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PARTITIONING OF POLY- AND DISACCHARIDES IN AQUEOUS BIPHASIC SYSTEM BASED ON A HYDROPHILIC IONIC LIQUID

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Abstract

Aqueous biphasic salt-salt system formed by the hydrophilic ionic liquid 3-methyl-1-octylimidazolium chloride and inorganic salt K_2HPO_4 are employed as alternative to volatile organic compounds for partitioning of poly- and disaccharides, both present in culture broths of EPS (Extracellular PolySaccharide) producing strains. The phase diagram of the aqueous biphasic system is determined. The selected composition of phase-forming components is used to carry out extractions on model aqueous solutions of xanthan and maltose. Based on the partition coefficients of both saccharides and the factors of saccharides' separation achieved in both phases, the effectiveness of the extraction process is evaluated. In all the equilibrium experiments performed, xanthan is almost completely transferred to the ionic liquid-rich phase, while the disaccharide separates in the aqueous K_2HPO_4-rich phase.

Keywords: *polysaccharide*, *disaccharide*, *extraction*, *separation*, *ionic liquid*, *aqueous biphasic system*

1. INTRODUCTION

Separation of bioproducts from the bioreaction media is an essential task in biotechnology. The demand for fast, cost-effective and environmental-friendly separation and purification processes has lead to a significant effort to develop clean manufacturing methods and easily scaled-up industrially relevant techniques to increase selectivity and capability and reduce overall costs.

In a case, the method for separation of poly- and disaccharides has not been addressed yet to a novel "green" issue. The carbohydrate di- and polymers are both present in the final fermentation broth of the EPS (Extracellular PolySaccharide) producing strains, as main substrate (carbon source) and target product (Kambourova et al., 2009; Pavlova et al., 2010). Depending on the strain productivity, at the best particular culture conditions, the maximal EPS yield may vary in two orders, from *ca.*0.1 to 10 g dm^{-3} (Lee et al., 2001; Takita et al., 2004; Xu et al., 2006; Kambourova et al., 2009). While the sugar substrate is added in *ca.*2 to 10%, in majority, finally about 3-4 g dm^{-3} remains unutilized; thus becoming comparable to the EPS yield in the medium. The crude EPS is isolated from the cell free culture supernatant usually by precipitation applying a polar organic solvent (ethanol, isopropyl alcohol) in proportions up to four volume aliquots, followed by two-three washes with the organic solvent. Thus at the isolation step, a great waste stream of volatile organic compounds is discharged in the environment. After the recognition of the detrimental effect of such example processes, a new class of solvents, ionic liquids (ILs), has emerged revealing bright future as environmentally benign and tunable pretenders to replace conventional organic solvents.

Aqueous biphasic system (ABS) composed of a hydrophilic ionic liquid, carbohydrate, and water was first described in the literature by Zhang et al. (2007) and since then researchers have been exploring

sugars for tailoring the phase separation (Wu et al., 2008b; Chen et al., 2009). Along with the implementation of bio-friendly substances, the systems of ionic liquid/carbohydrate/water offer the convenience of adjustable pH of the aqueous phase which is of importance in partitioning of the charged substances, including biopolymers (such as proteins) and monomers (organic, nucleic, amino acids). However, only limited kinds of ionic liquids show capability of forming ABS with sugars, and the described ones are: imidazolium tetrafluoroborates, $[C_n mim]BF_4$ with carbon number of alkyl chain on imidazolium ring, $n \geq 3$ (Chen et al., 2009) and allyl-substituted imidazolium halides, [Amim]Cl/Br (Wu et al., 2008a). There is much more variety among the ionic liquids that can form ABS adding inorganic salts with salting-out inducing behavior (Ventura et al., 2009) and their applications on extraction and purification of distinct compounds have been already documented for amino acids (L-tryptophan - Ventura et al., 2009), proteins (bovine serum albumin - Pei et al., 2009), phenolic compounds (vanillin – Cláudio et al., 2010), alkaloids (Li et al., 2005), and others summarized in a recent review by Oppermann et al. (2011).

The aim of this study is to investigate the partitioning and separation of EPS and disaccharides, a model system xanthan/maltose, by means of an extracting system based on a hydrophilic ionic liquid. To the best of the author's knowledge, no such information has been reported yet and it would broaden the area of green downstream processing.

2. EXPERIMENTAL SECTION

2.1. Materials

The ionic liquid, 3-methyl-1-octylimidazolium chloride ($[C_8 mim]Cl$, min. 98%) employed in this work was purchased from Merck (Darmstadt, Germany). The inorganic salt used to form ABS was K_2HPO_4 (min. 99%, Fluka, Buchs, Switzerland). The carbohydrates, xanthan gum from *Xanthomonas campestris* (practical grade) and D-(+)-maltose monohydrate (min. 95%) were products of SIGMA. All other chemicals were of analytical grade and were used as received.

2.2. Preparation of phase diagram

The binodal curve of the system $[C_8 mim]Cl/K_2HPO_4$ was determined at 22 °C by the turbidimetric titration method. Briefly, an IL aqueous solution of known concentration was weighted in a volume graduated glass test tube. A K_2HPO_4 aqueous solution of known mass fraction was then added drop-by-drop to the tube. The tube was shaken, and the system behavior was observed. If a phase separation did not occurred, another known portion of the salt solution was added and so on until a phase change was noted. The volume fraction of each phase was measured, the weight of the system was noted (using analytical balance with a precision 0.0010 g), and the ABS composition was calculated. Water was then added dropwise to get a clear one-phase system, and further more K_2HPO_4 solution was added to obtain again an ABS. The above procedure was repeated and so on until sufficient data was collected to construct the phase diagram.

2.3. Extraction experiments

A specified amount of an aqueous solution of the single carbohydrate (maltose or xanthan gum) under study or a mixture of them was dissolved into a known amount of $[C_8 mim]Cl$. Then a specified amount of a K_2HPO_4 aqueous solution (known concentration) was added and the whole mixture in the test tube was vigorously shaken for five minutes and allowed to reach equilibrium by the separation of both phases overnight at 22 °C. In all the partitioning experiments, the solutions were prepared so that the final weights of $[C_8 mim]Cl$ and K_2HPO_4 were 0.6 g and 0.8 g, respectively, and the total volume of the aqueous solution introduced was 1.5 cm^3. This extracting composition is pointed on the phase diagram below (Figure 1). The volumes of the upper IL- and the lower aqueous (K_2HPO_4-rich) phases

were recorded. The samples from both phases were redrawn, diluted as appropriate and subjected to analysis as described in the next section.

2.4. Determination of the carbohydrates' concentration in both phases

The total carbohydrate content in each phase was measured by the colorimetric phenol-sulfuric acid method closely followed as described by Dubois et al. (1956) using maltose as standard. In order to avoid the interference from the other phase components, $[C_8mim]Cl$ and K_2HPO_4, the samples' absorbance was measured versus blanks prepared by contacting of the same phases but without carbohydrates, and subjected to the same (as samples were) analytical dilution. Further, in order to distinguish between di- and polymer carbohydrate content as the phenol-sulfuric acid method could not, the following procedure was accomplished:

(*1*) To the equilibrium aqueous phase, an equal volume of cool ethanol (96%) was added dropwise, under stirring in ice bath. The mixture was refrigerated at -4°C overnight to separate xanthan if any. The two phases formed were analyzed but xanthan was never seen to precipitate from either phase.

(*2*) The equilibrium IL-phase was chilled at -4°C overnight and two fractions were formed: (*i*) precipitated xanthan and (*ii*) a supernatant of clear liquid. The two fractions were analyzed and then

(*3*) equal volumes of cool ethanol (96%) were added dropwise, under stirring in ice bath and the resulted mixtures were refrigerated at -4°C overnight. Xanthan was never seen to precipitate from the mixture of ethanol and (*ii*) the supernatant of clear liquid. Contrary, from the mixture of ethanol and (*i*) precipitated xanthan, an amount of dissolved sugar (i.e. maltose) was determined in the liquid supernatant.

Thus differentiated, the concentrations of maltose and xanthan in each equilibrium phase (IL and aqueous) were recalculated independently. The overall mass balance was fulfilled within $\varepsilon \leq 7\%$ which is comparable to the material (volume) loss in manipulations.

2.5. Determination of the partition coefficients, extraction efficiencies and separation factor of the carbohydrates

The partition coefficients of xanthan (K_{Xan}) and maltose (K_{Mal}) were determined as the ratio of the concentrations of the respective carbohydrate in the IL- and the aqueous (K_2HPO_4-rich) equilibrium phases, as follows:

$$K_{Xan} = \frac{[Xan]_{IL}}{[Xan]_{AQ}} \qquad [-] \qquad (1);$$

$$K_{Mal} = \frac{[Mal]_{IL}}{[Mal]_{AQ}} \qquad [-] \qquad (2),$$

where [*Xan*] and [*Mal*] refer to the concentrations of xanthan and maltose, respectively, and "$_{IL}$" and "$_{AQ}$" indicate IL- and aqueous-rich equilibrium phase.

The extraction efficiencies of xanthan (E_{Xan}) and maltose (E_{Mal}) were calculated according to the equations (3) and (4), respectively:

$$E_{Xan}\,(\%) = \frac{[Xan]_{IL} \times V_{IL}}{[Xan]_{IL} \times V_{IL} \;+\; [Xan]_{AQ} \times V_{AQ}} \times 100 \qquad [\%] \qquad (3);$$

$$E_{Mal}\,(\%) = \frac{[Mal]_{IL} \times V_{IL}}{[Mal]_{IL} \times V_{IL} \;+\; [Mal]_{AQ} \times V_{AQ}} \times 100 \qquad [\%] \qquad (4),$$

where V is the volume of the equilibrium IL-rich phase ("$_{IL}$") or the equilibrium aqueous-rich phase ("$_{AQ}$").

The separation factors in the equilibrium IL-rich phase (S_{IL}) and the equilibrium aqueous-rich phase (S_{AQ}) were defined as the ratio between the concentrations of xanthan and maltose in the respective phase, as follows:

$$S_{IL} = \frac{[Xan]_{IL}}{[Mal]_{IL}} \quad [-] \quad (5);$$

$$S_{AQ} = \frac{[Mal]_{AQ}}{[Xan]_{AQ}} \quad [-] \quad (6).$$

3. RESULTS AND DISCUSSION

3.1. Phase diagram

Phase diagram data are needed for design of aqueous biphasic extraction process. The binodal curve of the system $[C_8mim]Cl/K_2HPO_4$ determined at 22°C is represented on Figure 1 in terms of molality and compared with the data for other similar systems found in the literature. In the studied concentration region of phase forming components, the system $[C_8mim]Cl/K_2HPO_4$ shows phase transition behavior close to the one of the less hydrophobic $[C_4mim]Br/K_2HPO_4$. Due to this similarity, the extracting composition employed in this study corresponded to that of $[C_4mim]Br/K_2HPO_4$ used by Pei and co-workers (2009) for protein extraction.

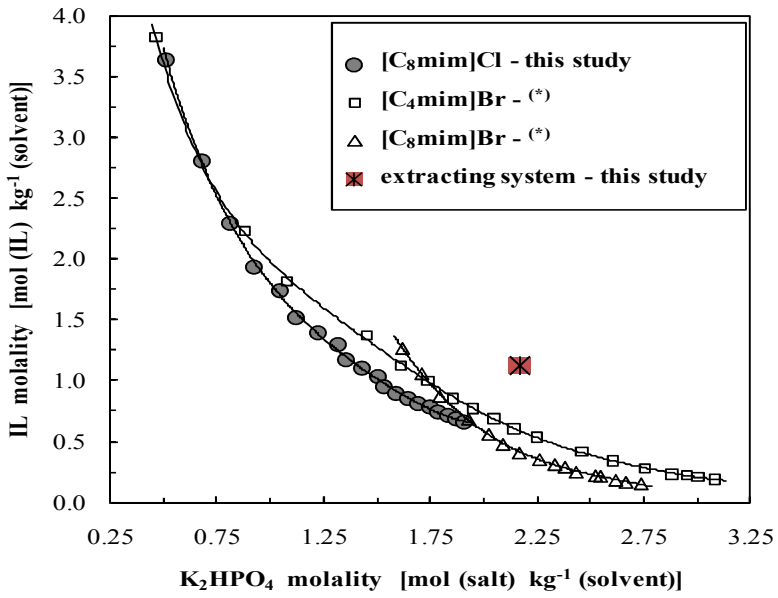

Figure 1. Phase diagram of $[C_8mim]Cl/K_2HPO_4$ aqueous biphasic system used in this study.

(*) Phase diagram data published by Pei et al. (2009)

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3.2. Phase volume fraction

Figure 2b represents the volume fraction of each equilibrium phase of the systems shown on the binodal curve in the order pointed on Figure 2a. The systems 1-7, which consist of ionic liquid molality higher than 1 mol kg^{-1}, separate in greater volume fraction of IL-rich phase. In system 7 the volume is almost equally partitioned in two phases. Further, the increase in the molal content of K_2HPO_4 raises the volume fraction of the aqueous-rich phase. The volume distribution in the extraction composition used corresponds to that of the system 8, namely 58% : 42% (lower to upper phase). However, as it is shown below, the introduction of different saccharides (in type and concentration) influences differently the volume partitioning.

On Figure 3 the phase volume fraction of five equilibrated systems containing saccharides is shown. The initial saccharide compositions of the studied systems were:

System **No 0** contained no carbohydrate. The other systems contained saccharides in the following concentrations with respect to the total volume of the aqueous solution of 1.5 cm^3:

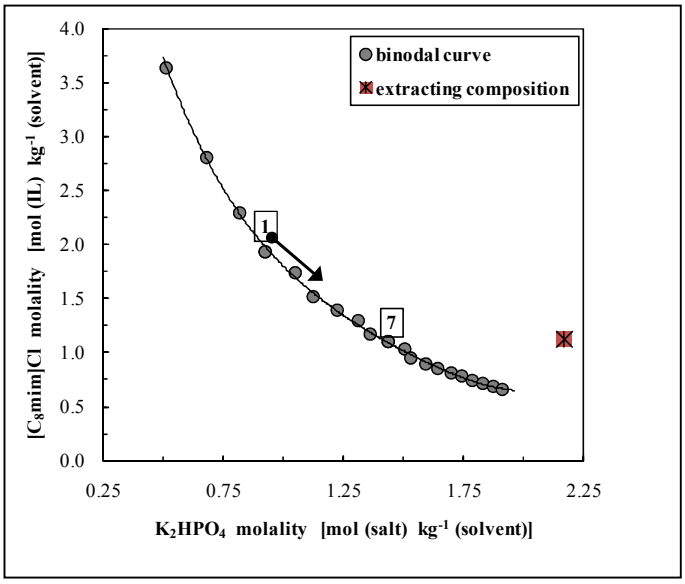
System **No 1** contained 10 g dm^{-3} of disaccharide, maltose;

System **No 2** contained 0.1 g dm^{-3} of polysaccharide, xanthan;

System **No 3** contained 1.0 g dm^{-3} of xanthan;

System **No 4** contained 1.0 g dm^{-3} of xanthan and 1.0 g dm^{-3} of maltose;

System **No 5** contained 1.0 g dm^{-3} of xanthan and 10 g dm^{-3} of maltose.



(2a)

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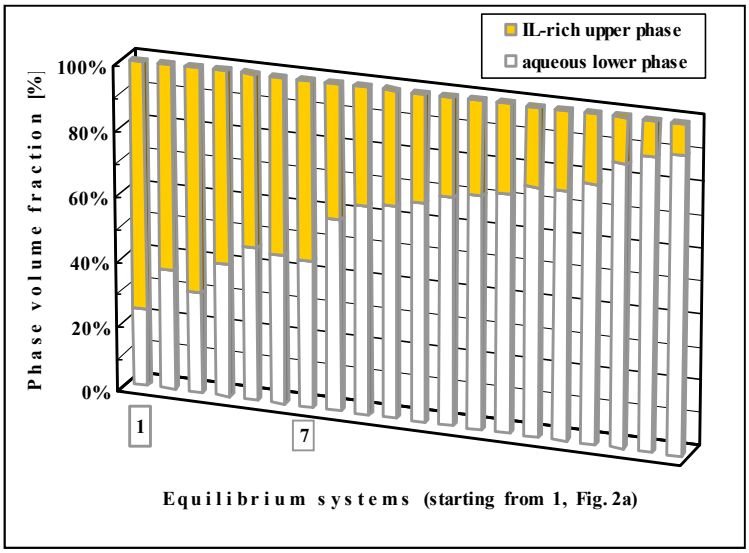

(2b)

Figure 2. Phase volume fraction of equilibrium $[C_8mim]Cl/K_2HPO_4$ aqueous biphasic systems **(2b)** in the order shown on the binodal curve **(2a)**

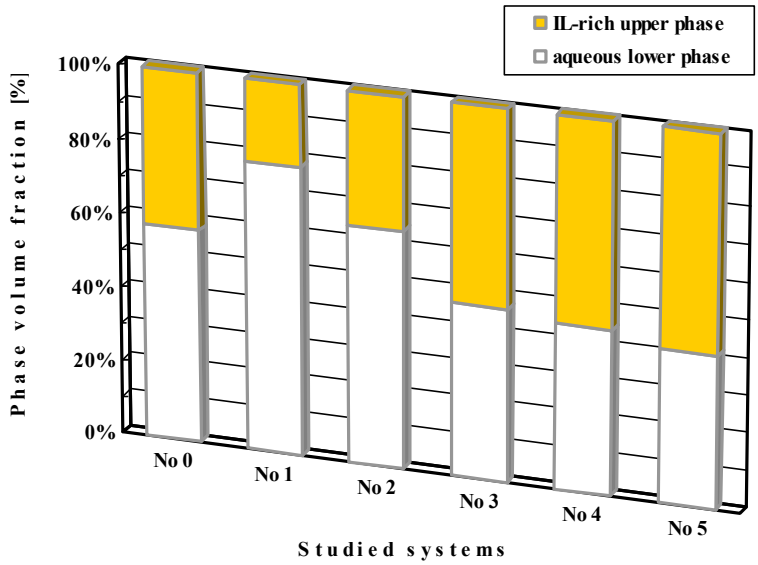

Figure 3. Phase volume fraction of equilibrium $[C_8mim]Cl/K_2HPO_4$ aqueous biphasic systems containing different type and concentrations of carbohydrates

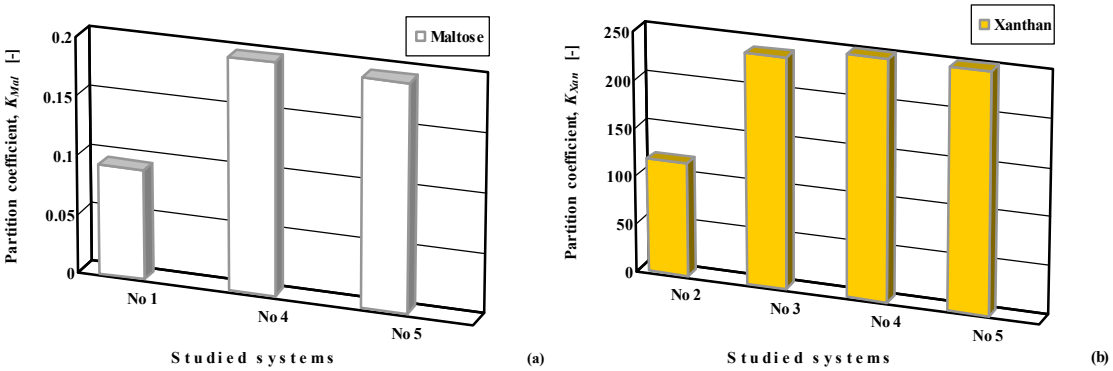

Figure 4. Partition coefficients of maltose, K_{Mal} **(a)** and of xanthan, K_{Xan} **(b)** in equilibrium $[C_8mim]Cl/K_2HPO_4$ aqueous biphasic systems

The introduction of disaccharide, which preferably partitions in the aqueous rich phase, is associated with an increase in the volume fraction of the lower phase as seen for System **No 1** on Figure 3. Inserting xanthan and raising its concentration resulted in enlargement of the volume of the upper IL-rich phase. The Systems **No 3**, **No 4**, and **No 5**, which share the same amount of xanthan, have almost the same volume distribution between the two phases, *ca.* 44% : 56% (lower to upper phase). Such fractioning is the opposite of the System **No 0**.

3.3. Partition coefficients, extraction efficiencies and separation factor of the carbohydrates

The partitioning of poly- and disaccharides in the five studied systems was examined. Figures 4 and 5 represent partition coefficients and extraction efficiencies for both carbohydrates, as described in section 2.5.

It is seen on Figure 4 that in all the studied systems the partition coefficients of the disaccharide, K_{Mal}, do not exceed the value of 0.2, while the partition coefficients of the polysaccharide, K_{Xan}, are 1000-fold higher.

As it was already mentioned (section 2.4), in the mixture systems (**No 4** and **No 5**) the amount of xanthan in the aqueous lower phase was below detectable limit, so its extraction efficiency approaches 100%, $E_{Xan} \approx 100\%$ (Figure 5). Although the initial maltose concentrations in these systems differ from each other in 1-order, maltose was extracted with the same efficiency of about 20%. The concentration of mutually extracted disaccharide is higher in System **No 5** compared to the System **No 4** which determines the lower separation factor in the IL-phase, S_{IL}(System **No 5**)=0.60. In the system of equal initial concentrations of maltose and xanthan (System **No 4**) the separation factor in the IL-phase is S_{IL}(System **No 4**)≈4. Regarding the aqueous-rich phase, as far as it contains only maltose and no detectable amount of xanthan, a complete separation between di- and polysaccharide is achieved therein, $S_{AQ} \rightarrow \infty$.

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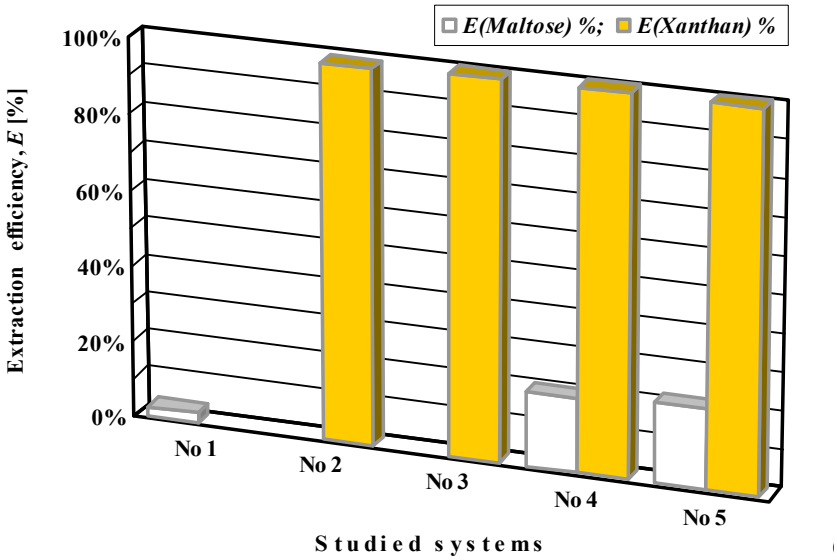

Figure 5. Extraction efficiencies of maltose and of xanthan in equilibrium $[C_8mim]Cl/K_2HPO_4$ aqueous biphasic systems

The main driving force for xanthan transfer from the aqueous (lower) to the IL-rich (upper) phase is induced by the salting-out effect of K_2HPO_4. The large amount of K_2HPO_4 present in the aqueous solution competes with the polysaccharide for water molecules, resulting in the reduction of the polymer hydration degree. Therefore, the introduction of a kosmotropic salt into an aqueous saccharide solution promotes the removal of the carbohydrate polymer form. This mechanism is analogous to the conventional dehydration by alcohols widely used for polysaccharide isolation. The fractioning of the polysaccharide in the IL-rich phase can be observed visually as seen on a camera picture representing System **No 4** (Figure 6). In the mixture systems maltose extraction in the IL-phase is probably due to an attachment to the polymer rather than particular transfer as depicted from its very low extraction efficiency in the single case (System **No 1**).

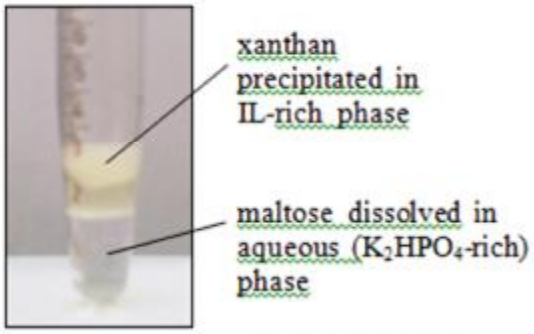

Figure 6. Camera picture of partitioning of xantan and maltose in $[C_8mim]Cl/K_2HPO_4$ aqueous biphasic system **No 4**

As xanthan is an anionic polysaccharide (García-Ochoa et al., 2000), other type of interactions, ex. electrostatic, could be also implemented in the extraction process. However, the electrostatic interactions between the appropriately charged polymer (through pH of the aqueous solution) and the ionic liquid ions require other type of systems different from ABS in order to avoid the strong electrostatic screening of the inorganic salt. Such alternative systems might be the biphasic systems based on hydrophobic ionic liquids and further research effort will be required.

4. CONCLUSIONS

Partitioning and separation of poly- and disaccharides have been achieved for the first time by means of aqueous biphasic system based on hydrophilic ionic liquid and inorganic salt. The phase diagram of the aqueous biphasic salt-salt system $[C_8mim]Cl/K_2HPO_4$ is determined and used for partitioning of a xanthan/maltose model carbohydrate mixture. Complete removal of xanthan from the aqueous phase is achieved. The polymer transfer to the IL-phase is attributed to the dehydration caused by the presence of a kosmotropic salt in the aqueous source, i.e. mechanism analogous to the conventionally applied precipitation by alcohols is exploited. Because of the extremely low extraction efficiency in the single case, maltose extraction in the IL-phase is probably due to an attachment to the polymer rather than a particular transfer. Considering the recognized "green aspect" of the ionic liquids with respect to their negligible vapour pressure, the latter system is an example of alternative to the application of organic solvents in biotechnology downstream processing.

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