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Aqueous two-phase extraction using thermoseparating polymer: a new system for the separation of endo-polygalacturonase

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Abstract

Aqueous two-phase partitioning of endo-polygalacturonase (endo-PG) produced by *Kluyveromyces marxianus* strains was studied, on systems containing the thermoseparating polymer Ucon 50-HB-5100 (a random copolymer of 50% ethylene oxide (EO) and 50% propylene oxide (PO)) as one of the phase-forming compounds. Ucon 50-HB-5100 (Ucon)–polyvinyl alcohol (PVA 10,000), Ucon 50-HB-5100–hydroxypropyl starch (Reppal PES100) and Ucon 50-HB-5100–$(NH_4)_2SO_4$ systems were tested. Ucon recycling was also investigated. Ucon–$(NH_4)_2SO_4$ system proved to be the most efficient system for aqueous two-phase extraction (ATPE) of endo-PG as when compared to total protein partition, the enzyme was strongly partitioned to the salt-rich phase.

Using Ucon–$(NH_4)_2SO_4$ system, a separation scheme consisting of three stages was proposed and tested at laboratory scale. In the first and second stages, operated in series, extractions were performed using the same Ucon–$(NH_4)_2SO_4$ system. Ucon recycling was done in each stage. In the third stage, removal of the Ucon and salt residues present in the enzyme-rich phase of the second extraction stage was done. The proposed scheme allowed for a 10-fold enzyme concentration and a purification factor close to the expected maximum while maintaining more than 95% of the initial enzyme activity.

Keywords: Downstream processing; Aqueous two-phase; Separation; Purification; Thermoseparating; Enzymes

1. Introduction

Pectic enzymes are used in food industry in two different ways: to completely clarify juice by removal of all the pectin from solution (by precipitation and/or by complete degradation of the polymer) and to reduce the viscosity of a solution without cloud loss [1].

In the first situation an enzyme preparation containing several pectic enzymes, including pectinmethylesterase, is the most efficient. However, when cloud is desirable an enzyme preparation free of pectinmethylesterase has to be required.

Kluyveromyces marxianus is one of the few yeasts with the ability to produce endo-polygalacturonase (endo-PG)—E.C.3.2.1.15—which degrades pectin [1,2] and has been widely studied [3–5]. This enzyme meets all the requirements for the two situations mentioned above.

Aqueous two-phase systems (ATPS) provide very mild environment for biological materials and can be used for the partition of biomolecules [6,7], since both phases contain 70–90% water. Preliminary studies in our laboratory have showed the possibility of using polyethylene glycol (PEG)–$(NH_4)_2SO_4$, PEG–polyvinyl alcohol (PVA), and/or a combination of these two systems to purify and concentrate endo-PG from its fermentation broth [8,9]. However, difficulties arise when considering the separation efficiency and/or economics of the extraction processes. For example, when a combination of PEG–PVA and PEG–$(NH_4)_2SO_4$ systems was used to separate endo-PG, the enzyme can be well purified and concentrated. However, the economics associated with the separation scheme are low. PEG has to be recycled at an extra cost of $(NH_4)_2SO_4$ consumption (PEG is concentrated by adding more salt into the PEG-rich phase to cause phase splitting again), while the recycling of PVA and salt is very difficult to achieve or not possible.

PEG can also be recycled by thermoseparation. However, the economics for thermoseparation of PEG might be even worse than PEG recycling at an extra cost for salt consumption, due to the high temperatures required. As a matter of fact, PEG cloud point temperature is around 180 °C for the lower molecular weights and decreases with increasing molecular weights, reaching a value of approximately 95 °C for molecular weights of 200,000 Da or more [10].

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Aqueous two-phase systems with new thermoseparating polymers have been recently introduced. Most systems are formed by the use of a random copolymer of ethylene oxide (EO) and propylene oxide (PO) [11]. This EOPO random copolymer has a cloud point temperature as low as 47 °C making possible to perform temperature induced phase separation where a target protein can be separated from the polymer [12–14]and the polymer can be recycled [15]. The cloud point temperature of these aqueous polymer solutions is a linear function of the mass fraction of PO in the copolymer [16,17].

In this work, the extraction of endo-PG and total protein from a *K. marxianus* fermentation broth in an aqueous two-phase system containing hydroxypropyl starch (Reppal PES100), PVA 10,000 or $(NH_4)_2SO_4$ as one of phase-forming species, and a random copolymer of 50% EO and 50% PO as another phase-forming species was studied with the aim of finding a suitable system for endo-PG separation.

2. Materials and methods

2.1. Chemicals

Polyvinyl alcohol (M_r = 10,000, 88% hydrolyzed) was obtained from Scientific Polymer Products (New York, USA), hydroxypropyl starch (Reppal PES100, M_r = 100,000) from REPPE AB (Sweden), and Ucon 50-HB-5100, a random copolymer (M_r = 3900) of 50% ethylene oxide and 50% propylene oxide, from the Union Carbide (New York, USA). Other chemicals are of analytical reagent grade.

All polymer and salt concentrations were calculated as percentage of weight. Stock solutions of salt 40%, PVA 30% and Reppal PES100 40% were previously prepared to be used in ATPS.

2.2. Production of endo-polygalacturonase

Endo-polygalacturonase was produced in a shaking flask incubation of *K. marxianus* CCT3172 or CH0-1 or CH4-1 at 30 °C in the following media (l^{-1}): 3.0 g $(NH_4)_2SO_4$, 4.5 g KH_2PO_4, 0.25 g $MgSO_4$, 0.25 g $CaCl_2$ and 1.0 g yeast extract. Glucose or sucrose or molasses cane was used as carbon source. The concentrations of these sugars were indicated when necessary.

2.3. Phase diagram determination

The phase diagram of Ucon–$(NH_4)_2SO_4$ was determined by titrimetry and refractive index measurements of the phases. The refractive index method was used to determine the total concentrations of Ucon and $(NH_4)_2SO_4$ in the top and bottom phases, respectively. Standard curves for the refractive index of Ucon and $(NH_4)_2SO_4$ at 25 °C were determined using a refractometer 3T (Atago, Japan). Since the refractive index of a polymer and salt solution at a low total concentration (<10%) is additive [6], if the concentration of $(NH_4)_2SO_4$ can be measured accurately, the copolymer concentration can be calculated by determining the refractive index of a phase at 25 °C and subtracting the contribution of salt. The formaldehyde reaction mechanism was applied to measure the $(NH_4)_2SO_4$ concentration,

$$2(NH_4)_2SO_4 + 6HCHO = N_4(CH_2)_6 + 2H_2SO_4 + 6H_2O \quad (1)$$

a previously calibrated 0.1 M NaOH solution was used to titrate the sulfuric acid produced using phenolphthalein as indicator. The analysis showed that the presence of copolymer did not interfere with the determination, and the accuracy of the titration was within 0.4%. A tie-line measured by this methodology was also verified by using a combination of freeze-drying and refractive index measurements [6,9].

The tie-line length (TLL) is defined as follows:

$$\mathrm{TLL}(\%) = \sqrt{(W_1^{\mathrm{T}} - W_1^{\mathrm{B}})^2 + (W_2^{\mathrm{T}} - W_2^{\mathrm{B}})^2} \quad (2)$$

where W_i^{T}, W_i^{B} represent the weight percentages of phase-forming component i in top and bottom phases, respectively.

2.4. Partition coefficients determination

Enzyme samples were prepared from the fermentation broth of strain CCT3172 on 10 g/l glucose medium. The fermentation broth was clarified by centrifugation, dialyzed using 14,000 molecular weight cut-off membranes, and lyophilised. The enzyme solutions were finally obtained at a concentration eight times higher than the original broth, by re-dissolving the lyophilised sample.

In these experiments, appropriate amounts of polymer and salt stock solutions were mixed with 0.2 ml of the enzyme sample and water to form a 1 g total solution. After mixing, the solution was centrifuged at 3000 rpm for 3 min and put overnight in an air bath at a given temperature to ensure that equilibrium was attained. Samples of top and bottom phases were taken for quantifying enzyme activity and total protein concentration. The partitioning coefficients of enzyme and total protein, K_e and K_p, were usually defined as the ratio of enzyme activity or protein concentration in the top and bottom phases. However, in the case of Ucon–$(NH_4)_2SO_4$ ATPS, as phase inversion occurs at high temperatures (30 and 40 °C), the partitioning coefficients K_e and K_p are redefined as the ratio of enzyme activity or protein concentration in the Ucon-rich and salt-rich phases. The protein concentration was measured with a Coomassie Protein assay reagent (Pierce, USA) according to Bradford [18]. The enzyme activity was determined by measuring the release of reducing groups from polygalacturonic acid using cyanoacetamide reagent [19], and the activity unit

(U/ml) was expressed as μmol galacturonic acid equivalents released per minute per milliliter enzyme solution.

2.5. Extraction of endo-PG by the Ucon–$(NH_4)_2SO_4$ system

On the optimization experiments for endo-PG extraction using Ucon–$(NH_4)_2SO_4$ systems, the clarified fermentation supernatant was used without further processing. In the case of Ucon–$(NH_4)_2SO_4$ systems, the aqueous two-phase systems were prepared by adding solid salt and the pure copolymer into fermentation supernatant. If recycled copolymer was used to form the phase systems, a small amount of pure copolymer should be added to meet the desired compositions for the ATPS. In both situations, 64–72% of the total systems consist of fermentation broth. The solution was mixed thoroughly and then put into a bath at a given temperature until complete phase splitting occurred. Top and bottom phases were separated for data determination and analysis.

2.6. Copolymer recycling

For the recycling studies, the copolymer-rich phase from a primary extraction system was removed after phase separation and was weighed. This phase was put in a water bath at 80 °C for 1 h. After temperature induced phase separation, phases were separated and weighed again. The phase compositions were analyzed using the same method as in the phase diagram determination. The recovery of the copolymer was calculated by knowing the weight and compositions of the copolymer phase. Fresh fermentation broth and salt was added into this recycled copolymer phase for the next extraction experiment. Some pure copolymer had to be added to compensate for the loss in the last extraction. About 5% of the total copolymer was added in each recycling.

3. Results and discussion

3.1. Phase diagram

The phase diagram of Ucon–$(NH_4)_2SO_4$ ATPS at 30 °C was determined and shown in Fig. 1(a). In Fig. 1(b), the binodal curves for Ucon–$(NH_4)_2SO_4$ ATPS at 22 and 40 °C were also given.

For Ucon–$(NH_4)_2SO_4$ ATPS, the higher the temperature, the lower the polymer and salt concentrations for the formation of two phases. These phase diagrams are similar to those found in literature [20,21] where systems formed by a copolymer of EOPO with molecular weight 4000 and $(NH_4)_2SO_4$ were calculated at 25 °C. This new Ucon–salt ATPS is also similar to the well-known polyethylene glycol–salt systems. However, if the polymer molecular weight and temperature of the systems are kept the same, only about half of the salt concentration is required for the formation of two phases for this new system, which benefits the process economics. For example, the minimum salt concentration needed for the formation of two phases for PEG4000–$(NH_4)_2SO_4$ ATPS at 20 °C [6] is about 10% and for the Ucon–$(NH_4)_2SO_4$ ATPS at 22 °C is only 5%. Another interesting point is that phase inversion occurs at 30 and 40 °C. In this case, the salt-rich phase becomes the top phase. In general, the Ucon–$(NH_4)_2SO_4$ ATPS has the characteristics of low salt concentration, thermoseparating polymer and phase inversion at higher operation temperatures (30 °C or above).

3.2. Partitioning of endo-PG

Endo-PG showed a very strong tendency to be concentrated in the bottom salt phase in Ucon–$(NH_4)_2SO_4$ system at 22 °C, as shown in Fig. 2. The reciprocal of the

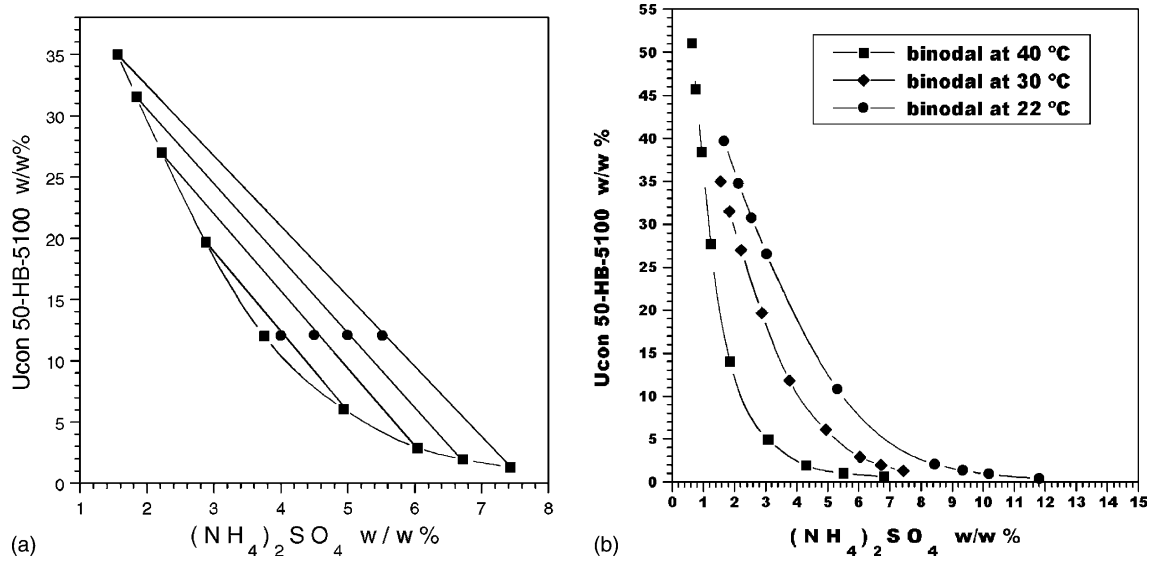

Fig. 1. Phase diagram at 30 °C (a) and binodals at 22, 30 and 40 °C (b) for the system Ucon–$(NH_4)_2SO_4$.

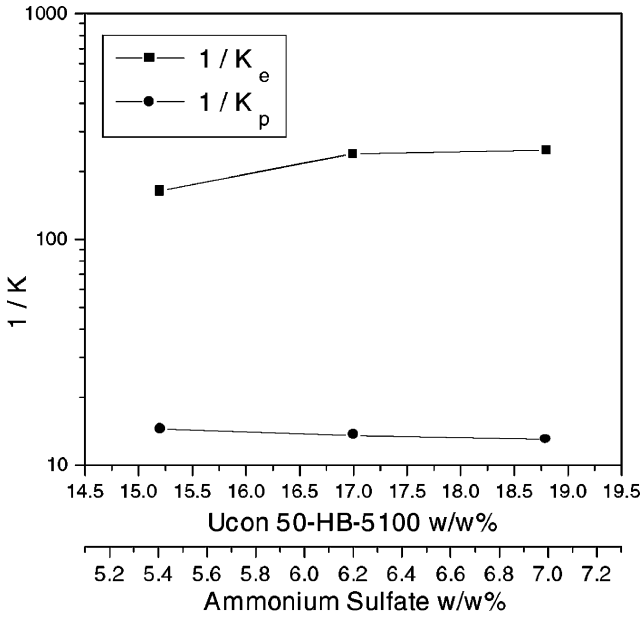

Fig. 2. Partitioning of endo-PG and total protein in Ucon–$(NH_4)_2SO_4$ system at 22 °C.

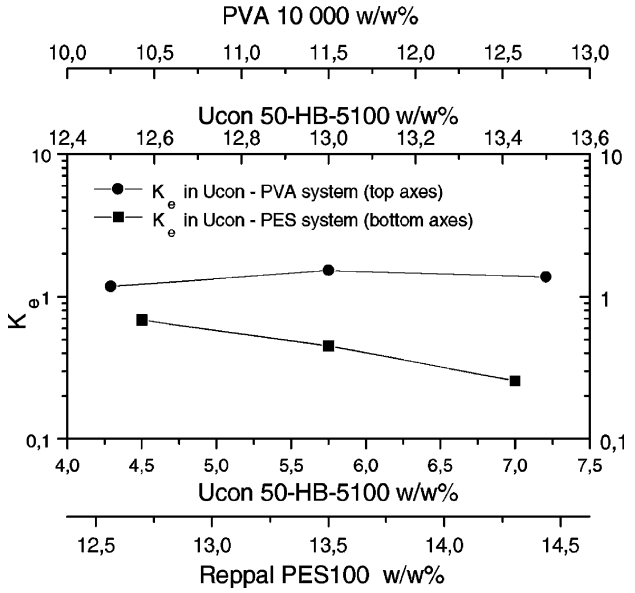

Fig. 3. Partitioning of endo-PG in Ucon–PVA 10,000 and Ucon–PES100 systems at 22 °C.

partitioning coefficient increases with increasing polymer and salt concentrations and can go up to 250. The partitioning of total protein from the fermentation broth of strain CCT3172 was also measured and is shown in Fig. 2. It was found that the reciprocal of partitioning coefficient of total protein is kept constant at about 14 for phase systems ranging from 15.2% Ucon–5.4% salt to 18.8% Ucon–7.0% salt (tie-line length is from 22 to 40%). The difference in partition coefficient between enzyme and total protein still showed the possibility of enzyme purification using the Ucon–$(NH_4)_2SO_4$ system.

In addition to the enzyme partition experiments in Ucon–$(NH_4)_2SO_4$ system at 22 °C, the partitioning coefficients of endo-PG and total protein from a fermentation broth of strain CCT3172 in Ucon–$(NH_4)_2SO_4$ system at 30 and 40 °C were also determined and compared with each other in Table 1. As shown in this table, increasing temperature slightly increases the values of $1/K_e$ and $1/K_p$ when the tie-line lengths of the systems were kept nearly at the same level.

As to the partitioning of endo-PG and total protein in Ucon–PES100 and Ucon–PVA 10,000, shown in Fig. 3, either endo-PG tends to be more equally partitioned between the top and bottom phases, or the partitioning of endo-PG and total protein does not differ largely, which makes these systems of no actual use in the enzyme purification.

Table 1
Effect of temperature on the partitioning of endo-PG and total protein

Temperature (°C)	System	Tie-line length	$1/K_e$	$1/K_p$
22	27.6% Ucon–3.2% salt	29.8	201	14.0
30	27.0% Ucon–2.4% salt	27.7	215	14.5
40	29.5% Ucon–1.4% salt	30.1	272	15.6

3.3. Extraction of endo-PG

3.3.1. Characterization of extraction procedure on Ucon–$(NH_4)_2SO_4$ ATPS

Laboratory-scale aqueous two-phase extraction experiments were performed to determine the separation efficiency and find optimized operation conditions for the endo-PG recovery from the fermentation supernatant. Three aqueous two-phase systems at 22, 30 and 40 °C were selected to perform the extraction experiments. These systems, either using recycled or fresh polymer, have similar tie-line length (about 28%) and phase volume ratio (5–7). The phase volume ratio was defined as the volume ratio of polymer-rich phase to salt-rich phase.

The fermentation supernatant from the production of strains CH4-1, CCT3172 and CH0-1 were used for the extraction experiments at 22 and 30 °C. Glucose, sucrose or molasses cane were used as the carbon source. The sugar concentration was either 10 or 30 g/l (in the case of molasses cane, 10 g/l represents the sucrose concentration). At 40 °C only one extraction experiment was done. In this case, the fermentation supernatant was from strain CCT3172 using 10 g/l glucose as carbon source. All separation results, as well as the fermentation supernatants and phase systems used for each extraction experiment, were summarized in Table 2. The percentage of endo-PG enzyme in total protein of the supernatant, analyzed by gel filtration as described by Schwan et al. [4], was also given.

As can be seen in Table 2, after the first extraction step, in any of the three aqueous two-phase system tested, an

Table 2
Summary of separation results by Ucon–$(NH_4)_2SO_4$ aqueous two-phase extraction

Fermentation supernatant						Aqueous two-phase system[a]			After aqueous two-phase extraction (ATPE)				
Strain	Substrate[b] (g/l)	Volume (ml)	Activity (U/ml)	Protein (mg/l)	Content (%) [c]	Ucon[d] (wt.%)	$(NH_4)_2SO_4$ (wt.%)	Temperature (°C)	Enzyme-rich phase	Volume (ml)	Activity recovery (%)	Protein recovery (%)	Concentration factor[e]
CH4-1	Glucose (30)	55.7	5.13	42.4	55 ± 10	27.6 (0)	3.0	22	Bottom	11.6	98.7	74.5	4.8
CCT3172	Glucose (10)	52.5	0.52	13.3	45 ± 10	27.6 (2)	3.0	22	Bottom	12.3	99.5	71.7	4.3
CH0-1	Molasse (10)	55.7	0.86	NA	NA	27.6 (0)	3.0	22	Bottom	14.0	97.9	NA	4.0
						Second ATPE step[f]			Bottom	12.8	95.6	NA	4.4
CH4-1	Glucose (30)	56.7	5.13	42.4	55 ± 10	27.0 (0)	2.2	30	Top	12.3	97.3	74.2	4.6
CCT3172	Glucose (10)	53.4	0.59	12.7	45 ± 10	27.4 (3)	2.1	30	Top	9.7	97.1	72.2	5.5
						Second ATPE step[f]			Top	10.8	95.8	60.2	4.9 (1.6)
CH0-1	Sucrose (10)	53.4	3.40	20.0	80 ± 10	27.0 (4)	2.2	30	Top	12.2	97.4	85.0	4.4
						Second ATPE step[f]			Top	14.3	95.6	79.8	3.7 (1.2)
CCT3172	Glucose (10)	57.2	0.55	12.4	45 ± 10	27.3 (0)	1.3	40	Top	14.5	99.0	76.0	3.9

[a] The total solution is 100 g.
[b] In the case of molasses cane 10 g/l represents the sucrose concentration.
[c] The content is the percentage of enzyme in total protein by gel filtration.
[d] The number in bracket is the number of polymer circulations, "0" denotes that the polymer is used for the first time, "1" for the first recycled polymer, and so on.
[e] The concentration factor is accumulative and the data in bracket are purification factors after two steps of ATPE.
[f] In the second ATPE step, the same volume of a fresh polymer-rich phase is mixed with the salt-rich phase of the first ATPE step.

enzyme recovery of more than 97% and a concentration factor, defined as the ratio between the volume of fermentation supernatant used and the volume of enzyme-rich phase, of 4–6 can be obtained. The enzyme recovery and the concentration factor are little affected by the operation temperature, the use of recycled polymer, the fermentation supernatant source (different strains and substrates), and the enzyme concentration (from 0.5 to 5 U/ml). As for the recovery of total protein and the purification factor, the results are strongly dependent on the enzyme content of total protein in the original fermentation supernatant. For example, for the fermentation supernatants produced by strains CH4-1 and CCT3172, with a similar enzyme content in total protein (about 50%), a protein recovery of 72–76% could be obtained in one extraction step. As enzyme purification needs further improvement, the introduction of a second extraction step is required. As also shown in Table 2, with two extraction steps, the recovery of total protein is 60% for the fermentation supernatant produced by strain CCT3172 in 10 g/l glucose, very close to the initial enzyme content (about 50%) in the original supernatant. In the case of fermentation supernatant produced by strain CH0-1 in sucrose, two steps of extraction lead to a protein recovery to 80%, nearly the same as the enzyme content in the original supernatant. These results demonstrate the usefulness of Ucon–$(NH_4)_2SO_4$ aqueous two-phase systems for the purification of extra cellular enzymes produced by yeast fermentation.

The laboratory-scale extraction experiments can also be used to select an optimized aqueous two-phase system for the endo-PG recovery. Since the separation efficiency is not significantly affected by the phases composition and temperature of the system, the selection of the optimal aqueous two-phase system can be done based on the simplicity of the operation and the economics of the process. Noting that:

(1) the optimized fermentation process was usually operated at 30 °C;
(2) the higher the temperature, the less the salt concentration for constructing the phase systems, and the higher asymmetric the phase diagram which will cause difficulties in extraction operation;
(3) the enzyme-rich salt phase becomes the top phase for the system operated at 30 and 40 °C, making easier the separation of endo-PG from some solid impurities, which usually precipitate in the bottom of the separator;
(4) the aqueous two-phase system at 30 °C, having a tie-line length of 28 % and a phase volume ratio of 5–7, can be selected as the best one.

3.3.2. Polymer recycling

Ucon 50-HB-5100 is a thermoseparating copolymer in water. Above 47 °C, Ucon aqueous solution containing 10–40% copolymer can form a two-phase system with most of the Ucon concentrated in the bottom phase.

Experiments have shown that the polymer-rich phase from an adequate Ucon–$(NH_4)_2SO_4$ system should have a polymer concentration of 25–35% and a salt concentration lower than 2.5%. Raising the temperature of this Ucon-rich phase to 80 °C, a new ATPS is formed. The newly formed Ucon-rich phase has a copolymer concentration of 83–87% and salt concentration of less than 0.2%. Also, any small amount of impurities from the fermentation broth, such as alcohol, salt, enzyme and other protein remaining in the Ucon-rich phase has little influence on the final concentration of the recycled Ucon phase after thermoseparation. Experiments showed that these impurities caused a maximum deviation of 1–2% in the concentration of recycled Ucon when comparing with the case of impurities free. Up to 95% of the total Ucon in a primary extraction can be recycled.

3.3.3. Separation scheme for endo-PG recovery

Laboratory-scale separation experiments have shown the efficiency in separating endo-PG by two in series aqueous two-phase extraction (ATPE) steps. Based on these data, a separation scheme for the enzyme recovery was proposed, as shown in Fig. 4.

The scheme consists of three main stages. In the first stage, the fermentation broth was concentrated about five-fold and partially purified using one ATPE step in 27% Ucon–2.2% $(NH_4)_2SO_4$ ATPS with a phase volume ratio of about 6. The operating temperature was 30 °C. The top enzyme-rich salt phase was pumped to the second extraction step, while the bottom polymer-rich phase was heated to 80 °C for thermoseparating the copolymer from water and other impurities. The thermoseparated copolymer was then recycled within this stage. About 5% of the total Ucon polymer needed in this stage should be added to compensate the polymer loss.

The second stage is similar to the first one, but the amount of fluid greatly decreased due to the concentration that occurred in the first stage. Polymer and fresh water was also added to make the final compositions of the separating system. No salt is added in this second stage. The top enzyme-rich phase from the first stage was thus further concentrated about 2.5-fold and purified again into a salt-rich phase, while the copolymer is again heated for thermoseparation and recycling.

The third stage is the refining process. The top fluid from the second stage was mixed with a small amount of fresh polymer (for the purpose of easy phase splitting) and heated to 40 °C, allowing for phase separation to occur. After centrifugation, the top enzyme-rich phase, which has a low polymer concentration (about 0.5%) was collected and then dialyzed to remove the salt. The purpose of this third stage is mainly to remove most of the phase-forming polymer and salt, so that the concentrated and purified enzyme solution can be directly used in food industry. For the two first stages, the time required for the enzyme to be extracted is about half an hour for each stage. In the third stage only 10 min are required. This means that the total time needed for the concentration and purification of endo-PG using the described process takes about 1.5 h.

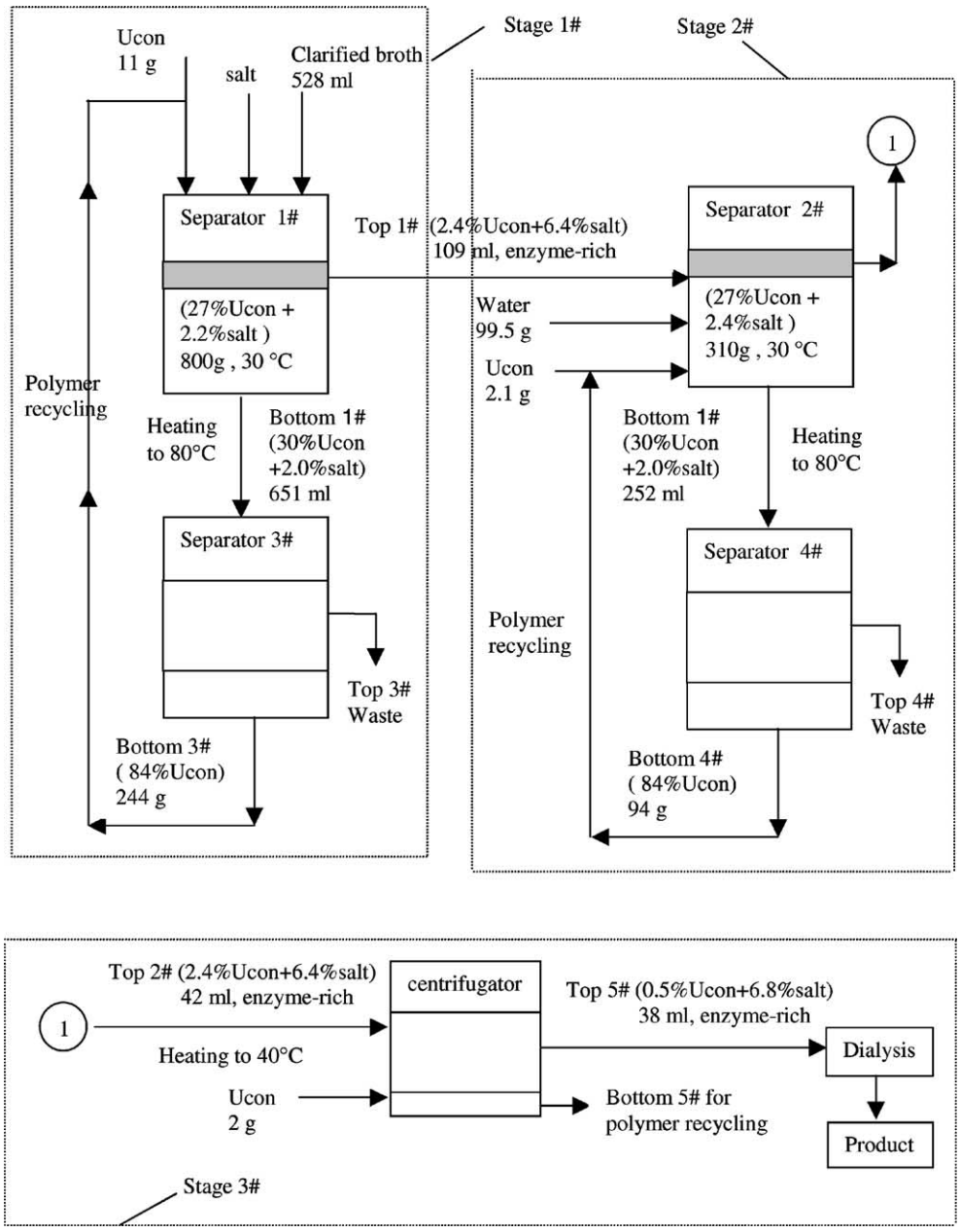

Fig. 4. Schematic view of the proposed separation scheme for endo-PG recovery. The data in bracket are phase compositions, and others are the amounts of fluid.

In order to verify the feasibility of the scheme, one test experiment was done. The volume (528 ml) of fermentation supernatant, as well as the phase compositions and other operation conditions used in each stage, were shown in the scheme (Fig. 4). The supernatant used was obtained from a CH0-1 fermentation using 75 g/l sucrose as carbon source. The initial enzyme activity was 11 U/ml and protein concentration was 62.4 mg/l. After the first two extraction stages, 38 ml of a salt-rich phase was obtained, containing an enzyme activity of 147.4 U/ml and a protein concentration of 692 mg/l. In the third stage, no losses in enzyme activity and protein were observed. So, after these three stages of aqueous two-phase extraction in Ucon–$(NH_4)_2SO_4$ ATPS, enzyme was concentrated 13.4-fold, while maintaining 96% of the initial enzyme activity.

The attractiveness of Ucon–$(NH_4)_2SO_4$ ATPS for endo-PG purification is reinforced by the small amounts of Ucon (13.1 g) and salt (17.6 g) consumed during the exemplified extraction process.

4. Conclusions

Thermoseparating aqueous two-phase systems containing the Ucon 50-HB-5100 polymer were tested for the purification of endo-polygalacturonase produced by

Kluyveromyces yeast fermentation. From the tested systems: Ucon 50-HB-5100–polyvinyl alcohol (10,000), Ucon 50-HB-5100–hydroxypropyl starch (Reppal PES100) and Ucon 50-HB-5100–$(NH_4)_2SO_4$, the last one was found to be the most efficient as enzyme, in comparison with total protein, strongly partitioned to the salt-rich phase.

Endo-PG purification experiments carried out with the Ucon–$(NH_4)_2SO_4$ ATPS with different fermentation supernatants demonstrated that the separation efficiency of the process is little influenced by the type of fermentation broth, operation temperature (although phase inversion occurs at 30 and 40 °C when compared to 22 °C), the use of recycled copolymer and the initial enzyme concentration.

The proposed separation scheme for endo-PG purification consisting of three in series extraction stages enables a 10-fold enzyme concentration while maintaining more than 95% of the initial enzyme activity. It overcomes the economic difficulty in most polymer–polymer and polymer–salt aqueous two-phase extractions, and its integration with the fermentation process opens new opportunities for the purification of yeast secreted enzymes.

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