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Extractive bioconversion of xylan for production of xylobiose and xylotriose using a PEG6000/sodium citrate aqueous two-phase system

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Abstract−Aqueous two-phase system (ATPS) was applied for extraction bioconversion of xylan by xylanase from *Trichoderma viride*. Phase diagrams for poly (ethylene glycol) (PEG) and sodium citrate were determined at room temperature. The ATPS composed of 12.99% (w/w) PEG6000 and 12.09% (w/w) sodium citrate was favorable for partition of xylanase and used for extraction bioconversion of xylan. Batch hydrolysis demonstrated that higher concentrations of xylobiose and xylotriose were obtained in the PEG6000/sodium citrate ATPS compared to those in the aqueous system. These results present the potential feasibility of production of xylo-oligosaccharides by extraction bioconversion in ATPS.

Key words: Extractive Bioconversion, Xylanase, Xylobiose, Xylotriose, Aqueous Two-phase System

INTRODUCTION

Xylan, the main constituent of hemicellulose in lignocellulose, gains an attractive interest for production of xylo-oligosaccharides as functional foods and feed additives [1]. Intestinal *bifidobacteria*, as an indigenous bacterium to human health, was firstly observed to be stimulated for growth by xylo-oligosaccharides [2]. And xylobiose and xylotriose were proven to promote the growth of intestinal *bifidobacteria* [3]. Xylo-oligosaccharides were produced from hemicellulose in lignocellulose by chemical method [4,5], direct enzymatic hydrolysis [6] and chemical-enzymatic methods [7-9]. The chemical-enzymatic process has been successfully scaled up for production of xylo-oligosaccharides. The process of the chemical-enzymatic method was a combination of extraction of xylan from lignocellulose, enzymatic hydrolysis of xylan and purification of xylo-oligosaccharides [10]. In the manufacturing process of xylo-oligosaccharides, biocatalyst was mixed with products in hydrolyzate. For refining xylo-oligosaccharides, protein should be removed from hydrolyzate by some methods, such as CaO flocculation [10]. This procedure would give a burden of downstream separation and increase the cost of production of xylo-oligosaccharides.

An aqueous two-phase system (ATPS) is formed when two water-soluble polymers or a polymer and an inorganic salt are mixed above critical concentrations. The phase separation of ATPS is attributed to the thermodynamic properties of components in solution described by Flory-Huggins theory [11]. The driving force is the enthalpy accompanied with the interactions between the components, which are opposed by the loss of entropy associated with the segregation of the components [12]. A series of bioproducts were partitioned in ATPS, such as α-amylase inhibitor [13], xylanase [14], and alkaline protease [15]. Extractive bioconversions in ATPS have proved to be a promising alternative to the conventional bioconversion process, such as production of prednisolne from hydrocortisone [16], casein hydrolysis [17], starch hydrolysis [18], and production of akirin [19]. As biocatalyst may be present in only one phase in ATPS, it is easy to recover products without losing biocatalyst when the products are either distributed in the two phases or partitioned in the biocatalyst-free phase.

In the present work, extraction bioconversion of xylan for production of xylobiose and xylotriose using xylanase was investigated in a PEG/sodium citrate ATPS. The aim of the work was to examine the effectiveness of ATPS applied to the enzymatic hydrolysis of xylan. Phase diagrams were first determined to find an operating region for forming ATPS. Then, partition behavior of xylanase in PEG/sodium citrate ATPS was studied for ATPS selection. The enzymatic hydrolysis of insoluble xylan in ATPS was carried out for efficient production of xylobiose and xylotriose, which was compared to those in aqueous system.

MATERIALS AND METHODS

1. Materials

Brichwood xylan and xylanase from *Trichoderma viride* (EC 3.2.1.32) was purchased from Sigma-Aldrich (St. Louis). Xylobiose and xylotriose were purchased from Megazyme (Wicklow, Ireland). PEG2000, 4000, 6000 and sodium citrate were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Other chemicals were of analytical grade without further treatment.

2. Phase Diagrams and ATPS Selection

Phase systems were prepared from stock solutions of PEG2000 (40%, w/w), PEG4000 (30%, w/w), PEG6000 (30%, w/w) and sodium citrate (40%, w/w). Phase diagrams of PEG/sodium citrate ATPSs were determined using the method as described previously

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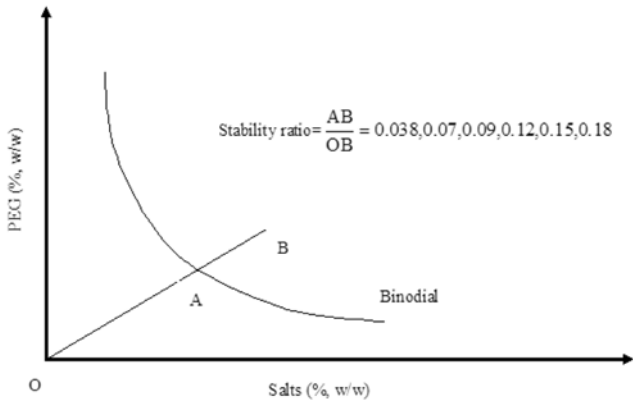

Fig. 1. Illustration of stability ratio in ATPS. O: original point, A: point on the binodial, B: selected ATPS point.

[13]. The selection of ATPS is a key for extractive bioconversion. The PEG-citrate salt system chosen for experiments was at specific distances from the binodials defined as stability ratio (Fig. 1) [20]. The stability ratio is defined as

$$\text{Stability ratio} = \frac{AB}{OB} \quad (1)$$

where B point is selected when the volume ratio of top and bottom phase volume is approximately 1. When shifting B point along with OB, the stability ratios are recalculated as assigned in Fig. 1. Table 1 shows the compositions of PEG/sodium citrate ATPS used in this study.

3. Determination of Xylanase Activity

Xylanase activity was determined by mixing 0.5 mL of an appropriately diluted enzyme solution with 1.0 mL of 1.0% (w/v) birchwood xylan in 50 mM citrate buffer pH 4.8 at 50 ℃ for 30 min. The concentration of reducing sugar was assayed by the dinitrosalicylic acid (DNS) method with xylose as standard [21]. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the liberation of 1 μmol of reducing sugar per min.

4. Partition of Xylanase in ATPS

Aqueous two-phase systems were prepared in 15 mL graduated centrifuge tubes by adding appropriate quantities of stock solutions in citrate-phosphate buffer (40 mM, pH 5.6). Enzyme (15 U/mL) was then piped into the systems, adjusting the total amount of the system to 10 g with buffer. All partition experiments were carried out at room temperature. After vortexing for 3 min, two phases were formed by centrifugation at 1,268 g for 10 min. The volumes of top and bottom phase were measured. And then both phases were separated for xylanase activity assay. All experiments were performed in duplicate and results represented the mean values of two independent experiments. The partition coefficient of xylanase is defined as

$$K = \frac{U_{top}}{U_{bottom}} \quad (2)$$

where U_{top} and U_{bottom} are the enzyme activity in top and bottom phase, respectively.

The phase volume ratio is defined as

$$R = \frac{V_{top}}{V_{bottom}} \quad (3)$$

where V_{top} and V_{bottom} are the top and bottom phase volume, respectively.

The recovery of xylanase in top phase is defined as

$$Y_t(\%) = \frac{U_{top}}{U_{total}} \times 100 \quad (4)$$

where U_{top} and U_{total} are the enzyme activity in top phase and the total enzyme activity added in ATPS, respectively.

5. Extractive Hydrolysis of Xylan

Extractive hydrolysis was conducted at 50 ℃ and 80 rpm in a

Table 1. Partitions of xylanase in the three PEG/sodium citrate ATPSs

Systems	Stability ratio	PEG (%, w/w)	Sodium citrate (%, w/w)	K	R	Y_t (%)
PEG2000/Citrate ATPS	0.038	14.67	15.15	8.32	0.62	83.77
	0.07	15.17	15.67	8.00	0.59	82.52
	0.09	15.51	16.02	5.56	0.62	77.51
	0.12	16.04	16.56	5.22	0.46	70.58
	0.15	16.60	17.15	3.64	0.62	69.30
	0.18	17.21	17.77	4.73	0.44	67.53
PEG4000/Citrate ATPS	0.038	14.61	12.75	19.16	0.72	93.24
	0.07	15.11	13.19	17.17	0.70	92.32
	0.09	15.45	13.48	12.65	0.65	89.16
	0.12	15.97	13.94	6.86	0.62	80.96
	0.15	16.54	14.44	4.46	0.66	74.65
	0.18	17.14	14.96	3.58	0.65	69.96
PEG6000/Citrate ATPS	0.038	12.99	12.09	47.35	0.69	97.03
	0.07	13.44	12.51	18.91	0.69	92.88
	0.09	13.73	12.78	13.26	0.69	90.15
	0.12	14.20	13.22	6.73	0.69	82.28
	0.15	14.70	13.68	5.14	0.68	77.74
	0.18	15.24	14.19	4.61	0.65	74.98

flask (100 mL) using PEG-citrate aqueous two-phase system formed in 40 mM citrate-phosphate buffer, pH 5.6. Birchwood xylan (0.5 g) and an appropriate amount of the two-phase system were poured in the flask. And then, addition of 20 U xylanase/g xylan was quickly followed by adding buffer to adjust the total amount of the system to 50 g. Samples were collected at regular time intervals. The samples were heated in a boiling water bath for 5 min to inactivate the enzyme. The pretreated samples were cooled to room temperature and centrifuged at 10,000 rpm for 5 min for further analysis.

6. Analytical Method

Xylose, xylobiose and xylotriose were determined using an ICS-3000 ion chromatography system (Dionex) with PAD and CarboPac PA200 column (3×250 mm) at 30 ºC. A dual mobile phase system (A: 100 mM NaOH, B: 500 mM sodium acetate) was applied. Saccharides were eluted using a linear sodium acetate gradient (B: 0-24% in 40 min; 0.3 mL/min), followed by elution with 100 mM NaOH (15 min; 0.3 mL/min).

RESULTS AND DISCUSSIONS

1. Phase Diagrams for PEG/Sodium Citrate Systems

Binodial curves were obtained for the PEG/citrate ATPSs formed by PEG2000, PEG4000, PEG6000 and sodium citrate at pH 5.6, which was the optimal pH for xylanase (data not shown) (Fig. 2). Single phase and two-phase systems were separated by the binodial curve. Two phases were formed only above the binodial curve. As the compositions of ATPS below the binodial curve, the system becomes homogeneous. So, it is important for the choice of an operating point in a particular ATPS. A chosen point, too far or too near from the binodial, might result in a failed operation of ATPS [22]. The concept of stability ratio was applied to select an appropriate ATPS. It was similar to the tie line length and easier to be determined [22]. The stability ratios used in this study were 0.038, 0.07, 0.09, 0.12, 0.15 and 0.18.

2. Partition of Xylanase in PEG/Sodium Citrate ATPSs

Some previous experiments were carried out to prove that xylanase stability kept over 95% within 24 h (data not shown). Partitions of xylanase in three PEG/citrate ATPSs are shown in Table 1 with systems of different stability ratio. An increase of stability ratio resulted from the increase of PEG and sodium citrate concentrations among the three ATPSs. A similar relationship between stability ratio and phase compositions was reported when a PEG4000/phosphate ATPS was applied for investigation of kinetics of phase separation [20].

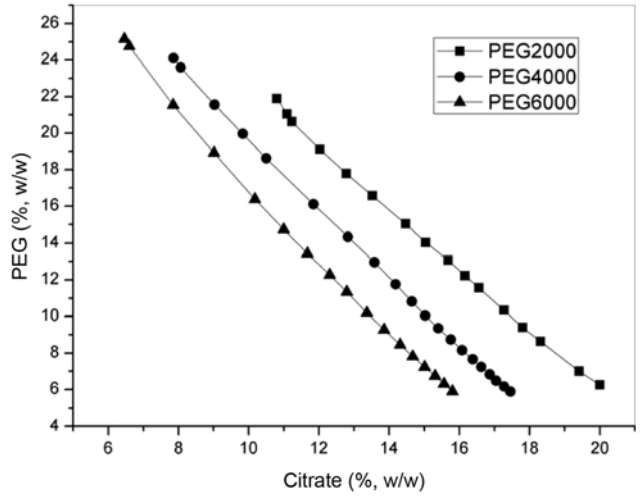

Fig. 2. Phase diagrams for PEG/citrate ATPSs at room temperature.

Since stability ratio is similar to tie line length, increasing of stability ratio is corresponding to increasing tie line length. Longer tie line length usually favored partition of protein in PEG-rich phase [23]. Oppositely, the recovery of xylanase in top phase (Y_t) and partition coefficient (K) decreased with the increment of the stability ratio in the three PEG/sodium citrate ATPSs (Table 1). A protein's partition coefficient might increase or decrease as polymer concentration was changed [11]. And increase or decrease of phase compositions also resulted in different partition behaviors of recombinant phenylalanine dehydrogenase using a set of different PEG6000/salt ATPSs [24]. These results indicated that partition of xylanase was influenced not only by phase compositions but also by other variables.

Partition behavior of biomolecules in ATPS is usually relative to the PEG molecular weight. As shown in Table 1, an increase in PEG molecular weight resulted in increase of Y_t at the same of stability ratio among the three ATPSs. The highest K (47.35) and Y_t (97.03%) were obtained at the stability ratio of 0.038 in the PEG6000/sodium citrate ATPS. Generally, due to an excluded volume effect, increasing molecular weight of phase polymer will cause a favored partition of biomolecule in polymer-poor phase. But opposite results were obtained in some studies. It was found that partition coefficient and yield of phenylalanine dehydrogenase increased when the PEG molecular weight increased from 2000 to 6000 [24]. Partition of elastase and xylanase also had similar results using a PEG/salt aqueous two-phase system [25,26]. Partition of a biomolecule in an aqueous two-phase system was influenced by many variables, such as electrochemistry, hydrophobicity, size, biospecificity, conformation, ionic composition, charge, and phase polymer and its molecular weight [27]. Except for excluded volume effect, other variables should be taken into account for partition of xylanase in PEG-rich phase when increasing PEG molecular weight. As the highest K and Y_t obtained at the stability ratio of 0.038, the 12.99% (w/w) PEG6000/12.09% (w/w) sodium citrate aqueous two-phase system was selected for further extraction bioconversion of xylan.

3. Extractive Bioconversion of Xylan by ATPS

As xylobiose and xylotriose were more effective in proliferation of *Bifidobacteria* [7], extraction bioconversion by ATPS was investigated to prepare xylobiose and xylotriose. Based on the results of xylanase partition, batch hydrolysis of xylan was carried out both in the PEG6000/sodium citrate ATPS and aqueous system (see Fig. 3). As shown in Fig. 3, the concentrations of xylobiose (2.12 g/L) and xylotriose (1.32 g/L) in ATPS were higher than 1.08 g/L xylobiose and 0.52 g/L xylotriose in aqueous system after 24 h hydrolysis. The hydrolysis yield of xylan for ATPS and aqueous system was 30.27% and 15.68%, respectively. For hydrolysis in the aqueous system, xylobiose and xylotriose reached a maximum concentration after 1-2 h hydrolysis. Correspondingly, xylotriose and xlyobiose reached a maximum concentration in ATPS at 8 h and 20 h hydrolysis, respectively. And it was obvious that time to peak concentra-

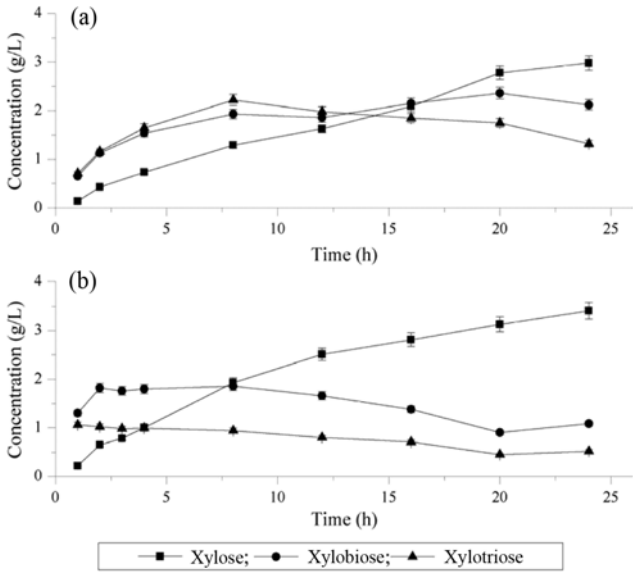

Fig. 3. Xylose, xylobiose and xylotriose produced from extraction bioconversion of xylan in PEG6000/sodium citrate ATPS (a) and aqueous system (b) at pH 5.6 and 50 °C. The ATPS composition was 12.99% (w/w) PEG and 12.09% (w/w) sodium citrate. Other conditions: 1% (w/w) xylan, 20 U xylanase/g xylan and 80 rpm.

tion of xylobiose and xylotriose in ATPS was later than those in the aqueous system. The difference between ATPS and aqueous system might result from the properties of ATPS and the existence of PEG. On one hand, ATPS dragged enzyme in PEG-rich phase [11]. On the other hand, excluded volume effect of polymers moderately reduced adsorption of enzyme on substrates [28]. Insufficient contact between xylanase and substrate in ATPS was responsible for incomplete hydrolysis of xylan, which gave higher concentrations of xylobiose and xylotriose and kept a lower level of xylose. Comparably, lower concentrations of xylobiose and xylotriose and a higher concentration of xylose were found in the aqueous system. Additionally, the partition coefficients of xylose, xylobiose and xylotriose were defined as the ratio of their concentrations between top and bottom phase. The partition coefficients of xylose, xylobiose and xylotriose were 0.29, 0.22 and 0.19, respectively. For the low partition coefficients of xylose, xylobiose and xylotriose, the products could be recovered into the bottom phase. Because xylanase and products were partitioned into different phase, extraction bioconversion made it possible to separate biocatalyst and products after enzymatic hydrolysis. Therefore, extraction bioconversion of xylan in a PEG6000/sodium citrate ATPS was more suitable for preparation of xylobiose and xylotriose.

CONCLUSION

Extraction bioconversion in ATPS has proven to be suitable for preparation of xylobiose and xylotriose compared to that in an aqueous system. The ATPS composition of 12.99% (w/w) PEG6000 and 12.09% (w/w) sodium citrate was a potential system for extractive bioconversion of xylan. Batch hydrolysis of xylan indicated that extraction bioconversion in the PEG6000/sodium citrate ATPS resulted in a higher concentration of xylobiose and xylotriose compared to control experiment. This result demonstrated the potential feasibility of production of xylo-oligosaccharides by extraction bioconversion in ATPS. Further researches are also needed for distribution of xylo-oligosaccharides in ATPS and their separation.

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