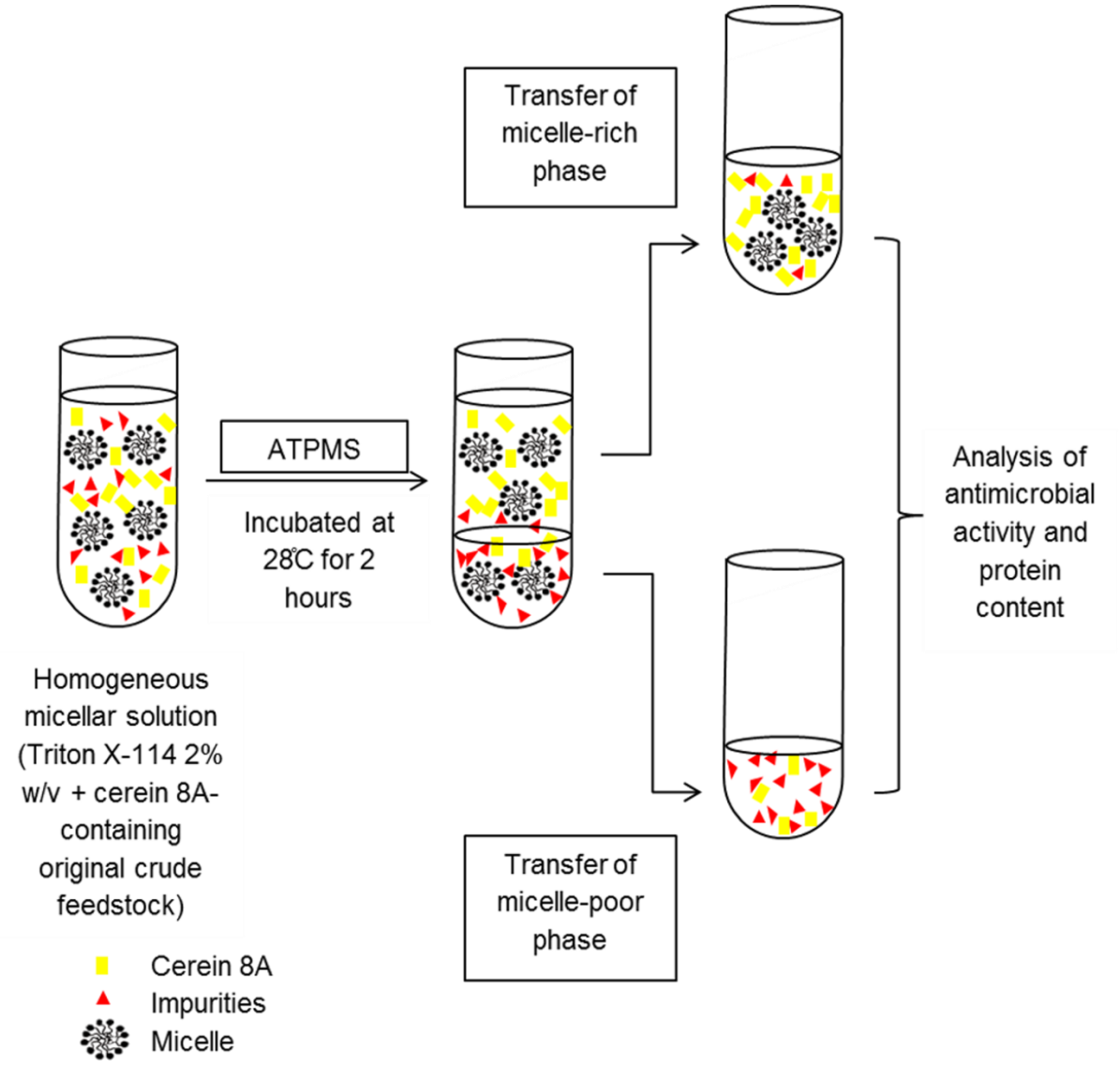

Figure 3: Schematic diagram of the cerein 8A purification via ATPMS according to the method of Lappe et al. (2012)