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Bacteriocin partitioning from a clarified fermentation broth of *Lactobacillus plantarum* ST16Pa in aqueous two-phase systems with sodium sulfate and choline-based salts as additives

Sabrina da Silva Sabo[a,1], André Moreni Lopes[b,**,1], Valéria de Carvalho Santos-Ebinuma[b], Carlota de Oliveira Rangel-Yagui[a], Ricardo Pinheiro de Souza Oliveira[a,*]

[a] Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo – FCF/USP, Brazil
[b] Department of Bioprocess and Biotechnology, School of Pharmaceutical Sciences, State University of São Paulo – FCFar/UNESP, Brazil

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ABSTRACT

The partitioning of bacteriocin, a promising alternative to chemical preservatives, in a novel inexpensive and stable aqueous two-phase system (ATPS) comprising poly(ethylene glycol) (PEG) and sodium polyacrylate (NaPA) was studied. The ATPS was generated by mixing both polymers with Na_2SO_4 or choline chloride ([Ch]Cl) and a bacteriocin extract from the fermented broth of *Lactobacillus plantarum* ST16Pa. Bacteriocin showed stability at different pH values (3.0–8.0) and temperatures (50–80 °C), as well as in the presence of ATPS components. Hydrophobic and electrostatic interactions were found to be the major driving forces for bacteriocin partitioning. The peptide partitioned preferentially to the PEG-rich phase (partition coefficient, $K_{Bact} > 1$). However, the highest partition coefficient was achieved in the polymeric-based ATPS using [Ch]Cl as additive, as follows: 8 wt% PEG 10,000 g/mol/8 wt% NaPA 8,000 g/mol/0.5 M [Ch]Cl, resulting in a K_{Bact} equal to 32. Moreover, these conditions promoted high selectivity ($S = 62.7$), since the greater part of total proteins partitioned into the NaPA-rich phase ($K_{prot} = 0.51$). In conclusion, ATPS composed of PEG/NaPA using choline-based salts as additive can be considered mainly as a first step for bacteriocin extraction from fermented broth by applying low polymer content and mild conditions.

1. Introduction

The wide use of chemical preservatives, such as nitrites and sulfur dioxide, to extend the shelf life of foods may cause adverse effects on human health and on the nutritive value of food [1]. For this reason, consumers are increasingly worried about the amount of chemical additives present in their diet, which has led to a growing demand for natural or chemical-free food [2]. This demand, coupled with the increasing desire for minimally processed food with long shelf life, has attracted research interest in finding natural preservatives [3]. In this sense, bacteriocins, mostly produced by lactic acid bacteria (LAB), are a promising alternative to the preservatives available in the market [4].

Bacteriocins are defined as bacterially produced, heat-stable peptides that are active against bacteria except its producer [5]. Several purification protocols have been developed to extract bacteriocin from LAB cultures [6]. Generally, the first step involves the concentration of bacteriocin from the culture supernatant using ammonium sulfate precipitation [7]. This method does not provide a high degree of purity; therefore, further steps using preparative isoelectric focusing and/or multiple chromatographic techniques, including cation exchange, gel filtration, hydrophobic interaction, and reverse-phase liquid chromatography, are necessary to achieve highly pure bacteriocins [8]. However, these methods have their own drawbacks, such as high operation costs and protocols that include several steps, resulting in low yield [9].

In the search for alternative methods, liquid–liquid extractions conducted in various types of aqueous two-phase complex-fluid systems have been investigated for bioseparation [10]. An aqueous two-phase system (ATPS) can be applied for bacteriocins extraction directly from the fermented medium, leading to a simplification of the overall purification protocol [11]. Some studies have demonstrated that an ATPS

* Corresponding author at: Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, Av. Lineu Prestes 580, São Paulo, 05508-900, Brazil.
** Corresponding author at: Department of Bioprocess and Biotechnology, School of Pharmaceutical Sciences, State University of São Paulo, Rodovia Araraquara Jaú, Km 01 – s/n Campos Ville, Araraquara, 14800-903, Brazil.
E-mail addresses: amorenilopes@gmail.com (A.M. Lopes), rpsolive@usp.br (R.P.d.S. Oliveira).
[1] These authors contributed equally.

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can be formed from polyethylene glycol (PEG) and sodium polyacrylate (NaPA), resulting in a PEG-enriched top-phase and a NaPA-enriched bottom phase [12–19]. PEG/NaPA systems have some advantages, such as low viscosity, ease of handling, rapid phase separation, relatively low-cost chemicals, and are environmentally friendly, compared with traditional polymer-polymer systems (PEG/dextran) [13,14]. PEG is an uncharged polymer, while NaPA is negatively charged, thus the entropic penalty of counter-ion compartmentalization leads to a minimal concentration of salt being required to form the two aqueous phases [14].

In previous studies, PEG/NaPA based ATPSs have been used to partition different biomolecules, such as green fluorescent protein [15], hemoglobin, lysozyme, glucose-6-phosphate dehydrogenase [16], myoglobin, ovalbumin [17], cytochrome c [18], proteases [13], and amyloglucosidase [19]. Remarkably, the use of a third component, *i.e.* salts, has been proposed to improve ATPS potential. The addition of salts may result in changes to the polymer-polymer interactions because of electrostatic screening. Although salts distribute almost evenly between the two phases of an ATPS, there is a small, but significant, difference in the partition coefficient of electrolytes, which may generate an electrostatic potential difference between the phases, thereby improving the partitioning of charged molecules [20–22]. Parallelly, the effect of electrolytes might also induce changes in the solution properties, *i.e.* causing salting in and salting out effects [23–25].

Although a few studies using bacteriocin partitioning/recovery using PEG/salt-based ATPSs [26–30], and with an aqueous two-phase micellar system (ATPMS) [31,32], have been evaluated, PEG/NaPA systems composed of inorganic salts or choline-based salts have not been proposed before. In this context, and based on the advantages mentioned above, the present study investigated a PEG/NaPA based ATPS with Na_2SO_4 or choline chloride ([Ch]Cl) as additives to extract bacteriocin from the fermented broth of *Lactobacillus plantarum* ST16Pa. Preliminary experiments were performed to evaluate the stability of bacteriocin in solutions of ATPS components (PEG, NaPA, and salts). Subsequently, a multifactorial experimental design was used to estimate the effects of pH and temperature on bacteriocin partitioning in ATPS. To the best of our knowledge, this is the first study on the partitioning of bacteriocin produced by *L. plantarum* ST16Pa in a polymer-polymer system.

2. Material and methods

2.1. Materials

PEG with molar masses of 2,000, 6,000, and 10,000 g/mol, were purchased from Merck (Hohenbrunn, Germany). Polyacrylic acid (NaPA) 8,000 g/mol (45 wt%) and the salts, sodium sulfate (Na_2SO_4) and choline chloride ([Ch]Cl), were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solutions were prepared in sodium acetate 0.1 M buffer, pH 5.0, with water purified using a Millipore Milli-Q system (Bedford, MA, USA). The glassware used was washed in a 50:50 ethanol:1 M sodium hydroxide bath, followed by a 1 M nitric acid bath, rinsed copiously with Milli-Q water and, finally, dried in an oven at 70 °C for 1 h. All other reagents were of analytical grade and used as received.

2.2. Microbial cultures and fermentation conditions

The microorganism used was the *L. plantarum* strain ST16Pa, isolated by Todorov et al. [33] from a papaya species. As a microorganism indicative of bacteriocin antimicrobial activity, it was used the *Listeria innocua* strain 6a CLIST 2860 (AL224/07), isolated from a dry-fermented sausage sample and provided by Collection of *Listeria* (CLIST) from Fundação Oswaldo Cruz (FioCruz, Rio de Janeiro, Brazil).

For bacteriocin production by *L. plantarum* ST16Pa, first, the strain was reactivated by adding 1 mL of cryopreserved strain stock into a 250 mL Erlenmeyer flask containing 100 mL of Man, Rogosa and Sharpe (MRS) broth (DIFCO, Detroit, MI, USA) and incubating at 30 °C on a rotatory shaker at 100 rpm for 24 h. Subsequently, 10 vv% of this culture was used to inoculate 1 L Erlenmeyer flasks containing 500 mL of MRS broth, also incubated at 30 °C for 24 h.

To grow the bioindicator strain, *L. innocua* 6a CLIST 2860, 10 mL of the Brain Heart Infusion (BHI) broth (DIFCO, Detroit, MI, USA) was inoculated with 1 mL of cryopreserved strain stock and incubated overnight at 37 °C on a rotatory shaker at 100 rpm.

2.3. Determination of bacteriocin antimicrobial activity

The 24 h culture of *L. plantarum* ST16Pa was centrifuged at 25,750 × *g* for 15 min. The pH of the resulting cell-free supernatant (CFS) was adjusted to 6.0–6.5 with 1 M NaOH to eliminate organic acids, and heated to 80 °C for 10 min to inactivate proteases. Finally, the CFS was filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA), and later tested against the bioindicator *L. innocua* strain 6a CLIST 2860. The test was performed by the agar diffusion method, in which the bioindicator culture broth was diluted 100-fold approximately 10^7 Colony Forming Units per mL (CFU/mL). One milliliter of this dilution was transferred to a Petri dish (90 × 15 mm) containing 10 mL of melted BHI soft agar (containing 0.75 wv% agar). Then, 20 μL of the CFS was spotted onto the agar surface. A period of 3 h was allowed for the diffusion of the supernatant at 25 °C and then the plates were incubated at 37 °C for 24 h. Subsequently, the inhibition halos were measured in four directions using a digital caliper gauge (Lee Tools, model 684132) and the antimicrobial activity of bacteriocin (A_{Bact}), defined in arbitrary units (AU), was calculated based on the method of Sidek et al. [30]. The equation was as follows:

$$A_{Bact} = \left(\frac{\pi R^2}{v}\right) = \text{AU/mL} \quad (1)$$

where πR^2 is the inhibition (clear) zone area of the halo (in cm^2) and V is the volume (in mL) of the CFS (sample) dropped onto the agar surface. Some results were presented as $\%A_{Bact}$, considering the initial bacteriocin activity (around 68.35 AU/mL) as 100%. All experiments were performed in triplicate and average values were presented.

2.4. Determination of the total protein concentration

The total protein concentration was determined using the bicinchoninic acid method (BCA), which is compatible with the polymers used. Samples containing proteins (100 μL) and 2 mL of the BCA working reagent, prepared according to the manufacturer's instructions, were added to a test tube. After 30 min, the optical density at 562 nm was determined in a spectrophotometer, using deionized water as a blank. Absorbance values were correlated with protein concentration based on a calibration curve using bovine serum albumin (BSA) solutions from 0 to 1,000 μg/mL (equation obtained: y (ABS) = 0.0011 x (μg/mL) + 0.0245, R^2 = 0.99).

2.5. Bacteriocin activity in different conditions

The effect of pH and temperature on bacteriocin activity after 1 h was studied using a 2^2 central composite design. Bacteriocin activity was defined as the residual antimicrobial activity after exposure to the pH and temperatures investigated; these values (AU/mL) were correlated in terms of bacteriocin stability. A set of 12 experiments, which contained a factorial or fractional factorial matrix, with center points and star points to allow the estimation of the curvature, was performed. The range and levels of the components under study are given in Table 1. The pH of the solutions was adjusted using 5 M HCl or 5 M NaOH. Subsequently, we evaluated the influence of the ATPS components, namely PEG 10,000 g/mol, NaPA 8,000 g/mol, Na_2SO_4, and

Table 1
Factor levels of the 2^2 central composite design to study the bacteriocin activity of *L. plantarum* ST16Pa as a function of independent variables (pH and temperature).

Factors	Coded Levels				
	Axial (−1.414)	Lower (−1)	Center (0)	Higher (+1)	Axial (+1.414)
pH	3.2	4.0	6.0	8.0	8.8
Temperature (°C)	37.6	50	80	110	122.4

[Ch]Cl, on the activity of bacteriocin after 1 and 24 h at 25 °C. In this set of experiments, different concentrations of the compounds were investigated as follows: PEG 10,000 g/mol and NaPA 8,000 g/mol – 10 and 20 wt%; Na_2SO_4 and [Ch]Cl − 0.20, 0.35, 0.50, and 0.90 M.

2.6. Bacteriocin partitioning in ATPS

ATPSs were prepared in 15 mL graduated glass tubes by adding PEG, NaPA, salts (Na_2SO_4 or [Ch]Cl) in sodium acetate buffer (pH 5.0), together with 150 μL of the sample containing bacteriocin (CFS), which was the last component added, resulting in a 5 g total mass system. The system components were added after weighing and then the tubes were sealed with PARAFILM® and homogenized in an orbital shaker (Barnstead/Thermolyne, Ramsey, MN, USA; model 400110) at 8 rpm for 15 min at 25 °C, to form a homogeneous single-phase solution. Then, the systems were kept in a thermo-regulated device for 1 h at 25 °C, to reach partition equilibrium. Afterwards, the two coexisting polymer phases (top and bottom phases) were carefully collected using disposable syringes. The volume of each phase was measured and the sample analyzed for protein concentration and bacteriocin activity. Each measurement was performed in triplicate.

The influence of PEG molar mass, PEG concentration, and NaPA concentration (independent variables) on the bacteriocin partition coefficient was investigated using 2^3 full factorial designs (Table 2). Two 2^3 full factorial designs were performed, one with Na_2SO_4 in the ATPS and the other with [Ch]Cl; both salts were used at a concentration of 0.35 M. The molecular weight of NaPA was fixed at 8,000 g/mol. All systems were evaluated in terms of the effect (interference) on halo inhibition and their values were subtracted from the same system in the presence of the sample. These assays were considered as blanks and were performed in triplicate.

2.7. Partitioning parameters

The partitioning behavior of bacteriocin in the ATPS was quantified by the partition coefficient, K_{Bact}:

$$K_{Bact} = \frac{A_{top}}{A_{bot}} \quad (2)$$

where A_{top} and A_{bot} are the bacteriocin activity in the top (PEG-rich) phase and bottom (NaPA-rich) phase, respectively. The activity balance ($\%AB_{Bact}$) was calculated according to Eq. (3), and the recovery of bacteriocin in both phases ($\%REC_{top}$ and $\%REC_{bot}$) was calculated according to Eqs. (4) and (5), respectively:

Table 2
Factor levels of the 2^3 full factorial design to study the partitioning of bacteriocin in an aqueous two-phase system (ATPS).

Factors	Coded levels		
	Lower (−1)	Center (0)	Higher (+1)
PEG (g/mol)	2,000	6,000	10,000
PEG (wt%)	8	12	16
NaPA 8,000 (wt%)	8	12	16

$$\%AB_{Bact} = \left(\frac{A_{top}V_{top} + A_{bot}V_{bot}}{A_i V_i}\right)\times 100 \quad (3)$$

$$\%REC_{top} = \left(\frac{A_{top}V_{top}}{A_i V_i}\right)\times 100 \quad (4)$$

$$\%REC_{bot} = \left(\frac{A_{bot}V_{bot}}{A_i V_i}\right)\times 100 \quad (5)$$

where A_i is the bacteriocin activity in the stock solution added to the system, and V_{top}, V_{bot}, and V_i are the volumes of the top (PEG-rich) phase, bottom (NaPA-rich) phase, and the bacteriocin stock solution initially added to the system, respectively.

The partition of bacteriocin (P_{Bact}) relative to the two systems (PEG/NaPA with Na_2SO_4 and [Ch]Cl) was quantified according to the equation:

$$P_{Bact} = \frac{K_{Bact-PEG/NaPA/[Ch]Cl}}{K_{Bact-PEG/NaPA/Na_2SO_4}} \quad (6)$$

The partition coefficient of total proteins (K_{prot}) was also evaluated according to Eq. (7), as follows:

$$K_{prot} = \frac{C_{top}}{C_{bot}} \quad (7)$$

where C_{top} and C_{bot} are the total proteins concentration (μg/mL) in the top (PEG-rich) phase and bottom (NaPA-rich) phase, respectively.

The selectivity in terms of proteins (S) was also evaluated according to Eq. (8), as follows:

$$S = \frac{K_{Bact}}{K_P} \quad (8)$$

2.8. Determination of the electrical conductivity in the ATPS

The electrical conductivity of each phase of the ATPS was determined using a conductivity device (METTLER TOLEDO, Columbus, OH, USA; model MPC 227). Before the measurements, the apparatus was calibrated by reading a standard solution of 12.88 mS/cm and reading a sample of the air (*i.e.* without solution). Each phase was read in triplicate.

2.9. Statistical analysis

For the statistical analysis of both experimental designs presented above, the coded values of each of the independent variable were used. The 'Statistica' software, Version 7.0 (Statsoft, Tulsa, OK, USA) was used for data regression and graphical analysis. The statistical significance of the regression coefficients was determined using Fischer's test for analysis of variance (ANOVA) at a significance level (p-value ≤ 0.05). The coefficient of determination (R^2), which is a measure for fitting a generalized linear statistical model in relation to the observed values, was used to indicate how well the model could explain the observed values. To minimize the ANOVA error, the tests corresponding to the central point were replicated four times. The experimental and predicted values were compared to determine the validity of the models.

3. Results and discussion

3.1. Effects of pH, temperature, and ATPS components on bacteriocin activity

The main application of bacteriocin is as a preservative in processed food, and these products are usually produced with temperature and pH variations [34]; therefore, the evaluation of bacteriocin stability under extreme pH and temperature conditions is necessary. Statistical tools

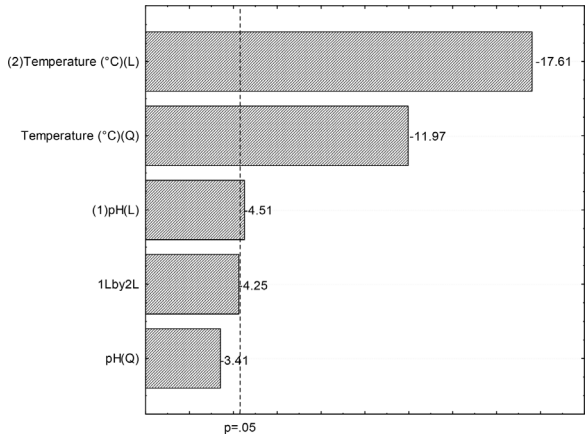


Fig. 1. Pareto chart for the effects of pH (1) and temperature (2) on bacteriocin stability according to a 2^2 central composite design. The number in front of each bar corresponds to the magnitude of the level that could improve the variable response.

allow the evaluation of several assay combinations with a reduced number of experiments; therefore, the effects of pH (3.2–8.8), and temperature (37.6–122.4 °C) on bacteriocin activity after 1 h of incubation were determined employing factorial design (Table 1). The choice of main variable values was based on the literature describing the pH tolerance and thermoresistance of bacteriocins [35], and, in view of the possible application of bacteriocins as food preservatives, the usual levels during the processing operations (for both variables).

The Pareto chart (Fig. 1) shows an estimative effect of the variables and their interactions on the response variable. Therefore, each bar length is proportional to the standardized effect and the vertical line shows the statistically significant variables (p-value > 0.05) [36]. As can be seen in Fig. 1, the temperature effect was greater than the pH effect, because the bar length for the first variable was longer for both the linear and quadratic levels. However, linear and quadratic temperature effects, as well as the linear effect of pH were significant at 95% confidence limit (p-value < 0.05) on bacteriocin activity. A synergistic effect between temperature and pH remained within the limit, considering the 95% confidence limit, and showed a lower effect on bacteriocin activity. The quadratic pH effect was not significant at the 95% confidence limit. The temperature variable (both linear and quadratic effects) showed a negative effect on bacteriocin activity, within the range evaluated. This negative effect of temperature means that lower temperatures would increase and/or maintain the peptide's activity. According to these results, we considered that the synergistic effect between temperature and pH did not have a pronounced effect on the bacteriocin activity, and the change in the temperature is more important than the change in pH, within the range evaluated.

Our results showed that temperatures lower than 122.4 °C promote bacteriocin stability. The lowest stability was observed at pH 6.0 and 122.4 °C, indicating that extreme conditions of heat lead to a loss of bacteriocin activity. To demonstrate the effect of temperature on bacteriocin activity, considering the pH 6.0 and keeping this variable at 37.6–80 °C (run 7 and central point) the activity was 79.59 and 75.98 AU/mL, respectively. Similar results were achieved at the others pHs evaluated. The fitted surface presented in Fig. 2 shows that bacteriocin is more stable at temperatures from 50 to 80 °C in the pH range of 3.0–8.0. According to Fig. 2, a higher bacteriocin activity would be achieved at a pH of 6.0 and 60–70 °C, which would theoretically result in an activity higher than 70 AU/mL.

Some researchers have demonstrated that the overall initial bacteriocin activity was significantly retained at temperatures up to 90 °C, low pH (around 5.0), and short incubation times (around 10 min) [35]. Guerra et al. [37] found similar results for the effect of pH/temperature on the stability of pediocin and nisin produced by *Pediococcus acidilactici* NRRLB5627 and *Lactococcus lactis subsp. lactis* CECT 539, respectively. Thermal stability in slightly acidic media has been described for most bacteriocins [33,37]. According to the literature, the optimal pH range for bacteriocin production is around 4.5–5.5 [35,38]. This behavior is attributed to their high content of glycine and to the formation, at a molecular level, of globular structures and strong hydrophobic interactions in the bacteriocin molecule [39].

The results reported in the present work are in agreement with the ones achieved by Todorov et al. [33], using the same bacteriocin-producing strain (*i.e. Lactobacillus plantarum* ST16Pa), in which bacteriocin was not affected by pH in a range from 2.0 to 10.0 after 24 h of exposure. Hu et al. [40] also showed that the antimicrobial activity of bacteriocin produced by *L. alimentarius* FM-MM$_4$ increased at acidic pH while it decreased at pH higher than 8.0. Here we did not evaluated pH values higher than 8.0. However, according to Yi et al. [41], under extreme pH conditions instability or dissociation of amino and carboxyl groups is observed, resulting in protein denaturation.

Anyway, our results also suggested that the bacteriocin evaluated here can tolerate the conditions normally encountered in food processing and would particularly be useful in medium acid fermented food products, including a number of fermented and ripened dairy and meat products.

Additional studies were conducted to evaluate the stability of bacteriocin in the presence of the ATPS components. We investigated the effect of electrolytes Na_2SO_4 and [Ch]Cl at four different concentrations (0.2, 0.35, 0.5, and 0.9 M), for two different periods of time (1 h and 24 h) (Fig. 3). Based on the results for 1 h, the influence of both salts on the percentage of bacteriocin activity (%A_{Bact}) was relatively low under the conditions evaluated. In the presence of Na_2SO_4, the %A_{Bact} varied between 95.1% (at 0.2 M) and 89.7% (at 0.9 M). In the case of [Ch]Cl, the %A_{Bact} varied between 92.6% (at 0.2 M) and 86.8% (at 0.9 M).

The same bacteriocin behavior was observed after 24 h of exposure to the ATPS components. The %A_{Bact} slightly decreased with the increase in electrolytes concentration. For Na_2SO_4, the %A_{Bact} values varied between 91.3% (at 0.2 M) and 86.6% (at 0.9 M). Regarding [Ch]Cl, at the same two electrolyte concentrations, %A_{Bact} was 91.4% and 84.0%, respectively. In general, the assays for 1 h and 24 h showed similar results for the %A_{Bact} in the presence of both electrolytes, and only a small activity loss occurred at high electrolyte concentrations (0.9 M).

The effect of the polymers on bacteriocin stability was also investigated. In this case, we evaluated PEG at a higher molecular weight than that used in the above-mentioned experiments (10,000 g/mol) and NaPA 8,000 g/mol, both at two different concentrations: 10 and 20 wt % (Fig. 4). Bacteriocin was found to be stable in the presence of PEG 10,000, with %A_{Bact} values above 91% even after 24 h of exposure. By contrast, bacteriocin presented a significant loss of activity in the presence of NaPA. The %A_{Bact} values were 91.1% (10 wt% NaPA) and 88.3% (20 wt% NaPA) after 1 h, and 76.0% (10 wt% NaPA) and 47.5% (20% NaPA) after 24 h. The NaPA polymer is strongly negatively charged [16] and can interact with oppositely charged sites of biomolecules through electrostatic interactions, destabilizing or changing their molecular structure and causing loss of activity.

Although experiments at pH 5.0 were not performed, from the results achieved and Fig. 2 we can consider that at this pH bacteriocin presents great stability and even the presence of Na_2SO_4, [Ch]Cl and PEG do not interfere with its stability under the conditions evaluated.

3.2. Bacteriocin partitioning in PEG/NaPA/Na_2SO_4 systems

An ATPS comprising PEG/NaPA and Na_2SO_4 under different conditions was investigated using a 2^3 full factorial design. This ATPS is of particular interest because these polymers are nontoxic, biodegradable, and approved by the Food and Drug Administration (FDA). Furthermore, they have been used to solubilize and stabilize pharmaceutical and biomedical products [42]. Table 3 presents the experiments of the factorial design with the response-variable partition

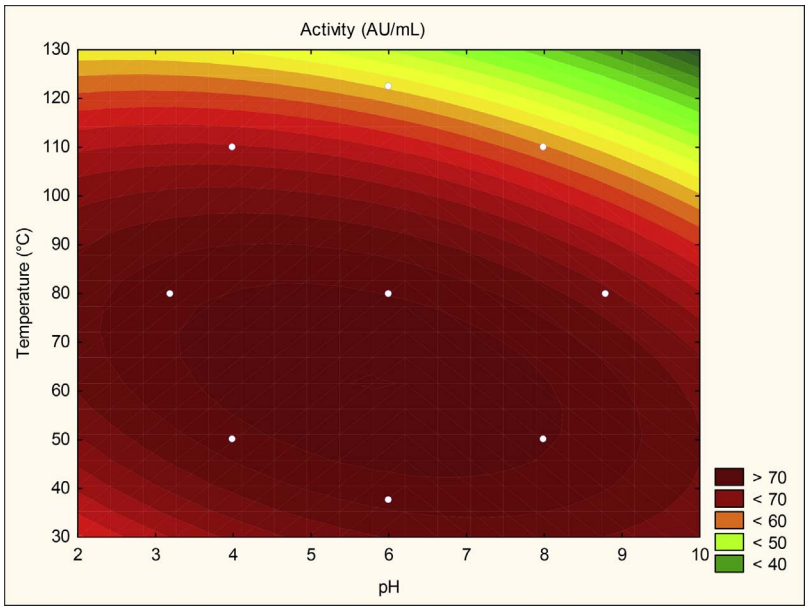
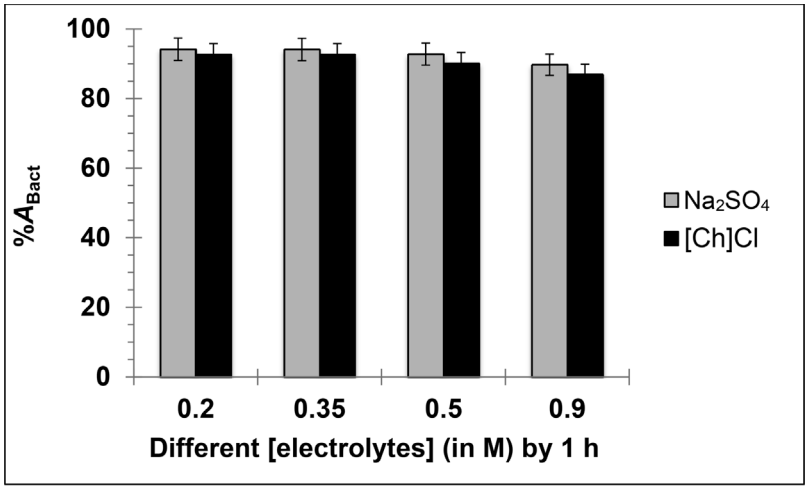
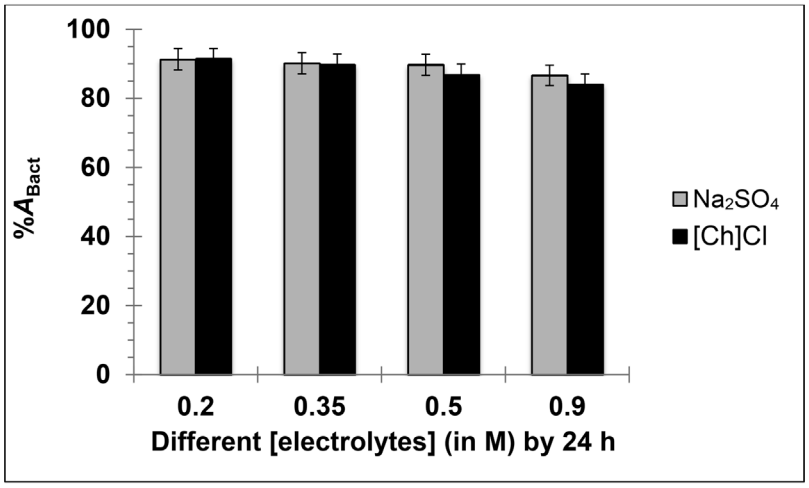


Fig. 2. Fitted surface showing the simultaneous effect of pH (from 3.2 to 8.8) and temperature (from 37.6 to 122.4 °C) on bacteriocin stability according to a 2^2 central composite design (Table 1). The deeper the red color, the greater the bacteriocin activity obtained. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

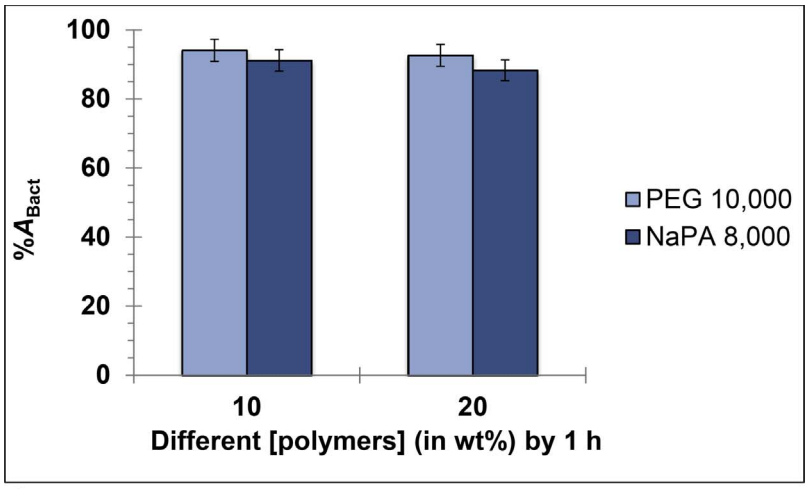


a)

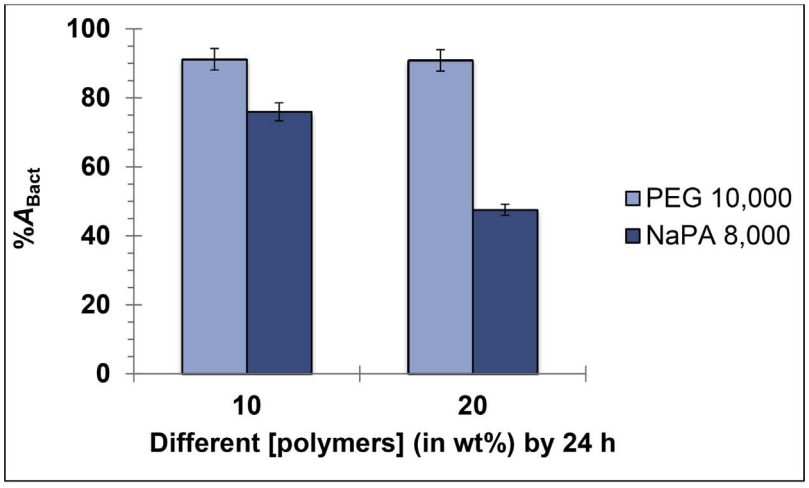


b)

Fig. 3. Bacteriocin activity (%A_{Bact}) in the presence of different electrolyte (Na_2SO_4 and [Ch]Cl) concentrations: 0.2, 0.35, 0.5, and 0.9 M for 1 h (**a**) and 24 h (**b**). The error bars represent the 95% confidence levels for the measurements.



a)



b)

Fig. 4. Bacteriocin activity (%A_{Bact}) in the presence of different polymer (polyethylene glycol (PEG) 10,000 g/mol and sodium polyacrylate (NaPA) 8,000 g/mol) concentrations: 10 and 20 wt% by 1 h (**a**) and 24 h (**b**). The error bars represent the 95% confidence levels for the measurements.

Table 3
Results of the 2^3 full factorial design to study the partitioning parameters of bacteriocin in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/Na_2SO_4 systems.

Experiments	K_{Bact}	%AB_{Bact}	%REC_{top}	%REC_{bot}
16%PEG 2,000/16%NaPA	20.8	93	82	11
8%PEG 2,000/16%NaPA	13.4	98	81	17
8%PEG 2,000/8%NaPA	18.7	102	88	13
16%PEG 2,000/8%NaPA	11.5	68	44	24
[a]12%PEG 6,000/12%NaPA	10.3 ± 0.37	81.9 ± 8.1	59.4 ± 7.2	22.5 ± 1.1
16%PEG 10,000/8%NaPA	18.3	45	32	14
16%PEG 10,000/16%NaPA	20.3	91	79	12
8%PEG 10,000/8%NaPA	21.3	72	61	11
8%PEG 10,000/16%NaPA	23.5	112	104	9

[a] The central point of full factorial design was repeated three times.

coefficient (K_{Bact}), as well as the experimentally obtained values for the activity balance (%AB_{Bact}) and bacteriocin recovery at both phases of the system (%REC_{top} and %REC_{bot}).

From the data presented in Table 3, the bacteriocin partition coefficient was > 1 for all the conditions investigated, indicating that it preferentially partitions to the top (PEG-rich) phase of PEG/NaPA systems. The partition coefficient values varied between $10.3 < K_{Bact} < 23.5$. The Pareto chart (Fig. 5) shows that the main variables, NaPA concentration and PEG concentrations, had a statistically significant (p-value < 0.05) effect on K_{Bac}. In addition, the interactions between PEG concentration and molecular weight (1 by 2) and between PEG and NaPA concentration (1 by 3) also had statistically significant (p-value < 0.05) effects. According this analysis, in an ATPS formed using PEG/NaPA/Na_2SO_4, the polymer could be used at a lower molecular weight to achieve similar partitioning results. Considering the experimental results, keeping the concentrations of PEG and NaPA constant at 16 wt% and increasing the molecular weight of PEG from 2,000 to 10,000 helped in achieving a K_{Bac} of 20.8 and 20.3, respectively, illustrating the low influence of PEG molecular weight on bacteriocin stability. Furthermore, the factorial design showed that higher concentrations of NaPA (positive level in the Pareto chart) and lower concentration of PEG (negative level in the Pareto chart) could increase peptide partitioning. The highest K_{Bact} value was obtained with PEG 10,000 g/mol at the lower concentration (8 wt%) and a higher

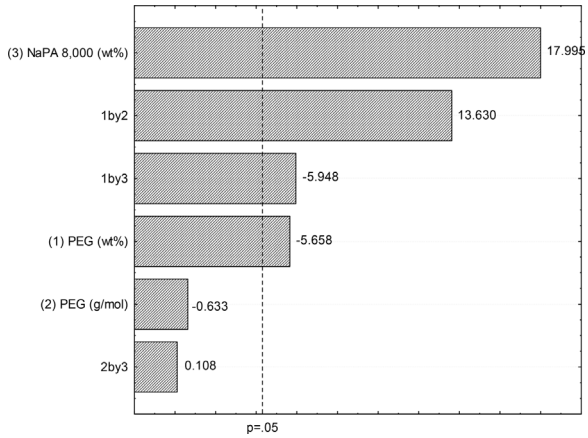


Fig. 5. Pareto chart of ANOVA to identify the variables and interactions that exhibited significant effects on bacteriocin partition coefficient (K_{Bact}) in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/Na_2SO_4 systems. The length of each bar is related to the standardized effect or interaction, and the vertical line corresponds to the significant effect for a confidence interval of 95%.

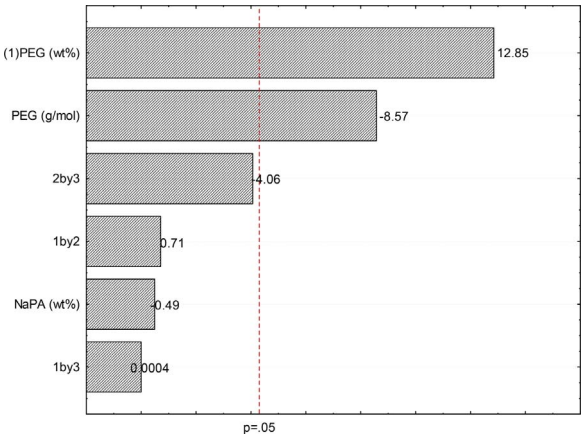


Fig. 6. Pareto chart of analysis of variance (ANOVA) applied to identify the variables and interactions with significant effect on the activity balance (%AB_{Bact}) in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/choline chloride ([Ch]Cl) systems. The length of each bar is related to the standardized effect or interaction, and the vertical line corresponds to the significant effect for a confidence interval of 95%.

concentration of NaPA (16 wt%).

NaPA is negatively charged; therefore, it can create repulsive or attractive interactions with the target biomolecule according to its charge. In this sense, previous studies demonstrated the influence of NaPA on the extraction of biomolecules such as clavulanic acid in PEG/NaPA systems, which partitions preferentially to the PEG-rich phase because of electrostatic repulsion [12]. Additionally, previous studies with hemoglobin [16] and green fluorescent protein [15] showed that electrolytes favor the partition of these negatively charged proteins to the PEG-rich phase; this behavior was more pronounced with Na_2SO_4 than with NaCl. Bacteriocin was strongly excluded from the bottom (NaPA-rich) phase; therefore, we hypothesized that it is negatively charged at pH 5.0, at which the ATPSs were performed. Similarly, pediocin PD-1 was also found to be hydrophobic and negatively charged, with an isoelectric point (p*I*) of approximately 3.5 and molecular mass of approximately 3.5 kDa [43]. Additionally, bacteriocin is approximately the same size as pediocin PD-1, based on electrophoresis analysis (data not shown). These features support the view that the bacteriocin studied in this work could be classified as a pediocin-like bacteriocin, although studies on the amino acid sequence need to be performed to confirm this hypothesis [5].

Based on in these considerations, the partitioning of bacteriocin in the ATPS probably results from a combination of electrostatic repulsion by the negatively charged bottom (NaPA-rich) phase and hydrophobic interactions with the polymeric top (PEG-rich) phase (with a molecular weight of 10,000 g/mol, the most hydrophobic PEG tested).

The activity balance (%AB_{Bact}) of bacteriocin was close to 100% for all the conditions studied, except for 16 wt% PEG 10,000/8 wt% NaPA (%AB_{Bact} = 45%). Under these conditions, the concentration of PEG

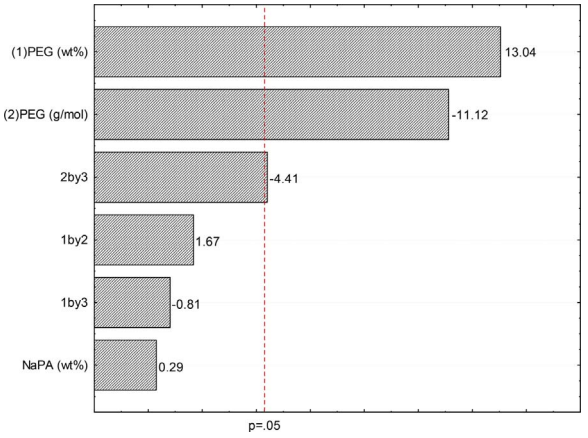


Fig. 7. Pareto chart of ANOVA applied to identify the variables and interactions with significant effects on the recovery of bacteriocin (%REC_{top}) in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/choline chloride ([Ch]Cl) systems. The length of each bar is related to the standardized effect or interaction, and the vertical line corresponds to the significant effect for a confidence interval of 95%.

10,000 together with the effect of the presence or combination of all ATPS components led to a denaturing effect on the molecule. The recovery of the target molecule in the PEG-rich phase (%REC_{top}) was greater than 60% for most of the conditions investigated, which confirmed that the molecule preferentially partitioned into the top (PEG-rich) phase. The highest value of 104% was observed using the 8 wt% PEG 10,000/16 wt% NaPA system. As expected, the %REC_{bot} showed

Table 4
Factor levels used in the 2^3 full factorial designs to study the partitioning parameters of bacteriocin in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/choline chloride ([Ch]Cl) systems.

Assays	K_{Bact}	P_{Bact}	%AB_{Bact}	%REC_{top}	%REC_{bot}
16%PEG 2,000/16%NaPA	26.9	1.3	99	94	5
8%PEG 2,000/16%NaPA	19.3	1.4	66	56	10
8%PEG 2,000/8%NaPA	29.1	1.5	82	76	5
16%PEG 2,000/8%NaPA	20.6	1.8	113	108	5
[a]12%PEG 6,000/12%NaPA	9.2 ± 0.39	0.9 ± 0.05	99.5 ± 3.5	82.2 ± 3.5	17.3 ± 0.0
16%PEG 10,000/8%NaPA	28.7	1.5	121	116	4
16%PEG 10,000/16%NaPA	29.5	1.4	89	83	6
8%PEG 10,000/8%NaPA	29.3	1.3	97.12	91.21	5.91
8%PEG 10,000/16%NaPA	30.2	1.2	53.94	46.94	7.00

[a] The central point of full factorial design was repeated three times.

Table 5
Analysis of variance (ANOVA) for the dependent variable the activity balance (%AB_{Bact}) of bacteriocin partitioning in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/choline chloride ([Ch]Cl) systems.

Source	*SS*	*df*	*MS*	*F*	*p*
(1) PEG (wt%)	2032.55	1	2032.55	165.09	*0.006
(2) PEG (g/mol)	904.36	1	904.36	73.45	*0.013
(3) NaPA (wt%)	2.98	1	2.98	0.24	0.671
1 by 2	6.20	1	6.12	0.50	0.552
1 by 3	0.00	1	0.00	0.00	0.999
2 by 3	203.20	1	203.20	16.50	0.056
Pure Error	24.62	2	12.31		
Total SS	3721.77	10			

* Significant level $p < 0.05$; $R^2 = 85\%$. *SS* = Sum of squares; *df* = degrees of freedom; *MS* = Mean squares; *F* = F-test.

Table 6
Analysis of variance (ANOVA) for the dependent variable the recovery of bacteriocin (%REC_{top}) during bacteriocin partitioning in polyethylene glycol (PEG)/sodium polyacrylate (NaPA)/choline chloride ([Ch]Cl) systems.

Source	*SS*	*df*	*MS*	*F*	*p*
(1) PEG (wt%)	2095.03	1	2095.03	170.17	*0.006
(2) PEG (g/mol)	1523.29	1	1523.29	123.73	*0.008
(3) NaPA (wt%)	1.07	1	1.07	0.09	0.796
1 by 2	34.47	1	34.47	2.80	0.236
1 by 3	8.14	1	8.14	0.66	0.502
2 by 3	239.02	1	239.02	19.41	*0.048
Pure Error	24.62	2	12.31	24.62	
Total SS	3986.79	10			

* Significant level $p < 0.05$; $R^2 = 95\%$. *SS* = Sum of squares; *df* = degrees of freedom; *MS* = Mean squares; *F* = F-test.

lower values (< 24%) in the condition of the central point (12 wt% PEG 6,000/12 wt% NaPA system) of the 2^3 factorial designs.

Given the promising results with PEG/NaPA/Na_2SO_4, we also evaluated a new PEG/NaPA system comprising a choline-based salt in an attempt to increase the partitioning parameters of bacteriocin from the fermentation broth.

3.3. Bacteriocin partitioning in PEG/NaPA/[Ch]Cl systems

Table 4 shows that, in general, higher K_{Bact} were observed in PEG/NaPA systems in the presence of [Ch]Cl compared with those obtained using Na_2SO_4. The electrolyte nature affects bacteriocin partitioning to the PEG phase and, in this sense, [Ch]Cl boosted partitioning towards the upper phase. We believe the electrostatic forces involved in systems using choline-based salts are more pronounced than those in the presence of Na_2SO_4, as [Ch]Cl may have a more pronounced screening effect on NaPA charge (see the electrical conductivity studies below).

We also calculated the relative partitioning of bacteriocin between the two ATPS (P_{Bact}), according to Eq. (6). Except for the central point, for all conditions evaluated, the P_{Bact} was > 1, which was expected based on the higher partition coefficients in the presence of [Ch]Cl.

Overall, %AB_{Bact} values closer to 100% were obtained in the presence of [Ch]Cl, with only two values being less than 75% under the following conditions: 8 wt% PEG 2,000/16 wt% NaPA with %AB_{Bact} = 66%; and 8 wt% PEG 10,000/16 wt% NaPA with %AB_{Bact} = 54%. In terms of bacteriocin recovery in the PEG-rich phase, high values (above 75%) were obtained, except for the conditions at which the %AB_{Bact} was low, probably indicating bacteriocin denaturation at this PEG (top-phase) concentration. Values of %REC_{top} of approximately 100% were observed with 16 wt% PEG 2,000 or 10,000/8 wt% NaPA systems.

The parameters %AB_{Bact} and %REC_{top}were also analyzed as response variables for the PEG/NaPA/choline-based salt. The statistical analysis for %AB_{Bact} and %REC_{top} are presented in Figs. 6 and 7, respectively. The factorial design showed that in an ATPS comprising PEG/NaPA and [Ch]Cl as electrolyte, the main variables that were statistically significant (p-value < 0.05) for %AB_{Bact} and %REC_{top} were the PEG concentration (wt%) and molecular size (g/mol). The NaPA concentration and the interactions among the main variables were not statistically significant under the conditions evaluated. This set of experiments demonstrated that the type of electrolyte used to produce an ATPS has a marked influence on bacteriocin recovery. ANOVA for these parameters was performed (Tables 5 and 6), and the PEG concentration and molecular weight were significant for the bacteriocin partitioning in an ATPS.

Overall, the best results for bacteriocin partitioning were obtained with higher concentrations of both polymers. For both polymeric aqueous systems, the volume ratio was < 1, indicating that the PEG phase had a smaller volume compared with the NaPA phase. For the PEG/NaPA/Na_2SO_4 systems, the response variables (%AB_{Bact} and %REC_{top}) were also analyzed, but no statistically significant differences were found.

3.4. Comparison between the PEG/NaPA systems

Analyzing the values of K_{Bact} for both systems, the bacteriocin partitioning followed the trend: [Ch]Cl > Na_2SO_4. According to Pereira et al. [12], for clavulanic acid partitioning in PEG/NaPA/Na_2SO_4, the entropic driving force cannot play an important role because this molecule partitions to the PEG-rich phase, despite this phase having a higher polymer concentration. Bacteriocin is a small peptide, with a molecular size of approximately 3.5 kDa; therefore, it is only weakly affected by the entropic exclusion effect. To understand the forces that drive bacteriocin partitioning, the conductivity and pH of each phase after separation were analyzed (Table 7).

Based on the conductivity measurements, we observed that the electrostatic potential difference between top and bottom phases is higher in the presence of [Ch]Cl (3.72 mS/cm) than in the presence of Na_2SO_4 (2.78 mS/cm) and, therefore, electrostatic interactions play a

Table 7
Experimental values for conductivity and pH of top (polyethylene glycol (PEG)-rich) phase (TP) and bottom (sodium polyacrylate (NaPA)-rich) phase (BP) after separation. The partitioning parameters of bacteriocin in PEG/NaPA/salt systems, prepared in sodium acetate buffer at pH 5.0 and 25 °C, are also presented.

8%PEG 10,000/8%NaPA/salt systems	Phases	Conductivity (mS/cm)	pH	K_{Bact}	%AB_{Bact}	%REC_{top}	%REC_{bot}
0.35 M of Na_2SO_4	TP	0.46	5.45	21.3*	71.66*	60.65*	11.01*
	BP	3.2	5.65				
0.35 M of [Ch]Cl	TP	0.38	5.45	29.3**	97.12**	91.21**	5.91**
	BP	4.1	5.75				
Additional PEG/NaPA/[Ch]Cl systems evaluated							
0.2 M of [Ch]Cl	TP	0.41	5.50	25.0	95.85	89.64	6.21
	BP	3.6	5.55				
0.5 M of [Ch]Cl	TP	0.3	5.45	32.0	99.21	93.36	5.85
	BP	4.7	5.80				

* And ** results were already showed in Tables 1 and 2, respectively.

Table 8
Partitioning parameters of bacteriocin from different microorganisms by an aqueous two-phase system (ATPS) obtained in several conditions found in the literature.

ATPS employed	Antimicrobial peptide studied and producer microorganism	K_{Bact}	$\%REC_{top}$	Reference
26.5 wt% PEG 8,000/11 wt% sodium citrate at pH 7. Centrifugation for 10 min at 2,860 × *g*	Bacteriocin from *Pediococcus acidilactici* Kp10	5.0	83	[26]
19 wt% PEG 8,000/14 wt% sodium citrate at pH 7 for 30 min		10.7	70.3	[30]
11 wv% PEG 20,000/3.5 wv% $MgSO_4.7H_2O$ at pH 3.0 at 30 °C for 40 min	Nisin from *Lactococcus lactis* ATCC 11454	< 2.0	–	[29]
15.99 wt% PEG 4,000/15.85 wt% Na_2SO_4 at pH 2. The systems were centrifuged at 30 °C		–	110.17	[28]
20 wv% PEG/20 wv% $(NH_4)_2SO_4$ system at pH 7.0. Centrifugation for 10 min at 3,000 xg after systems were placed at 4 °C for 3 h	Cerein 8A from *Bacillus cereus* 8A	9.0	81	[27]
8 wt% PEG 10,000/8 wt% NaPA/0.5 M (7 wt%) [Ch]Cl	Bacteriocin from *Lactobacillus plantarum* ST16Pa	32.0	93.36	Results obtained in this work by our group

"–" Results not presented by the authors.

major role in bacteriocin partitioning to the PEG-rich phase. To confirm this behavior, a new series of experiments were performed using the PEG/NaPA/[Ch]Cl system at the optimal conditions (8 wt% PEG 10,000/8 wt% NaPA/[Ch]Cl), but varying electrolyte concentration (0.2 and 0.5 M). It is clear from the results shown in Table 7 that the electrostatic potential difference between the top and bottom phases increases with [Ch]Cl concentration (3.19 mS/cm for 0.2 M, 3.72 mS/cm for 0.35 M, and 4.4 mS/cm for 0.5 M), consequently increasing the partitioning of bacteriocin into the top (PEG-rich) phase.

Bacteriocin extraction was previously evaluated in PEG/salt-based ATPSs. To improve our discussion, we summarized the main results obtained using ATPSs in Table 8.

Despite the interesting results found using ATPSs to purify bacteriocin from different microorganisms, the main disadvantages were the high PEG and salt concentrations used in the ATPS, in some cases more than double that used in our experiments. In addition, the results presented by other groups showed lower values for all partitioning parameters compared with those presented in our data. PEG/NaPA is one of the most promising combinations for ATPSs with electrolytes. The PEG/NaPA/salts systems resulted in high recovery yields, and are a rapid (only 1 h), and convenient method for bacteriocin extraction from fermented medium. When both polymers are mixed together, they phase separate at quite low polymer concentrations and at low salt concentrations [14]. Another positive aspect is the possibility of recycling the polymers, *i.e.* PEG, through salting-out, and NaPA via precipitation at a pH below 3.

To evaluate the purification of bacteriocin with respect to the total proteins in the fermented broth, the partition coefficient of total proteins was also calculated for the optimal ATPS, *i.e.* 8 wt% PEG 10,000/8 wt% NaPA with 0.5 M of [Ch]Cl (Table 7). The protein partition coefficient (K_{prot} = 0.51 ± 0.07) showed that the proteins present in the fermented broth are preferentially concentrated in the bottom (NaPA-rich) phase, achieving a selectivity (*S*) of 62.7. Thus, our results are very promising, because ideally, in a purification process, proteins should partition oppositely to the target molecule, in our case, bacteriocin, which partitioned preferentially to the top (PEG-rich) phase.

In addition, the salts used in this study, a neutral salt (Na_2SO_4) and a choline-based salt ([Ch]Cl), were very mild for the target molecule. Furthermore, for the [Ch]Cl salt when applied in an ATPS, traces of [Ch]Cl in the bacteriocin extracted by the PEG/NaPA/[Ch]Cl ATPS would not be a concern because choline has been widely used as a feed additive for decades [44], as well as for the bacteriocins produced by lactic acid bacteria.

4. Conclusions

The stability and partitioning of bacteriocin from the fermented broth of *L. plantarum* ST16Pa in PEG/NaPA/electrolytes aqueous two-phase systems was studied. Bacteriocin showed high stability after 1 h in temperatures from 50 °C to 80 °C and at a pH ranging from 3.0 to 8.0, as well as in the presence of ATPS components, with exception of NaPA. Bacteriocin lost up to 47.5% of its activity after 24 h in the presence of 20 wt% NaPA as a result of the electrostatic interactions with the negatively-charged polymer. Additionally, the synergistic effect of pH and temperature on the bacteriocin stability was not statistically significant under the conditions evaluated. All the partitioning studies showed that the bacteriocin partitions preferentially to the top (PEG-rich) phase (K_{Bact} > 9.2), demonstrating that hydrophobic and electrostatic interactions are the major driving forces of partitioning. The highest partition coefficient (K_{Bact} = 32) was obtained with 8 wt% PEG 10,000/8 wt% NaPA/0.5 M [Ch]Cl. At the same time, total proteins were removed into the bottom (NaPA-rich) phase (K_{prot} = 0.51). The factorial design showed that the presence of different electrolytes had a marked influence on bacteriocin partitioning. In the systems comprising PEG/NaPA/Na_2SO_4 the NaPA concentration was the main variable and the interaction among some variables influenced bacteriocin partitioning statistically, while in the systems comprising PEG/NaPA/[Ch]Cl, only the PEG concentration and molecular size were statistically significant. Our results show the potential of ATPSs using choline-based salt as an initial step for bacteriocin recovery and purification, by applying a low-cost (low polymer content) and ecologically friendly (mild conditions) PEG/NaPA/[Ch]Cl system. Complementary studies on chromatographic processes after bacteriocin recovery in an ATPS are being conducted for high-resolution bacteriocin purification, aiming at its complete characterization.

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References

[1] M. Rai, R. Pandit, S. Gaikwad, G. Kövics, Antimicrobial peptides as natural bio-preservative to enhance the shelf-life of food, J. Food Sci. Technol. 53 (2016) 3381–3394.
[2] J.I. Boye, Y. Arcand, Current trends in green technologies in food production and processing, Food Eng. Rev. 5 (2013) 1–17.
[3] M.P. Castro, N.Z. Palavecino, C. Herman, O.A. Garro, C.A. Campos, Lactic acid bacteria isolated from artisanal dry sausages: characterization of antibacterial compounds and study of the factors affecting bacteriocin production, Meat Sci. 87 (2011) 321–329.
[4] G. Giraffa, N. Chanishvili, Y. Widyastuti, Importance of lactobacilli in food and feed biotechnology, Res. Microbiol. 161 (2010) 480–487.
[5] P.D. Cotter, C. Hill, R.P. Ross, Bacteriocins: developing innate immunity for food, Nat. Rev. Microbiol. 3 (2005) 777–788.
[6] S.S. Sabo, M. Vitolo, J.M.D. González, R.P.S. Oliveira, Overview of *Lactobacillus*

plantarum as a promising bacteriocin producer among lactic acid bacteria, Food Res. Int. 64 (2014) 527–536.
[7] R. Yang, M.C. Johnson, B. Ray, Novel method to extract large amounts of bacteriocins from lactic acid bacteria, Appl. Environ. Microbiol. 58 (1992) 3355–3359.
[8] D. Guyonnet, C. Fremaux, Y. Cenatiempo, J.M. Berjeaud, Method for rapid purification of class IIa bacteriocins and comparison of their activities, Appl. Environ. Microbiol. 66 (2000) 1744–1748.
[9] J.-M. Berjeaud, Y. Cenatiempo, Purification of antilisterial bacteriocins, Methods Mol. Biol. 268 (2004) 225–233.
[10] P.G. Mazzola, A.M. Lopes, F.A. Hasmann, A.F. Jozala, T.C.V. Penna, P.O. Magalhaes, C.O. Rangel-Yagui, A. Pessoa-Junior, Liquid–liquid extraction of biomolecules: an overview and update of the main techniques, J. Chem. Technol. Biotechnol. 83 (2008) 143–157.
[11] J.V. Dutra-Molino, V.A. Feitosa, L.C. de Lencastre-Novaes, V.C. Santos-Ebinuma, A.M. Lopes, A.F. Jozala, D.A.V. Marques, L.P. Malpiedi, A. Pessoa-Junior, Biomolecules extracted by ATPS: practical examples, Rev. Mex Ing Quim. 13 (2014) 359–377.
[12] J.F.B. Pereira, V.C. Santos, H.-O. Johansson, J.A.C. Teixeira, A. Pessoa-Junior, A stable liquid–liquid extraction system for clavulanic acid using polymer-based aqueous two-phase systems, Sep. Purif. Technol. 98 (2012) 441–450.
[13] K.V.G. Barros, P.M. Souza, M.M. Freitas, E.X.F. Filho, A. Pessoa-Junior, P.O. Magalhães, PEG/NaPA aqueous two-phase systems for the purification of proteases expressed by *Penicillium restrictum* from Brazilian Savanna, Process Biochem. 49 (2014) 2305–2312.
[14] H.-O. Johansson, E. Feitosa, A. Pessoa-Junior, Phase diagrams of the aqueous two-phase systems of poly(ethylene glycol)/sodium polyacrylate/salts, Polymers 3 (2011) 587–601.
[15] H.-O. Johansson, M. Ishii, M. Minaguti, E. Feitosa, T.C.V. Penna, A. Pessoa-Junior, Separation and partitioning of green fluorescent protein from *Escherichia coli* homogenate in poly(ethylene glycol)/sodium-poly(acrylate) aqueous two-phase systems, Sep. Purif. Technol. 62 (2008) 166–174.
[16] H.-O. Johansson, F.M. Magaldi, E. Feitosa, A. Pessoa-Junior, Protein partitioning in poly(ethylene glycol)/sodium polyacrylate aqueous two-phase systems, J. Chromatogr. A 1178 (2008) 145–153.
[17] S. Saravanan, J.R. Rao, B.U. Nair, T. Ramasami, Aqueous two-phase poly(ethylene glycol)-poly(acrylic acid) system for protein partitioning: influence of molecular weight, pH and temperature, Process Biochem. 43 (2008) 905–911.
[18] J.H.P.M. Santos, F.A.E. Silva, J.A.P. Coutinho, S.P.M. Ventura, A. Pessoa-Junior, Ionic liquids as a novel class of electrolytes in polymeric aqueous biphasic systems, Process Biochem. 50 (2015) 661–668.
[19] L.A.P. Alcântara, K.S. do Nascimento, C.A. Mourão, V.P.R. Minim, L.A. Minim, Aqueous two-phase poly(ethylene glycol)-sodium polyacrylate system for amyloglucosidase purification: equilibrium diagrams and partitioning studies, Sep. Purif. Technol. 118 (2013) 888–894.
[20] L.A. Sarubbo, L.A. Oliveira, A.L.F. Porto, H.S. Duarte, A.M.A. Carneiro-Leão, J.L. Lima-Filho, G.M. Campos-Takaki, E.B. Tambourgi, New aqueous two-phase system based on cashew-nut tree gum and poly(ethylene) glycol, J. Chromatogr. B 743 (2000) 79–84.
[21] H.N. Xu, An aqueous anonic/nonionic surfactant two-phase system in the presence of salt. 2. Partitioning of ice structuring proteins, RSC Adv. 2 (2012) 12251–12254.
[22] N.R. da Silva, L.A. Ferreira, L.M. Mikheeva, J.A. Teixeira, B.Y. Zaslavsky, Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000–sodium sulfate two-phase system, J. Chromatogr. A 1337 (2014) 3–8.
[23] X. Rico-Castro, M. González-Amado, A. Soto, O. Rodríguez, Aqueous two-phase systems with thermo-sensitive EOPO co-polymer (UCON) and sulfate salts: effect of temperature and cation, J. Chem. Thermodyn. 108 (2017) 136–142.
[24] M. González-Amado, E. Rodil, A. Arce, A. Soto, O. Rodríguez, The effect of temperature on polyethylene glycol (4000 or 8000)–(sodium or ammonium) sulfate aqueous two phase systems, Fluid Phase Equilibr. 428 (2016) 95–101.
[25] A.M. Lopes, V.C. Santos-Ebinuma, A. Pessoa Júnior, C. Rangel-Yagui, Influence of salts on the coexistence curve and protein partitioning in nonionic aqueous two-phase micellar systems, Braz. J. Chem. Eng. 31 (2015) 1057–1064.
[26] S. Abbasiliasi, J.S. Tan, T.A.T. Ibrahim, S. Kadkhodaei, H.S. Ng, F. Vakhshiteh, Z. Ajdari, S. Mustafa, T.C. Ling, R.A. Rahim, A.B. Ariff, Primary recovery of a bacteriocin-like inhibitory substance derived from *Pediococcus acidilactici* Kp10 by an aqueous two-phase system, Food Chem. 151 (2014) 93–100.
[27] R. Lappe, V.S. Anna, A. Brandelli, Extraction of the antimicrobial peptide cerein 8A by aqueous two-phase systems and aqueous two-phase micellar systems, Nat. Prod. Res. 26 (2012) 37–41.
[28] C. Li, J. Bai, W. Li, Z. Cai, F. Ouyang, Optimization of conditions for bacteriocin extraction in PEG/Salt aqueous two-phase systems using statistical experimental designs, Biotechnol. Prog. 17 (2001) 366–368.
[29] C. Li, F. Ouyang, J. Bai, Extractive cultivation of *Lactococcus lactis* using a polyethylene glycol/$MgSO_4.7H_2O$ aqueous two-phase system to produce nisin, Biotechnol. Lett. 22 (2000) 843–847.
[30] N.L.M. Sidek, J.S. Tan, S. Abbasiliasi, F.W.F. Wong, S. Mustafa, A.B. Ariff, Aqueous two-phase flotation for primary recovery of bacteriocin-like inhibitory substance (BLIS) from *Pediococcus acidilactici* Kp10, J. Chromatogr. B 1027 (2016) 81–87.
[31] A.F. Jozala, A.M. Lopes, P.G. Mazzola, P.O. Magalhães, T.C.V. Penna, A. Pessoa-Junior, Liquid-liquid extraction of commercial and biosynthesized nisin by aqueous two-phase micellar systems, Enzyme Microb. Technol. 42 (2008) 107–112.
[32] A.F. Jozala, A.M. Lopes, L.C. Lencastre-Novaes, P.G. Mazzola, T.C.V. Penna, A. Pessoa-Junior, Aqueous two-phase micellar system for nisin extraction in the presence of electrolytes, Food Bioprocess Technol. 6 (2012) 3456–3461.
[33] S.D. Todorov, H. Prévost, M. Lebois, X. Dousset, J.G. LeBlanc, B.D.G.M. Franco, Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*) – from isolation to application: characterization of a bacteriocin, Food Res. Int. 44 (2011) 1351–1363.
[34] J. An, W. Zhu, Y. Liu, X. Zhang, L. Sun, P. Hong, Y. Wang, C. Xu, D. Xu, H. Liu, Purification and characterization of a novel bacteriocin CAMT2 produced by *Bacillus amyloliquefaciens* isolated from marine fish *Epinephelus areolatus*, Food Control 51 (2015) 278–282.
[35] Z.B. Belgacem, A. Rehaiem, P.F. Bernárdez, M. Manai, L.P. Castro, Interactive effects of pH and temperature on the bacteriocin stability by response surface analysis, Microbiology 81 (2012) 195–200.
[36] Z.D.V.L. Mayerhoff, I.C. Roberto, T.T. Franco, Purification of xylose reductase from *Candida mogii* in aqueous two-phase systems, Biochem. Eng. J. 18 (2004) 217–223.
[37] N.P. Guerra, L. Pastrana, Enhanced nisin and pediocin production on whey supplemented with different nitrogen sources, Biotechnol. Lett. 23 (2001) 609–612.
[38] M. Calderón-Santoyo, P.G. Mendoza-García, M.A. García-Alvarado, B.I. Escudero-Abarca, Effect of physical factors on the production of bacteriocin from *Pediococcus acidilactici* ITV 26, J. Ind. Microbiol. Biotechnol. 26 (2001) 191–195.
[39] Microbiology, genetics and applications, in: L. De Vuyst, E.J. Vandamme (Eds.), Bacteriocins of Lactic Acid Bacteria, Blackie Academic and Professional, London, 1994, pp. 91–142.
[40] Y. Hu, X. Liu, C. Shan, X. Xia, Y. Wang, M. Dong, J. Zhou, Novel bacteriocin produced by *Lactobacillus alimentarius* FM-MM_4 from a traditional Chinese fermented meat Nanx Wudl: purification, identification and antimicrobial characteristics, Food Control 77 (2017) 290–297.
[41] L. Yi, J. Dang, L. Zhang, Y. Wu, B. Liu, X. Lü, Purification, characterization and bactericidal mechanism of a broad spectrum bacteriocin with antimicrobial activity against multidrug-resistant strains produced by *Lactobacillus coryniformis* XN8, Food Control 67 (2016) 53–62.
[42] V.C. Santos-Ebinuma, A.M. Lopes, A. Pessoa-Junior, M.F.S. Teixeira, Extraction of natural red colorants from the fermented broth of *Penicillium purpurogenum* using aqueous two-phase polymer systems, Biotechnol. Prog. 31 (2015) 1295–1304.
[43] G. Green, L.M.T. Dicks, G. Bruggeman, E.J. Vandamme, M.L. Chikindas, Pediocin PD-1, a bactericidal antimicrobial peptide from *Pediococcus damnosus* NCFB 1832, J. Appl. Microbiol. 83 (1997) 127–132.
[44] S.H. Zeisel, K.-A. da Costa, Choline: an essential nutrient for public health, Nutr. Rev. 67 (2009) 615–623.