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Phase diagrams of a CTAB/organic solvent/buffer system applied to extraction of enzymes by reverse micelles

E. Feitosa [a,*], K.T. Catelam [a], F.A. Hasmann [c], Hans-Olof Johansson [a], I.C. Roberto [c], A. Pessoa Jr. [b]

[a] *Department of Physics, São Paulo State University, São José do Rio Preto, SP, Brazil*
[b] *Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, SP, Brazil*
[c] *Department of Biotechnology, Engineering School of Lorena, University of São Paulo, Lorena, SP, Brazil*

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Abstract

A partial pseudo-ternary phase diagram has been studied for the cethyltrimethylammonium bromide/isooctane:hexanol:butanol/potassium phosphate buffer system, where the two-phase diagram consisting of the reverse micelle phase (L_2) in equilibrium with the solvent is indicated. Based on these diagrams two-phase systems of reverse micelles were prepared with different compositions of the compounds and used for extraction and recovery of two enzymes, and the percentage of enzyme recovery yield monitored. The enzymes glucose-6-phosphate dehydrogenase (G6PD) and xylose redutase (XR) obtained from *Candida guilliermondii* yeast were used in the extraction procedures. The recovery yield data indicate that micelles having different composition give selective extraction of enzymes. The method can thus be used to optimize enzyme extraction processes.

Keywords: Phase diagram; Liquid–liquid extraction; Reverse micelles; CTAB; Enzymes; Glucose-6-phosphate dehydrogenase; Xylose redutase

1. Introduction

Liquid–liquid extraction (LLE) is a well-established method based on the phase separation of a liquid system in a polar- and an apolar-rich environment, between which biomolecules such as enzymes can be partitioned [1]. A reverse micelle (apolar-rich) phase in equilibrium with an aqueous (polar-rich) phase can thus be used for enzyme extraction/purification [2–8]. A two-phase system like this can be easily provided through the construction of ternary phase diagrams, as those reported in this communication for the pseudo-ternary system cethyltrimethylammonium bromide (CTAB)/isooctane: hexanol:butanol/potassium phosphate buffer and selected fractions of the alcohols. Based on these phase diagrams, reverse micelles having different compositions of the compounds can be tested in the enzyme extraction.

Reverse micelles are thermodynamically stable surfactant aggregates comprising a polar micro-domain immersed in an apolar solvent with the surfactant molecules interfacing the polar–apolar environments, as a monolayer, such that the surfactant polar head group and the hydrocarbon chain face, respectively, the polar and apolar moieties [9]. The aqueous core of the reverse micelles allows the solubilization of small polar molecules, including enzymes that are insoluble in organic solvents [1]. The size of the reverse micelles thus plays an important role in the enzyme solubility in the micellar core. The enzyme solubility in reverse micelles is the first step for enzyme extraction from different sources. Furthermore, phase diagrams provide regions of different compositions of the system compounds that constitute reverse micelles (and other structures) with different characteristics, including size and surface charge density.

Cethyltrimethylammonium bromide, CTAB (molecular formulae $CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$), is a cationic surfactant that self-assembles as micelles, and other structures and phases depending on the concentration and solvent characteristics [9]. In the presence of excess organic solvents and moderate aqueous solution CTAB forms reverse micelles having a size as determined by the composition of the compounds, temperature, ionic strength, etc.

* Corresponding author at: Physics Department, IBILCE/UNESP, Rua Cristovão Colombo, 2265 São José do Rio Preto, CEP: 15054-000, SP, Brazil. Tel.: +55 17 3221 22 38; fax: +55 17 3221 22 47.
E-mail address: eloi@ibilce.unesp.br (E. Feitosa).

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In spite of recent efforts in reverse micelle studies for liquid–liquid extraction (LLE), to our knowledge there is no phase diagram for CTAB reported in the literature specially built for this purpose. These kinds of phase diagrams indicate that the reverse micelle phase is quite suitable for enzyme extraction, therefore, being important in the LLE of biomolecules. The majority of the studies are focused on the anionic surfactant AOT (sodium di-2-ethylhexyl sulfosuccinate). However, some authors, although under optimized conditions, observed low recovery when using AOT, due to enzyme denaturation [10,11]. Additionally, previous reports [12–14] point out that CTAB is appropriate for extraction of biomolecules, since its reverse cationic micelles are stable over a wide range of pH, being thus suitable for biopatitioning, and the phase diagrams can be used to optimize the extraction process.

Reverse micelle systems can be applied in LLE of biomolecules, including proteins and enzymes. They are suitable, versatile, and economic tools for large-scale extractions of the macromolecules. The method exploits the phase partition of target solute molecules between the aqueous and the organic phases, followed by a transfer of the macromolecules to a stripping aqueous phase [1,2].

Reverse micelles have high potential application in industries as they provide a suitable environment for protein/enzyme solubility in an organic phase preserving their native structure and activity function [6]. Furthermore, the extraction of biomolecules is closely related to the development of biotechnology and a high degree of purity of the extracted biomolecules is often required. In food industry, for example, there is an increasing use of biomolecules, specially in beer production and fermentation [15].

The LLE by reverse micelles can be performed in two stages: the extraction and the back extraction of the biomolecules. In the extraction, the biomolecules are transferred from their original moiety to the polar core of the reverse micelles, or micelle phase (MPI), while in the back extraction they are removed from the micelle interior to a bulk aqueous phase (APII). The extraction process is dominated by electrostatic attraction between the surface charges of the enzymes and micelles. Thus, the pH of both the micelle aqueous core and the enzyme solution, play an important role in the extraction process as they determine the surface charge of these compounds. The back extraction process is governed mainly by electrostatic repulsion, and the surface charges of the enzymes and micelles should have the same charge. The pH or ionic strength of the micelle system might thus be adjusted to obtain such a condition [1,2]. Overall, low ionic strength in the micelle aqueous phase favors the enzyme transfer to the micelle core, whereas, high ionic strength favors the release of the enzyme from the micelle to the desired solvent [16,17].

Glucose-6-phosphate dehydrogenase (G6PD) is an important intracellular enzyme commonly used in biochemical and medical studies as analytical reagent for the measurement of creatin-kinase and hexokinase activities, ATP concentrations, and as a marker for enzyme immunoassays [18].

Xylose redutase (XR), found mainly in *Candida guilliermondii* yeasts, is of great importance as a catalyst in the xylose-to-xylitol conversion. Xylitol is important owing to its sweetening characteristics and insulin-independent metabolism, dentistry formulations for its anticariogenicity, tooth rehardening and remineralization properties, and pharmaceutical formulations for its capability of preventing otitis and its possibility of being used as a sweetener or excipient in syrups, tonics, and vitamin formulations [19,20].

Even though the composition of the reverse micelle compounds plays an important role in the micelle properties, which in turn, determine their efficiency in the protein extraction, the construction of phase diagrams for the purpose of biomolecule extraction/recovery has not been explored yet. Once known, the phase diagrams allow the visualization of the two-phase region, of interest for biomolecule extraction by reverse micelles having different characteristics. The phase diagrams can be used to optimize the biopartitioning of macromolecules in the way we report here.

Thus, this work aims to evaluate the effectiveness of CTAB-based reverse micelles having different compositions (selected from phase diagrams), in purifying the G6PD and XR enzymes. The extraction and recovery of these enzymes have been investigated with special attention to the recovery of the enzymatic activities.

2. Materials and methods

2.1. Chemicals

CTAB (Sigma) was used as received. Isooctane (Vetec), butanol (Synth) and hexanol (Merck) were analytical grade. Ultra pure milli-Q quality water was used in the sample preparations. All other reagents were of analytical grade.

2.2. Construction of the phase diagrams

The pseudo-ternary phase diagrams were built up such that one of the vortexes of the triangle account for a mixture of the organic solvents isooctane/hexanol/butanol and the other two vortexes account for CTAB and 0.078 M potassium buffer pH 6.0.

The diagrams were built up by preparing a mixture x wt.% surfactant/y wt.% organic solvent such that $x + y = 100$ wt.%. Microvolumes of the buffer solution were added to the x:y mixtures such that the new solutions were on a dilution line and the molar ratio of the surfactant to organic x:y was constant. The samples were observed through crossed polarizers at room temperature, ca 25 °C, to identify birefringency. The non-birefringent reverse micelle phase (L_2) and a two-phase (WinsorII), one rich (top) and the other poor (bottom) of reverse micelles were identified for use in the enzyme extraction. Three diagrams were obtained for the following composition of the organic solvents: 80/05/15, 76/06/18, 72/07/21, respectively for isooctane/hexanol/butanol.

2.3. Enzymes

2.3.1. Candida guilliermondii cells

C. guilliermondii FTI 20037 (ATCC 201935) was maintained at 4 °C on slants of yeast malt agar [21,22].

2.3.2. Hemicellulosic hydrolysate

The hemicellulosic hydrolysate was obtained from rice straw under optimized conditions of acid hydrolysis described by Gurpilhares et al. [23]. The obtained hydrolysate (xylose at 19 g L^{-1}) was filtered and the liquid fraction (hemicellulosic hydrolysate) was treated with active charcoal for impurities removal in the following conditions: a proportion of charcoal:hydrolysate of 3% (wt.%/v), 45 °C and 30 min of agitation. Solids were removed by centrifugation (2000 × *g*) and the hydrolysate was further concentrated under vacuum at 65 °C to increase the xylose concentration. The vacuum procedure was necessary to avoid sugar degradation.

2.3.3. Preparation of inoculum

The inoculum for batch fermentation was prepared by adding a yeast suspension to a 500 mL shaking flasks containing 200 mL of hydrolysate, 30 g L^{-1} of xylose, and pH 6.2 adjusted with solid NaOH. After 29 h incubation in a rotary shaker (Tecnal Te 420) at 200 rpm and 30 °C, the culture was harvested by centrifugation at 2000 × *g* for 20 min at 4 °C and resuspended in distilled water aiming for a concentrated cell suspension. From this suspension, the volume necessary to obtain the initial concentration of cell suspension (1 g L^{-1}) in fermentation medium using an optical density (O.D.) curve was determined.

2.3.4. Fermentation

The concentrated hydrolysate was diluted with distilled water to obtain the initial xylose concentration of 70 g L^{-1}, approximately. After that, the pH was adjusted using solid NaOH (pH 7.5). Fermentation medium was composed only by hydrolysate, without nutrient supplementation [23].

2.4. Cell disruption

The yeast cells *C. guilliermondii* obtained from bioprocess for xylitol production were harvested by centrifugation at 2000 × *g*, washed with sterile distilled water, centrifuged and resuspended in 0.071 M Tris–HCl buffer, pH 7.5 resulting in a cell suspension with determined concentration (3 g L^{-1}). These suspensions were mechanically disrupted in centrifuge tubes under vortex agitation using glass beads (0.5 mm of diameter). Aliquots of 3 mL of cell suspension were added to 3 mL of glass beads (proportion of 1:1, v/v) in a centrifuge tube under vortex agitation at regular time (5 min). The disruption period was 1 min separated by a 30 s interval in an ice bath. The samples were then centrifuged at 6725 × *g*, 15 min, and 5–8 °C (Jouan Centrifuge, Model BR 4i, St. Herblain, France) and the supernatants (called initial sample) were assayed for the enzyme (G6PD and XR) activity.

2.5. The enzyme extraction by reverse micelles in the L_2 phase

Reverse micellar solutions for enzyme extraction were prepared and had the following compositions: (1) 90 wt.% organic solvent, 5.0 wt.% CTAB and 5.0 wt.% buffer, (2) 75 wt.% organic solvent, 12.5 wt.% CTAB, and 12.5 wt.% buffer, (3) 15 wt.% organic solvent, 2.5 wt.% CTAB, and 72.5 wt.% buffer, (4) 57.5 wt.% organic solvent, 2.5 wt.% CTAB, and 40 wt.% buffer, (5) 38.5 wt.% organic solvent; 5 wt.% CTAB, and 57.5 wt.% buffer, and (6) 95 wt.% organic solvent, 2.5 wt.% CTAB and 2.5 wt.% buffer.

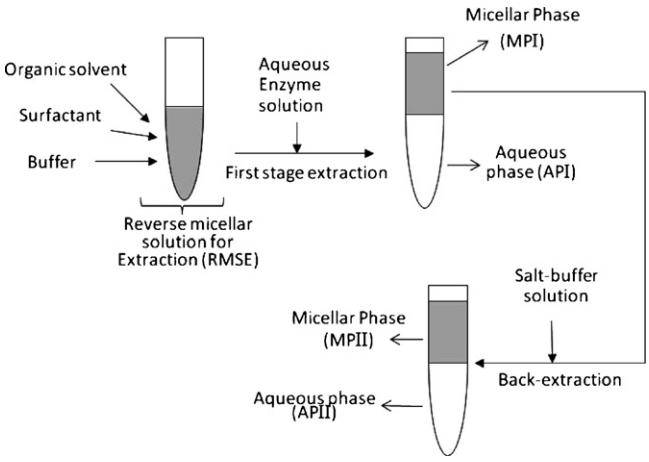

Fig. 1. Flow-scheme for enzyme extraction by reverse micelle.

All organic solvents had the following composition in wt.%: isooctane:hexanol:butanol 76:06:18.

The extraction process was performed in two stages: first, within the extraction stage, 5 mL of the aqueous solution containing the enzyme was mixed to 5 mL of a reverse micelle solution, according to the following flow-scheme shown in Fig. 1.

After vortexing the sample for 1 min the mixture was centrifuged (Jouan Centrifuge Mod.1812, Saint Herblain) at 1677 × *g* for 10 min to accelerate the phase separation into MP I and AP I. In the second stage, the top phase (MP I) containing the enzymes in the micelle core was removed in the back extraction process performed by mixing 2.0 mL of MP I with an equal amount of a sodium acetate buffer 1.0 M pH 5.5 in presence of 1.0 M NaCl in order to transfer the enzymes from the micelle interior to an aqueous phase (AP II). Within this stage, the sample was vortexed and centrifuged under the same condition as above. The enzyme was then recovered by partitioning to this second aqueous phase (AP II). Fig. 1 shows the flow-scheme of the enzyme extraction process.

The final results of the back extraction were expressed as a percentage of the enzyme activity.

2.6. Determination of the G6PD and XR activities

The activity of G6PD was determined by monitoring the reduction of the cofactor $NADP^+$ through absorbance measurements at 340 nm. The composition of the reduction moiety was: 600 μL buffer Tris–HCl (0.071 M, pH 7.5) in presence of $MgCl_2$ (35 mM); 5 μL $NADP^+$ (0.131 M); 10 μL G6PD (500 mM), and 100 μL sample. The unity of activity (U) of G6PD is defined as the amount of enzyme necessary to catalyze the reduction of 1 μmol of $NADP^+$ per minute. XR activity was determined spectrophotometrically at 340 nm and at room temperature using NADH as cofactor. Enzyme unit was defined as the amount of

enzyme which catalyzes the oxidation of 1 μmol of NADH per minute. The activities were determined for the initial aqueous phase as well as for API and APII.

The extraction data were expressed as percentage of activity in API and/or APII relative to the activity of the initial enzyme aqueous phase (recovery). Eq. (1) was used to determine the enzyme recovery (R):

$$R = \frac{A_2 v_2}{A_1 v_1} \times 100 \quad (1)$$

where A_1 is the initial enzyme activity [U]; v_1, volume of initial aqueous phase [L]; A_2, enzyme activity after extraction, API or APII [U]; v_2, volume of aqueous phase after extraction [L].

3. Results and discussion

3.1. Construction of the phase diagrams

Fig. 2 shows the pseudo-ternary phase diagrams for the system CTAB/isooctane/hexanol/butanol/phosphate buffer, for the following compositions of the organic solvents isooctane/hexanol/butanol: 80/05/15, 76/06/18 and 72/07/21. The diagrams indicate the two-phase region, the top one rich in reverse micelles and the bottom phase rich in buffer solution that probably contains a few normal micelles. The equilibrium of these two single phases is necessary for the enzyme extraction process.

For the isooctane/hexanol/butanol ratios = 80/05/15, 76/06/18 and 72/07/21 it was obtained, respectively 13.9, 17.1 and 16.3% of the whole area of the phase diagram for the two-phase region. Thus, 76/06/18 is the composition that gives the largest area for two-phase region and samples from this region were then used for enzyme extraction, in order to find the more efficient composition for the extraction.

3.2. The enzyme extractions

The enzyme extractions and back extractions were performed at room temperature (ca 25 ± 1 °C) and the reproducibility of the results was checked by double running the activity tests and recovery yield experiments.

When cationic surfactants are used, the transport of enzymes to the interior of the reverse micelles is favored by their negative electric charge, which is reached at pH values higher than the enzyme p*I* (isoelectric point). In case of G6PD and XR, the p*I* values are about 6.5 and 6.8, respectively. In this work, pH 6.0 (lower than the p*I* of both enzymes) was used, and so the transfer of the enzyme to the micellar phase was governed by hydrophobic interactions. Tables 1 and 2 summarize the mean values obtained for the activity and recovery yield of the enzymes G6PD and XR as extracted using six different samples of CTAB-based reverse micelles having compositions as indicated in the phase diagrams (Fig. 2).

Some discrepancies in the data reported in Tables 1 and 2 could be explained. It is worth mentioning that samples 1 and 2 phase separate and exhibit clear phases API and APII; the same was observed for samples 3, 5, and 6, except that for these samples the phase API was turbid. Sample 4, on the other hand, also phase separates but in addition it exhibits flocculation.

The recovery values in APII varied from 0 to 43% and from 0 to 89% for G6PD and XR, respectively. According to these data, the best results for the enzyme recovery yield were 44 and 89% for G6PD and XR, respectively. The data also indicate that the extraction does not depend much on the CTAB concentration, as in these assays the lower surfactant concentration of 2.5% was used, meaning that the extraction can be performed at quite a low surfactant concentrations also.

Cortez et al. [1] using CTAB obtained a recovery yield for XR of about 121% in an optimized extraction, at pH 7.0,

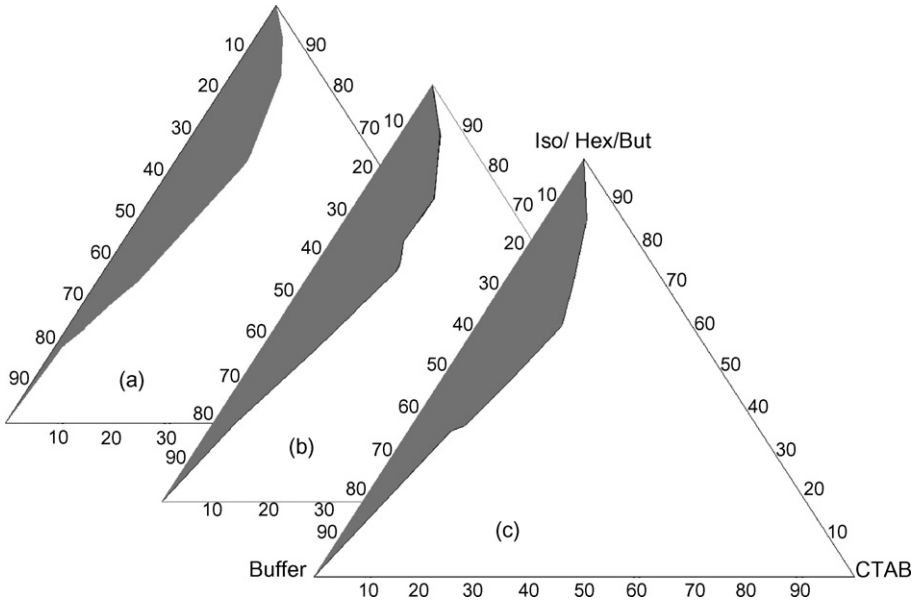

Fig. 2. Pseudo-ternary phase diagrams for the system CTAB/isooctane/hexanol/butanol/potassium phosphate buffer 0.078 M, pH 6.0, for the following compositions of the organic solvents: isooctane/hexanol/butanol = 72/07/21 (a), 76/06/18 (b) and 80/05/15 (c). Samples were prepared and investigated at 25 °C.

Table 1
Values of the activity and recovery of glucose-6-phosphate dehydrogenase (G6PD) by *Candida guilliermondii* after the extraction and back extraction using the CTAB reverse micelles

Samples	Systems composition[a] (wt.%)	Volumetric activity (U/L)		Volume sample (L)		Recovery yield (%)	
		AP I	AP II	AP I	AP II	AP I	AP II
Initial 1	–	1810.49	–	0.005	0.002	–	100
Initial 2	–	189.10	–	0.005	0.002	–	100
1	90/5.0/5.0	23.34	25.90	0.0052	0.0019	12.83	13.01
2	75/12.5/12.5	36.55	22.06	0.0034	0.0018	68.00	10.5
3	15/2.5/72.5	42.07	797.52	0.0048	0.002	1.24	44.05
4	57.5/2.5/40	833.17	788.65	0.0068	0.002	1.75	43.56
5	38.5/5/57.5	36.55	829.4	0.007	0.0019	1.80	43.52
6	95/2.5/2.5	86.10	0.0	0.0054	0.0019	108	0

[a] Organic solvents/CTAB/buffer.

Table 2
Values of the activity and recovery of glucose xylose redutase (XR) by *Candida guilliermondii* after the extraction and back extraction using the CTAB reverse micelles

Samples	Systems composition[a] (wt.%)	Volumetric activity (U/L)		Volume sample (L)		Recovery yield (%)	
		AP I	AP II	AP I	AP II	AP I	AP II
Initial 1	–	270.74	–	0.005	0.002	–	100
Initial 2	–	253.69	–	0.005	0.002	–	100
1	90/5.05.0	12.99	217.95	0.0052	0.0019	5.73	87.85
2	75/12.5/12.5	204.50	100.09	0.0034	0.0018	59.00	38.22
3	15/2.5/72.5	193.38	76.59	0.0048	0.002	68.57	28.29
4	57.5/2.5/40	208.49	0.00	0.0068	0.002	104.73	0.0
5	38.5/5/57.5	54.53	185.21	0.007	0.0019	28.20	64.99
6	95/2.5/2.5	9.71	221.05	0.0054	0.0019	4.45	89.10

[a] Organic solvents/CTAB/buffer.

this result is similar to the data shown in Table 2. Hasmann [24], using the same surfactant studied the G6PD extraction and obtained low recovery values (from 4%) and a maximum G6PD recovery (at pH 5.5) of 15%, under optimized conditions. Such a low recovery yield was attributed to the enzyme denaturation. In this communication it is reported higher recovery yield values, possibly due to the use of more suitable composition of the compounds that favors the formation of reverse micelles with well appropriate characteristics (e.g., micelle size) for the enzyme extraction/recovery. This more suitable composition was only attained after constructing the pseudo-ternary phase diagrams for the system CTAB/isooctane/hexanol/butanol/potassium phosphate buffer.

4. Concluding remarks

The phase diagrams shown in this communication indicate two-phase regions of reverse micelles in equilibrium with a solvent rich solution that can be explored in enzyme the extraction/recovery processes. Points in the phase diagram correspond to samples having different composition with potential application in the enzyme extraction. Extraction tests reveal the best composition that gives more efficient extraction. The micelle size determined by the composition of the mixtures thus plays an important role in the extraction process.

Most probably, the pretty good recovery yield data in the present report are related to the self-assembly of the surfactants into reverse micelles that preserve the enzyme activity in the micelle core from the external organic solvent, as suggested by Luisi et al. [6].

In conclusion, the extraction data here reported look very promising, as reverse micelles having different composition give selective extraction of enzymes, as is the case for assays 4 and 6 (Tables 1 and 2) in which higher recovery values for G6PD and XR were attained using different conditions. The surfactant and organic solvent composition thus plays an important role in the enzyme extraction/recovery and ternary phase diagrams offer an advantageous way to represent the reverse micelle containing two-phase region of interest to enzyme extraction.

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