

Hyaluronic Acid Generated by *Streptococcus zooepidemicus* is Recovered Utilising PEG-Citrate Aqueous Two Phase Systems

Alok Kumar Srivastav^{1*}, P.C. Suriyakala²

¹Department of Biotechnology, Faculty of Science, Lincoln University College, Petaling Jaya, Selangor, Malaysia, MY 47301

²Faculty of Medicine, Lincoln University College, Petaling Jaya, Selangor, Malaysia, MY 47301

ABSTRACT

Due to its biocompatibility, rheological, and physiological qualities, hyaluronic acid (HA) has gained significant attention as a biomaterial with several medical and aesthetic uses. In the last several decades, microbial fermentations have emerged as a significant source for the industrial production of HA. To maintain clinical and cosmetic-grade purity, microbial HA must undergo rigorous and time-consuming purifying procedures owing to its eventual usage. The use of Aqueous Two Phase Systems (ATPS) for the primary recovery of high value biomolecules has been shown to be an effective approach. Nonetheless, their use in HA post-production has been mostly unexplored. In this study, polyethylene glycol (PEG)-citrate ATPS were employed for the first time in the main recovery of HA generated by a *Streptococcus equi subsp. zooepidemicus* designed strain. With a clarified fermentation broth as the feed material, the effects of PEG molecular weight (MW), tie-line length (TLL), volume ratio (V_R), and sample load on HA recovery and purity were examined. HA was recovered from the salt rich bottom phase, and its recovery enhanced when a PEG molecular weight of 8000 g mol^{-1} was employed. Lower V_R values (0.38), on the other hand, favored HA recovery, while a high V_R increased purity (3.50). In the meanwhile, sample weight had a detrimental effect on both recovery and purity. The ATPS with the greatest performance consisted of PEG 8000 g mol^{-1} , TLL 43% (w/w), and V_R 3.50, with a HA recovery of 79.4% and a purity of 74.5%. This work indicated for the first time that PEG-citrate ATPS has the potential to be a successful main recovery technique for the downstream processing of microbial HA.

Keywords: Hyaluronic acid, Primary recovery, Aqueous Two Phase Systems, Downstream process, *Streptococcus zooepidemicus*.

INTRODUCTION

In 2020, the worldwide hyaluronic acid (HA) market size was evaluated at USD 9.2 billion, and it is anticipated to expand primarily as a result of rising aesthetic awareness and ageing populations (Grand View Research, 2020). HA is a naturally occurring polysaccharide with a molecular weight between 105 and 107 Da (Toole, 2002). It is a glycosaminoglycan composed of repeated units of d-glucuronic acid and d-N-acetylglucosamine, connected by alternating β -1,4 and β -1,3 glycosidic linkages (Stick and Williams, 2009). HA is a primary constituent of the extracellular matrix (ECM), making it a pervasive chemical.

However, it is present in high quantities in connective tissue, such as hyaline cartilage and skin dermis, as well as specialized bodily fluids, such as the vitreous humour of the eye and synovial fluid (Falcone et al. 2006). HA serves several functions in the body,

including cellular support, tissue hydration and healing, viscoelasticity, and cellular signaling (Cowman and Mat-suoka 2005).

Due to its viscoelastic qualities, water retention capacity, bio-compatible, biodegradability, and non-immunogenicity, HA has become an intriguing biomaterial with several uses in medicine, cosmetics, and food (Sudha et al. 2014).

Address for Correspondence:

Dr. Alok Kumar Srivastav,
Department of Biotechnology, Faculty of Science,
Lincoln University College, Petaling Jaya, Selangor,
Malaysia, MY 47301
Email: pdf.alok@lincoln.edu.my

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Viscosupplementation for arthritis, ocular surgery, prevention of post-surgical adhesion, drug delivery systems, scaffolds for tissue engineering, wound healing, dermal fillers, and skin moisturizers are all applications of HA-based products in the medical and aesthetic fields (Bukhari et al. 2018; Huang and Huang 2018). The ultimate use of HA depends on the molecular weight of the polymer, with high molecular weight HA (> 2 MDa) being favored in medicine and low molecular weight HA (0.8–800 kDa) being chosen in cosmetics (Ghodke et al. 2018).

Extraction from animal sources, primarily rooster combs, bovine cartilage, synovial fluids, and vitreous humour, is used to produce HA on an industrial scale (Vázquez et al., 2010). However, HA obtained from these sources is susceptible to contamination by proteoglycans generated from the extracellular matrix (ECM), which might be allergens if not removed from the final HA product (Murado et al. 2012). In the past two decades, microbial fermentation has become a viable alternative for commercial production of HA due to immunogenicity issues, inconsistent product quality, and the high cost of HA obtained from animals (Sze et al. 2016). Furthermore, microbial fermentation may create HA with specified properties, such as a certain molecular weight (MW). Microorganisms that naturally produce HA, such as *Streptococcus equi subsp. zooepidemicus*, or heterologous expression systems, such as *Bacillus subtilis*, *Pichia pastoris*, *Lactococcus lactis*, and *Corynebacterium glutamicum*, have been genetically or metabolically engineered to create strains that produce high molecular weight HA and increased product yield (Cheng et al. 2019; Jeong et al. 2014; Jia et al. 2013; Kaur and Jayaraman 2016; Wang et al. 2020).

HA, whether obtained from animals or microorganisms, must be subjected to stringent purification techniques in order to provide a highly pure product that fulfils the requirements for therapeutic and aesthetic purposes. For microbiological HA, the downstream process often includes alcohol precipitation, adsorption on silica gel, and/or activated charcoal and diafiltration stages (Patil et al. 2011). Proteins are the most prevalent contaminants in microbial HA (Cavalcanti et al., 2020), but endotoxins from pathogenic bacteria, such as *S. zooepidemicus*, can provide potential safety problems (Liu et al. 2011). Obtaining highly pure HA is difficult, and given its rising market demand, there is an urgent need to create a more effective purification procedure.

Aqueous two-phase systems (ATPS) are a liquid-liquid extraction technique that is generated by combining two components beyond a critical concentration, resulting in two immiscible aqueous phases (González-Valdez et al. 2018). Polymer-polymer and polymer salt combinations are the most prevalent kinds of ATPS. ATPS provide various benefits over traditional liquid-liquid extraction techniques, including a high water content, cheap component costs, simplicity of scaling, process integration capability, and high yields of the target product (Gómez and Macedo 2019; Loureiro et

al. 2017). When a solute is introduced into the ATPS, the partition between the two phases reacts to various factors, including the type and concentration of phase-forming components, the molecular weight of polymers, the system pH, temperature, and the inherent physicochemical characteristics of the solute (Iqbal et al. 2016). Due to the amount of interactions between the aforementioned elements controlling partition behavior, the creation of an optimum ATPS extraction stage is a difficult process.

ATPS, because of their aqueous environment and mild conditions, have been widely utilized for the recovery and separation of a wide variety of biomacromolecules, such as proteins, enzymes, antibodies, peptides, and genetic material (Sánchez-Trasvia et al. 2019), as well as low molecular weight compounds with biological activity (Enriquez-Ochoa et al. 2020). The implementation of ATPS in the downstream processing of polysaccharides from microbial fermentations is largely unexplored. To present, just one research has been published on the use of ATPS for the recovery of HA. (Rajendran et al. 2016) used ATPS as the first step in the downstream processing of HA (> 1.8 MDa) generated in recombinant *L. lactis*. An ATPS composed of 18% (w/w) PEG and 7% (w/w) potassium phosphate had a recovery of 97% and purity of 29.4%. Even though certain protein contaminants had been eliminated, more purification processes were needed to attain the desired purity.

This is the first research to investigate the use of PEG-citrate ATPS for the main recovery and partial purification of HA generated by *S. zooepidemicus*. Sodium citrate was chosen due to its biodegradable and non-toxic properties (Lu et al., 2010), which make citrate salts disposal on an industrial scale more environmentally friendly than phosphate salts. Initially, the effects of PEG MW, tie-line length (TLL), and volume ratio (V_R) on the partition behavior and recovery of pure HA samples were examined. Afterwards, the PEG-citrate ATPS with the greatest recovery was chosen and tested directly from *S. zooepidemicus* fermentation broth, where TLL, V_R , and sample load were evaluated.

MATERIALS AND METHODS

Materials

PEG with nominal molecular masses of 6000 (PEG6000) and 8000 (PEG8000), Bovine Serum Albumin (BSA), Sodium Dodecyl Sulphate (SDS), Cetyltrimethylammonium Bromide (CTAB), Sodium Chloride, Acetic Acid, BCA Protein Assay Kit, Sodium Acetate Anhydride and Sodium Citrate Dihydrate. Chemico Especialidades Quimica cosmetic grade HA (0.8 MDa) was utilized as a reference for HA measurement. The remaining reagents were all of analytical grade. There was no further purification of any chemicals. All solutions were prepared using water of Milli Q quality. Two pure samples of *S. zooepidemicus* with varying molecular weights: 1–2 MDa (HA1.5) and 3–4 MDa (3.5HA), as well as a

sample of *S. zooepidemicus* fermentation broth (MW 3 MDa). As solutions (0.15 M NaCl), pure samples were obtained and used without additional purification. Prior to usage, each sample was kept at 4°C.

Preparation of Crude Extract

The possibility of employing ATPS as a main recovery and partial purification step of microbial HA was investigated using a sample of fermentation broth containing *S. zooepidemicus* cells. The fermentation broth was diluted with a 20% (w/v) SDS solution at a 10:1 ratio (final SDS concentration of 2% w/v) and swirled for 15 minutes to complete cell lysis and release HA. The mixture was then centrifuged for 60 minutes at 4°C and 10,000 rpm using a centrifuge to separate cell debris. The clarified supernatant was collected, and the sample, known as the crude extract, was kept at 4°C until use.

Hyaluronic Acid Quantification

The concentration of HA was measured using the turbidimetric technique with CTAB, as described by Song et al. (2009), with a few adjustments. 50 L of standard or sample was combined with 50 L of 0.2 M acetate buffer (pH 6.0). Following 5-minute incubation at 37°C, 100 L of 2.5% (w/v) CTAB in 0.5 M NaOH was added (at 37°C). The mixture was incubated at 37°C for ten minutes, and the absorbance was immediately measured at 400 nm using a microplate reader. Using HA standard solutions ranging from 0 to 150 µg mL⁻¹, the calibration curve was generated.

Protein Quantification

Using BCA Protein Assay Kit according to the microplate protocol, the protein concentration was determined. Briefly, 25 L of standard or sample was combined with 200 L of newly made BCA working reagent (Reagent A-Reagent B, 50:1) for 30 seconds. The reaction was incubated at 37°C for 30 minutes, cooled to room temperature for 5 minutes, and the absorbance was immediately measured at 562 nm using a microplate reader. Using BSA solutions ranging from 0.0 to 2.0 mg mL⁻¹, the calibration curve was generated.

Aqueous Two Phase Systems Partition of Pure Hyaluronic Acid

The partitioning behavior of HA in PEG–citrate ATPS was studied using pure HA samples. First, the impact of PEG MW (6000 and 8000 g mol⁻¹) and TLL (23–43% w/w) was analysed at a V_R of 1.0. Then, the PEG MW with the greatest HA recovery was chosen to examine various V_R (0.38 and 3.50) at identical TLLs. A total of twelve ATPS were evaluated.

In transparent, graded 2-mL microcentrifuge tubes, ATPS were produced by combining stock solutions of either PEG6000 (50%) or PEG8000 (50%) with sodium citrate (25%). The TLL and compositions of the ATPS were determined based on the previously published PEG–citrate binodal curves (Ghaf-fari et al., 2019) and are summarized in Table 1. The sample was

added to account for the 10 (w/w) of the ATPS, and the ATPS's ultimate mass was corrected to 2.0 g using water. The tubes were vigorously mixed for 15 minutes at room temperature after all components were introduced. Using a 5417R centrifuge, phase separation was aided by 10 minutes of spinning at 10,000 rpm and 25°C. The volume of each phase was calculated using tube graduation to compute the ATPS V_R (top phase volume/bottom phase volume), and phases were separated meticulously to measure the HA concentration. As a control, ATPS containing pure water rather than HA were employed. Prior to performing the HA quantification process, aliquots from both phases of the ATPS were diluted by a factor of ten, since excessive salt concentrations inhibit the formation of the CTAB-HA complex, hence hindering the analysis (Oueslati et al. 2014). Using the following formula, the percentage of recovery for each phase was determined.

$$\text{Recovery (\%)} = \frac{C_i V_i}{m_{\text{initial}}} \times 100,$$

Where C_i represents the concentration of HA in phase I (top or bottom), V_i represents the volume of phase I and m_{initial} represents the initial mass of HA delivered into the system.

Table 1: Composition of polyethylene glycol (PEG)–citrate aqueous two-phase systems (ATPS) employed for the main recovery of microbial hyaluronic acid in this study (HA)

PEGMW (g mol ⁻¹)	V _R	TLL (%w/w)	PEG (%w/w)	Citrate (%w/w)
6000	1.00	23	12.13	9.91
	1.00	35	16.45	10.48
	1.00	42	19.56	10.87
8000	0.38	24	6.95	11.80
	0.38	36	8.20	14.36
	0.38	43	7.74	16.36
	1.00	24	10.79	9.75
	1.00	36	16.31	10.94
	1.00	43	19.41	11.51
	3.50	24	17.62	7.20
	3.50	36	24.50	6.90
	3.50	43	28.91	6.85

MW: molecular weight; V_R: volume ratio; TLL: tie line length

HA Recovery and Partial Purification from Crude Extract

Using a crude extract, the possibility of using PEG–citrate ATPS for the main recovery and partial purification of HA was examined. On the basis of the findings obtained with pure HA, PEG8000–citrate systems were used for this purpose. The impact of TLL (24, 36, and 43% w/w) and V_R (0.38 and 3.50) on the recovery and purity of HA was analysed. The impact of increasing sample load (10, 12, and 14 w/w) on recovery and purity was subsequently evaluated. The

ATPS were produced using the same method outlined in the preceding section. In addition to quantifying HA, the quantity of protein, the most prevalent impurity in the crude extract, was measured. Before conducting the protein quantification technique, aliquots from both stages of the ATPS were diluted tenfold. Similar to Eq. (1), protein recovery was assessed, and HA purity was computed using the following equation:

$$HA \text{ purity}(\%) = \frac{C_{HA}}{C_{HA} + C_P} \times 100, \quad (2)$$

Where C_{HA} and C_P are the concentrations of HA and protein, respectively, in the bottom phase.

Statistical Analyses

Triplicates of separate experiments were conducted, and the findings were reported as the mean standard deviation (SEM). Minitab® was used to do statistical analysis (19.2020.1). To determine if there were significant differences between groups, a one-way ANOVA and Tukey's HSD test were conducted with a significance threshold of 0.05.

RESULTS

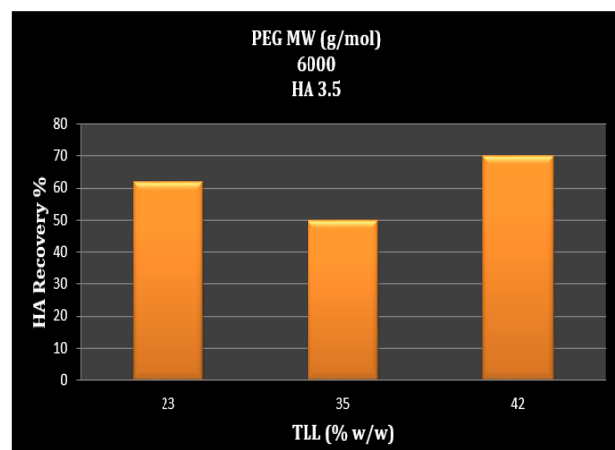
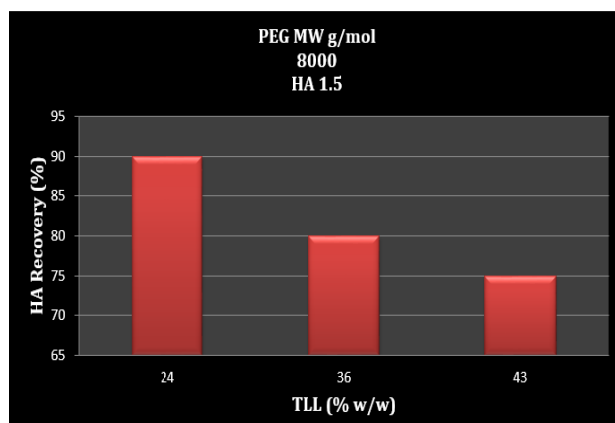
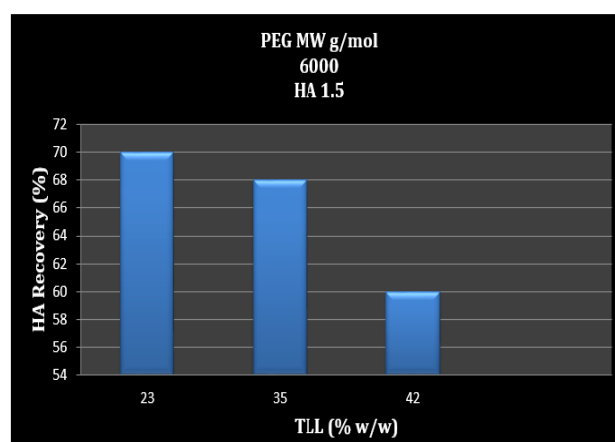
Effect of TLL and PEG MW on HA Recovery of Pure Samples

HA partition behavior in PEG–citrate ATPS has not previously been investigated; so, its partition behavior utilizing PEG6000 and PEG8000 at three different TLLs and V_R 1.0 was first investigated (Fig. 1). HA1.5 (MW: 1–2 MDa) and HA3.5 (MW: 3.5 MDa) pure HA sample solutions of varied molecular weights were employed for this purpose (Table 2). (MW: 3–4 MDa). Despite the composition of the ATPS, HA moved to the salt-rich bottom phase. Since insignificant levels of HA were identified in the PEG-rich upper phase (data not shown), further investigations focused on HA recovery in the lower phase. Nonetheless, HA was checked regularly during both periods.

TLL demonstrated contradictory impacts on HA recovery between the two samples (Fig. 1). For HA1.5, an increase in TLL had a detrimental effect on HA recovery, with a significant decrease from 72.0 ± 3.1 to $58.0 \pm 0.7\%$ when TLL increased from 23 to 42% (w/w) while using PEG6000. For HA3.5, increasing TLL from 35% to 42% (w/w) with PEG6000 resulted in a substantial 20% improvement in HA recovery. This showed that bigger TLLs favored the recovery of HA with a high molecular weight; nevertheless, it is difficult to draw this conclusion as the difference in HA recovery between TLLs 23 and 42% (w/w) was not statistically significant. Similar to 70%, the greatest HA recovery for both samples was around 23% w/w for HA1.5 and 42% w/w for HA3.5. Since not all HA could be collected in the bottom phase and its presence in the top phase was insignificant, it is possible that the remaining HA was kept at the interface, but this area

could not be analysed owing to the impracticality of extracting HA from there.

When comparing each set of identical TLLs (23 vs. 24, 35 vs. 36, and 42 vs. 43%), a 12.1% average increase in HA recovery was seen when the PEG MW increased from 6000 to 8000 g mol^{-1} in both samples. HA1.5 exhibited the same association between HA recovery and TLL for both PEG8000 and PEG6000; however, for HA3.5, the beneficial influence of TLL on HA recovery was more pronounced when using PEG8000; the maximum HA recovery was achieved at TLL 43% (w/w). In general, PEG8000 yielded better HA recovery percentages for both samples, but at different TLLs in each instance. The greatest achievable HA recoveries were $87.4 \pm 5.3\%$ for HA1.5 and $85.0\% \pm 2.7\%$ for HA3.5. Consequently, PEG8000 was created for the assessment of the V_R impact.



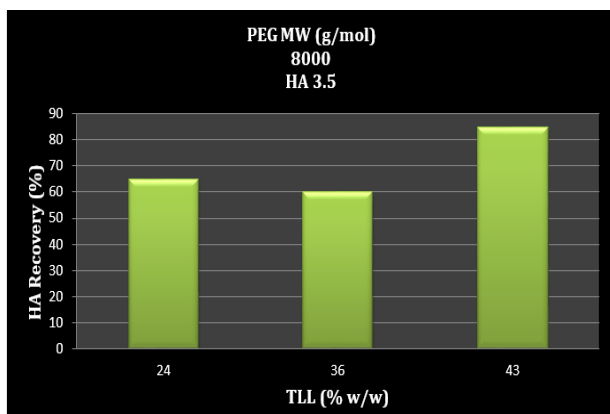


Figure 1: Effect of polyethylene glycol (PEG) molecular weight (MW; 6000 and 8000 g mol⁻¹) and tie-line length (TLL; 23–43% wt) on hyaluronic acid (HA) recovery in the bottom phase of PEG–citrate aqueous two-phase systems (ATPS). The molecular weights of 1–2 MDa (HA1.5) and 3–4 MDa (HA3.5) pure HA solutions were investigated. The ultimate concentration of HA solutions put onto the ATPS was 10% (w/w). The volume ratio of the ATPS (V_R) was one. The bars depict the sample mean standard error of the mean (SEM) for triplicates of an experiment. Bars in the same graph that do not share the same capital letter are statistically distinct ($p < 0.05$).

Effect of V_R on HA Recovery of Pure Samples

After selecting the PEG MW (8000 g mol⁻¹), the influence of the ATPS V_R on HA recovery was determined by evaluating two further V_R (0.38 and 3.50) at the same established TLLs (24, 36, and 43% w/w). While a V_R of 1 showed that the top and bottom phases had the same volume, an ATPS with a V_R 1 had a bigger bottom phase volume and one with a $V_R > 1$ had a higher top phase volume. Figure 2 illustrates the outcomes of HA recovery from pure samples. In general, both samples exhibited the same V_R -induced effect. Sample HA1.5, for instance, exhibited minor gains in HA recovery as V_R declined, from an average of 73.3% at V_R 3.50 to an average of 83.5% at V_R 0.38. For sample HA3.5, the influence of V_R on HA recovery was more pronounced, with the maximum recoveries (> 90%) occurring at V_R 0.38 at TLL 24 and 43% (w/w) (w/w). At a TLL of 24% (w/w), V_R 0.38 demonstrated substantial gains of 25% and 18% in HA recovery compared to V_R 1.0 and V_R 3.50, respectively. On the basis of these findings, lower V_R values favored greater HA recoveries.

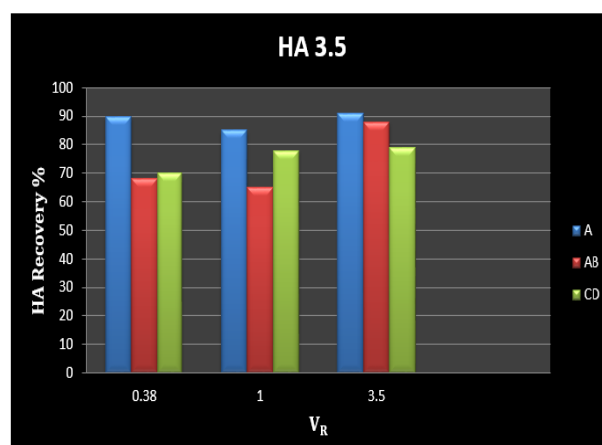
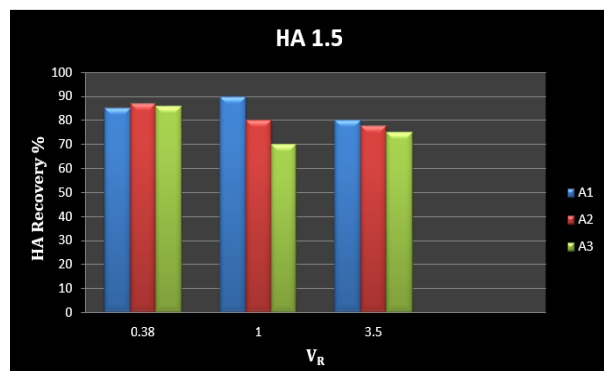


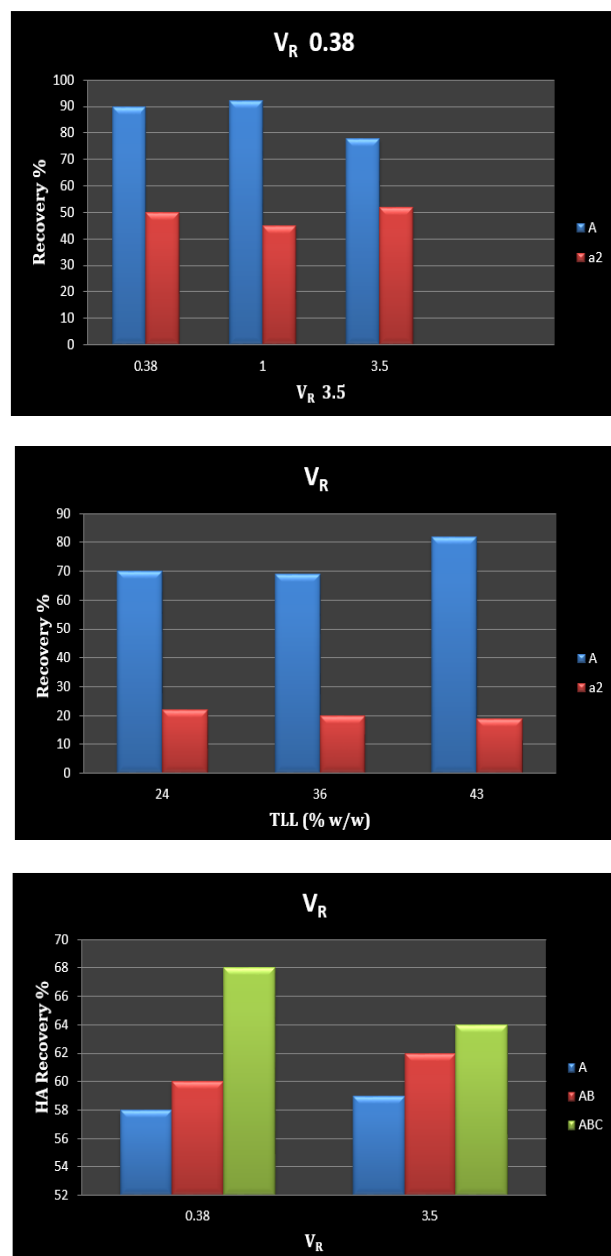
Figure 2: Effect of volume ratio (V_R , 0.38, 1.00, and 3.50) and tie-line length (TLL, 24, 36, and 43% w/w) on hyaluronic acid (HA) recovery in the bottom phase of polyethylene glycol 8000–citrate aqueous two-phase systems (ATPS). Both 1–2 MDa (HA1.5) and 3–4 MDa (HA3.5) pure HA solutions with varying molecular weights were investigated. At a final concentration of 10% (w/w), HA solutions were fed into the ATPS. The bars depict the sample mean SEM for triplicates of an experiment. Bars within the same graph that do not share the same uppercase letter vary considerably ($p < 0.05$).

Hyaluronic Acid Recovery and Partial Purification from a Crude Extract

The possibility of employing an ATPS as the major recovery step and partial purification for microbial HA downstream processing was evaluated using a crude extract (MW 3 MDa). In addition to measuring HA recovery, the protein content in the bottom phase of these ATPS was measured to evaluate protein removal ability and quantify HA purity after separation. Initial characterization of this sample (Table 2) indicated that following clarity, the HA purity was 43.4%. The selection of PEG8000–citrate ATPS was based on the higher HA recoveries seen with pure samples. Figure 3 depicts the outcomes of evaluating two V_R values (0.38 and 3.5) at three TLLs (24, 36, and 43%). At V_R 0.38, HA and protein recovery were similar across the three TLLs (no significant differences were identified for either measure), with average values of 85.9 and 48.4%, respectively, resulting in an average HA purity of 58.2%. At V_R 0.38, HA recovery values were comparable to those found for pure sample HA3.5 (MW: 3–4 MDa) at the same concentration (Fig. 2). Increasing the V_R to 3.50 led to an overall loss of HA. However, the resulting increase in the volume of the top phase resulted in a near twofold decrease in the protein concentration in the bottom phase, compared to V_R 0.38, which resulted in HA purities of more than 65 percent for all TLLs. As found with pure samples, HA recovery was greater at V_R 0.38, whereas protein removal in the bottom phase was higher at V_R 3.50. PEG8000–citrate, TLL 43% (w/w), and V_R 3.50 (HA recovery 79.4 1.5%, HA purity 74.5 0.8%) was the ATPS with the best performance based on HA purity data. This system was chosen for further testing.

Table 2: Composition of pure and crude samples of hyaluronic acid (HA) produced from *Streptococcus equi subsp. zooepidemicus*.

Sample	HA MW (MDa)	HA Concentration (g L ⁻¹)	Protein Concentration (g L ⁻¹)
HA 1.5	1-2	3.70	-
HA 3.5	3-4	3.40	-
Crude Extract	3.0	3.50	4.60

**Figure 3:** Effect of volume ratio (V_R , 0.38 and 3.50) and tie-line length (TLL) on a hyaluronic acid (HA) and protein recovery in the bottom phase of polyethylene glycol (PEG) 8000–citrate aqueous two-phase systems (ATPS); and on b HA purity (TLL 24, 36, and 43% w/w). The ATPS was loaded with a 10% (w/w) concentration of the crude extract (molecular weight: 3 MDa). The bars depict the sample mean standard error of the mean (SEM) for triplicates of an experiment. Bars on the same graph that do not have

the same capital (HA) or lowercase (protein) letter are statistically distinct ($p < 0.05$).

DISCUSSION

HA has become an amazing biomaterial with several uses, particularly in the medical and cosmetics industries. To avoid unwanted side effects, the manufacture of HA-based medicines for these applications needs a high-purity HA starting material that complies with clinical requirements. Precipitation by alcohols, often ethanol or isopropanol in ratios ranging from 1:1 to 3:1 v/v alcohol: broth and/or many consecutive phases, is one of the most frequent operations in the first stages of microbial HA downstream processing. Even if the toxicity of these short-chain alcohols is lower than that of other organic solvents, the use of large quantities still poses a danger in terms of their manipulation, environmental problems, and expensive disposal at industrial scales. Given their aqueous environment, ATPS are an ecologically preferable alternative to liquid extractions utilizing organic solvents; their use in the downstream processing of high-value biomolecules derived from microbial fermentations is also well-documented. Thus, the goal of this study was to evaluate the viability of PEG–citrate ATPS as a key recovery step in the synthesis of *S. zooepidemicus* derived HA, based on its product recovery and impurity removal capacities.

Regardless of ATPS composition, HA of both MWs was primarily partitioned to the salt-rich bottom phase, confirming earlier observations (Rajendran et al. 2016). This partitioning behavior may be explained by many factors. Due to the large MWs of both polymers (6 and 8 kDa for PEG, 1–2 and 3–4 MDa for HA), steric hindrance arises. HA is partitioned towards the bottom phase because the free volume in the PEG-rich phase is insufficient for HA molecules to migrate to the top phase. In polymer-polymer (such as PEG-dextran) ATPS, steric exclusion causes the separation of the two polymers into distinct phases (Asenjo and Andrews 2011). Additionally, the solubility of HA in PEG-rich or salt-rich environments impacts its partitioning. The pKa of the carboxyl groups in HA is between 3 and 4 (Dosio et al. 2016); therefore, at the pH of the salt-rich bottom phase (8.0) (Ghaffari et al. 2019), the carboxyl groups are deprotonated, turning HA into a poly-anion with a preference for the more hydrophilic phase, where ionic species are easily solvated by free water molecules. Additionally, electrostatic interactions between the biomolecule and the components of the two phases play a role in the partitioning of biomolecules in ATPS (Yang et al. 2010). As a result of a buildup of anions, the salt-rich bottom phase of PEG-salt ATPS has a larger negative charge than the PEG-rich top phase; hence, positively charged proteins tend to partition into the bottom phase, while negatively charged proteins are driven to the top phase (Herculano et al. 2012). Although different salts may influence the electrostatic contacts of ATPS in various ways. Due to sulphate group

repulsion, 500 kDa sulphated dextran partitioned mainly to the PEG-rich top phase in a PEG1000–ammonium sulphate ATPS. When this top phase was recovered and another salt, such as sodium citrate, was introduced to stimulate the development of a second ATPS, sulphated dextran partitioned mostly to the salt-rich bottom phase (Du et al. 2018). Both sulphated dextran and HA are examples of negatively charged polysaccharides with a high molecular weight.

Polysaccharide partitioning in PEG-salt ATPS is a complicated process that is variably impacted by all system factors. TLL demonstrated opposing effects on HA recovery in PEG6000–citrate and PEG8000–citrate ATPS at V_R 1.0, depending on the MW of the polysaccharide. In general, when TLL grew, the free volume in the top phase decreased (Sánchez-Trasvia et al. 2019); hence, as TLL increased, HA was compelled to move to the bottom phase. HA3.5, but not HA1.5, exhibited this characteristic. By analyzing the partitioning behavior of dextran with various MWs in PEG-ammonium sulphate ATPS at different TLLs, Du et al. (2018) found that neither TLL nor dextran MW had a discernible effect on the recovery of dextran in the bottom phase. Consequently, TLL and HA MW may not have a direct effect on HA recovery.

Inversely, the volume exclusion effect may also explain the beneficial impact of PEG MW on HA recovery. When previously indicated, as the PEG MW grew, so did the volume filled by the polymer (Silva et al., 2018), forcing the migration of HA to the bottom phase. In addition, increasing the PEG MW improved the hydrophobicity of the top phase (Iqbal et al., 2016), which facilitated the migration of the highly water-soluble HA towards the bottom phase.

Low V_R levels improved HA recovery. A V_R 1 indicated that the volume of the bottom phase was greater than that of the top phase; this meant that there was more space available to solubilise the same quantity of HA given to the system, hence overcoming phase saturation concerns (Gómez-Loredo et al., 2014). Furthermore, only at V_R 1.0 was HA recovery neither sensitive to TLL, but neither at V_R 0.38 nor 3.50 was recovery impacted by TLL. This is feasible because a high HA recovery may be achieved with an ATPS containing a minimal concentration of PEG and citrate provided V_R is maintained at 0.38. ATPS at V_R 0.38 and 3.50 were more susceptible to disturbance than ATPS at V_R 1.00, as shown by a minor cloudiness in the top phase during manual separation. Therefore, these systems need extra handling precautions to avoid re-mixing of phases and resulting recovery loss. This behavior may be due to the compositions of these systems being closer to the binodal curve of the PEG8000–citrate ATPS, which defines the border between the one- and two-phase zones.

V_R 0.38 demonstrated better HA recoveries while dealing with the crude extract than V_R 3.50 did when working with pure samples. Overall, the HA recovery was reduced at both V_R s when compared to sample HA3.5, which had a molecular weight that was closer to that of the HA in the crude extract. The crude extract

is the viscous, cell-free fermentation broth that is contaminated mostly with proteins. In fact, these systems exhibited a visible interface with suspended particles that grew in thickness as TLL rose. This thick interface enabled phase separation to be maintained throughout handling. However, the presence of proteins exceeding $> 2 \text{ mg mL}^{-1}$ impedes the recovery of HA through phase saturation (Benavides and Rito-Palomares, 2008), which explains the disparity between the HA recoveries in pure samples and the crude extract. When the PEG/salt ratio (V_R 3.50) was raised, much more protein was extracted from the bottom phase, lowering the protein concentration by half compared to V_R 0.38. By increasing the amount of the PEG-rich phase, more proteins were able to migrate to the top phase. The HA recovered from PEG8000–citrate, TLL 43% (w/w), V_R 3.50 ATPS was 31% more pure than the HA in the crude extract. This increase in HA purity was larger than that seen in a prior study with PEG6000–phosphate systems (Rajendran et al. 2016). In contrast, the crude extract load of Rajendran et al. (2016) was much higher (between 64% and 80% w/w, depending on the PEG/salt mix) and nucleic acids were also measured as contaminants.

Lastly, the resilience of the chosen ATPS (PEG8000–citrate TLL 43% w/w, V_R 3.50) was evaluated by increasing the quantity of crude extract put into the system. Even at 14% (w/w) crude extract, this resulted in a significant loss of HA. Due to the limited volume of this system's bottom phase, phase saturation might be attained more quickly. Moreover, not only did the quantity of HA rise with increasing crude extract loading, but so did the number of impurities, which may have impeded the recovery of HA, as previously noted. In fact, protein recovery in the bottom phase remained consistent from 10% to 14% (w/w) crude extract, indicating that there are proteins in the combination with a stronger affinity for the salt-rich bottom phase than HA, therefore competing with the polysaccharide for the same area.

Despite the benefits ATPS provide as an extraction method, their use in the post-extraction processing of high-value microbial polysaccharides has not been well investigated. This study was the first to evaluate the possibility of PEG–citrate ATPS for the main recovery and partial purification of HA generated by *S. zooepidemicus*. Screening various system parameters (PEG MW, TLL, V_R , and sample load) revealed that PEG MW and system V_R were critical variables that controlled the recovery of HA, which was improved with PEG of high MW (8000 g mol^{-1}) and V_R 0.38. Probably due to the high concentration of proteins, increasing the quantity of crude extract put into the system drastically decreased HA recovery and purity. Using PEG8000–citrate, TLL 43% (w/w), and V_R 3.50 ATPS, HA was recovered with a recovery rate of 79.4% and purity of 74.5%; from the cell-free fermentation broth. Moreover, the low salt content of this system (6.85% w/w) was favourable in that fewer diafiltration stages would be required to desalt, if necessary, HA in downstream processes. It would be

interesting to see whether PEG with a higher MW may enhance HA recovery beyond what was achieved in this work; however, PEG viscosity rises with MW, as does its cost; hence, technical and economic feasibility must be addressed for large-scale purification. In order to assess the total yield and purity of HA, more research should focus on integrating the APTS phase into the downstream processes. In the context of the purification of microbial HA, PEG–citrate APTS provide a viable, scalable option as an initial step to minimize or even replace alcohol precipitation. In addition, environmental issues about its disposal were addressed by employing sodium citrate as the phase-forming salt.

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