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Research Article

Use of Tunable Copolymers in Aqueous Biphasic Systems for Extractive Bioconversion Aimed at Continuous Fructooligosaccharide Production

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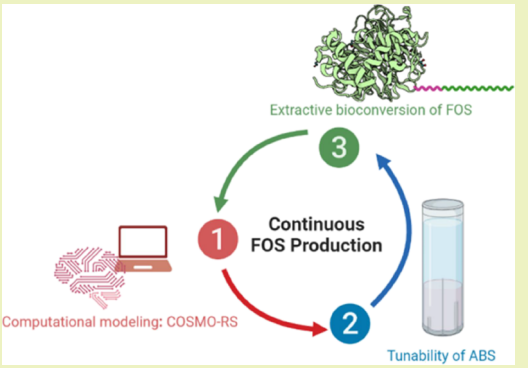
ABSTRACT: Aqueous biphasic systems (ABSs) based on sodium polyacrylate (NaPA), ethylene oxide/propylene oxide (EO/PO) polymers, and $(EO)_x$-$(PO)_y$-$(EO)_x$ triblock copolymers were prepared and applied aiming at continuous fructooligosaccharide (FOS) production and separation. EO/PO hydrophilicity/hydrophobicity balance had a significant effect on ABS formation. To develop an integrated process including the continuous enzymatic (levansucrase) production of FOSs and their purification while improving the production yield by further glucose separation, the potential of these novel polymer-based ABSs as alternative platforms was investigated. They were used for the partitioning of different carbohydrates (FOS, sucrose, D-fructose, and D-glucose) and levansucrase. Results revealed a highly polymer-dependent partition of carbohydrates and a poorly dependent one of the enzymes. Changing EO/PO and copolymers, FOS was purified with high yields (72.94−100.0%). Using polypropylene glycol 400 + NaPA 8000-based ABS, the FOS was precipitated in the interphase and separated from the other components. Pluronic PE-6800 + NaPA 8000 was identified as the best ABS for FOS continuous production and in situ purification, while minimizing levansucrase inhibition by D-glucose. This system allowed selective partition of FOSs and D-glucose toward the top phase and that of levansucrase and its substrates toward the bottom one. COnductor-like Screening MOdel for Real Solvent (COSMO-RS) suggested that ABS formation may have been due to NaPA and polymer/copolymer competition to form hydrogen bonds with water molecules. Moreover, the partition of FOSs and sugar may have been the result of a subtle balance between hydrogen bonding of sugar and polymer/copolymer and electrostatic misfit of solute with NaPA. Finally, two integrated processes were proposed to be applied with real FOS extracts obtained by chemical or enzymatic hydrolysis of inulin or by transfructosylation of concentrated sucrose solutions using bacterial levansucrases.



KEYWORDS: *fructooligosaccharides, levansucrase, aqueous biphasic systems, polymers, sodium polyacrylate, extractive bioconversion*

1. INTRODUCTION

In recent years, the increased demand of the food and nutraceutical industry for functional fibers has led to renewed interest in diverse types of exopolysaccharides (EPSs) from vegetables, microalgae, and microbial sources.[1,2] From the large plethora of EPSs, fructooligosaccharides (FOSs) have gained special recognition by the scientific community and industry due to their health benefits[2−4] and caloric profiles.[5] Generally regarded as safe for human consumption,[6,7] FOSs have been classified as prebiotics since they (i) are not hydrolyzed/absorbed by the upper part of the gastrointestinal tract, (ii) are a selective substrate for one or a limited number of probiotics, and (iii) are able to alter the colonic microbiota toward a potentially healthier composition and/or activity.[8−10] Since their Food and Drug Administration approval, FOSs entered the food and feed international market as a functional ingredient.[9−11] With daily consumption of 1−4 g in the USA and 3−11 g in Europe,[12] FOS acceptance and application in different food products have extensively increased in the last decades. Based on such consumption trends, the global FOS market was forecast to grow at a rate of 10.4% during the period of 2016−2027 and to reach USD 3.88 billion in 2027.[13] FOSs are industrially produced by either chemical or enzymatic hydrolysis of inulin or by enzymatic trans-

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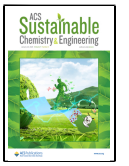

fructosylation of concentrated sucrose solutions using bacterial and fungal levansucrases.[2,14]

Depending on the catalyst nature, substrate structure, reaction conditions, and hydrolysis time, mixtures of FOSs with different degrees of polymerization (DPs) and high contents of monosaccharides are obtained by inulin hydrolysis.[15] On the other hand, sucrose transfructolysation using levansucrase leads to a mixture leaving the reactor composed of FOSs with different DPs, glucose in large amounts, unreacted sucrose, and some fructose.[16] Although the amount of mono- and disaccharides formed depends on the process, their removal is required to satisfy FOS functional properties as well as their typical caloric and sweetness profiles. To meet market demand and food industry requirements, industrial-scale FOS separation processes are paramount. Despite sugars' electroneutrality in a wide pH range, selective small-scale FOS separation has been accomplished by chromatography techniques[16−18] based on size exclusion and sugar complexation with ions of ion exchangers.[19] Separation profiles were shown in different comparative studies on carbohydrate equilibrium isotherms using ion-exchange resins in the Ca^{2+}, K^+, and Na^+ forms.[15,16] Partitioning of carbohydrates between the stationary and the mobile phases was explained by differences in strength, selectivity, and stability of their coordination complexes with functional resin ions.[19]

Although several batch and continuous production processes using microbial levansucrase have been reported in the literature and patents,[1,4,20] productivity limitations associated with a large reduction of enzyme activity (34−45%) due to end-product inhibition by D-glucose are yet an industrial challenge to be overcome.[4,21,22] In this sense, some novel approaches to overcome glucose inhibition by removing it from the reaction mixture have been proposed.[23−27] Among the downstream processing techniques used, nanofiltration[25,27] and microfiltration[24] have been shown to be efficient tools to remove low-molecular-weight carbohydrates from a mixture of oligosaccharides. However, considerable sucrose and fructose amounts are wasted in this process, affecting the FOS yield. Innovative approaches using immobilized enzymes,[28,29] such as glucose oxidase and peroxidase, have also been explored. However, despite the promising results, the high-cost associated with this additional step is believed to have a negative impact on the economic feasibility of the biocatalytic process. Therefore, the development of alternative bioconversion approaches is still needed to tackle the aforementioned limitations, while ensuring a continuous FOS production in an efficient and feasible way.

The application of in situ aqueous biphasic systems (ABSs) has been shown to be a cost-effective, sustainable, and efficient downstream strategy that can be integrated with upstream processes in order to selectively purify biomolecules from byproducts/inhibitors.[30−32] In recent years, the application of ABSs has been extended to the selective extraction of biomolecules, mainly enzymes, as well as to their fractionation and separation from products.[33−35] Pavlovic et al.[33] used polyethylene glycol (PEG) + dextran ABS to accomplish enzyme partitioning and investigate its influence on the kinetics of enzymatic reactions.[33] These findings help to understand the dynamic regulation of enzyme reactivity in macromolecular-crowded nanoenvironments similar to those occurring in the cell. Ferreira et al.[35] showed the applicability of thermoreversible ABSs composed of ammonium-based zwitterions in integrated bioreaction−separation processes. The authors demonstrated the separation of laccase from the products after the biocatalytic process in a single-step process. The recovery and reuse of laccase in these systems were effective, turning this platform into a sustainable biocatalytic process. Additionally, the PEG-cholinium salt ABS proved to be a promising integrated reaction−separation platform for laccase-catalyzed oligomerization of rutin, reinforcing its sustainability and feasibility in extractive purification processes.[34]

Among the wide range of ABS types, polymer-based ABSs using polyelectrolytes (e.g., sodium polyacrylate, NaPA) have been applied to the extraction and purification of an extensive group of biomolecules.[36−39] These systems are generally formed by NaPA in combination with PEG.[40] Taking into consideration the mechanisms underlying sugar separation and partitioning by ion-exchange chromatography, liquid−liquid sugar fractionation exploring carbohydrate complexation with sodium polyacrylate was studied in the present work using the NaPA-PEG ABS. In order to increase the hydrophilicity/hydrophobicity range of NaPA-based ABSs and enhance FOS purification, novel ABSs formed by combining NaPA with other EO/PO Pluronic copolymers and polypropylene glycol were additionally tested. The small amount of polymer required by these novel ABSs to promote phase separation is a further advantage in addition to biocompatibility (i.e., suitable environment for enzyme activity, high water content), low cost, low viscosity, and fast phase separation. Moreover, the mechanism behind the phase separation and sugar partition in these ABS systems has been understood thanks to the use of COSMO-RS.

To the best of our knowledge, direct application of ABSs to FOS purification and simultaneous selective separation of glucose from levansucrase, sucrose, and fructose in different aqueous phases have never been attempted. By preventing glucose inhibition, this innovative integrated process might lead to an increase in FOS production yield and its effective purification.

2. MATERIALS AND METHODS

2.1. Materials. The polymers used in the ABS systems, namely, polypropylene glycol (PPG 400), polyethylene glycol (PEG 2000 and 8000), and sodium polyacrylate (NaPA 8000), were acquired from Sigma-Aldrich (St. Louis, MO, USA), while the block ethylene oxide/propylene oxide (EO/PO) copolymers of the Pluronic PE type (PE 6800, PE 6400 and PE 6200) were acquired from BASF (Ludwigshafen, Germany). Ultrapure water by reverse-osmosis (Millipore, Bedford, MA, USA) was used in all experiments.

2.2. Microbial Strains, Culture Conditions, and Genomic DNA (gDNA) Extraction. The *Bacillus subtilis* strain ATCC 6051 was obtained from Fundação André Tosello (Campinas, SP, Brazil) and cultured for 24 h at 30 °C in a Petri dish containing nutrient agar medium consisting of beef extract (3 g/L), peptone (5 g/L), and agar (15 g/L), pH 6.6. For DNA extraction, a single colony of *B. subtilis* was used to inoculate a 500 mL Erlenmeyer flask with 100 mL of nutrient broth (same composition without agar) and was cultivated for 24 h at 30 °C and 180 rpm. Subsequently, the culture was centrifuged in a 250 mL centrifuge bottle for 30 min at 1789 × *g*, the supernatant was discarded, and 10 mg of cell pellet were used for gDNA extraction using the Wizard Genomic DNA Purification kit (Promega, Madinson, WI, USA). *Escherichia coli* ArcticExpress (DE3) competent cells (Agilent, Santa Clara, CA, USA) were cultivated in lysogenic broth (LB) medium at 37 °C and used for plasmid propagation and heterologous protein expression.[41]

2.3. Gene Cloning of *B. subtilis* Levansucrase. The gene sequence encoding the *B. subtilis* levansucrase (accession number

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CBI68350) was obtained from the National Center for Biotechnology Information database. The amino acid sequence was sent to SignalP-5.0 Server of the Center for Biological Sequence Analysis (CBS, Lyngby, Denmark) to analyze the secretion signal peptide, and it was removed in oligonucleotide design.[42] The levansucrase gene was amplified from *B. subtilis* gDNA by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with the oligonucleotides BsGH68 forward (5′ − **CAGGGCGCCATG**AAAGAAACGAACCAAAAGCCATATAA − 3′) and BsGH68 reverse (5′ − **GACCCGACGCGGTTA**TTATTTGTTAACTGTTAATTGTCCTTGTTC − 3′). The used oligonucleotides contained specific regions (in bold) to allow insertion of the amplicon into pET-TRX1-a/LIC vector (Novagen, Madison, WI, USA) by Ligase Independent Cloning (LIC), allowing the expression of recombinant protein with a His_6-thioredoxin tag at the *N*-terminus.[43] For the LIC reaction, the vector was linearized by PCR using the oligonucleotides pET-LIC forward (5′ − TGGCGCCCTGAAAATAAA − 3′) and pET-LIC reverse (5′ − CCGCGTCGGGTCAC − 3′) as previously described.[44] The resulting reaction was used to transform the chemically competent *E. coli* expression system, and positive colonies were selected on LB medium with kanamycin and gentamicin (50 μg/mL, each). Experimental details of the heterologous expression and purification of levansucrase can be seen in the Supporting Information.

2.4. Levan-Type FOS Production and Purification. Since pure FOSs are available only as highly expensive chromatographic standards, their partitioning profiles were assessed using a mixture of levan-type FOSs produced by *Paenibacillus* sp. #210 isolated from Brazilian crude oil (details of the isolation of the microorganism could be found in a previous paper of the authors).[45] Levan-type FOSs were produced in mineral salt solution (MSS) supplemented with sucrose as a carbon source. The composition of the MSS medium was the following (g L^{-1}): NaCl 10.0; sucrose 10.0; Na_2HPO_4 5.0; NH_4NO_3 2.0; KH_2PO_4 2.0; $MgSO_4{\cdot}7H_2O$ 0.2. After FOS production, biomass was removed from the medium by centrifugation at 25 °C and 2795 × *g* for 20 min. The cell-free supernatant was mixed with three volumes of ethanol and incubated overnight at 4 °C. The FOS-containing precipitate was separated by centrifugation at 4 °C and 2795 × *g* for 20 min and subsequently dissolved in a minimum volume of demineralized water, and dialyzed using a semipermeable regenerated cellulose membrane (Orange Scientific, Braine-l'Alleud, Belgium) with a cut-off of 6000−8000 Da and a width of 25 mm. Dialysis was performed using 4 L of deionized water for four 2 h cycles and a final step of 12 h at 4 °C. The dialysate was subsequently filtered in a vacuum filtration system using a filter with 0.45 μm pore diameter, frozen, and lyophilized (L101, Liobras, São Carlos, SP, Brazil). Full FOS structural and physicochemical characterization was already performed and discussed in a previous study.[45] Summing up, the *Paenibacillus* sp. # 210 FOS was characterized as a predominantly linear oligofructan with $\beta(2 \rightarrow 6)$ linkages and $\beta(2 \rightarrow 1)$ branching points, with an estimated average DP of approximately 18 fructosyl units and a ramification ratio of approximately 20.

2.5. Purification Platform: ABS Formed by Water, NaPA 8000, and Polymers/Copolymers. *2.5.1. Determination of the Phase Diagrams of Ternary Systems.* Experimental phase diagrams of ABS formed by NaPA 8000 and polymers/copolymers in water were determined gravimetrically, within an uncertainty of $\pm 10^{-4}$ g, using the cloud point titration method[46] at room temperature (25 ± 1 °C) and atmospheric pressure. Diagrams were determined for several neutral EO/PO polymers (PEG 2000, PEG 8000, and PPG 400) and copolymers (Pluronic PE 6800, PE 6400, and PE 6200) combined with NaPA as a polyelectrolyte polymer. Briefly, two stock aqueous solutions were prepared, namely, 50 wt % EO/PO polymer/copolymer and 45 wt % NaPA 8000. NaPA 8000-containing solution was added dropwise to a solution of PEG EO/PO polymer/copolymer until visual detection of two phases. Subsequently, water was added dropwise until the two phases disappeared, highlighting a monophasic region. This procedure was repeated several times under constant stirring to obtain phase diagrams. The experimental data were correlated using the equation proposed by Merchuk et al.:[47]

$$[\mathrm{EO/PO}] = A\exp[(B \times [\mathrm{NaPA}]^{0.5}) - (C \times [\mathrm{NaPA}]^3)] \quad (1)$$

where [EO/PO] and [NaPA] are the weight percentages of EO/PO polymer/copolymer and NaPA 8000, respectively, while *A*, *B*, and *C* are parameters estimated by regression of the experimental data. This equation was selected because it has a low number of adjustable parameters to correlate experimental data and is the most applied to fit solubility curves of the polymer-based ABS.[36,48,49]

Each tie-line (TL), which gives the composition of each phase for a given mixture point, was determined by solving the following system of four equations for the four unknown values of $[\mathrm{EO/PO}]_T$, $[\mathrm{EO/PO}]_B$, $[\mathrm{NaPA}]_T$, and $[\mathrm{NaPA}]_B$:

$$[\mathrm{EO/PO}]_T = A\exp[(B \times [\mathrm{NaPA}]_T^{0.5}) - (C \times [\mathrm{NaPA}]_T^3)] \quad (2)$$

$$[\mathrm{EO/PO}]_B = A\exp[(B \times [\mathrm{NaPA}]_B^{0.5}) - (C \times [\mathrm{NaPA}]_B^3)] \quad (3)$$

$$[\mathrm{EO/PO}]_T = \frac{[\mathrm{EO/PO}]_M}{\alpha} - \frac{1-\alpha}{\alpha} \times [\mathrm{EO/PO}]_B \quad (4)$$

$$[\mathrm{NaPA}]_T = \frac{[\mathrm{NaPA}]_M}{\alpha} - \frac{1-\alpha}{\alpha} \times [\mathrm{NaPA}]_B \quad (5)$$

where the subscripts M, T, and B refer to the initial mixture, the top phase, and the bottom phase, respectively, while α is the ratio between the mass of the top phase and the total mass of the mixture. The system solution allowed estimating the polyelectrolyte and EO/PO polymer/copolymer concentrations in the bottom and top phases.[50]

The tie-line length (TLL) was calculated by the equation:

$$\mathrm{TLL} = \sqrt{([\mathrm{NaPA}]_T - [\mathrm{EO/PO}]_B)^2 + ([\mathrm{EO/PO}]_T - [\mathrm{EO/PO}]_B)^2} \quad (6)$$

2.5.2. Partitioning of FOSs and Simple Sugars in the Polymer-Based ABS. In order to assess the extraction capacity of the ABS under study, partition experiments were carried out with different carbohydrates by fixing the mixture point at the biphasic zone of the ternary phase diagrams. Considering the distinct abilities to undergo phase separation, the following ternary mixture composition was selected: 10 wt % NaPA 8000 + 10 wt % EO/PO polymers or copolymers +80 wt % water. Each carbohydrate was added separately to ternary mixtures in a proportion of 1.25 mg per gram of total mass in independent assays. Vigorous agitation was applied, and at least 12 h of equilibration time was allowed. After separating the phases, they were weighed, and the carbohydrate contents in each phase were determined by the phenol-sulfuric acid method using a UV−vis spectrophotometer.[51] At least three replicates were prepared for subsequent duplicate analysis aimed at determining the partition coefficient of each carbohydrate (K_{Carb}) and its recovery yield (Y_{Carb}), which were expressed as mean ± standard deviation. The same ternary mixtures without any addition of carbohydrate were used as blank controls to exclude any possible interference of the ABS phase-forming compounds. K_{Carb} and Y_{Carb} were calculated by the equations:

$$K_{Carb} = \frac{[\mathrm{Carb}]_T}{[\mathrm{Carb}]_B} \quad (7)$$

$$Y_{Carb}(\%) = \frac{[\mathrm{Carb}]_{rich-phase} \times V_{rich-phase} \times f}{[\mathrm{Carb}]_T \times V_T \times f + [\mathrm{Carb}]_B \times V_B \times f} \times 100 \quad (8)$$

where *V* is the aqueous phase volume, [Carb] is the concentration of each individual carbohydrate, i.e., FOS, sucrose, D-glucose or D-fructose, *f* is the dilution factor of the respective aqueous phase, while the subscript "rich phase" refers to the phase in which the carbohydrate is enriched.

The total carbohydrate concentration was determined by the phenol-sulfuric acid method.[51] In brief, an aliquot (80 μL) of top or bottom phase, 150 μL of 5% (w/v) aqueous phenol solution and 1.0 mL of concentrated sulfuric acid were added to a long test tube, stirred, and heated at 100 °C for 10 min in a water bath. The

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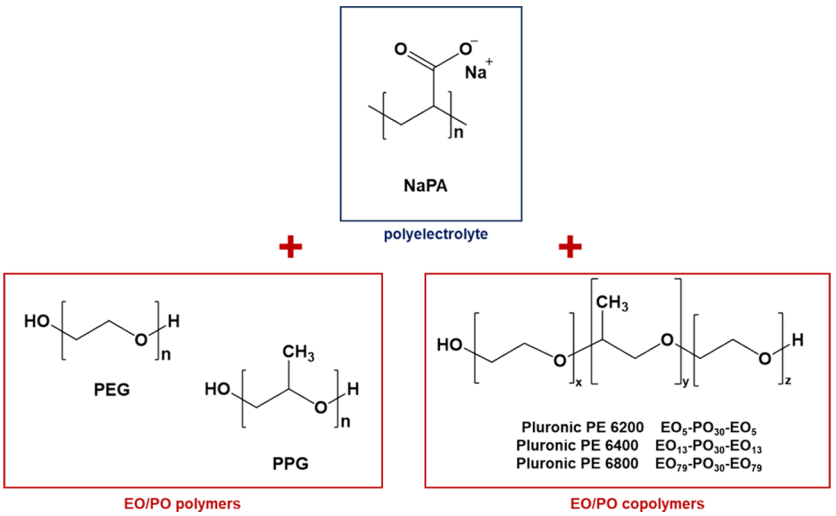

Figure 1. Chemical structures of the phase-forming components of ABS based on polyelectrolyte and EO/PO polymers or copolymers.

absorbance of the resulting yellow-orange colored solutions was measured at 490 nm using a BioTek Eon spectrophotometer (BioTek, Winooski, VT, USA), and the concentration was determined with the aid of calibration curves (0.05−0.30 mg mL^{-1}) previously prepared using each carbohydrate. The pH (±0.003) and conductivity (±1.0% mS cm^{-1}) at 25 (±1) °C of top and bottom phases for all ABSs were measured with a portable pH meter and conductometer Metrohm/Model 914 (Herisau, Switzerland). The pH was calibrated with two standard buffers (pH values of 7.00 and 4.01), and the calibration of the conductometer was performed using a standard solution of KCl (0.1 mol L^{-1}).

2.5.3. Partitioning of Levansucrase in the Polymer-Based ABS. Levansucrase partitioning experiments were performed at the same ternary mixture point and using the same procedure as for carbohydrates. The partition coefficient of levansucrase (K_{Lev}) and its recovery yield (Y_{Lev}) were calculated in the same way as those of carbohydrates but using levansucrase concentration instead of [Carb], with each system prepared in triplicate. The mass balance of levansucrase [MB_v (%)] was calculated by the equation below:

$$MB_V(\%) = \frac{[Lev]_T \cdot V_T + [Lev]_B \cdot V_B + [Lev]_I \cdot V_I}{[Lev]_0 \cdot V_0} \times 100 \quad (9)$$

where $[Lev]_0$, $[Lev]_T$, $[Lev]_B$, and $[Lev]_I$ are the concentrations of levansucrase in the initial solution, top phase, bottom phase, and interface (when there was protein precipitation in the interface) expressed in mg mL^{-1}, while V_0, V_T, V_B, and V_I are their respective volumes (after resuspension of protein precipitate for quantification) expressed in mL.

The concentration of the initial solution of levansucrase was quantified by two total protein quantification protocols, namely, Bradford protein assay (using Sigma Bradford reactant and following the manufacturer's instructions and the Enspire multimode plate reader from PerkinElmer) and intrinsic fluorescence of proteins. Bovine serum albumin (BSA, Sigma) was used as a protein standard. The levansucrase in the ABS was quantified by fluorescence spectroscopy with a calibration curve using the initial solution of levansucrase, after the definition of its concentration. The levansucrase in the interface for the systems with precipitate (ABS with PE 6400 and PE 6800) was resuspended in 1 mL of milliQ water before quantification. To define the best wavelengths for levansucrase quantification by fluorescence with minimal interference of the ABS phase-forming agents, the enzyme and the different ABS phases were evaluated by 3D fluorescence spectroscopy. The 3D spectra were acquired at 25 °C with a variable wavelength range of excitation from 220 to 550 nm and emission from 280 to 560 nm (interval of 2.0 nm for the excitation and emission range), excitation bandwidth of 10 nm, and emission bandwidth of 1 nm, using the spectrofluorophotometer RF-6000 (Shimadzu, Kyoto, Japan).[52,53] The 3D spectrum of levansucrase is presented in Figure S1, while the spectra of the different ABS phases and comparison with levansucrase are shown in Figure S2 (Supporting Information). The calibration curves of the levansucrase quantification methods are illustrated in Figure S3.

2.6. Computational Modeling: COSMO-RS. Computational modeling, namely, COSMO-RS, is a quantum chemical-based thermodynamic model that can be used to predict the thermophysical properties of a chemical compound in the pure and mixture state.[54] This model has recently been proven as a valuable tool to provide insight into the molecular interactions occurring in pure and mixture systems of interest, including aqueous biphasic systems.[55,56] The advantage of using COSMO-RS is that the model relies solely on the chemical structure of the compounds used. Therefore, it does not require experimental data to perform the predictions.

In general, the computational modeling using COSMO-RS consisted of two main steps. In the first step, the COSMO files for each molecule were obtained using the TURBOMOLE V7.3 2018 software.[57] The molecular geometry of water, polyelectrolyte (NaPA 8000), and polymer/copolymer was optimized using density functional theory, applying the BP functional B88-p86 with a triple-ζ valence polarized basis set (TZVP) and the resolution of identity standard approximation to produce the required cosmo file. The optimized structures of molecules of interest were also confirmed by the absence of imaginary frequency. Due to the large chemical structures of the studied polymers, it took 6 to 7 days to optimize and produce the cosmo file. In the second step, the obtained cosmo files were used as input in the COSMOtherm software (COSMOlogic, Levekusen, Germany) using the BP_TZVP C30_1801 parameter.[58] The COSMO-RS calculation was used to estimate the interaction energy between water and polymer in excess energy, aimed at getting insight into the molecular interaction that rules the formation of the ABS studied in this work. The detailed calculation of excess energy can be found in the literature.[59]

3. RESULTS AND DISCUSSION

3.1. Characterization of Polymer-Based ABSs. Although polymer-polymer ABSs are the oldest type of ABS, understanding the molecular-level mechanisms underlying their phase separation remains considerably uncertain compared to other ABS types, such as the polymer-salt and ionic-liquid-salt ones. Despite the lower number of experimental and theoretical studies concerning the complex interaction network of intra and intermolecular driving forces, polymer-based ABSs composed of polyelectrolytes such as NaPA are relatively well described in the literature.[36,46,48]

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However, although the number of phase diagrams available for polymer-based ABSs is relatively high, the study of the combination of NaPA with EO-PO Pluronic copolymers and PPG has never been accomplished. Considering partitioning profiles of biomolecules in the copolymer/salt-based ABS,[60] the possibility of modulating the polarity of these new systems using different Pluronic ethylene oxide/propylene oxide (EO/PO) ratios is expected to be successful as regards the partition of complex sugar mixtures.

Based on the above considerations, the experimental phase diagrams of each ternary system formed by water, polyelectrolyte (NaPA 8000), and polymer/copolymer were determined. Three different polymers/copolymers were tested to promote phase separation, namely, PEG (an EO polymer), PPG (a PO polymer), and different Pluronic PE copolymers belonging to the EO/PO family. The chemical structures of ABS components and composition of EO/PO copolymers are given in Figure 1 and Table 1, respectively.

Table 1. Main Characteristics of $(EO)_x$-$(PO)_y$-$(EO)_x$ Triblock Copolymers Used to Prepare ABS Based on Polyelectrolyte and EO/PO Polymers or Copolymers

pluronic	MW (g mol^{-1})[a]	average formula[b]	EO monomers (%)[c]
PE 6200	2450	EO_5-PO_{30}-EO_5	20
PE 6400	2900	EO_{13}-PO_{30}-EO_{13}	40
PE 6800	8000	EO_{79}-PO_{30}-EO_{79}	80

[a]Average molecular weight. Values provided by the manufacturer (BASF SA, Ludwigshafen, Germany). [b]Average formula designated by the EO/PO ratios. Values provided by the manufacturer (BASF SA, Ludwigshafen, Germany). [c]Percentage of EO monomers estimated from the EO/PO ratios. Values provided by the manufacturer (BASF SA, Ludwigshafen, Germany).

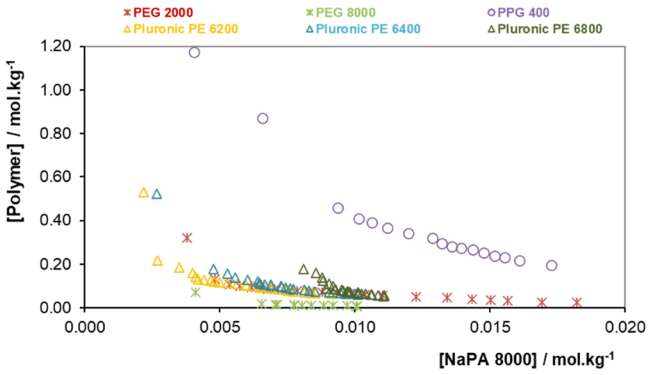

Figure 2. Phase diagrams for the systems composed of NaPA 8000 and different polymers (PEG 2000, PEG 8000, and PPG 400) or copolymers (Pluronic PE 6200, Pluronic PE 6400, and Pluronic PE 6800) at 25 ± 1 °C, expressed in mol/kg.

Figure 2 illustrates the binodal curves of each ABS determined by the cloud point titration method,[61] being given in molality units to avoid differences directly related to the polymer's molecular weight. Compositions of NaPA and EO/PO polymers or copolymers above each binodal curve result in two-phase systems, whereas mixture compositions below fall into the monophasic region. As a rule of thumb, the larger the biphasic region, the higher the ability of a given polymer or copolymer to induce phase separation, thus the lower the amount needed to create ABS. Detailed information on experimental weight fractions, correlation parameters (*A*, *B*, and *C*) in eq 1, and phase diagrams is reported in the Supporting Information (Tables S1−S3 and Figure S4). The results concerning the ability of polymers or copolymers to induce phase separation, at 0.010 mol kg^{-1} of NaPA, showed the following trend: PEG 8000 > Pluronic PE 6200 ≈ Pluronic PE 6400 ≈ Pluronic PE 6800 ≈ PEG 2000 > PPG 400.

According to literature data, the main driving force behind the NaPA-based ABS phase separation mechanism is the aqueous phase electroneutrality, which is particularly relevant for systems involving electrolytes, which is not the case of the current work. In these systems, the minimum free energy of the mixture is ensured by the compartmentalization of components between the co-existing phases, which due to coulombic long-range interactions become electroneutral at the equilibrium.[46] However, without other charged species in equilibrium, in this work the type of noncharged polymer/copolymer also showed a significant effect on the phase diagrams (Figure 2), suggesting the contribution of additional driving forces during two-phase separation. As for the effect of PEG molecular weight, it is noteworthy that an increase in molecular weight caused the binodal curve to move toward the water-rich corner, and a wider biphasic region was obtained. Such a behavior, observed for PEG 8000, is justified by its higher hydrophobicity, thereby promoting the easier separation of the two polymers.

When compared to more hydrophilic polymers (e.g., EO polymers like PEG), the PPG binodal curve moved toward the polymer-rich region, showing the need for larger polymer contents to reach segregation. Although PPG and PEG are composed of similar end groups, the lateral repeated methyl groups present in the PPG chain hinder the access of water molecules to the chain ether group, limiting the formation of hydrogen bonds between water and ether oxygen atoms, thus resulting in a more hydrophobic polymer. However, it should be considered that PPG has lower molecular weights than the PEG counterparts. In such a scenario, the polyelectrolyte may behave more like a charged polymer, thus favoring polymer association and reducing polyacrylate tendency to compartmentalize and to undergo liquid−liquid demixing. Based on these results, it is possible to infer that the different hydrophilicity of EO/PO polymers plays a predominant role in the segregation of polymers into two coexisting phases.

As for the block copolymers, which have an intermediate hydrophobicity, their behavior was much more similar to that of PEG than that of PPG. However, the effect of the EO/PO ratio of Pluronics (belonging to the PE family, with the same MW of PO monomers = 1750 g mol^{-1}) was negligible, as revealed by an almost coincident tendency of the systems to form ABSs. Still, the obtained results reinforce the importance of EO monomers in promoting phase separation. In comparison with Pluronics-salt,[62] PEG-salt,[63] and PPG-salt[64]-based ABSs, the use of NaPA as a counterphase-forming compound greatly enlarged the biphasic region, providing a wider range of different mixture points. This allows obtaining more versatile, low-cost, and biocompatible ABSs, with a high water content (about 80 vs 50 wt %).[60,65] The results obtained are in line with those of Johansson et al.[46] on the PEG-NaPA-salt-based ABS, who observed that, in the absence of electrolytes, ABS formation required no less than 10 wt % of each polymer.

Further discussion about the formation of the biphasic system at the molecular level is presented below in Section 3.4.

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Table 2. Weight Fraction Compositions of TLs, with Their Respective Ratios between the Mass of the Top Phase and the Total Mass of the Mixture (α) and Length (TLL), Referred to NaPA 8000 + EO/PO Polymer/Copolymer + Water ABS, at the Top (T) and Bottom (B) Phases and at the Initial Biphasic Composition of the Mixture (M) at 25 °C and Atmospheric Pressure

	weight percentage composition of TL (%wt)										
EO/PO copolymer or polymer	$[NaPA]_T$	$[EO/PO]_T$	$[water]_T$	$[NaPA]_M$	$[EO/PO]_M$	$[water]_M$	$[NaPA]_B$	$[EO/PO]_B$	$[water]_B$	α	TLL
PEG 2000	4.47	19.63	75.90	10	10	80	13.98	3.07	82.95	0.419	19.09
PEG 8000	4.18	20.29	75.53	10	10	80	15.44	0.38	84.18	0.483	22.87
Pluronic PE 6800	5.12	41.83	53.05	10	10	80	11.26	1.78	86.96	0.205	40.52
Pluronic PE 6400	4.08	22.09	73.83	10	10	80	13.68	2.49	83.83	0.383	21.82
Pluronic PE 6200	2.55	28.02	69.43	10	10	80	13.11	2.47	84.42	0.295	27.64
PPG 400	7.83	13.70	78.47	10	10	80	12.05	6.50	81.45	0.486	8.35

3.2. Partitioning of Carbohydrates in the Polymer-Based ABS. Taking into consideration the requirements imposed by the food industry regarding functional/caloric properties of FOSs, the development of proper FOS separation systems requires yields of at least 95% for refined FOS and even higher purity for standard formulations. The design of such systems has to contemplate the ability of selectively separating FOS from mono- and disaccharides either present in the medium after sucrose transfructosylation by levansucrase or released by chemical or enzymatic hydrolysis of inulin. Considering that, in the case of levansucrase-catalyzed FOS synthesis, the sugar mixture after FOS production is usually composed of (wt %) 55 FOS, 31 glucose, 12 sucrose, and 2 fructose,[16] the development of effective purification systems is a difficult challenge. In this context, the application of ABS technology as a strategy to purify FOSs or overcome levansucrase inhibition by D-glucose requires a careful study on the driving forces related to the partitioning behavior of each sugar.

To perform the partitioning of carbohydrates in systems containing polymer-based phase-forming compounds, ternary mixtures with fixed compositions of (wt %) 10 NaPA 8000, 10 EO/PO polymer/copolymer, and 80 water were used. The phase composition was characterized through the TL calculated for each system (Table 2). ABSs were composed of two aqueous phases, i.e., a (co)polymer-rich top phase and an NaPA 8000-rich bottom phase, with a well-defined interface in the middle. Partitioning of each carbohydrate was carried out at the selected mixture point, using the partition coefficient (K) and recovery yield (Y) to evaluate the partitioning performance of biomolecules and ABS effectiveness in fractionating them. From the collected results, shown in Figure 3 and Table S4 (Supporting Information), it is possible to see a different partitioning scenario for each carbohydrate among the selected polymer-based ABS.

The partition mechanisms of different carbohydrates in the polymer-based ABS can be appraised according to an ascending order of polymer hydrophobicity, namely, PEG, Pluronics, and PPG. Since carbohydrates are neutrally charged, no electrostatic ion−ion interactions may have occurred with the negatively charged NaPA, unlike charged biomolecules like proteins.[36,38] Nonetheless, two distinct partitioning profiles were observed for the studied carbohydrates, i.e., a highly polymer-dependent and a hardly polymer-dependent partition.

FOS partitioning was shown to be highly polymer-dependent. Polymer replacement strongly influences its partitioning toward one phase or another or even promotes its precipitation at the interphase. When PEG was used as a phase-forming component, the FOS was partitioned toward

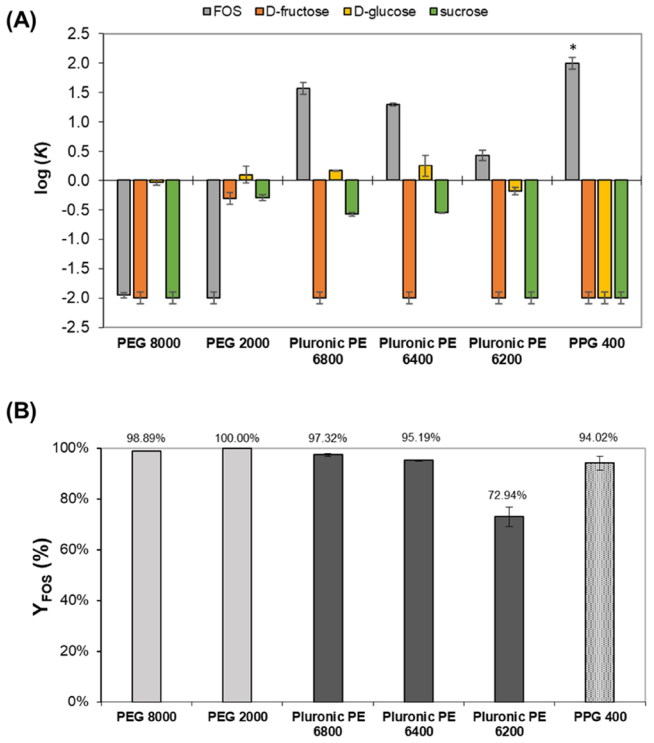

Figure 3. (A) Partition coefficient (log$K$) of D-fructose (orange bars), D-glucose (yellow bars), sucrose (green bars), and FOS (gray bars) in ABS at the mixture point composed of 10 wt % NaPA 8000 + 10 wt % EO/PO (co)polymers +80 wt % water. *log(K) calculated taking into consideration the amount of FOS precipitated and present in the interphase by the equation $K = \frac{[FOS]_I}{[FOS]_{B+T}}$, where the subscripts I, B, and T do refer to the interphase, bottom phase, and top phase, respectively. (B) Recovery yield of FOS (Y_{FOS}, bars) in the same polymer-based ABS at the same mixture point. The bar color corresponds to the phase in which the FOS was concentrated: bottom (light gray), top (dark gray), and interphase (speckled).

the bottom phase, i.e., the NaPA 8000-rich phase. This effect was already observed for other biomolecules in several polymer-polymer systems, in which PEG caused large biomolecules to be partitioned to the other phase, due to saturation in the PEG-rich aqueous phase.[66,67] Furthermore, no major differences were observed between the two systems composed of PEG with different molecular weights. Although the FOS solution used in partitioning experiments (1.25 mg mL^{-1}) was a complex mixture of FOS with different DPs, this partitioning behavior (Y_{FOS} = 98.89 ± 0.12% with PEG8000 and Y_{FOS} = 100 ± 0% with PEG 2000) can be ascribed to the

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high ability of levans to self-assemble in water into micellar aggregates with large size and molecular weight (up to 2200 kDa).[68] It has been reported that the occurrence of such an aggregation phenomenon when the critical aggregation concentration (CAC) threshold is reached (0.05 mg mL^{-1} for levan-type FOS from microbial sources) is due to the FOS amphiphilic nature.[68] As the FOS concentration in the recovered bottom phase was about 50-fold its CAC value reported above, its partition toward the NaPA-rich bottom phase was likely to occur due to its high water content. Contrariwise, when EO/PO copolymers were used as phase-forming agents, the FOS concentrated mostly in the top phase probably due to the amphiphilic nature of the copolymer itself, and possible creation of mixed and stable micelles. Depending on Pluronics hydrophilic−lipophilic-balance (HLB), different copolymer aggregate types, as well as different surrounding hydration layers, are known to occur in aqueous solution.[69] Moreover, changes in the hydrodynamic radius and breakdown of copolymer micelle clusters were observed in the presence of amphiphilic molecules.[70] Therefore, in the presence of copolymers, FOS-FOS inter/intramolecular interactions may have been partially replaced by the FOS-copolymer ones, thereby resulting in FOS accommodation among copolymer hydrophobic/hydrophilic regions, resulting in the formation of mixed micelle aggregates and/or simply interaction with copolymer disaggregated monomers. Such justifications are supported by a more pronounced FOS partitioning toward the top (copolymer-rich) phase with increasing the percentage of EO monomers in the copolymer (Y_{FOS} = 72.94 ± 3.91% with Pluronic PE 2000, Y_{FOS} = 95.19 ± 0.24% with Pluronic PE 4000, and Y_{FOS} = 97.32 ± 0.57% with Pluronic PE 8000). Finally, the use of PPG as a phase-forming compound, even at low concentration (i.e., 6.50 wt % PPG 400 in the bottom phase) (Table 2), forced FOS exclusion from NaPA-rich phase and induced the formation of a white cloudy precipitate in the interphase without affecting FOS recovery (Y_{FOS} = 94.02 ± 2.70%) (Table S4, Supporting Information), thus confirming its high efficiency to selectively precipitate high-molecular-weight biomolecules.[71]

Contrarily to the FOS partitioning profile, D-fructose and sucrose partitioning behaviors were shown to be less dependent on the EO/PO polymer ratio. Despite the influence of this ratio and molecular weight on system polarity, partitioning of both sugars occurred preferentially toward the NaPA 8000-rich phase, even though that of D-fructose was in general more effective than that of sucrose, with a complete recovery (Y = 100%) in five of the six ABSs studied. For sucrose, complete partitioning toward the NaPA-rich phase was obtained only in three of the studied systems, namely, PPG400, Pluronic PE 6200, and PEG 8000. However, recovery yields were satisfactory also in the remaining cases and even in the less effective PEG 2000 ABS (Y = 62.3 ± 5.49% for fructose and 61.81 ± 1.88% for sucrose) (Table S4, Supporting Information). These results indicate that, regardless of the carbohydrate hydrophobicity, the higher the PEG molecular weight, the weaker the attractive interactions between the PEG-rich phase and carbohydrate molecules. Partitioning of both sugars to the NaPA-rich phase cannot be justified solely by the NaPA availability to form hydrogen bonds and the high water content of this phase (approximately 84 wt % water), because the hydrophilicity and water content of the PEG-rich phase (approximately 76 wt % water) (Table 2) were close to those of the polyelectrolyte. Thus, the reasons for such a partitioning behavior may have been a possible more stable relative orientation and/or a less hydrated state of these sugars in the NaPA-rich phase, which were likely to enable their complexation with the polyelectrolyte Na^+ counterion. The formation of these complexes may have reduced the system's overall mixing free energy and driven the transfer of both sugars toward the NaPA-rich phase, resulting in a lower influence of the polymer/copolymer on the partitioning process. The ability of sugars to form complexes with metal cations has been previously explored in solid−liquid separation.[15,16,19] As in the chromatographic process, the stability of coordination complexes, which depends on the sugar hydration extent, number and space disposition of hydroxyl groups, pyranose or furanose form, and then anomeric C position, might have driven the separation process between ABS coexisting phases.[19,72]

As for the D-glucose partitioning profile, a more extensive distribution between the two ABS phases was obtained with both PEG and Pluronic PE ($-0.18 \pm 0.06 < \log K < 0.25 \pm 0.18$). The main interactions acting between glucose and these agents may be: (a) dipole−dipole interactions and hydrogen bonds between −OH groups and ether oxygen atoms of glucose and polymers/copolymers, respectively, (b) hydrogen bonds between glucose −OH groups and PEG/Pluronic PE −OH terminal groups, and (c) short-range dispersion forces between glucose and polymer/copolymer hydrocarbon moieties. Compared to fructose and sucrose, the non-one-way partitioning behavior of glucose between the two phases may be justified by differences at conformational isomerism level, which, however, would require a broader systematic investigation on the driving forces influencing its partitioning in NaPA 8000-based ABS. Complete recovery of D-glucose toward the NaPA-rich phase was obtained using PPG ($\log K = -2.00 \pm 0.10$), likely due to interference of PPG methyl groups on the attractive interactions with glucose and the high hydration state of this sugar.[72]

Finally, in view of the enzymatic FOS production, the partition profiles of the three sugars taken together allowed us to select the ABS constituted by NaPA and Pluronic PE 6800 or PE 6400 as the most efficient systems to separate levansucrase inhibitor (D-glucose) from reagents (D-fructose and sucrose), while the PPG 400-based ABS was the best one to perform FOS downstream separation from simple sugars, followed by Pluronic PE 6800- and PE 6400-based systems.

3.3. Partitioning of Levansucrase in the Polymer-Based ABS. In order to further explore the application of ABS technology to overcome glucose inhibition of enzyme activity,[73] the partitioning profile of microbial levansucrase was investigated in the same polymer-based ABS at the same mixture point as for the carbohydrates (Figure 4). More information regarding the detailed weight fraction compositions and physicochemical characteristics [pH, conductivity (mS cm^{-1} at 25 °C)] of each ABS is shown in Table S5 (Supporting Information), while values of the partition coefficient (K), log (K), Yield [Y_{Lev} (%)] and Mass Balance [MB_v (%)] (with respective standard deviations) of each ABS are listed in Table S6 (Supporting Information).

One can see that levansucrase partitioning between the two phases was driven by copolymer nature in PEG- and PPG-based ABSs ($-1.27 \pm 0.06 < \log K < 1.20 \pm 0.20$), but it was more directed toward the bottom phase in the high-molecular-weight polymers/copolymers (PE-6800 and PEG-8000). Such a partitioning profile can be explained by a balance between

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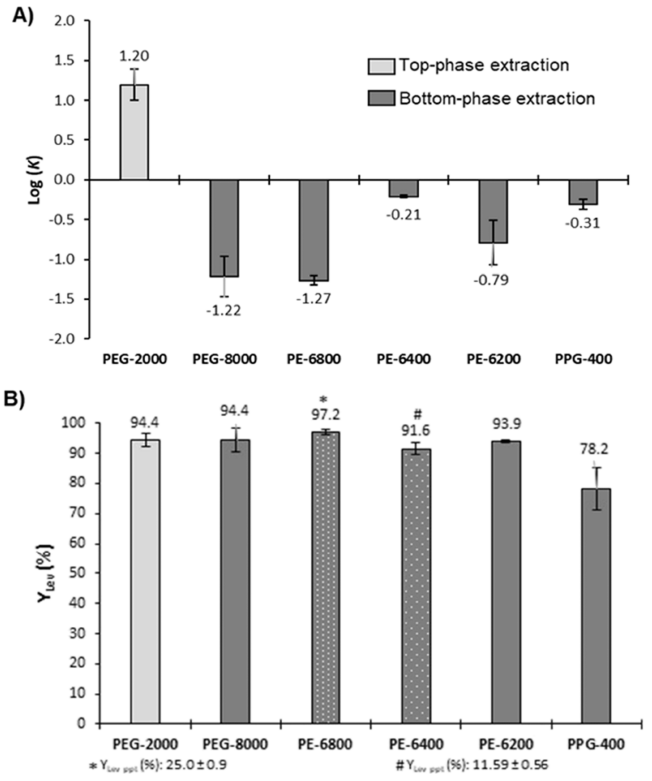

Figure 4. (A) Partition coefficient [Log (K)] of levansucrase in the ABS at the mixture point composed of 10 wt % NaPA 8000 + 10 wt % EO/PO (co)polymers +10 wt % water +10 wt % enzyme solution (0.1 mg mL^{-1}). (B) Recovery yield of the levansucrase (Y_{Lev} bars) in the same polymer-based ABS at the same mixture point. The bar color corresponds to the phase in which levansucrase was concentrated: in panel A, top (light gray), bottom (dark gray). The results represent the average of a duplicate with the respective standard deviations.

different driving forces acting on levansucrase in both aqueous phases, namely, the NaPA 8000−levansucrase electrorepulsive forces, exclusion volume effect, and other interactions occurring between the enzyme and the phase-forming components. Since the isoelectric point of levansucrase (pI) is 5.84 (calculated by Expasy Software through GenBank: CBI68350 FASTA sequence), at the ABS pH (pH 7−8) levansucrase had an overall negative charge, which should have favored partitioning toward the EO/PO polymer-rich phase; however, this did not occur in the majority of the systems, indicating that other attractive interactions between NaPA and enzyme may have played a more significant role. Furthermore, gravitational forces and the volume exclusion effect for high-molecular-weight polymers (which is well described in the literature for high-molecular-weight PEG-salts[66] and PEG polymer systems[74]) can explain the highest enzyme partitioning profile toward the NaPA-rich phase in the ABS with the larger co/polymers (PE-6800 and PEG8000). In the case of the PEG 2000 ABS, levansucrase partitioned toward the top PEG-rich phase, likely because the small molecular weight and hydrophilic nature of the polymer hindered the enzyme exclusion, and the repulsive electrostatic interaction between NaPA 8000 and enzyme became predominant.

The overall partitioning trend indicates that glucose separation from fructose, sucrose, and levansucrase was successful; therefore, acting on the tunability of polymers, two integrated processes can be designed to effectively produce FOSs. The main goal of this selective partitioning of glucose and levansucrase is to avoid inhibition of enzyme activity along the FOS production, thus increasing the production yield.

3.4. Computational Modeling Using COSMO-RS.

3.4.1. ABS Formation. In this work, COSMO-RS was used to get insights into the molecular mechanisms that rule the phase separation and sugar partitioning in the NaPA 8000-based ABS under investigation. In previous studies, the excess enthalpy predicted using COSMO-RS has been shown to be a novel tool to understand the molecular interaction playing a role in phase separation in the ABS composed of polymer and ionic liquid/salt.[55,56] In addition, the COSMO-RS model has been proven to differentiate the impact of PEG polymer end groups on the formation of ABS.[55,56] These findings claim its reliability to shed light on the primary molecular mechanism underlying the phase separation observed in the current work.

Values of the excess enthalpy of binary systems (H_2O + NaPA8000/polymer/co-polymer) predicted by COSMO-RS are given in Table S7 (Supporting Information). This parameter was calculated as the contribution of the electrostatic misfit ($H_{E,MF}$), hydrogen bond ($H_{E,HB}$), and van der Waals forces ($H_{E,VdW}$) of each component. The spontaneous interaction, reflected by the negative value of the predicted excess enthalpy, arises from the newly formed hydrogen bonding between water and NaPA8000/polymer/co-polymer. On the other hand, water contribution to the hydrogen bond is positive, as its molecules need to break the water intermolecular network to accommodate the new bond with NaPA8000/polymer/co-polymer. To get a better visualization, Figure 5 depicts the contribution of a hydrogen bond between

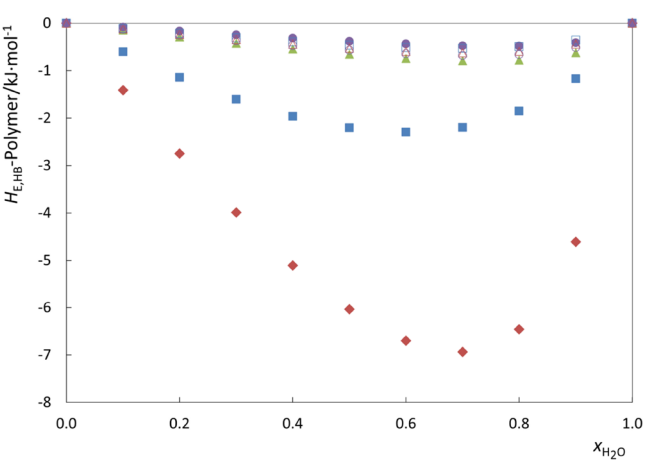

Figure 5. Contribution of hydrogen bonding on the H_2O + NaPA8000/polymer/co-polymer binary system predicted using COSMO-RS at 25 °C. Symbols: (solid box), H_2O + NaPA 8000; (solid tilted square), H_2O + PPG 400; (solid upward facing triangle), H_2O + PEG 2000; (solid circle), H_2O + PEG 8000; (open square), H_2O + Pluronic 6200; (open upward pointing triangle), H_2O + Pluronic 6400; and (open circle), H_2O + Pluronic 6800.

water and NaPA8000/polymer/co-polymer in the binary system. It is evident that all the studied polymers have a significant influence on the attraction of water molecules. Specifically, the hydrogen bond interaction of NaPA8000 with water molecules primarily occurs in the polymer-end acrylate group, while that of the other polymers in their hydroxyl functional groups. The small size of PPG400 compared to the other polymers and copolymers results in the highest capability

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of forming hydrogen bonds with water molecules, thus justifying its lower observed ability to form ABSs with NaPA. On the other hand, the strong ability of PEG-8000 to induce phase separation could be ascribed to its weak hydrogen bond interactions with water molecules. Thus, it is evident that the different strengths of polymers and copolymers to form hydrogen bonding with water molecules lead to diverse capability of inducing phase separation.

To delve further into the molecular mechanism ruling the phase separation, the COSMO-RS model was used to estimate the partial molar excess enthalpies in the ternary mixtures composed of H_2O + NaPA 8000 + Polymer/co-polymer, whose values are given in Table S8 (Supporting Information). As expected, the contribution of NaPA 8000−water and polymer/copolymer−water molecules to hydrogen bonding significantly affects the exothermicity of the ternary mixture. With the exception of PPG 400, NaPA 8000 formed a stronger hydrogen bond with water compared to the studied polymer/co-polymer combinations. Consequently, water would preferentially migrate to hydrating NaPA 8000 and leave the polymer/co-polymer-rich phase.

The strong interaction of water molecules with NaPA 8000, which is somehow expected given its charged nature, could be due to the localized negative charge of acrylate compared to the hydroxyl functional group carried by the other polymers. On the other hand, the stronger hydrogen bond between PPG 400-water compared to NaPA 8000-water could be attributed to the significantly smaller size of the former polymer. Thus, by keeping NaPA 8000, as depicted in Figure 6, the hydrogen

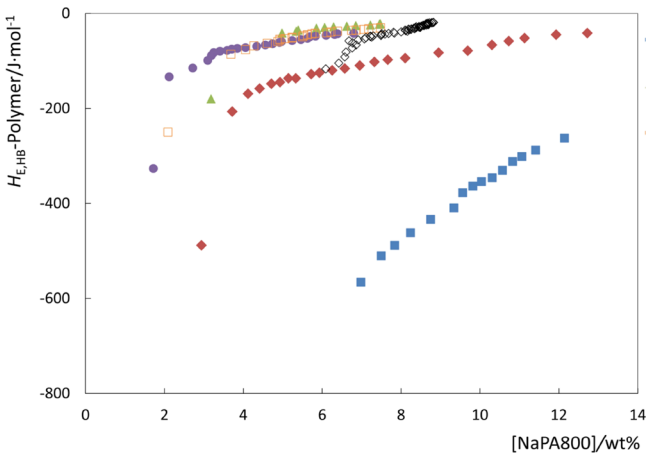

Figure 6. Contribution of hydrogen bonding on the H_2O + NaPA8000 + polymer/co-polymer ternary system predicted using COSMO-RS at 25 °C. Symbols: (solid square), H_2O + NaPA 8000 + PPG 400; (solid tilted square), H_2O + NaPA 8000 + PEG 2000; (solid upward pointing triangle), H_2O + NaPA8000 + PEG 8000; (solid circle), H_2O + NaPA 8000 + Pluronic 6200; (open square), H_2O + NaPA 8000 + Pluronic 6400; and (open tilted square), H_2O + NaPA 8000 + Pluronic 6800.

bond strength of water molecules with the other polymers can be ranked as follows: PEG 8000 < Pluronic PE 6800 ≈ Pluronic PE 6400 ≈ Pluronic PE 6200 ≈ PEG 2000 < < PPG 400. This order follows the ability trend of the polymer to induce phase separation with NaPA 8000.

Based on the excess enthalpies of binary and ternary mixtures predicted by COSMO-RS, the scenario of phase separation could then be explained as follows: (i) due to higher localized charge distribution, water would preferentially hydrate the NAPA8000 than the polymer/co-polymer, (ii) as a result, water molecules will migrate into the NAPA8000-rich phase and leave the polymer/co-polymer-rich phase. The extent of this migration process depends on the affinity of the other polymer for water molecules. Polymers with a stronger affinity for water molecules, for instance PPG 400, are less capable of inducing phase separation in NaPA-polymer ABS, as observed experimentally. On the other hand, PEG 8000 has the weakest interaction with water molecules, as indicated by the lowest negative partial excess enthalpy, and ultimately, it has the highest ability to induce ABS formation.

3.4.2. Partition of Carbohydrates in ABS. Previously, it was shown that the hydrogen bonding competition between NaPA 8000-water and polymer-water molecules plays a significant role in the formation of ABSs. As these ABSs are proposed to partition sugar molecules, it is essential to understand their impact as well as that of their phase-forming components on sugar partitioning. The estimated partial excess enthalpy for the experimental recovery data listed in Table S4 is given in Table S9 (Supporting Information). It is quite challenging to see a direct correlation between the individual estimated partial excess enthalpy estimated using COSMO-RS and the observed Log*K* or the recovery yield. In general, for the partition of sugars, the unfavorable contribution (positive values) arises from the sugar molecules themselves against NaPA 8000. The sugar molecule displays repulsive electrostatic misfit interaction with the negative charge of acrylate of NaPA 8000; therefore, it goes to the other phase with less NaPA 8000 amount. On the other hand, favorable interactions are observed between sugar molecules and polymer/co-polymer, which may be due to hydrogen bonding between their hydroxyl groups. These findings suggest that sugar recovery is governed by a subtle balance between electrostatic misfit of sugar against highly negatively charged NaPA 8000 and hydrogen bonding established between sugar and polymer/co-polymer.

3.5. Conceptual Design of Two Integrated Processes for FOS Production and Separation. After investigating the partitioning patterns of sugars and levansucrase in each ABS, we are able to propose two integrated processes for FOS production, i.e., one purification approach conceiving sugar mixtures obtained by either chemical or enzymatic hydrolysis of inulin, and the continuous FOS synthesis and purification using *B. subtilis* levansucrase and concentrated sucrose solutions. As previously discussed, distinct profiles of extraction and separation can be obtained with the NaPA 8000-based ABS containing either conventional polymers or copolymers.

The most performing ABS able to purify FOS with the highest purity and yield was that using PPG 400. An integrated process using this system can be proposed (Figure 7), starting with FOS production by chemical hydrolysis of inulin. Such a process considers a complex FOS mixture rich in glucose and fructose, which is subsequently purified by FOS precipitation in the ABS interphase using PPG 400 + NaPA 8000 ABS.

On the other hand, the Pluronic PE 6800-based ABS was the most effective in preventing the inhibition exerted by D-glucose on levansucrase-catalyzed sucrose transfructosylation to FOSs, being able to partition D-glucose (albeit with a low log*K*) toward the aqueous phase, i.e., oppositely to reagents (sucrose and D-fructose) and biocatalyst; even though FOS would be partitioned with high yields in this system toward the same phase as D-glucose, the molecular weights of these sugars are

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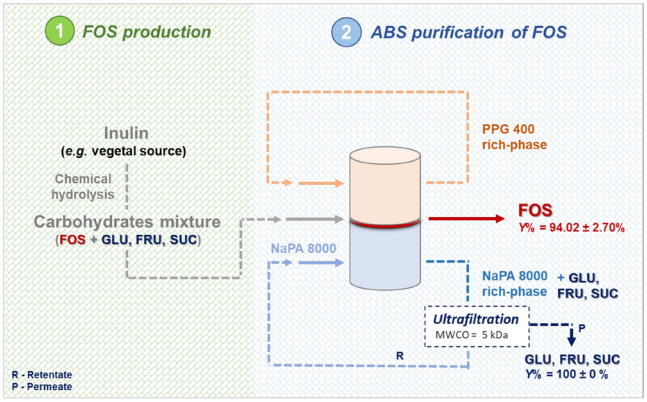

Figure 7. Schematic representation of the integrated two-step process of FOS production by chemical hydrolysis of inulin and their sequential extraction and separation by the PPG 400-based ABS.

different enough to be easily separated by ultrafiltration. Figure 8 provides a schematic representation of the overall process consisting of batch microbial production of levansucrase (gene cloning, heterologous expression, and chromatographic purification) followed by in situ Pluronic PE 6800 + NaPA 8000 ABS for continuous FOS production and recovery. According to such an operating strategy, continuous FOS production might take place in the bottom phase by continuous feeding of sucrose and partial removal of D-glucose toward the top phase. Moreover, the proposed protocol would allow in situ FOS partition toward the top phase and partial purification by ultrafiltration.

Although outside of the scope of the present experimental study, some suggestions to recycle and reuse the phase-forming compounds are also provided in Figures 7 and 8 (dashed lines) as a way to stress the sustainable character of the proposed processes.

Pluronic PE 6800 has a low critical solution temperature,[75] above which it could be separated, releasing FOS in aqueous solution, thus allowing for FOS recovery and copolymer recycling. Although referring to Pluronic F68, the above authors claimed that this copolymer was indistinguishable from PE 6800, having almost the same composition. NaPA 8000 could be easily recovered from the NaPA-rich phase by ultrafiltration, as done elsewhere.[76] The small amount of catalyst partitioned by the Pluronic PE 6800-based ABS could be additionally recovered by an additional step of ultrafiltration and recycled in the system.

The proposed processes would allow for a significant advance in the development of new, more sustainable, and efficient strategies to extract and separate FOS from their natural sources and culture broth. The conventional process for FOS production includes liquid−liquid extraction[77] with organic solvents followed by chromatographic separations.[78] The high amounts of hazardous solvents, low product recovery, poor selectivity for the different carbohydrates, and low productivity are the main drawbacks to be overcome. As previously highlighted, alternative purification methods should gain a place in the food and biotechnology industries; this innovative work can be part of such a technological advance aiming at a more sustainable and profitable process, through the application of scaled-up polymer-based ABSs.

4. CONCLUSIONS

Motivated by the tunable nature of EO/PO block copolymers and polymers, novel ABS composed of NaPA 8000 and EO/PO copolymers were studied, disclosing for the first time their high ability to purify FOS. Initially, the ternary phase diagrams were determined at 25 °C and used to study the ABS ability to selectively recover FOS from complex carbohydrate mixtures and to separate, at the same time, D-glucose (a levansucrase inhibitor) from the enzyme and its substrates. With a proper choice of the EO/PO polymer, good FOS purification results were obtained. Despite its low molecular weight, the strong ability of FOS to self-assembly in water into very large aggregates proved to be behind its partition profile. The excluded volume effect induced by PEG drove the partitioning of FOS aggregates toward the NaPA 8000-rich phase. An ability loss when using PPG 400, even at low concentrations in the bottom phase, forced FOS aggregate exclusion, and their selective precipitation at the ABS interface. Due to their amphiphilic nature, when EO/PO copolymers were used, the FOS was partitioned toward the top phase. From the increase in copolymers hydrophilic−lipophilic-balance, it was possible

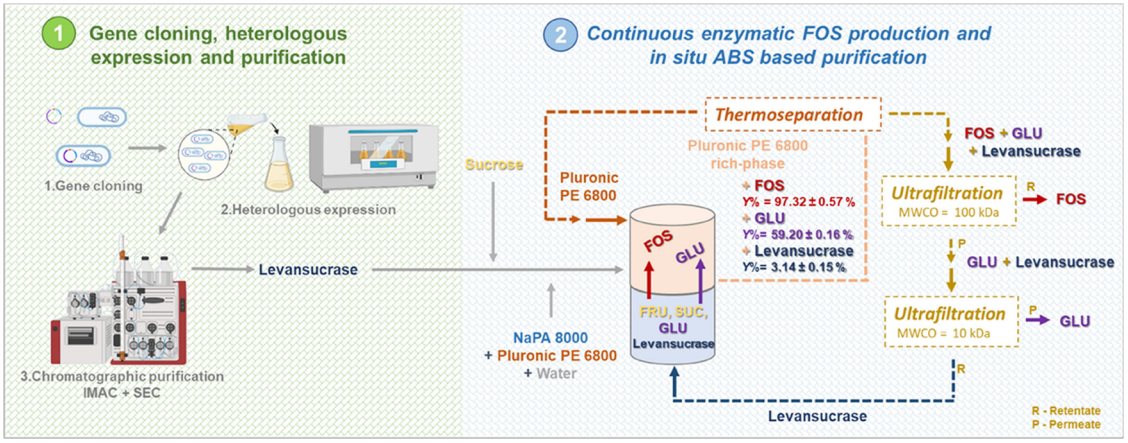

Figure 8. Schematic representation of the overall process combining batch microbial production of levansucrase and the in situ continuous enzymatic FOS production and purification using the Pluronic PE 6800-based ABS. (1) Gene cloning using pET-TRX1-a/LIC vector with levansucrase *Bacillus subtilis* inserted gene (CBI68350); heterologous expression in chemically competent *E. coli* (2 L Erlenmeyer batch) and levansucrase purification in IMAC and SEC (FPLC AKTApure). (2) NaPA 8000/Pluronic PE 6800-based ABS for selective continuous enzymatic FOS production with a proposed step (dashed lines) of catalyst recycling using thermoseparation and ultrafiltration steps.

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to identify the ratio of EO/PO segments capable of maximizing FOS aggregate disassembly and accommodation in its individual chain form in the copolymer-rich phase. By means of the developed fractionation platforms, glucose was effectively separated from the remaining carbohydrates and levansucrase. Different forces were shown to drive the partitioning of each compound between ABS top and bottom phases, namely electrostatic repulsion by NaPA negative charges, and hydrogen bonding. Based on the known ability of sugars to form adducts with metal cations, their complexation with the polyelectrolyte Na^+ counterion was postulated as the main key force behind the different partition profiles of mono- and disaccharides. However, due to the complexity of the events involved, a broader systematic investigation should be done in the future to deeply understand its role.

Given the extraction patterns obtained using the distinct classes of ABSs studied, two novel processes were proposed: (i) integrated two-step FOS production by chemical hydrolysis followed by sequential extraction and separation using the PPG 400-based ABS, and (ii) continuous enzymatic FOS production integrated with in situ ABS purification using the Pluronic PE 6800-based ABS. In summary, this work proposes two novel and sustainable processes to overcome the FOS limitation problems related to low productivity (caused by D-glucose inhibition of levansucrase) and low effectiveness of FOS downstream processes, through which new platforms may gain a place in the food and biotechnology industries. Additionally, the separation principles explored in the present work might represent an important step forward in the fractionation and biotransformation of sugars from lignocellulosic sources, which are fundamental in the biorefinery context.

To assess the feasibility of continuous cycles of FOS production, further studies are needed on the stability of the levansucrase in the phase-forming aqueous solutions, i.e., in terms of activity and structural conformation. The scalability of the proposed process will be an important next step to be evaluated with the possibility to use as a scale-up model of ABS: (i) higher volumes of aqueous phases, maintaining the same mixture point and best operational conditions, (ii) centrifugal partition chromatography, or (iii) high-speed counter-current chromatography based on the optimized ABS conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.2c04147.

Experimental details of heterologous expression and purification of levansucrase; the weight fraction data of the binodal curves; the correlation Merchuk parameters of the phase diagrams; fractionation parameters and characteristics of the aqueous biphasic system mixture points applied; in silico data obtained by COSMO-RS (i.e., excess enthalpies); and 3D fluorescence spectra and calibration curves (PDF)

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Notes

The authors declare no competing financial interest.

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