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Purification of hyperthermophilic archaeal amylolytic enzyme (MJA1) using thermoseparating aqueous two-phase systems

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Abstract

Purification of a recombinant, thermostable α-amylase (MJA1) from the hyperthermophile, *Methanococcus jannaschii*, was investigated in the ethylene oxide–propylene oxide random copolymer (PEO–PPO)/$(NH_4)_2SO_4$, and poly(ethylene glycol) (PEG)/$(NH_4)_2SO_4$ aqueous two-phase systems. MJA1 partitioned in the top polymer-rich phase, while the remainder of proteins partitioned in the bottom salt-rich phase. It was found that enzyme recovery of up to 90% with a purification factor of 3.31 was achieved using a single aqueous two-phase extraction step. In addition, the partition behavior of pure amyloglucosidase in polymer/salt aqueous two-phase systems was also evaluated. All of the studied enzymes partitioned unevenly in these polymer/salt systems. This work is the first reported application of thermoseparating polymer aqueous two-phase systems for the purification of extremophile enzymes.

Keywords: Aqueous two-phase systems; *Methanococcus jannaschii*; Partitioning; Thermoseparation; α-Amylase; Amyloglucosidase

1. Introduction

An aqueous two-phase system (ATPS) is formed when low concentrations of two incompatible polymers (or of one polymer and an inorganic salt) are mixed such that two immiscible phases coexist [1]. These systems are high in water content (70–90%, w/w), have low interfacial tension, and are safe, non-toxic, non-flammable, and relatively environmentally benign as extraction or reaction media. ATPSs have been successfully applied to the separations of proteins, nucleic acids, microorganisms, and animal and plant cells [1,2]. Application of ATPS has also been extended to paper pulp processing for deligninfication [3–5]. Most recently, novel aqueous two-phase systems have been developed based on stimuli-responsive polymers, i.e., polymers capable of reversible phase transition in response to small changes in the environment, such as changes in pH or temperature [6–8]. The ethylene oxide–propylene oxide random copolymer (PEO–PPO), which is linear and non-ionic, has a decreased solubility in water at higher temperatures. When heated above the lower critical solution temperature (LCST), this copolymer can be separated from the aqueous solution. A two-phase system composed of a PEO–PPO bottom phase and an aqueous top phase is thus formed. The temperature at which this phenomenon occurs is known as the cloud point (CP) of the polymer. Several studies have been performed on the thermoseparation of PEO–PPO copolymers [9–18]. After ATPS protein isolation, a protein-free PEO–PPO copolymer is produced, which can be recycled to the primary phase system for a new extraction [19]. There are several advantages to using such a system. Not only can low salt concentrations be used thereby lowering the risk for salting out and precipitation of protein, but also the target protein is recovered in an aqueous solution after the thermoseparation of PEO–PPO copolymer. This facilitates further downstream processing.

Starch hydrolysis is a key process in the production of foods, natural sweeteners, pharmaceuticals, detergents, paper, and animal feeds [20,21]. Industrial glucose production from starch by enzymatic hydrolysis is a two-step process involving a number of amylolytic enzymes. In the first

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step, pretreatment of starch is initiated by a gelatinization step where a starch slurry (30–40%) is heated at 105 °C followed by the liquefaction at 90 °C by α-amylase (AMY) (E.C. 3.2.1.1) to convert a concentrated starch suspension into a solution of soluble dextrins having different degrees of polymerization. The subsequent saccharification step at 56 °C requires the addition of amyloglucosidase (AMG) (E.C. 3.2.1.3) to hydrolyze these dextrins to produce glucose. Enzymatic hydrolysis of starch is usually inhibited by glucose production [22–24]. It has been shown that aqueous two-phase systems facilitate the partitioning of products to a separate phase from the enzyme and substrates [25–27]. Our previous studies [28,29] successfully demonstrated that PEO–PPO/$MgSO_4$ aqueous two-phase reactor system (ATPRS) enhanced the starch hydrolysis reaction by decreasing the glucose inhibition. Since both α-amylase and amyloglucosidase are strongly maintained in the bottom salt phase and starch is completely partitioned into the bottom phase, the enzyme is, in a certain sense, in a temporarily immobilized status. Due to the fact that glucose partitions between different phases during the hydrolysis process, this is especially advantageous in extractive reactions with severe product inhibition.

Since starch processing usually requires multiple high-temperature steps and the gelatinization, liquefaction, and saccharification steps are energy intensive, with energy accounting for a significant proportion of manufacturing cost, it is of great interest to use intrinsically thermostable enzymes that would increase starch-processing yields and decrease costs. The MJA1 enzyme is a novel, extremely thermostable amylase from the hyperthermophilic methanogen, *Methanococcus jannaschi* [30]. This novel enzyme would most likely result in more effective ATPRS-mediated starch hydrolysis. MJA1, which was cloned *Escherichia coli*, has been demonstrated to have optimal activity over 100 °C. Coupling a continuous ATPS bioreactor for enzymatic starch hydrolysis with the extremely stable hyperthermophilic MJA1 along with amyloglucosidase could result in reduced energy consumption by eliminating the traditional gelatinization step.

Despite the fact that numerous examples of extremophilic proteins that have been cloned and expressed into mesophilic hosts, no attempts to purify these extremophilic enzymes by using ATPS are found in the literature. As such, in this work, the use of ATPS in the purification of a cloned extremophilic protein away from the mesophilic proteins of the expression host was evaluated. The partitioning of a recombinant α-amylase (MJA1) and of total protein from *E. coli* cell-free extract was studied in PEO–PPO-2500/$(NH_4)_2SO_4$ and poly(ethylene glycol) (PEG)/$(NH_4)_2SO_4$ aqueous two-phase systems. These partitioning studies were performed such that suitable conditions for enzyme purification could be determined. The potential for enzyme purification in the novel thermoseparating systems was compared to that in more conventional PEG/salt aqueous two-phase systems.

2. Materials and methods

2.1. Chemicals

The chemicals, random copolymer of ethylene oxide and propylene oxide with molecular weight of 2500 (PEO–PPO-2500), poly(ethylene glycol) with three molecular weights 2000, 4000, and 8000, $(NH_4)_2SO_4$, K_3PO_4, and soluble starch were obtained from Aldrich (Milwaukee, WI, USA). Ampicillin was obtained from Sigma (St. Louis, MO, USA), and yeast extract and Bacto tryptone were from DIFCO (Detroit, MI, USA). All other chemicals were of reagent grade and obtained from Fischer Scientific (Hanover Park, IL, USA). All water used was purified using a Barnstead commercial deionization system (Boston, MA, USA).

Purified amyloglucosidase (E.C. 3.2.1.3) from *Aspergillus niger* (A-3042) were purchased from Sigma. Recombinant α-amylase, MJA1, was used in the form of an *E. coli* cell-free extract prepared as described previously [30].

2.2. Preparation of cell-free MJA1 enzyme solution

The extremely thermostable α-amylase, MJA1, was cloned on the plasmid, pMW200 [30] and expressed in *E. coli*. An isolated colony of transformed *E. coli* was cultured in Luria–Bertani (LB) medium (tryptone, 10 g l^{-1}; yeast extract, 5 g l^{-1}; NaCl, 5 g l^{-1}; pH 7.0) supplemented with 100 mg l^{-1} ampicillin and transferred to 500 ml of Terr–Broth (tryptone, 12 g l^{-1}; yeast extract, 24 g l^{-1}; glycerol, 4 g l^{-1}; KH_2PO_4, 2.31 g l^{-1}; K_2HPO_4, 12.54 g l^{-1}; pH 7.4) supplemented with 100 mg l^{-1} ampicillin. The culture was grown at 37 °C in a rotary shaker (200 rpm) for 15 h. The resulting biomass was harvested by centrifugation at 6000 × *g* (20 °C) for 30 min. The pellet obtained from centrifugation was suspended in sodium phosphate buffer (0.02 M; pH 7) and disintegrated using a 550 Sonic Dismembrator (Fisher Scientific). Disruption was performed at 0 °C for 5 min with 1 min intervals after each 1 min exposure. After removal of cell debris by centrifugation at 15 000 × *g* for 30 min, the protein extract was heated at 100 °C for 15 min, centrifuged at 15 000 × *g* for 10 min, and filtered through a 0.2 μm pore-size filter (Millipore, Bedford, MA, USA). The cell-free extract was stored at −20 °C until further use.

2.3. Partitioning and purification of enzymes in aqueous two-phase systems

Partitioning and purification were performed in 15 ml graduated centrifuge tubes by weighing out appropriate quantities of the polymer and salt stock solutions in sodium phosphate buffer (0.02 M; pH 7.0) at room temperature. The total compositions of the phase systems prepared are given in Tables 1 and 2. All concentrations were given in weight per weight percentage (w/wt.%). Enzyme (0.2 ml

Table 1
Laboratory-scale purification of MJA1 in PEO–PPO-2500/$(NH_4)_2SO_4$ aqueous two-phase systems

Phase system (w/wt.%)		TLL (w/wt.%)	Phase ratio	K_e	K_p	Specific activity (units mg^{-1} protein)	Recovery (%)	Purification factor
PEO–PPO	Salt							
20	7	22.71	2.00	2.26	0.21	6.62	81.88	2.89
20	10	26.16	1.25	7.49	0.32	7.59	90.35	3.31
20	12	32.48	0.80	5.03	0.34	9.34	80.11	4.08
20	15	45.20	0.56	2.72	0.52	7.63	60.18	3.33

Table 2
Laboratory-scale purification of MJA1 in PEG-2000/$(NH_4)_2SO_4$ aqueous two-phase systems

Phase system (w/wt.%)		Phase ratio	Specific activity (units mg^{-1} protein)	Recovery (%)	Purification factor
PEG	Salt				
20	15	1.00	6.65	83.21	2.90
10	18	0.54	7.48	87.17	3.27

of amyloglucosidase, A-3042, or 0.5–1.0 ml of cell-free MJA1 solution) was then added to the system. The systems thus prepared were vortex mixed for 1 min, centrifuged at 2000 × *g* for 5 min to speed up phase separation, and then allowed to equilibrate for 30 min. After the phase volumes were measured, top and bottom phases were withdrawn separately with pipettes. Samples of top and bottom phases were then diluted 20–50-fold with 0.02 M sodium phosphate buffer (pH 7.0) and analyzed for enzyme activity and protein concentration. The partitioning of enzyme and total protein can be described by their respective partition coefficients, K_e and K_p, defined as the ratio of the enzyme activity or protein concentration in the top phase to that in the bottom phase (Eq. (1)):

$$K = \frac{C_{top}}{C_{bottom}} \quad (1)$$

The phase volume ratio is defined as:

$$R = \frac{V_{top}}{V_{bottom}} \quad (2)$$

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

2.4. Assays

The amount of protein was measured with a Bio-Rad protein assay kit, using bovine serum albumin (BSA) as standard. MJA1 activity and amyloglucosidase activity were determined by the starch–iodine method [31] with some modifications. The assay solution consisted of 0.1 ml enzyme-containing sample and 1.0 ml starch solution (1%, w/v) in sodium phosphate buffer (0.02 M; pH 7.0). Assay tubes were incubated for 5 min at 37 °C for amyloglucosidase, and at 100 °C for MJA1 in a dry bath incubator. Appropriate blanks (without enzyme) were made in all cases. One unit of enzyme activity was defined as the amount of enzyme, which hydrolyzed 1 mg of starch min^{-1} under specified conditions.

Sugars produced were identified and quantified by a Shimadzu HPLC system (liquid chromatograph LC-10AT, diode array SPD-M10A, and RID 6A) equipped with a Rezex RCM column (300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA). The column was maintained at 85 °C using a Bio-Rad column heater. Samples were eluted isocratically with water at a flow rate of 0.4 ml min^{-1}. Maltooligosaccharides were purchased from Sigma as a standard.

3. Results

3.1. Partitioning and purification of MJA1 in polymer/salt ATPS

The discovery of thermophilic archaea has led to some of the most significant recent contributions to science. Characterization and application of archaeal enzymes has contributed considerably to understanding the evolution and engineering of protein stability. Further, the identification of new catalysts based on archaeal genome sequence data has been a significant advance over the last decade. The MJA1 enzyme is a novel, extremely thermostable amylase from the hyperthermophilic methanogen, *M. jannaschii*. The advent of extremely thermoactive and thermostable enzymes is significant to the enhancement of saccharification technologies. Coupling novel extremophilic catalysts with thermoseparating systems is of interest in process development. The MJA1 activity (expressed in cell-free extracts of recombinant *E. coli*) has a temperature optimum of 120 °C and a optimum pH between 5.0 and 8.0. This amylase also exhibits extreme thermostability at 100 °C with a half-life of 52 h [30]. This novel enzyme would most likely result in more effective ATPRS-mediated starch hydrolysis. The pursuit of advanced catalysts coupled with the recycling

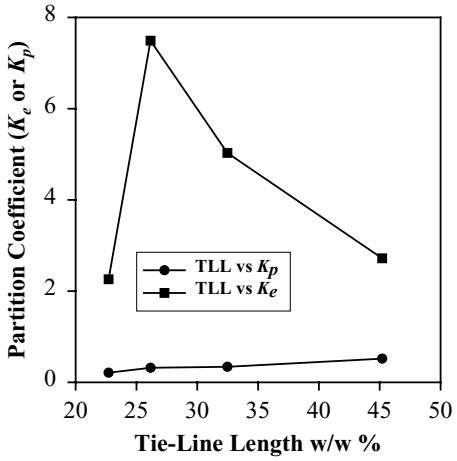

Fig. 1. Partitioning of MJA1 in PEO–PPO-2500/$(NH_4)_2SO_4$ ATPS: (●) total protein; (■) MJA1.

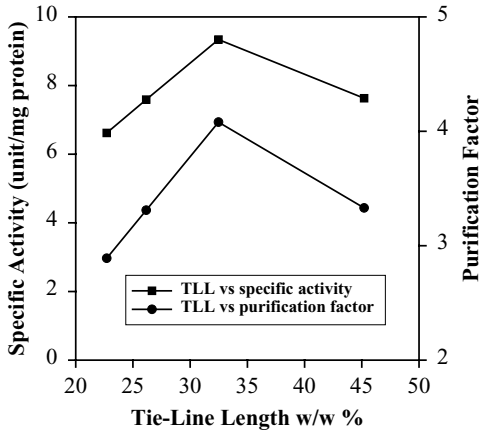

Fig. 2. Purification factor and specific activity of MJA1 in PEO–PPO-2500/$(NH_4)_2SO_4$ ATPS: (●) purification factor; (■) specific activity of MJA1.

of PEO–PPO polymer and enzyme in the process, will directly benefit the starch-processing industries. Along these lines, the partition behavior of MJA1 and total protein in PEO–PPO-2500/$(NH_4)_2SO_4$ and PEG-2000/$(NH_4)_2SO_4$ was experimentally investigated.

Fig. 1 shows the partitioning of MJA1 in the PEO–PPO-2500/$(NH_4)_2SO_4$ systems. Phase compositions of 20% polymer, and 7–15% salt were chosen based on the phase diagrams of PEO–PPO-2500/salt ATPS that has previously been measured [28]. It was observed that MJA1 shows a tendency to partition in the top polymer-rich phase with the partitioning coefficients ranging from 2.3 to 7.5. In addition, the partitioning of total protein is also shown in Fig. 1. The values of total protein concentration are accounted for in the fraction relative to the enzyme being extracted. As can be seen, the total protein in enzyme solution mainly partitioned to the bottom salt-rich phase. The partition coefficient increased from 0.21 to 0.52 when the total phase composition was increased from 20% polymer/7% salt ATPS to 20% polymer/15% salt ATPS (tie-line length is from 22.71 to 45.20%). These results indicated that the separation and purification of MJA1 could be achieved by ATPS since total protein and enzyme had different affinity towards different phases.

Although the partition coefficients of MJA1 in the PEO–PPO-2500/$(NH_4)_2SO_4$ system were not very high, partitioning yield (Y) of more than 90% in the top phase can be achieved, as given by Eq. (3) [32] by adjusting the phase volume ratio. By changing the total phase compositions lying on the same tie line, the phase volume can be adjusted while maintaining the top and bottom phase compositions:

$$Y\,(\%) = \frac{100}{(1 + (V_{\text{bottom}}/V_{\text{top}}))(1 + (1/K))} \quad (3)$$

Laboratory-scale aqueous two-phase purification experiments were performed to determine the separation efficiency and find optimized operation conditions for the MJA1 purification. The initial specific enzyme activity was 2.29 units mg^{-1} protein. Fig. 2 shows the specific activity and purification factor of MJA1 in PEO–PPO-2500/$(NH_4)_2SO_4$ ATPS. For example, for 20% PEO–PPO-2500/$(NH_4)_2SO_4$ and 10% PEO–PPO-2500/$(NH_4)_2SO_4$ ATPS (corresponding TLL of 26.16%), after equilibrium, the enzyme partitioned into the top polymer-rich phase with a volume ratio of 1.25. The total recovered activity in the top phase was 2.23 units as compared to the initially added 2.47 units. Up to 91% of the MJA1 loaded to this separator could be recovered in the PEO–PPO-rich top phase with specific activity of 7.59 units mg^{-1} protein as compared to the initial specific activity of 2.29 units mg^{-1} protein. The purification factor of 3.31 was thus obtained, where 72.73% of unwanted proteins were removed. Table 1 summarizes the results of such experiments indicating the percentage recovery of MJA1 by ATPS. As can be seen in Table 1, in any of the four ATPSs tests, an enzyme recovery of from 60 to 90% and a purification factor of 2.89–4.08 can be obtained in one extraction step. Thus, by employing ATPS and manipulating the system parameters, the required degree of purification can be obtained.

Previous studies showed that amylase partitioned to the bottom phase of polymer/$MgSO_4$ ATPS [28,29]. In addition, Gouveia and Kilikian used starch as a free bioligand to separate amyloglucosidase from clarified broth of *Aspergillus awamori* [33]. It was found that AMG and contaminants were partitioned in the top PEG phase without starch while the partition coefficient of AMG was decreased nine-fold without altering the partition of contaminants upon starch addition at 0.1% (w/w). As inspired by our previous results as well as the results from Gouveia and Kilikian, we further tested a separation/purification scheme consisting of two consecutive ATPS extraction steps: (1) a first extraction in PEO–PPO/$(NH_4)_2SO_4$ ATPS, where MJA1 is partitioned into the top polymer phase; and (2) followed by a second extraction in PEO–PPO-2500/$MgSO_4$ ATPS with the addition of starch, in hoping that this would make separation of MJA1 and recycling of PEO–PPO polymer possible.

However, the enzyme was still found in the top polymer phase even the addition of starch as free bioligand (data not shown). Thus, an attempt to further purify MJA1 by this two-step method did not improve the purification. Previous studies with PEO–PPO polymer recycle in amylolytic enzyme systems proved successful [34]. Further studies on thermoseparation with the MJA1 system may enable the development of appropriate recovery conditions.

The purification of MJA1 was also conducted in PEG-2000/$(NH_4)_2SO_4$ ATPS, which has a phase volume ratio of 0.54–1 and similar results were obtained as summarized in Table 2. Purification factors of 2.9–3.27 were achieved.

It should be noted that ATPS is not a total solution to separation, recovery and final purification of enzymes since usually it is not sufficiently selective to provide the extreme purity desired. In order to achieve final product purity specifications, the finishing steps need to be accomplished by other downstream techniques such as chromatography or electrophoresis, which are highly selective yet more expensive. Thus, ATPS has been recognized as a potential and powerful *primary* purification step in the overall enzyme/protein recovery/purification train. In ATPS, as these biphasic systems form specific environments suitable for maintaining enzymes in their native structure, phase-forming polymers or polymer and salt can be added to diluted enzyme extract containing the product to form ATPS so that the desired product is selectively partitioned into one of the phases, thus considerably reducing the volume to be handled in the following purification steps. For example, endo-polygalacturonase can be concentrated by 10-fold in thermoseparating UCON 50-HB-5100/$(NH_4)_2SO_4$ ATPS [18]. The cost of overall downstream processing can be reduced considerably. Use of ATPS can be considered as a rapid, simple and effective first step in the MJA1 purification procedure.

3.2. Partitioning of amyloglucosidase in polymer/salt ATPS

Partitioning of amyloglucosidase in PEG-300, PEG-600, PEG-1500, PEG-4000 and PEG-6000/potassium phosphate (a mixture of KH_2PO_4 and K_2HPO_4) has been reported [35]. Here we report the partitioning of amyloglucosidase in PEG-2000, PEG-4000, PEG-8000/potassium phosphate systems.

The variation of partition coefficient of AMG with increasing tie-line length is shown in Fig. 3. In all cases with PEG/potassium phosphate systems, AMG had affinity towards the bottom phases. The partition coefficients increased from 0.07 to 0.12 when the tie-line length was increased from 5.56 to 35.71% in the PEG-2000/potassium phosphate system.

The effect of molecular weight of PEG on the partition coefficient was studied. The partition coefficient decreased from 0.1 to 0.02 as the PEG molecular weight increased from 2000 to 4000. The phase-forming polymer molecular weight influences the partitioning both by altering the phase diagram (i.e., by influencing the composition of the phases) and by changing the interactions of polymer and enzyme in general. An increase in molecular weight of the phase-forming polymer in one phase will cause the biomaterial to partition more strongly into the other phase. Increasing the polymer molecular weight decreases the amount of solvent in the polymer-rich phase available to solubilize the enzyme and the salt. The effective enzyme and salt concentration in "free" solvent would therefore increase with molecular weight, causing the enzyme to partition more strongly into the salt-rich phase. However, the magnitude of this effect decreases with increasing polymer chain length and the AMG partitioning became complex when PEG molecular weight became large (for example, to PEG-8000). In PEG-8000/potassium phosphate ATPSs, the partition coefficient of AMG became higher than those in PEG-4000/potassium phosphate systems, while partition coefficients were located between those in PEG-2000 and PEG-4000 systems.

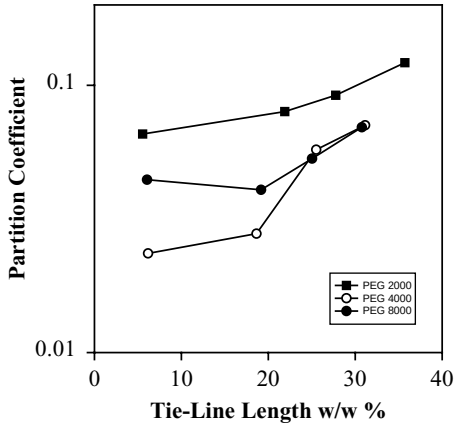

Fig. 3. Partitioning of AMG in PEG/potassium phosphate ATPS: (■) PEG-2000; (○) PEG-4000; (●) PEG-8000.

4. Conclusions

Aqueous two-phase extraction employing a PEO–PPO/salt and PEG/salt was used for the concentration and purification of MJA1. This technology could be successfully applied for primary purification of the extremophilic protein. The effects of TLL, molecular weight of polymer on the partitioning of AMG were also studied. AMG partitions to the bottom salt phase and therefore might be successfully immobilized for ATPS liquefaction operations.

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References

[1] P.A. Albertsson, Partition of Cell Particles and Macromolecules, third ed., Wiley, New York, 1986.
[2] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses, and Application to Biotechnology, Academic Press, Orlando, FL, 1985.
[3] H.D. Willauer, J.G. Huddleston, M. Li, R.D. Rogers, J. Chromatogr. B 743 (2000) 127.
[4] M. Li, H.W. Willauer, J.G. Huddleston, R.D. Rogers, Sep. Sci. Technol. 36 (5–6) (2001) 835.
[5] Z. Guo, M. Li, H.D. Willauer, J.G. Huddleston, G.C. April, R.D. Rogers, Ind. Eng. Chem. Res. 41 (10) (2002) 2535.
[6] H.-O. Johansson, G. Karlstrom, B. Mattiasson, F. Tjerneld, Bioseparation 5 (1995) 269.
[7] H.-O. Johansson, J. Peterson, F. Tjerneld, Biotechnol. Bioeng. 66 (1999) 247.
[8] N. Pietruszka, I.Y. Galaev, A. Kumar, Z.K. Brzozowski, B. Mattiasson, Biotechnol. Prog. 16 (2000) 408.
[9] P.A. Harris, G. Karlstrom, F. Tjerneld, Bioseparation 1 (1991) 237.
[10] P.A. Alred, A. Kozlowski, J.M. Harris, F. Tjerneld, J. Chromatogr. A 659 (1994) 289.
[11] P.A. Alred, F. Tjerneld, R.F. Modlin, J. Chromatogr. 628 (1993) 205.
[12] P.A. Alred, F. Tjerneld, A. Kozlowski, J.M. Harris, Bioseparation 2 (1992) 363.
[13] R.F. Modlin, P.A. Alred, F. Tjerneld, J. Chromatogr. A 668 (1994) 229.
[14] K. Berggren, H.-O. Johansson, F. Tjerneld, J. Chromatogr. A 718 (1995) 67.
[15] T. Farkas, H. Stalbrand, F. Tjerneld, Bioseparation 6 (1996) 147.
[16] M. Li, Z.Q. Zhu, L.H. Mei, Biotechnol. Prog. 13 (1997) 105.
[17] M. Li, Z.Q. Zhu, Y.T. Wu, D.Q. Lin, Chem. Eng. Sci. 53 (1998) 2755.
[18] M. Pereira, Y.-T. Wu, A. Venancio, J. Teixeira, Biochem. Eng. J. 15 (2) (2003) 131.
[19] J. Persson, A. Kaul, F. Tjerneld, J. Chromatogr. B 743 (2000) 115.
[20] H. Guzman-Maldonado, O. Paredes-Lopez, Crit. Rev. Food Sci. Nutr. 35 (1995) 373.
[21] P. Nigam, D. Singh, Enzyme Microb. Technol. 17 (1995) 770.
[22] K. Kusunoki, K. Kawakami, F. Shiraishi, K. Kato, M. Kai, Biotechnol. Bioeng. 24 (1982) 347.
[23] C. Akerberg, G. Zacchi, N. Torto, L. Gorton, J. Chem. Technol. Biotechnol. 75 (2000) 306.
[24] M. Fujii, Y. Kawamura, Biotechnol. Bioeng. 27 (1985) 260.
[25] M. Larsson, V. Arasaratnam, B. Mattiasson, Biotechnol. Bioeng. 33 (1989) 758.
[26] E. Andersson, A.C. Johansson, B. Hahn-Hagerdal, Enzyme Microb. Technol. 7 (1985) 333.
[27] M. Stredansky, L. Kremnicky, E. Sturdik, A. Feckova, Appl. Biochem. Biotechnol. 38 (1993) 269.
[28] M. Li, J.-W. Kim, T.L. Peeples, J. Biotechnol. 93 (2002) 15.
[29] M. Li, J.-W. Kim, T.L. Peeples, Biochem. Eng. J. 11 (2002) 25.
[30] J.-W. Kim, L.O. Flowers, M. Whitely, T.L. Peeples, Folia Microbiol. 46 (6) (2001) 467.
[31] Y.J. Yoo, J. Hong, R.T. Hatch, Biotechnol. Bioeng. 30 (1987) 147.
[32] H. Hustedt, K.H. Kroner, M.R. Kula, in: H. Walter, D.E. Brooks, D. Fisher (Eds.), Partitioning in Aqueous Two-Phase System: Theory, Methods, Uses, and Application to Biotechnology, Academic Press, New York, 1985, pp. 529–587.
[33] T. Gouveia, B.V. Kilikian, J. Chromatogr. B 743 (2000) 241.
[34] M. Li, J.-W. Kim, T.L. Peeples, Sep. Sci. Technol. 38 (2003) 2709.
[35] N.M. Minami, B.V. Kilikian, J. Chromatogr. B 711 (1998) 309.