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PARTITIONING OF CELLULOLYTIC ACTIVITY IN THE POLYETHYLENE GLYCOL/DEXTRAN TWO-PHASE SYSTEMS

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This study is concerned with the partitioning of cellulolytic activity in the polyethylene glycol/dextran two-phase systems. In the system of 10% (w/w) polyethylene glycol 1500/5% (w/w) dextran 500,000/80% (w/w) crude enzyme at the pH 5, 100%, yield of cellulolytic activity from Penicillium *sp. in the top phase was achieved in a single extraction step. Addition of KH_2PO_4 to this system at a concentration of 15 mmol/L improved the purification factor in the top phase for cellulolytic activity from crude preparation to a value of 2.6, although it had an adverse effect on the yield in the same phase.*

KEY WORDS: aqueous two-phase system, cellulolytic activity, partitioning, purification

INTRODUCTION

An aqueous two-phase system (ATPS) is the medium that enables selective partitioning of biomaterials such as proteins, nucleic acids, organelles and whole viable cells, from complex mixtures (1). This system is formed by mixing the solutions of two mutually incompatible polymers or polymer and salt above critical concentrations. The basis of separation is the uneven distribution of biomaterials between two phases, both having high water content. This high water content combined with the low interfacial tension of the system allows non-destructive partitioning of sensitive biomaterials and is often referred as biocompatibility. Even more, the biocompatibility of the phases allows preservation of biomolecules' native structure while the presence of polymer can even improve their stability (1). Partitioning is governed by numerous factors that can be manipulated to achieve desired separation and purification results, which makes ATPS very flexible for the application (1, 2).

Being the medium that is very well suited for the partitioning of biomaterials, ATPS has found wide and advantageous application in bioseparation of enzymes as well. There are numerous examples of extraction of enzymes in ATPS in downstream processing with the aim of their isolation and purification (1,3-5).

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Cellulases, enzymes belonging to the family of glycosyl hydrolases, play a key role in organic carbon turnover and have important and wide application in industry. At present, cellulases are used in the food, brewery and wine, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture (6). In addition, cellulases have recently gained additional attention because of their application in the production of biofuel from lignocellulosic substrates (7). So, the demand for these enzymes is growing rapidly, becoming a driving force for the research on cellulases production and downstream processing.

In this study, partitioning of cellulolytic activity in polymer/polymer two-phase systems was studied with the aim to establish the conditions in which the highest possible yield and purification factor in the top phase can be achieved. Several factors were investigated in polyethylene glycol/dextran ATPS - molecular weight of polyethylene glycol (PEG) and its concentration, as well as addition of different salts. Partitioning parameters of cellulolytic activity from commercial enzyme preparation and crude enzyme from *Penicillium* sp. were determined and compared.

EXPERIMENTAL

Preparation of ATPS

Polyethylene glycols having molecular weights 1500 g/mol (PEG 1500), 4000 g/mol (PEG 4000) or 6000 g/mol (PEG 6000) (Merck, Germany) and fractionated dextran with moleculat weight ~500,000 g/mol (Fluka, Switzerland) were used for the preparation of ATPS. Ten-gram phase systems were prepared by adding adequate quantities of PEG, dextran, enzyme solution and 10 mmol/L acetate buffer pH 5.0, to achieve desired concentrations (%, w/w). The mixtures were vortexed for 5 minutes and the phases were allowed to separate in graduated tubes for 12 hours. Then, the top phase was carefully removed with a pipette, leaving a small amount at the interface, and the bottom phase was then sampled through the interface. Samples of each phase were analysed for enzyme activity and protein.

Commercial enzyme

Commercial preparation Celluclast 1.5 L™ (Novozyme) was prepared for partitioning experiments by dilution in the 10 mmol/L acetate buffer pH 5.0 to make basal enzyme solution.

Crude enzyme from *Penicillium* sp.

Crude enzyme preparation was obtained by submerged cultivation of *Penicillium* sp. - 300 mL Erlenmeyer flasks, containing 100 mL medium with 1.5 g sugar beet extraction waste (particle size >400 μm) and 0.5 g $(NH_4)_2SO_4$ in 0.15 mol/L KH_2PO_4, pH 4.5, were inoculated with 10^6 spore/mL and incubated at 28°C and 200 rpm. After 4 days, the cultivation was stopped and content of flasks was filtered to obtain crude enzyme (CE).

Enzyme assay

Cellulolytic activity was determined according to König et al. (8) - 1.5 mL of 4% (w/v) carboxymethylcellulose in 0.1 mol/L acetate buffer, pH 5, and 0.5 mL enzyme solution were kept in a water bath at 40°C for 30 minutes. The reaction was stopped by addition of DNS reagents, followed by boiling at 100°C for 5 minutes and absorbance was read at 540 nm. One unit was determined as the amount of the enzyme catalysing the formation of 1 μmol of glucose per minute at 40 °C and pH 5.0. Protein concentration was determined by Bradford method (9) with bovine serum albumin as standard.

Partition parameters

The partition coefficient for cellulolytic activity in the ATPS systems was defined as

$$K = \frac{activity_{top\,phase}}{activity_{bottom\,phase}} \quad [1]$$

and the yield in the top and the bottom phase, respectively, as

$$Y_t(\%) = \frac{100 \cdot V_t \cdot K}{V_t \cdot K + V_b} \quad [2]$$

$$Y_b(\%) = \frac{100 \cdot V_b}{V_t \cdot K + V_b} \quad [3]$$

where V_t and V_b are the volumes of the top and bottom phase, respectively.

The purification factor of crude enzyme in the top phase was defined as

$$PF_t = \frac{specific\ activity_{top\ phase}}{specific\ activity_{CE}} \quad [4]$$

where specific activity represents the ratio between the enzyme activity and protein concentration in the sample.

The results are the mean value of at least three measurements of activity (the accuracy is considered to be ±5%) on a minimum of three replicas for every partition experimental point.

RESULTS AND DISCUSSION

The influence of molecular weight of PEG on the partitioning of cellulolytic activity

The selection of molecular weight of polymer is usually the first step in the partitioning experiments with the aim of finding a suitable phase system where selective separation of target material is achieved. Results of the distribution of cellulolytic activity from commercial preparation and crude enzyme between two phases of polyethylene glycol/dextran two-phase systems obtained at different molecular weights of the top phase polymer are given in Figure 1.

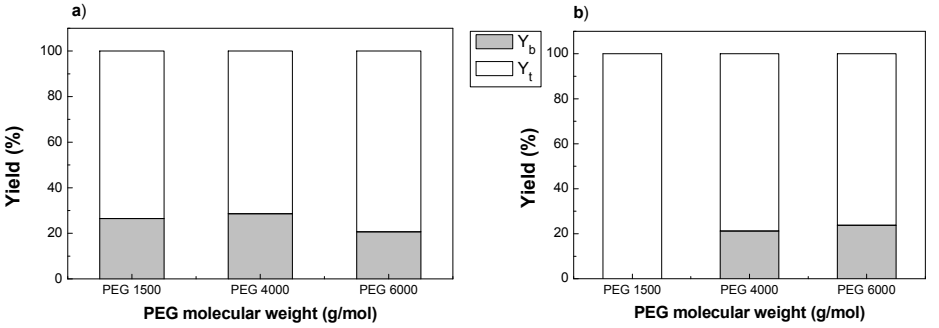

Figure 1. Influence of the molecular weight of PEG on the partitioning of cellulolytic activity between the top and the bottom phases from a) commercial enzyme preparation in 10% (w/w) PEG/5% (w/w) dextran/35% (w/w) basal enzyme solution ATPS and b) crude enzyme from *Penicillium* sp in 10% (w/w) PEG/5% (w/w) dextran/80% (w/w) crude enzyme ATPS

It is known that the phase polymer molecular weight influences the material partitioning both by altering the phase diagram, i.e. by influencing the composition of the phases, and by changing the number of polymer-enzyme interactions in general. Usually, the partition coefficient of enzyme and consequently top phase yield decrease as the PEG chain length increases (1), but in some cases, the partition parameters show just opposite dependence (10, 11). This was also the case with the results obtained with commercial enzyme preparation – although the the top phase yield did not change very much with the change of the moleculat weight of PEG (from approx. 71 to 79%), still the highest amount of cellulolytic activity was partitioned to the top phase of the system containing the longest investigated PEG molecule (Figure 1a). On the other hand, the decrease of the molecular weight of PEG was followed by an increase in the yield of cellulolytic activity from crude enzyme from *Penicillium* sp. and in the system containing PEG 1500 enzyme activity was completely partitioned to the top phase (Figure 1b). In addition, the top phase yields of the enzyme activity from crude preparation were in average higher in comparison to those from commercial preparation.

The influence of concentration of PEG 1500 on the partitioning of cellulolytic activity

Further investigations were carried out with PEG 1500 to establish the influence of the concentration of the top phase polymer on the partitioning of cellulolytic activity into the phases of ATPS (Figure 2).

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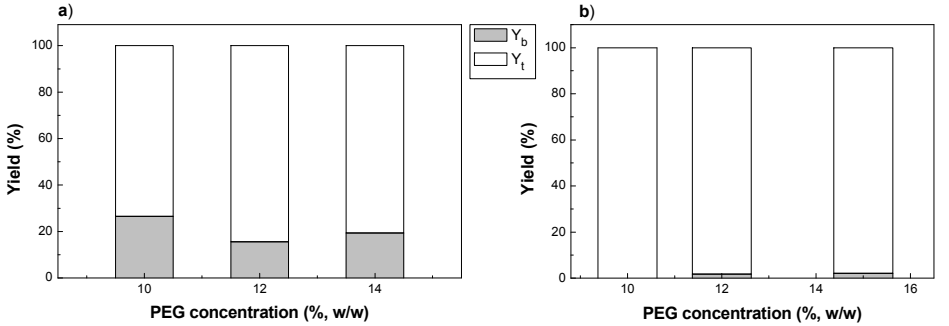

Figure 2. Influence of the concentration of PEG on the partitioning of cellulolytic activity between the top and the bottom phases from a) commercial enzyme preparation in PEG 1500/5% (w/w) dextran/35% (w/w) basal enzyme solution ATPS and b) crude enzyme from *Penicillium* sp. in PEG 1500/5% (w/w) dextran/80% (w/w) crude enzyme ATPS.

The most favourable conditions for cellulolytic activity from commercial enzyme to be partitioned in the top phase were in the system containing 12% PEG 1500 (Figure 2a). As for the enzyme activity from crude preparation, the highest obtained top phase yield of cellulolytic activity was achieved in the system with the lowest investigated PEG concentration, while at the other two concentrations small portions of the enzyme were partitioned to the bottom phase (Figure 2b).

The influence of concentration of added salt on the partitioning of cellulolytic activity

It is known that the addition of salt to a polymer-polymer two-phase system can influence the partition behaviour of the material (1) and that it may be a powerfull tool for the improvement of partitioning parameters. So, to the systems with highest cellulolytic activity from commercial and crude preparation partitioned in the top phase, observed in the previous experiments, three salts were added at concentrations that do not change equilibrium in ATPS (12).

The presence of the three tested salts in ATPS influenced only slightly the ratio between the top and bottom phase yields during the partitioning of cellulolytic activity from commersial preparation (Figure 3a). Contrary to that, the addition of the salts dramatically influenced distribution of cellulolytic activity from the crude enzyme between the phases in way that favoured its partitioning to the bottom phase of the system (Figure 3b). However, the system with 15 mmol/L KH_2PO_4 provided the most favourable conditions for the selective distribution of cellulolytic activity from crude preparation in the top phase, and hence the highest purification factor was obtained (Table 1).

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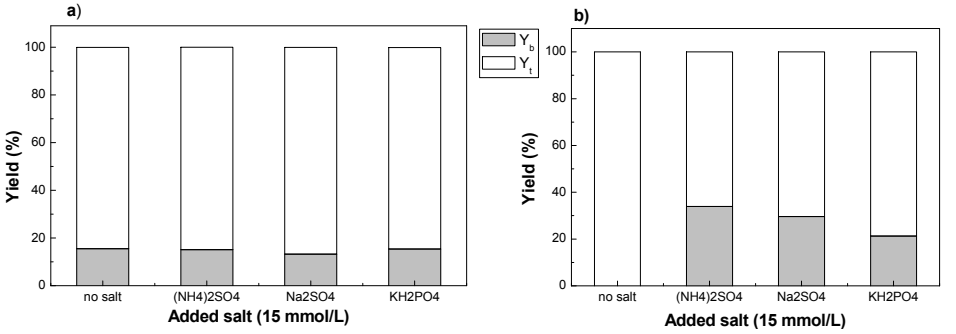

Figure 3. Influence of added salt on the partitioning of cellulolytic activity between the top and the bottom phases from a) commercial enzyme preparation of 12% (w/w) polyethylene glycol 1500/5% (w/w) dextran/35% (w/w) basal enyzme solution ATPS and b) crude enzyme from *Penicillium* sp. in 10% (w/w) polyethylene glycol 1500/5% (w/w) dextran/80% (w/w) crude enzyme ATPS

Purification of crude enzyme in ATPS

Since the aim of downstream processing of enzymes is not only to achieve high yield but also their separation from contaminants, purification factor of cellulolytic activity produced by cultivation of *Penicillium* sp. was also determined throughout all partitioning experiments. Its highest obtained values in the top phase along with corresponding compositions of ATPS are presented in Table 1.

Table 1. Purification factor of cellulolytic activity from crude enzyme from *Penicillium* sp. in the top phase of ATPS

Composition of ATPS	PF_t
10% (w/w) PEG 4000/5% (w/w) dextran/80% (w/w) crude enzyme	2.33
12% (w/w) PEG 1500/5% (w/w) dextran/80% (w/w) crude enzyme	2.45
10% (w/w) PEG 1500/5% (w/w) dextran/80% (w/w) crude enzyme in 15 mmol/L KH_2PO_4	2.60

By comparing the results presented in Figures 1b, 2b and 3b with those in Table 1, it can be noticed that the compositions of ATPS that enabled the highest partitioning into the top phase were not the same as those that provided the most appropriate conditions for the selective distribution of cellulolytic activity in the same phase. So, in the downstream processing of enzymes in ATPS, the factors influencing the partitioning have to be carefully selected to enable good balance between both bioseparation parameters.

CONCLUSION

The polyethylene glycol/dextran 500,000 two-phase system appeared to be a suitable medium for the partitioning of cellulolytic activity to the top phase. It was shown that the extraction of cellulolytic activity from crude enzyme in this system can be useful technique in the downstream processing for both isolation and purification. Appropriate conditions for the favourable and selective partitioning of enzyme activity to the top phase were created by selection of polyethylene glycol molecular weight and concentration, and by addition of salt to the system. The observed differences in responses to the changes of the factors influencing partitioning between cellulolytic activities originated from two sources can be explained by the differences in the complexity of the matrix in commercial, partially purified, enzyme preparation and, on the other side, in crude unpurified enzyme. It might be that the partitioning of contaminants also creates such environment in the phases which in turn may additionally influence partitioning behaviour of the enzyme activity.

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РАСПОДЕЛА ЦЕЛУЛОЛИТИЧКЕ АКТИВНОСТИ У ДВОФАЗНИМ СИСТЕМИМА ПОЛИЕТИЛЕНГЛИКОЛ/ДЕКСТРАН

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У раду је испитана расподела целулолитичке активности у воденим двофазним системима полиетиленгликол/декстран. Максимално могућ 100% принос целулолитичке активности добијене култивацијом *Penicillium* sp. постигнут је у двофазном систему састава 10% (m/m) полиетиленгликол 1500/5% (m/m) декстран 500000/ 80% (m/m) сирови ензим на pH 5 у само једном кораку екстракције. Додатак KH_2PO_4 у концентрацији 15 mmol/l у овај систем, иако је смањио расподелу целулолитичке активности из сировог ензимског препарата у горњу фазу система, побољшао је фактор пречишћавања у тој фази на вредност 2,6. Разлике у одзиву између целулолитичких активности из два испитивана извора на промене фактора који утичу на расподелу могу се објаснити различиом комплексошћу њихових матрикса – комерцијалног, делимично пречишћеног, и сировог непречишћеног препарата добијеног култивацијом. Наиме, и присуство самих контаминената може додатно утицати на расподелу ензимске активности.

Кључне речи: водени двофазни систем; целулолитичка активност; расподела; пречишћавање.

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