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Primary recovery of a bacteriocin-like inhibitory substance derived from *Pediococcus acidilactici* Kp10 by an aqueous two-phase system

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ABSTRACT

A polymer–salt aqueous two-phase system (ATPS) consisting of polyethylene–glycol (PEG) with sodium citrate was developed for direct recovery of a bacteriocin-like inhibitory substance (BLIS) from a culture of *Pediococcus acidilactici* Kp10. The influences of phase composition, tie-line length (TLL), volume ratio (V_R), crude sample loading, pH and sodium chloride (NaCl) on the partition behaviour of BLIS was investigated. Under optimum conditions of ATPS, the purification of BLIS was achieved at 26.5% PEG (8000)/11% sodium citrate with a TLL of 46.38% (w/w), V_R of 1.8, and 1.8% crude load at pH 7 without the presence of NaCl. BLIS from *P. acidilactici* Kp10 was successfully purified by the ATPS up to 8.43-fold with a yield of 81.18%. Given that the operation of ATPS is simple, environmentally friendly and cost-effective, as it requires only salts and PEG, it may have potential for industrial applications in the recovery of BLIS from fermentation broth.

1. Introduction

Increasing consumer awareness of the risks not only from food-borne pathogens but also from chemical preservatives has led to renewed interest in "green technologies". These include novel minimal processing methods and exploitation of bacteriocins for food biopreservation. Bacteriocins are small ribosomally synthesised antimicrobial peptides, which are mostly cationic, amphiphilic and membrane-permeabilising. Bacteriocins are unstructured in aqueous solution, but have the propensity to form an α-helical structure when exposed to structure-promoting solvents or membrane-mimicking media (Moll, Konings, & Driessen, 1999).

Numerous purification strategies have been reported for bacteriocins all with varying degrees of success, which may be attributable to the extremely heterogeneous nature of bacteriocin (Klaenhammer, 1993). The purification methods commonly employed include ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic chromatography coupled with Mono S cation-exchange column chromatography, reverse-phase high-performance liquid chromatography (HPLC), Amberlite XAD-2, Sephadex G-25 gel filtration, ultrafiltration and gel permeation chromatography and ethanol precipitation. Each purification method has its own drawbacks, which may include issues with low yield and purity, cost, and the requirement for a skilled operator (Abriouel, Valdivia, Martinez-Bueno, Maqueda, & Galvez, 2003).

The aqueous two-phase system (ATPS) has been proposed as an ideal purification technique for the separation, extraction and concentration of biomolecules because of its high productivity, simplicity, short processing time, cost effectiveness, scalability and versatility. ATPS, consisting of a polyethylene glycol (PEG)/salt system has been widely used for the bioseparation of proteins due to its low cost and the wide range of hydrophobic differences between the two-phase systems that allow enhancement of the partition selectivity of the target protein (Antov & Omorjan, 2009). ATPS has been applied in the extraction and purification of various compounds, such as enzymes, biopharmaceutics and natural products. The system has also been used in selected biotechnological fields possessing, medium industrial maturity and resolution

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potential (Przybycien, Pujar, & Steele, 2004). In biomanufacturing, ATPS allows simultaneous separation and concentration of the target product.

In view of the fact that ATPS is an ideal purification technique for the separation, extraction and concentration of biomolecules, this study evaluated the partitioning efficiency of a bacteriocin-like inhibitory substance (BLIS) from *Pediococcus acidilactici* Kp10 using ATPS comprised of PEG and sodium citrate. The effect of various parameters on the partitioning behaviour of the BLIS, such as the molecular weight of the polymer, tie-line length (TLL), volume ratio (V_R) and pH were investigated. The effect of the addition of sodium chloride (NaCl) on the efficiency of ATPS for the recovery of BLIS was also investigated in order to optimise BLIS purification.

2. Materials and methods

2.1. Supplies

PEG with various average molecular weights (MW), ranging from 2000 to 10,000 (g/mol), were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium citrate ($NaH_2C_6H_5O_7$) was sourced from SAFC (St. Louis, MO, USA). M17 broth and citric acid ($C_6H_8O_7$) were purchased from Merck (Darmstadt, Germany). The protein assay kit and albumin standard were supplied by Bio-Rad, USA and Thermo Scientific Pierce, respectively. All chemicals used in this study were of analytical grade.

2.2. Microorganism and BLIS production

The BLIS producing strain, *Pediococcus acidilactici* Kp10, was used in this study. This bacterium was isolated from dried curd and the details have been described in our earlier study (Abbasiliasi et al., 2012). The primary culture was prepared by taking a single bacterial colony from an agar plate and grown in a 50 ml tube containing 10 ml of M17 broth and incubated at 37 °C for 24 h without shaking. The inoculum was prepared by inoculating 1% (v/v) of the primary culture into a 50 ml tube containing 10 ml of M17 medium, incubating at 37 °C on a shaker agitated at 100 rev/min for 24 h.

2.3. Antimicrobial activity test

The antimicrobial activity of the isolates was determined by the agar well diffusion assay (Tagg, Dajani, & Wannamaker, 1976) using cell free culture supernatant (CFCS). The harvested cells were separated by centrifugation at 12,000×*g*, using a refrigerated centrifuge at 4 °C (rotor model 1189, Universal 22R centrifuge, Hettich AG, Switzerland) for 20 min. The aliquots of the supernatants (100 µl) were placed in wells (6 mm diameter) of cooled soft agar plates (25 ml) previously seeded (1% v/v) with actively growing test strains. The plates were incubated at 37 °C for 24 h for the growth of *L. monocytogenes* as the target microorganism. After 24 h, the diameters of the growth inhibition zones were measured and the antimicrobial activity was defined as AU (expressed as mm^2/ml), which is the unit area of inhibition zone per unit volume. The BLIS activity was calculated using Eq. (1):

$$\text{BLIS activity}\left(\frac{\text{mm}^2}{\text{mL}}\right) = \frac{L_z - L_s}{V} \quad (1)$$

where, L_z = clear zone area (mm^2), L_s = well area (mm^2), V = volume of sample (ml)

2.4. Determination of protein content

The total protein concentration was determined using a Bio-Rad protein assay kit with albumin as a standard protein (Bradford, 1976). A total of 20 µl of the sample was added to 200 µl of Bradford reagent in a microtitre plate and incubated at 37 °C for 15 min. The absorbance was measured at 595 nm.

2.5. Salt selection and binodal curve construction

The system compositions were achieved by weighing an equivalent amount of PEG and citrate stock solutions into the system. Five different salts, namely potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen phosphate (K_2HPO_4), magnesium sulphate ($MgSO_4.7H_2O$), ammonium sulphate ($(NH_4)_2SO_4$), sodium acetate (CH_3COONa), and sodium citrate ($NO_3C_6H_5O_7.2H_2O$) were tested. The pH of each system was adjusted to 7 using different acids and alkalis, such as hydrogen chloride, sodium hydroxide, acetic acid and citric acid.

A binodal line is the boundary of the phase separation where two or more phases are formed beyond the curve but only one phase exists below the curve. The binodal curve of PEG 4000, 6000, 8000 and 10,000 (g/mol) and sodium citrate salt were constructed using a turbidometric titration method according to Albertsson (1986). Mixtures of PEG standard solutions and salts of known concentrations were titrated by the addition of diluents until the turbid solution turned clear (Fig. 1).

2.6. Tie-line length

The binodal curves were estimated using a turbidometric titration method, as described by Albertsson (1986). TLL showed the compositions of the two phases, which are in equilibrium and calculated using Eq. (2):

$$\text{TLL} = \sqrt{\Delta P^2 + \Delta C^2} \quad (2)$$

where ΔP and ΔC are the differences between the PEG and sodium citrate concentration in the two phases, respectively.

PEG was analysed by refractive index while the salt concentrations were analysed by conductivity measurements (Albertsson, 1986). The salt concentration was determined from a calibration curve constructed with a range of standard salt concentrations. The PEG concentration in the phase was determined by subtracting the refractive index value contributed by the salts.

2.7. Construction of ATPS

ATPS was prepared from PEG stock solutions [(50% w/w) of different MWs (PEG 6000, 8000 and 10,000 g/mol)] and sodium citrate stock solutions (40% w/w). Centrifuge tubes (15 ml) were used to prepare the phase systems. Concentrations of PEG stock solution, citrate stock solution and 16% (w/w) crude samples were calculated and weighed into the centrifuge tubes. Distilled water was added into the centrifuge tubes to obtain a final 10 g system. The feed solutions were stirred thoroughly using a vortex mixer and then centrifuged at 2860×*g* for 10 min to complete the phase separations. Finally, the volumes of both phases were measured and samples from coexisting phases were obtained for antimicrobial and protein assays. The final system composition constructed for BLIS purification at different MWs of PEG is shown in Tables 1 and 2. The partition coefficient (K) of the BLIS was calculated as the ratio of BLIS activity in the two phases using Eq. (3):

$$K = \frac{A_T}{A_B} \quad (3)$$