Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Energy-Cost Reduction in Starch Processing Using Aqueous Two Phase Reactor Systems

Mian Li[a]; Jin-Woo Kim[b]; Tonya L. Peeples[a]
[a] Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, Iowa, USA [b] Department of Biological and Agricultural Engineering, The University of Arkansas, Fayetteville, Arkansas, USA

To cite this Article Li, Mian , Kim, Jin-Woo and Peeples, Tonya L.(2003) 'Energy-Cost Reduction in Starch Processing Using Aqueous Two Phase Reactor Systems', Separation Science and Technology, 38: 12, 2709 — 2724

To link to this Article: DOI: 10.1081/SS-120022568

URL: http://dx.doi.org/10.1081/SS-120022568

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

SEPARATION SCIENCE AND TECHNOLOGY
Vol. 38, Nos. 12 & 13, pp. 2709–2724, 2003

Energy-Cost Reduction in Starch Processing Using Aqueous Two Phase Reactor Systems

Mian Li,[1] Jin-Woo Kim,[1,2] and Tonya L. Peeples[1,*]

[1]Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, Iowa, USA
[2]Department of Biological and Agricultural Engineering, The University of Arkansas, Fayetteville, Arkansas, USA

ABSTRACT

Saccharification is an energy intensive process of particular industrial interest in agribusiness. Considerable improvements to the energy costs of processing can be made by the elimination of multiple high temperature steps during liquefaction. To facilitate technology development for more energy efficient saccharification, the effectiveness of thermoseparating polymer-based aqueous two-phase reactor systems (ATPRS) in the enzymatic hydrolysis of starch was investigated. The partition behavior of pure α-amylase, and a recombinant, thermostable α-amylase (MJA1) from the hyperthermophile, *Methanococcus jannaschii* and amyloglucosidase in PEO-PPO/salt aqueous two-phase systems was evaluated. All of the studied enzymes partitioned unevenly in these systems. Hydrolysis of soluble starch and corn starch into

---

[*]Correspondence: Tonya L. Peeples, Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, IA 52242, USA; Fax: +319-335-1415; E-mail: tonya-peeples@uiowa.edu.

DOI: 10.1081/SS-120022568 0149-6395 (Print); 1520-5754 (Online)
www.dekker.com

glucose by thermostable α-amylase and amyloglucosidase was performed in an ATPRS coupled with temperature-induced phase separation. Use of the ATPRS reduced the hydrolysis time to half of that for single-phase processing, thus reducing energy inputs. The hydrolysis time was 18 hours for 20% soluble starch with an amyloglucosidase concentration of 2.18 unit mL^{-1} in the aqueous system, but only 11 hours in aqueous two-phase reactor system. The extent of product inhibition is greatly reduced. These results reveal the potential for polymer-enhanced extractive bioconversion of starch as a practical technology.

INTRODUCTION

Starch hydrolysis is a key process in the production of foods, natural sweeteners, pharmaceuticals, detergents, paper, and animal feeds.[1,2] Two steps are usually required for enzymatic starch hydrolysis. In the first step, pretreatment of starch is initiated by a gelatinization step where a starch slurry is heated at 105°C followed by the liquefaction at 90°C by α-amylase (AMY) (E.C. 3.2.1.1) to randomly catalyze the hydrolysis of α-1,4-glucosidic linkage of α-1,4-glucans. The subsequent saccharification step at 56°C requires the addition of amyloglucosidase (AMG) (E.C. 3.2.1.3) to attack both exo-[1−4] and branch-point[1−6] linkages to produce glucose. The gelatinization, liquefaction, and saccharification steps are energy intensive, with energy accounting for a significant proportion of manufacturing cost. In addition, enzymatic hydrolysis of starch is usually inhibited by glucose production.[3−5] In conventional hydrolysis reactions, Akerberg et al.[4] demonstrated that the rate of starch hydrolysis by AMG was decreased by 58% at a starch concentration of 140 g dm^{-3}. This inhibition could be overcome by removing the product during the reaction through extraction of product into a liquid organic phase or the use of ultrafiltration membranes to separate reactants from products.[6,7] It has been shown that aqueous two-phase systems facilitate the partitioning of products to a separate phase from the enzyme and substrates.[8−11] Thus, an aqueous two-phase reactor system (ATPRS) may be well-suited for hydrolysis reactions in which the substrate has a high molecular weight, such as starch, and the product is known to inhibit the enzyme. Since the time required for hydrolysis is shorter due to the reduction of product inhibition, reduction in energy costs would be expected from the use of ATPRS.

An aqueous two-phase system (ATPS), formed when two incompatible polymers or one polymer and an inorganic salt are mixed at low concentrations, has low interfacial tension and is safe, nontoxic, nonflammable, and relatively environmentally benign as extraction media. Novel aqueous two-phase systems have been developed based on a wide variety of 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.[12–14] Among these, the ethylene oxide-propylene oxide random copolymer (PEO-PPO) 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 formed, which can be recycled to the primary phase system for a new extraction.[15] 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. Application of these biphasic systems has also been extended to paper pulp processing for delignification.[16,17]

In the present work, the use of ATPRS was explored for the potential to improve amylolytic starch processes. The enzymatic hydrolysis of soluble starch and corn starch as a model extractive bioconversion in the aqueous two-phase system was examined. The objective was to develop a cost-effective, environmentally acceptable processing system that consumes less energy than traditional processes.

EXPERIMENTAL

Materials

The chemicals, random copolymer of ethylene oxide and propylene oxide with molecular weight of 2500 (PEO-PPO-2500), $(NH_4)_2SO_4$, $MgSO_4$, soluble starch, and corn starch were obtained from Aldrich (Milwaukee, WI, 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 α-amylase (E.C. 3.2.1.1) from *Bacillus lichenoformis* (A-3403) and 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 *Escherichia coli* cell-free extract prepared as described previously.[18]

Hydrolytic Activity Assays

For starch processing experiments, α-amylase activity and amyloglucosidase activity were determined by the starch–iodine method[19] with some modifications. Detailed protocols are described elsewhere.[8] One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 mg of starch per minute under specified conditions.

Sugars produced by the enzymatic hydrolysis of starch were identified and quantified by a Shimadzu HPLC system (Liquid Chromatograph LC-10AT, Diode Array SPD-M10A, and RID 6A) equipped with an Aminex HPX-87H cation-exchange column (300 mm × 7.8 mm, Bio-Rad Laboratories, Richmond, CA, USA). The column was maintained at 50°C using a Bio-Rad column heater. Samples were eluted isocratically with 5-mM H_2SO_4 at a flow rate of 0.4 mL min^{-1}. Maltooligosaccharides were purchased from Sigma as a standard.

Partitioning of α-Amylase in Aqueous Two-Phase Systems

All partition experiments were carried out at room temperature. Phase systems were prepared 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). Enzyme (0.2 mL of α-amylase A-3403, 9820 units mL^{-1}, or 1.0 mL of cell-free MJA1 solution, 20 units mL^{-1}) was then added to the system. The systems were vortex mixed for 1 minute, centrifuged at 2000 × *g* for 5 minute to speed up phase separation, and then allowed to equilibrate for 30 minute. After the phase volumes were measured, top and bottom phases were withdrawn separately. Samples of top and bottom phases were then diluted 20- to 50-fold with 0.02-M sodium phosphate buffer (pH 7.0) and analyzed for enzyme activity.

Partition of enzyme between the two phases is expressed by the partition coefficient, K, defined as the ratio between the enzyme activities in the top and the bottom phases.

Starch Hydrolysis

Starch hydrolysis was carried out in aqueous two-phase and homogeneous one-phase systems at 300 rpm for comparison. Soluble starch or corn starch was used as substrate, and the system pH was initially adjusted to 7.0. After the starch was liquefied at 98°C for 1 hour by adding 30-ul AMY, it was loaded into ATPRS consisting of 20% PEO-PPO, and 4% $MgSO_4$. The process conditions were then adjusted to 55°C and pH 4.5 prior to addition of amyloglucosidase. Samples (1 mL) were taken at 10- to 60-minute intervals, and the reaction was stopped by quenching in ice and adding 1.0-mL HCl

(1.0 M). In the reaction system containing high starch concentration, after HCl addition, samples were heated in a boiling water bath for 10 minute to ensure the inactivation of the enzymes. Samples were further analyzed by HPLC as described previously.

Temperature-Induced Phase Separation and Partitioning of Glucose in Thermoseparating Aqueous Two-Phase Systems

After the starch hydrolysis, the top PEO-PPO copolymer phase was removed and put into a vessel. The container with this top phase was placed in a water bath at 66°C for 20 minute for a new two-phase system formation. After the new two-phase system had formed and separated into an aqueous top phase and a PEO-PPO-rich bottom phase, both phases were analyzed to determine glucose partitioning. The thermoseparated PEO-PPO-rich phase was used in further cycles of starch hydrolysis by the addition of fresh enzyme, starch and salt to form a new polymer/salt ATPRS.

RESULTS AND DISCUSSION

Partitioning of Pure α-Amylase in Polymer/Salt ATPS

Two types of PEO-PPO/salt ATPSs, PEO-PPO-2500/$(NH_4)_2SO_4$ and PEO-PPO/$MgSO_4$, were used. Phase diagrams of the two systems can be obtained elsewhere.[8] Figure 1 shows the partitioning of α-amylase in the PEO-PPO-2500/$(NH_4)_2SO_4$ system. It was found that increasing the degree of phase divergence of the systems, i.e., tie line length (TLL), as defined in eq. (1), generally increased the partition coefficients of α-amylase from $K = 1.1$ to $K = 19.4$. α-Amylase shows a strong tendency to partition in the top polymer phases. This indicates that the concentration process of α-amylase with higher recovery can be easily achieved at a higher TLL.

$$TLL = \left[\Delta polymer^2 + \Delta Salt^2\right]^{0.5} \tag{1}$$

In contrast to PEO-PPO/$(NH_4)_2SO_4$ system, α-amylase partitions more to the bottom salt phase in PEO-PPO/$MgSO_4$ systems. Figure 2 illustrates that the partition coefficients of α-amylase can be selectively increased by the addition of NaCl. α-Amylase can be transferred almost entirely from the bottom salt phase to the top polymer phase by increasing the NaCl

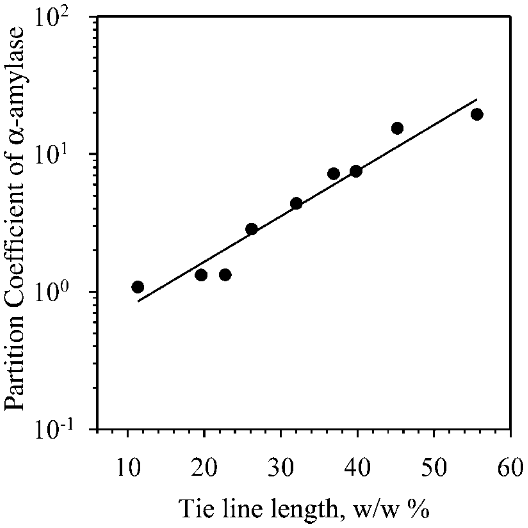

Figure 1. Tie line length effect on partitioning of α-amylase in PEO-PPO-2500/$(NH_4)_2SO_4$ system.

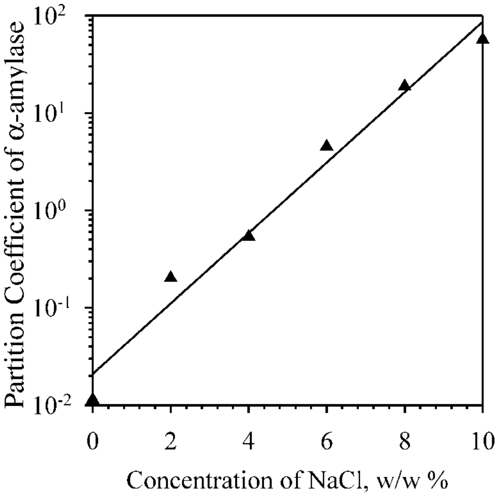

Figure 2. The effect of NaCl on partitioning of α-amylase in PEO-PPO-2500/$MgSO_4$ system. The system composition was 20% (w/w) PEO-PPO-2500 and 4% (w/w) $MgSO_4$.

concentration up to 10% (w/w) (NaCl does not salt out PEO-PPO polymer under the experimental conditions). At low concentration of NaCl (<2%, w/w), α-amylase partitioned mainly to the bottom phase ($K < 0.2$). At intermediate concentration of NaCl (2–5%, w/w), α-amylase partitioned evenly between the phases ($0.2 < K < 1$). When the concentration of NaCl was higher than 5%, α-amylase partitioned preferentially to the top phase. At 10% (w/w) NaCl, K value of 56 was achieved. A similar observation has been reported for PEG/salt ATPS.[20] These effects are due to the weak electrical potential difference over the phase interface created by orientation of ions at the interface.[20] Changing the salt composition causes a change in the potential difference, which in turn, influences the partitioning of α-amylase. High-ionic strength also improves the movement of the enzyme to other phase by electrostatic repulsion effects.

Starch Hydrolysis in ATPRS

Batch enzymatic starch hydrolysis of 20% soluble starch and corn starch by α-amylase and amyloglucosidase was carried out in an aqueous solution as a reference (without any addition of polymer and salt) and in an aqueous two-phase system. Table 1 shows the experimental conditions for starch hydrolysis in ATPRS. The reaction medium contained 20% PEO-PPO-2500 and 4% $MgSO_4$, and these concentrations correspond to a tie line length of 21.5 w/w%. The partition coefficients were 0.02 for α-amylase and 0.03 for amyloglucosidase, respectively. 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.

Table 1. Experimental conditions for enzymatic hydrolysis of starch in PEO-PPO-2500/$MgSO_4$ ATPRS.

	Partition coefficient
AMY	0.02
AMG	0.03
Starch	—
Maltose	0.37
Glucose	0.45

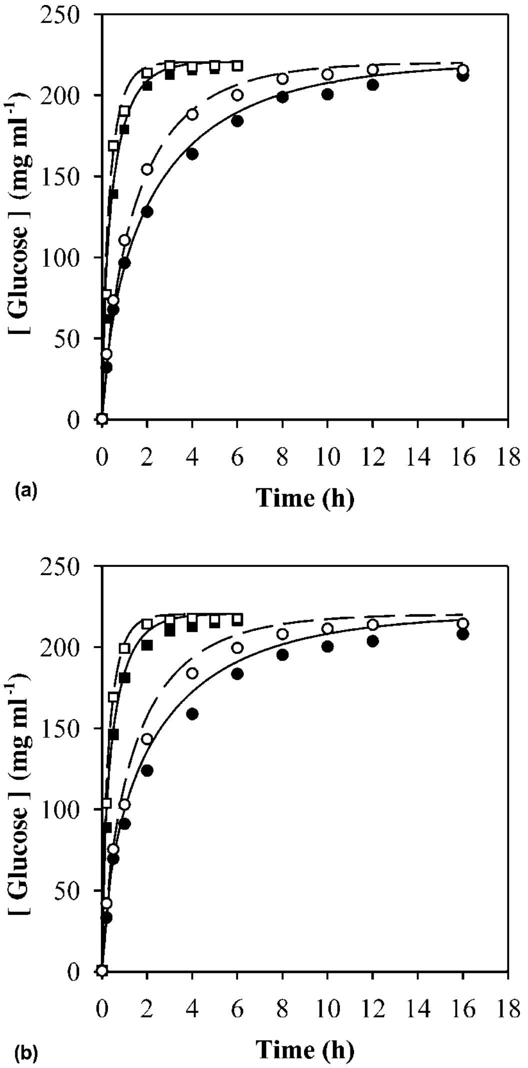

Figure 3. Comparison of the glucose profiles for the batch enzymatic hydrolysis of starches in the aqueous two-phase reactor systems with those in equivalent homogeneous one-phase systems. Closed symbols: control run; open symbols: PEO-PPO-2500/$MgSO_4$ aqueous two-phase system. The system composition was 20% (w/w) PEO-PPO-2500 and 4% (w/w) $MgSO_4$. (●, ○) 2.18 unit/mL; (■, □) 10.90 unit/mL. (A) 20% soluble starch; (B) 20% corn starch.

Figure 3 compares the glucose produced progress curve in the ATPRS system with that in the homogeneous one-phase reference system. Under the given conditions, the use of ATPRS reduced the hydrolysis time to almost half of that for the single-phase processing. For example, the hydrolysis reaction was completed in 2 hours in ATPRS; while taking 4 hours in a one-phase water system with 10.90 unit mL^{-1} amyloglucosidase. In addition, a conversion of 90% was achieved in 11 hours in the aqueous two-phase system; while similar conversion was reached in 18 hours with the single-phase aqueous solution with 2.18 unit mL^{-1} amyloglucosidase. It appears that the presence of PEO-PPO/salt enhanced the starch hydrolysis reaction by decreasing the glucose inhibition. In industrial-scale starch hydrolysis, 150-psi steam is usually used to maintain the temperature for saccharification.[21] The reduced hydrolysis time resulting from the use of ATPRS will clearly reduce the energy cost.

Laboratory-Scale Polymer Recovery After Thermoseparation

Significant savings in energy cost may be made by using ATPRS. However, this was achieved at the cost of the addition of polymer and salt to form two-phase systems. To make the process economically feasible, it is necessary to recycle the PEO-PPO to minimize chemical consumption. To address this issue, it is important to investigate the robustness of the two-phase system with recycled PEO-PPO copolymer in starch hydrolysis processing.

A simple scheme with polymer recycling was proposed, as shown in Fig. 4. This process consists of an ATPRS reaction followed by polymer recycling after temperature-induced separation. Partition coefficients of glucose in thermoseparating PEO-PPO-2500/water systems were measured. All of the glucose partitioned into the aqueous phase (data not shown). Thus, after the starch hydrolysis, glucose was easily recovered in the polymer-free aqueous phase in the PEO-PPO/water two-phase system without loss due to the temperature-induced phase separation. Hydrolysis, followed by temperature-induced polymer and product recovery, was performed for three cycles. For each reaction, fresh enzyme, salt, and substrate were added to the recycled PEO-PPO to form new ATPRS with almost the original total phase compositions, assuring the same tie line length and partitioning of glucose.

Table 2 shows that, in all experiments, yields of about 210 mg mL^{-1} of glucose were observed. The productivity was relatively constant during the recycling of PEO-PPO copolymer, indicating that the recycling of thermoseparating PEO-PPO polymer would not affect the efficiency of starch

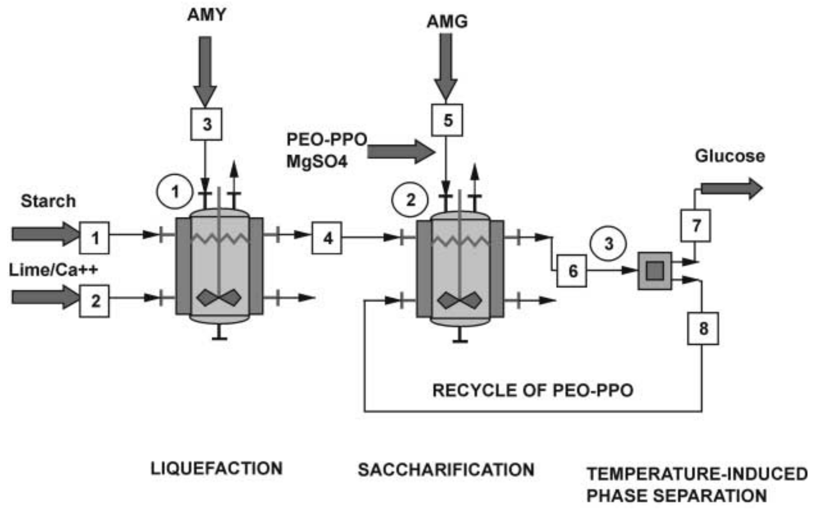

Figure 4. Scheme for enzymatic starch hydrolysis coupled with PEO-PPO recovery.

hydrolysis. Therefore, the recycling of copolymer facilitated the design of cost-efficient and robust aqueous two-phase system for large-scale starch hydrolysis. All of these factors contribute to lowering the operational costs. Since the top copolymer phase in the primary two-phase system contained a low concentration of salt, this would lead to an increase in the recovery of copolymer after thermoseparation.[15] Persson et al.[15] extensively investigated the PEO-PPO recovery and recycling in ATPS. It was found that the temperature, time, bottom phase polymer, salt, and protein addition affected the recovery of PEO-PPO copolymer. The recovery of PEO-PPO copolymer

Table 2. Glucose production by enzymatic starch hydrolysis during PEO-PPO recycling.

	Glucose concentration (mg/mL)
ATPRE using PEO-PPO and $MgSO_4$ stock solution	214.51
ATPRE using first recycling PEO-PPO	213.87
ATPRE using second recycling PEO-PPO	212.16
ATPRE using third recycling PEO-PPO	212.34

could be increased to 90% by optimizing the temperature and time for thermoseparation. The data from our current study are consistent with these polymer recovery values.

Energy Analysis of ATPRS

The kinetic model of starch hydrolysis allowing for competitive product inhibition can be given by[22]:

$$\frac{dP}{dt} = \frac{1.1 V_{\max} S}{K_m(1 + \frac{P}{K_i}) + S} \tag{2}$$

where P is product concentration (mg mL^{-1}), V_{max} (mg mL^{-1} min^{-1}) the maximum rate of reaction, K_m (mg mL^{-1}) the Michaelis–Menten constant, and K_i (mg mL^{-1}) the inhibition constant. Enzyme inactivation is assumed to be described by a first-order process. The synergistic effects of using AMY together with AMG for starch hydrolysis can be evaluated by using eq. 2. For the dual enzyme hydrolysis, changes in the rate of substrate consumption reveal apparent kinetic parameter values (K_m^{APP}, V_{max}^{APP}, and K_i^{APP}) that reflect the changes in the overall enzymatic catalysis.[23] Values for the kinetic parameters can be found in previous research.[22]

Reaction rates in an ATPRS relative to those in a homogeneous one-phase reactor can be simulated by including thermodynamic partitioning data in classical kinetic models.[22] Figure 3 shows the simulation results of concentration changes of glucose for the batch enzymatic hydrolysis of starch in the ATPRS with those in equivalent homogeneous one-phase systems. The model predictions were in good agreement with experimental data in all cases.

A model of a batch enzymatic hydrolysis of starch was constructed by incorporating the kinetic model into ChemCAD 5.0 for energy analysis. The scale-up (28,157 kg/hr corn starch feedstock, data from Ref.[21]) and energy analyses were performed to obtain a baseline for comparison of the homogeneous one-phase reaction with the ATPRS operation. The results show a potential for energy savings of 25% in the saccharification step. Furthermore, since the enzyme is in a temporarily immobilized status in the bottom salt phases, it is also possible to recycle the enzyme by combining the recycled PEO-PPO polymer with this original bottom phase, thus cutting the cost of amyloglucosidase.

For the work presented here, fresh bottom phase was used to decouple the effects of polymer recycle on starch hydrolysis from residual glucose inhibition. Similar results are expected when using recycled PEO-PPO with the recycled bottom salt phase, with addition of a smaller amount of fresh enzyme as determined by deactivation kinetics. Although, this enzyme recycling was not studied experimentally in the current study, enzyme recovery from cycle to cycle was predicted from thermodynamic and kinetic models. From partition data, enzyme has been shown to remain in the bottom salt phase.[8] Analysis of enzyme stability (deactivation kinetics) shows that AMG under the process operating conditions had a half-life of 3 days. Based on these numbers, for the succession of cycles, the percentage of the original enzyme activity recovered is 90%, 70%, and 60 to 65%, respectively.

MJA1 for Potential Starch Hydrolysis for Energy Cost Reduction

Starch processing usually requires multiple high temperature steps and thus is energy intensive. Therefore, 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 jannaschii*. The enzyme activity (expressed in cell-free extracts of recombinant *E. coli*) had a temperature optimum of 120°C and a pH optimum between pH 5.0 and pH 8.0. The amylase also exhibited extreme thermostability at 100°C with a half-life of 52 hours.[18] The partition coefficients of MJA1 in PEO-PPO-2500/$MgSO_4$ were measured, as shown in Table 3. The MJA1 partition coefficients were tenfold higher than those of *Bacillus* amylases. Possible explanations for the difference in the partitioning behavior relate to (1) the different biochemical characteristics of the enzymes, and/or (2) NaCl and other impurities possibly present in the MJA1 enzyme solution. The *M. jannaschii* amylase is a member of the family-57 glycosyl hydrolases unlike the other amylases, which belong to family-13.[24] Proteomic analysis[25] of enzyme sequences reveals predicted similarities and differences.

Table 3. Partitioning coefficients for MJA1 in the PEO-PPO-2500/$MgSO_4$ two-phase system.

PEO-PPO-2500 w/w%	$MgSO_4$ w/w%	Tie line length w/w%	K
20	4	21.48	0.23
20	6	31.93	0.45
20	8	39.30	0.76

Both enzymes have similar molecular weights (ca. 55,000 Da) and acidic pI (5.28 for MJA1 and 6.31 for A3403, respectively). The most striking differences from primary sequence data appear in the number of charged residues and the aliphatic indexes of the proteins. MJA1 has more charged residues and a higher-predicted aliphatic index. Alternatively, cell extract buffer components could alter the enzyme–polymer–salt interactions that govern phase distribution. Because the MJA1 enzyme was used in the form of a cell-free extract, further study is needed to distinguish the impact of impurities from biochemical driving forces that direct partitioning.

This novel enzyme would most likely result in more effective ATPRS-mediated starch hydrolysis. MJA1 was demonstrated to have optimal activity over 100°C. The pursuit of advanced catalysts, coupled with the recycling of PEO-PPO polymer and enzyme in the process, will make improved starch processing technologically and economically feasible.

CONCLUSION

Corn-based processes should be designed to minimize both energy and chemical consumption. The effectiveness of thermoseparating polymer-based aqueous two-phase reactor systems (ATPRS) in the enzymatic hydrolysis of starch was investigated. A marked reduction in the use of energy resulted from the use of ATPRS, which reduced the hydrolysis time to half of that for single-phase processing. The extent of glucose inhibition of enzymatic hydrolysis was reduced by partitioning between different phases during the hydrolysis process. This enhancement, coupled with the ease of PEO-PPO copolymer recycle through temperature-induced separation, makes aqueous two-phase reactor systems for enzymatic starch hydrolysis a technically feasible and environmentally benign alternative in the starch processing industry.

NOMENCLATURE

K	partition coefficient
K_i	the inhibition constant (mg mL^{-1})
K_m	the Michaelis–Menten constant (mg mL^{-1})
P	product concentration (mg mL^{-1})
PEO-PPO-2500	random copolymer of ethylene oxide and propylene oxide with molecular weight of 2500

S	substrate concentration (mg mL^{-1})
TLL	tie line length
V_{max}	maximum rate of reaction (mg mL^{-1} min^{-1})

ACKNOWLEDGMENTS

This work was supported in part by NSF Career grant BES 9702588 and by NASA NAG8-1593. Special thanks to Henry A. Terc for assistance with the CHEM CAD analysis.

REFERENCES

1. Guzman-Maldonado, H.; Paredes-Lopez, O. Amylolytic enzymes and products derived from starch: a review. Crit. Rev. Food Sci. Nutr. **1995**, *35*, 373–403.
2. Nigam, P.; Singh, D. Enzyme and microbial systems involved in starch processing. Enzyme Microb. Technol. **1995**, *17*, 770–778.
3. Kusunoki, K.; Kawakami, K.; Shiraishi, F.; Kato, K.; Kai, M. A kinetic expression for hydrolysis of soluble starch by glucoamylase. Biotechnol. Bioeng. **1982**, *XXIV*, 347–354.
4. Akerberg, C.; Zacchi, G.; Torto, N.; Gorton, L. A kinetic model for enzymatic wheat starch saccharification. J. Chem. Tech. Biotechnol. **2000**, *75*, 306–314.
5. Fujii, M.; Kawamura, Y. Synergistic action of α-amylase and glucoamylase on hydrolysis of starch. Biotechnol. Bioeng. **1985**, *27*, 260–265.
6. Lopez, J.L.; Matson, S.L.; Stanley, T.J.; Quinn, J.A. Liquid–liquid extractive membrane reactors. In *Extractive Bioconversions*; Mattiasson, B., Holst, O., Eds.; Marcel Dekker: New York, 1991; 27–66.
7. Roffler, S.R.; Blanch, H.W.; Wilke, C.R. Insitu extractive fermentation of acetone and butanol. Biotechnol. Bioeng. **1988**, *31*, 135–143.
8. Li, M.; Kim, J.-W.; Peeples, T.L. Amylase partitioning and extractive bioconversion of starch using thermoseparating aqueous two-phase systems. J. Biotechnol. **2002**, *93*, 15–26.
9. Larsson, M.; Arasaratnam, V.; Mattiasson, B. Integration of bioconversion and downstream processing: starch hydrolysis in an aqueous two-phase system. Biotechnol. Bioeng. **1989**, *33*, 758–766.

10. Andersson, E.; Johansson, A.C.; Hahn-Hagerdal, B. α-Amylase production in aqueous two-phase systems with Bacillus subtilis. Enzyme Microb. Technol. **1985**, *7*, 333–338.
11. Stredansky, M.; Kremnicky, L.; Sturdik, E.; Feckova, A. Simultaneous production and purification of *Bacillus subtilis* α-amylase. Appl. Biochem. Biotechnol. **1993**, *38*, 269–276.
12. Johansson, H.-O.; Karlstrom, G.; Mattiasson, B.; Tjerneld, F. Effects of hydrophobicity and counter ions on the partitioning of amino acids in thermoseparating UCON-water two-phase systems. Bioseparation **1995**, *5*, 269–279.
13. Johansson, H.-O.; Peterson, J.; Tjerneld, F. Thermoseparating water/-polymer system: a novel one-polymer aqueous two-phase system for protein purification. Biotechnol. Bioeng. **1999**, *66*, 247–257.
14. Pietruszka, N.; Galaev, I.Y.; Kumar, A.; Brzozowski, Z.K.; Mattiasson, B. New polymers forming aqueous two-phase polymer systems. Biotechnol. Prog. **2000**, *16*, 408–415.
15. Persson, J.; Kaul, A.; Tjerneld, F. Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide-propylene oxide copolymers. J. Chromatogr. B **2000**, *743*, 115–126.
16. Willauer, H.D.; Huddleston, J.G.; Li, M.; Rogers, R.D. Investigation of aqueous biphasic systems for the separation of lignins from cellulose in the paper pulping process. J. Chromatogr. B **2000**, *743*, 127–135.
17. Li, M.; Willauer, H.D.; Huddleston, J.G.; Rogers, R.D. Temperature effects on polymer-based aqueous biphasic extraction technology in the paper pulping process. Sep. Sci. Technol. **2001**, *36* (5&6), 835–847.
18. Kim, J.-W.; Flowers, L.O.; Whitely, M.; Peeples, T.L. Biochemical confirmation and characterization of the family-57-like α-amylase of *Methanococcus jannaschii*. Folia Microbiol. **2001**, *46* (6), 467–473.
19. Yoo, Y.J.; Hong, J.; Hatch, R.T. Comparison of amylase activities from different assay methods. Biotechnol. Bioeng. **1987**, *30*, 147–151.
20. Schmidt, A.S.; Ventom, A.M.; Asenjo, J.A. Partitioning and purification of α-amylase in aqueous two-phase systems. Enzyme Microb. Technol. **1994**, *16*, 131–142.
21. *Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks*, National Renewable Energy Laboratory Technical Report, NREL/TP-580-28893, October 2000.
22. Li, M.; Kim, J.-W.; Peeples, T.L. Kinetic enhancement of starch bioconversion in thermoseparating aqueous two-phase reactor systems. Biochem. Eng. J. **2002**, *11*, 25–32.

23. Fujii, M.; Kawamura, Y. Synergistic action of α-amylase and glucoamylase on hydrolysis of starch. Biotechnol. Bioeng. **1985**, *27*, 260–265.
24. Appel, R.D.; Bairoch, A.; Hochstrasser, D.F. A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server. Trends Biochem. Sci. **1994**, *19*, 258–260.
25. Janecek, S. Sequence of archaeal *Methanococcus jannaschii* alpha-amylase contains features of families 13 and 57 of glycosyl hydrolases: a trace of their common ancestor? Folia Microbiol. **1998**, *43*, 123–128.