Integration of Bioconversion and Downstream Processing: Starch Hydrolysis in an Aqueous Two-Phase System

Mats Larsson, Vasanthy Arasaratnam, and Bo Mattiasson
Department of Biotechnology, Chemical Center, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

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Integration of bioconversion and the first step(s) of downstream processing can be used as a means to increase the productivity of bioprocesses. This integration also gives the possibility to run the bioconversion in a continuous mode. We demonstrate the use of an aqueous two-phase system in combination with ultrafiltration to accomplish this. Conversion of native starch to glucose by α-amylase and glucoamylase was carried out in an aqueous two-phase system in connection with a membrane filtration unit. In this way, a continuous stream of glucose in buffer solution was obtained; the phase-forming polymers as well as the starch-degrading enzymes were recycled, and clogging of the ultrafiltration membrane was avoided. The process was carried out continuously in a mixer–settler reactor for a period of 8 days. The enzyme activities in the top and bottom phases and in the mixing chamber were monitored intermittently throughout the experiment. The optimum pH, temperature, and ionic strength for the activity of the enzyme mixture were determined. The settling time of phase systems containing varying amounts of PEG, crude dextran, and solid starch was studied. The activity and stability of enzyme mixtures was studied both in buffer medium and in the medium containing the polymers. The enzymes were found to be more active and stable in medium containing polymers than in the buffer solutions.

INTRODUCTION

Starch hydrolysis is one of the major enzyme-catalyzed processes in industrial operation today. As it deals with the degradation of a macromolecular or even particulate substrate, the use of soluble enzymes has been preferred. Since the enzymes used are rather inexpensive, it has been possible to get the process in operation under conditions that are economically acceptable in spite of the consumption of enzymes. Because the soluble enzymes are not recovered, the operating catalyst density is kept rather low, thereby keeping the reaction time long.

These characteristics, i.e., consumption of enzymes and long reaction times, are general features of processes when dealing with macromolecular or particulate substrates.

In earlier reports we have demonstrated the feasibility of carrying out enzymatic conversions in aqueous two-phase systems, performing the process in one of the phases and continuously extracting the product to the other phase.[1,2] This could also provide a convenient system which uses the soluble enzymes economically for the degradation of macromolecular substrates.

This article deals with enzymatic degradation of native starch to glucose using continuous extraction in an aqueous two-phase system and an integrated ultrafiltration step.

MATERIALS AND METHODS

Polyethylene glycol 20,000 (PEG 20M) from Union Carbide, crude dextran, a gift from Sorigona (Sweden), and waxy maize starch, a gift from Stadex (Sweden) were used in this experiment. α-Amylase (Termamyl 60 L; activity 67.5 KNU/g) and glucoamylase (Spiritamylase 150 L; activity 159.9 AGU/mL) were generously supplied by Novo (Denmark). A Millipore Pellicon ultrafiltration unit and membranes (PTGC, cutoff 10,000) were used for the separation of glucose from the polymers.

Enzyme Assays

The enzyme activities were determined according to Novo's analytical methods AF 9 and AF 22. One KNU is defined as the amount of enzyme which breaks down 5.26 g/h starch (Merck Amylum Solubile Erg.B.6 Batch 9947275) at 37°C and pH 5.6, whereas one AGU is defined as the amount of enzyme which hydrolyzes 1 μmol/min maltose at pH 4.3 and 25°C.

The activity of glycoamylase during stability and reactor studies was determined under conditions as described in the preceding, and that of α-amylase was measured by using Phadebas test tablets (Pharmacia, Sweden).

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PEG Assay

The PEG 20M was determined by measuring the absorbance at 280 nm. By this method it was possible to determine concentrations down to 0.05 g/l.

Separation Time for Phase Systems

The time for phase separation was studied for a series of two-phase systems, each containing the polymers in a constant ratio.

Equal volumes of aqueous solutions of 10% (w/w) PEG 20M and 6% (w/w) crude dextran were mixed. The mixture was diluted with varying amounts of water to 10 mL and mixed well. Native starch (1 g) was added to these polymer mixtures, and the time needed for the phases to separate was noted.

Effect of Ratio Between Activities of α-Amylase and Glucoamylase on Glucose Production

Solid starch (16% w/w) was hydrolyzed at 55°C in 0.1*M* acetate buffer, pH 5.0, by different ratios and amount of α-amylase (diluted with 0.1*M* acetate buffer, pH 5.0) and glucoamylase (diluted with the same buffer). The reaction mixture was continuously mixed. The glucose produced was determined by high-performance liquid chromatography (HPLC) using an ion exchange column (Sugar Pak 1, Waters).

Batch Experiments

Starch, 10% (w/w), was hydrolyzed by a mixture of α-amylase (0.2 KNU/mL) and glucoamylase (0.38 AGU/mL) at 55°C in an aqueous two-phase system and in 0.1*M* acetate buffer, pH 5.0. The two-phase system contained 5% (w/w) PEG and 3% (w/w) crude dextran dissolved in 0.1*M* acetate buffer, pH 5.0. The product formed was monitored intermittently.

Effect of pH, Ionic Strength, and Temperature on Enzyme Activity

pH

The activities of the enzyme mixture were studied at 55°C at different pH values in the range 2.6–8.0 (pH 2.6–7.0 citrate phosphate buffer and pH 7.6–8.0 phosphate buffer was used), both in buffer and in media containing the polymers.

Ionic Strength

The activities of the enzyme mixture in varying ionic strengths (0.005–0.4*M*) of acetate buffer with and without the polymers were compared at 55°C.

Temperature

Studies were carried out to determine the optimum temperature for the activity of the enzyme mixture in 0.05*M* acetate buffer, pH 5.0, with and without the polymers in the media.

In all the preceding cases the activities of the enzyme mixtures were measured and represented in terms of the product (glucose) formed.

Enzyme Stability

Mixtures of α-amylase and glucoamylase were incubated at 50°C in 10% (w/w) glucose syrup prepared in buffers with pH ranging from 4.6 to 5.6 in the presence as well as absence of polymers. Samples were withdrawn every 24 h and the activities of the enzymes were determined individually as mentioned in the preceding.

Enzyme Reactor Experiment

Equal volumes of 10% (w/w) PEG 20M solution and 6% (w/w) crude dextran solution in 0.05*M* acetate buffer, pH 5.0, containing 70 ppm Ca^{2+} were mixed in an enzyme reactor (Fig. 1) having a total volume of 3400 mL. The phase system was allowed to separate in the settling part of the reactor, and the temperature was maintained at 50°C.

A mixture of concentrated α-amylase (11.5 mL) and glucoamylase (8.5 mL) was added to the mixing chamber while the top and bottom phases were recirculated. The top phase in the right chamber (Fig. 1) was pumped through the ultrafiltration unit (Millipore Pellicon) containing a polysulfone membrane with a nominal molecular weight limit of 10,000 NMWL (PTGC, Millipore). The surface area of the membrane was 0.47 m^2. The retentate was returned to the same chamber. The filtrate was collected at a rate of 6 mL/min. Starch suspension (10% w/w) in 0.05*M* acetate buffer, pH 5.0, containing 70 ppm Ca^{2+} (added in the form of calcium acetate) was pumped into the mixing chamber. The addition of substrate was controlled by an inductive sensor fixed to the wall of the reactor. After the equilibration of the enzyme mixture in the reactor, samples from the top and the bottom phases and from the mixing chamber were taken for enzyme activity measurements. The activities of both the enzymes were studied individually throughout the operation. The glucose produced was quantified by HPLC.

The same experiment was repeated using 30% (w/w) starch suspension as the substrate.

RESULTS AND DISCUSSION

Effect of Ratio Between Activities of α-Amylase and Glucoamylase on Glucose Production

The synergistic effect of α-amylase and glucoamylase on the hydrolysis of starch is quite complex, especially

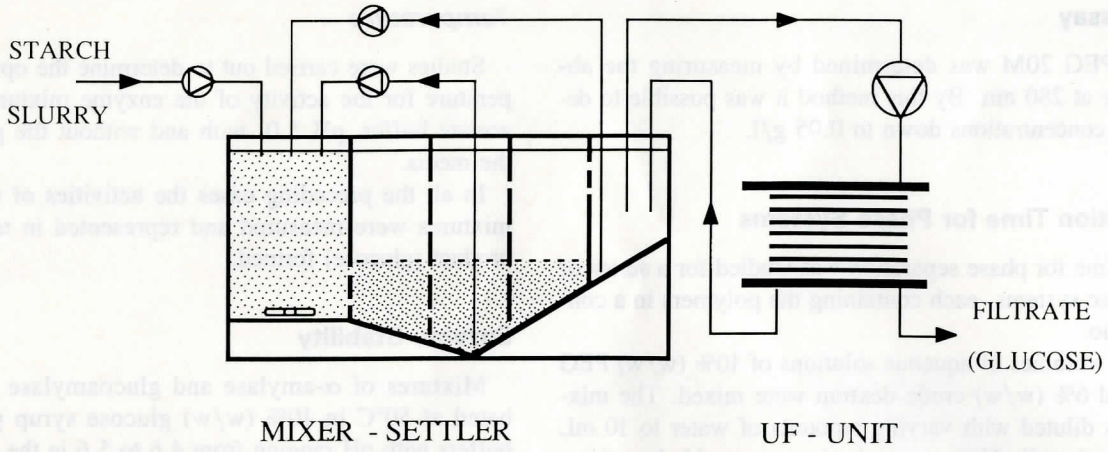

Figure 1. Experimental setup. Flow in pumps recirculating top and bottom phase, 25 mL/min. Pump feeding the mixer part with starch slurry controlled by level in reactor.

when using dissolved starch as a substrate.[3,4] With solid starch as a substrate the complexity is somewhat reduced due to lower concentration of competitive inhibitory oligosaccharides containing 1–6 glucose units.[5–7]

The additive effect of increasing amounts of glucoamylase to a fixed amount of α-amylase is shown in Figure 2.

As can be seen from Figure 2, a suitable ratio between glucoamylase and α-amylase for the optimal hydrolysis of solid starch is 0.8 AGU/KNU. This ratio was also found valid when performing the hydrolysis in the aqueous two-phase system. It is notable that the activity of the enzymes when used separately is relatively low compared to the synergistic effect. These observations are interpreted to be the result of the enzymatic activity of α-amylase (endo-amylase) splitting up the starch molecules at the surface of the starch granules to dextrins, and these dextrins are then degraded into glucose units by glucoamylase (exoamylase).

Effect of pH, Ionic Strength, and Temperature on Process

To achieve optimal conditions with respect to pH, ionic strength, and temperature for the mixed enzymes, experiments were performed using the enzyme ratio of 0.8 AGU/KNU (Figs. 3–5).

The pH activity profile for the enzymes in aqueous two-phase systems and in buffer showed the same pH optimum (Fig. 3). The same observation was made when studying the influence of ionic strength (Fig. 4).

The enzyme mixture showed optimal activity at 65°C in 0.025*M* acetate buffer, pH 4.5, both in a buffer system and the two-phase system. In all the cases the relative activities were higher in the phase system at optimal conditions. The broader temperature acceptance in polymer media is similar to immobilized preparations of glucoamylase.[8]

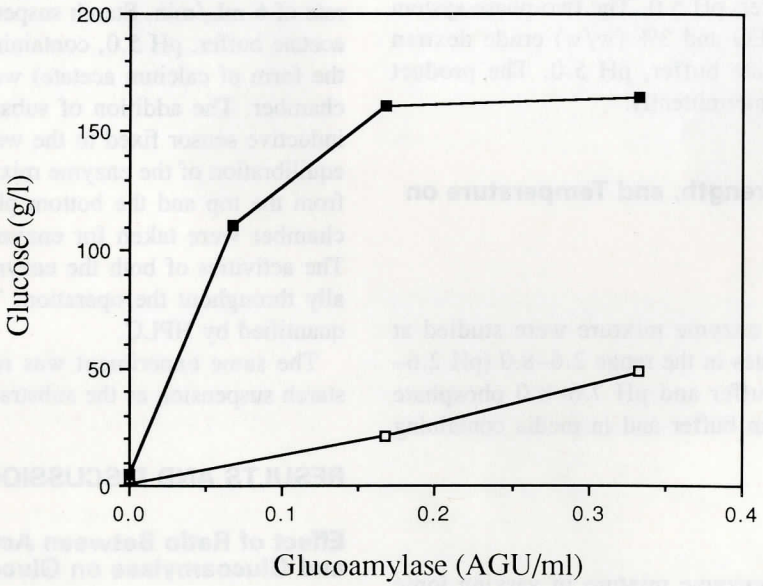

Figure 2. Glucose produced after 180 min using fixed amount of α-amylase (0.2 KNU/mL) and increasing amount of glucoamylase (filled symbols). Open symbols show action of glucoamylase only. Experimental conditions: 50°C, pH 5.0 (0.1*M* acetate buffer).

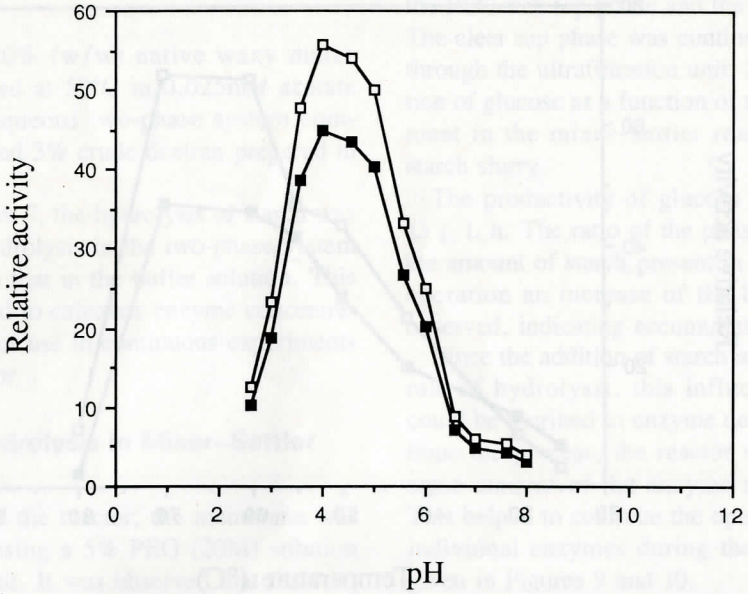

Figure 3. Effect of pH on enzyme activity in phase system with buffer (open symbols) and in buffer only (closed symbols). Temperature, 55°C. Buffers used were from pH 2.6 to 7.0 citrate phosphate buffer and from pH 7.6 to 8.0 phosphate buffer.

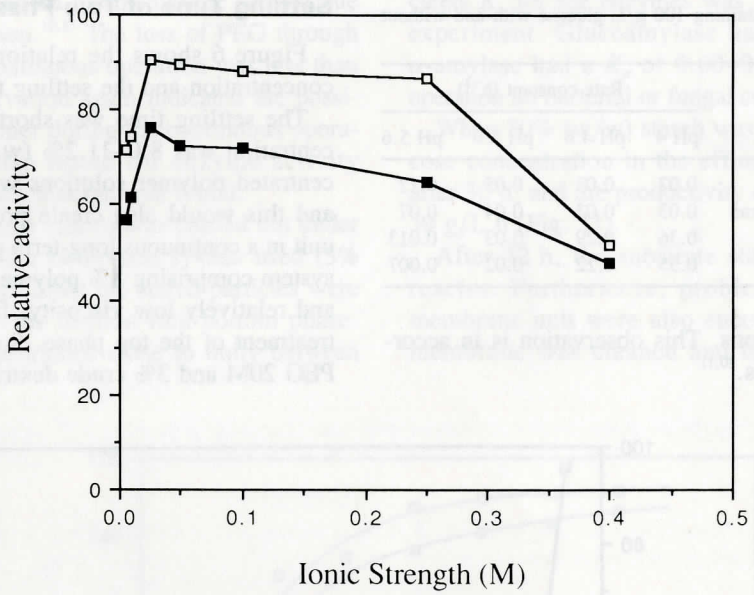

Figure 4. Effect of ionic strength (sodium acetate) at 55°C on enzyme activity in phase system with buffer (open symbols) and in buffer only (closed symbols).

When selecting operating conditions for the enzyme sequence, one has to consider both temperature and pH influence on the individual activities. Glucoamylase was reported to be stable at 60°C for 1 h and α-amylase considerably longer at this temperature (Novo product information). Since the process was operating at suboptimal pH values, a lower temperature was chosen. Table I shows the stability of glucoamylase and α-amylase at different pH values, both in the buffer system and the two-phase system, at 50°C.

At 50°C, the stability of glucoamylase was acceptable between pH 4 and 5, and α-amylase between pH 4 and 5.6. From these data it was decided to choose pH 5.0 for the hydrolysis of starch rather than pH 4.6 or 5.6, which are more suitable for the stability of individual enzymes. This selection of an intermediate pH value for optimal conditions is fully in line with earlier experiences when optimizing the performance of enzyme sequences.[9]

Both α-amylase and glucoamylase were found to be more active and stable in the media containing polymers

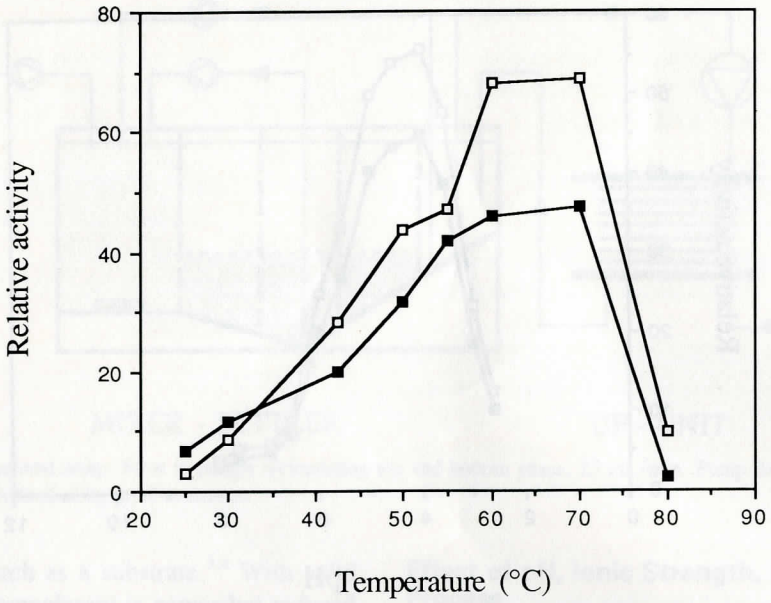

Figure 5. Effect of temperature in 0.05*M* acetate buffer, pH 5.0, on enzyme activity in phase system (open symbols) and in buffer (closed symbols).

Table I. Inactivation rate constants for glucoamylase and α-amylase at 50°C in citrate–phosphate containing 100 g/L glucose with and without PEG–dextran.

	Rate constant (h^{-1})			
	pH 4	pH 4.6	pH 5.0	pH 5.6
Glucoamylase in buffer	0.02	0.03	0.05	0.12
Glucoamylase with PEG–dextran	0.03	0.03	0.04	0.07
α-Amylase in buffer	0.36	0.29	0.03	0.013
α-Amylase with PEG–dextran	0.35	0.22	0.02	0.007

than in the buffer solutions. This observation is in accordance with earlier reports.[10,11]

Settling Time of Two-Phase System

Figure 6 shows the relationship between the polymer concentration and the settling time.

The settling time was shortest when the polymer concentration was 8.0–11.2% (w/w). The use of more concentrated polymer solutions would increase the viscosity, and this would also create problems in the ultrafiltration unit in a continuous long-term process. Hence, a two-phase system comprising 8% polymer would give fast separation and relatively low viscosity, facilitating the ultrafiltration treatment of the top phase. Such a system containing 5% PEG 20M and 3% crude dextran was chosen.

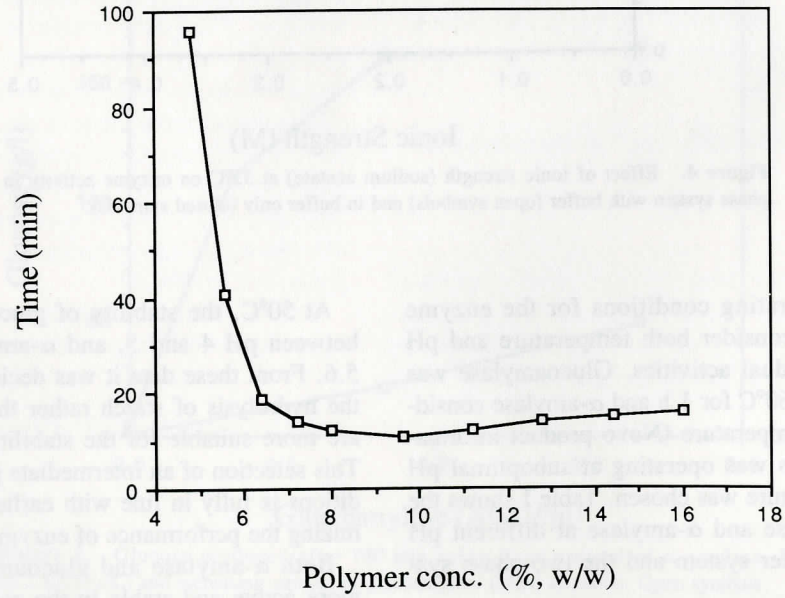

Figure 6. Separation time as function of polymer concentration (five-eights parts PEG 20*M* and three-eights parts crude dextran).

Batch Experiments

Batch hydrolysis of 10% (w/w) native waxy maize starch was then performed at 50°C in 0.025m*M* acetate buffer, pH 5.0, and in aqueous two-phase system composed of 5% PEG 20M and 3% crude dextran prepared in the same buffer.

As can be seen in Figure 7, the hydrolysis of starch was completed in 6 h. The hydrolysis in the two-phase system was somewhat faster than that in the buffer solution. This batch experiment was used to calculate enzyme concentrations and retention time for use in continuous experiments in the mixer–settler reactor.

Continuous Starch Hydrolysis in Mixer–Settler Reactor

Before the operation of the reactor, the membrane was tested for leakage by passing a 5% PEG (20M) solution through the membrane unit. It was observed that the concentration of PEG in the filtrate decreased with time during operation, starting with about 0.2 g/L, and after 2000 mL filtrate had passed through the membrane, the concentration had decreased to below 0.1 g/L. This indicated that a secondary layer was formed on the membrane surface due to concentration polarization.[12,13] The loss of PEG through the membrane during a continuous operation was less than 0.01% (w/w). This observation again indicated the possibility of reuse of the polymer during the continuous operation. The filtrate was also tested for enzyme activity indicating leakage. No such leakage was found.

The process in the enzyme reactor was carried out under optimized conditions. In the two-phase system used (3% crude dextran and 5% PEG 20M) the starch particles were completely partitioned to the dextran-rich bottom phase. Glucose produced was partitioned close to unity between the PEG-rich top phase and the dextran-rich bottom phase. The clear top phase was continuously removed and passed through the ultrafiltration unit. Figure 8 shows the production of glucose as a function of time in a continuous experiment in the mixer–settler reactor fed with 10% (w/w) starch slurry.

The productivity of glucose after the initial phase was 13 g/L h. The ratio of the phase volumes was affected by the amount of starch present in the system.[14] After 41 h of operation an increase of the bottom-phase volume was observed, indicating accumulation of starch.

Since the addition of starch was initially set to match the rate of hydrolysis, this influence on the phase volume could be ascribed to enzyme denaturation. In order to continue the process, the reactor was supplemented with the same amount of the enzyme mixture as added initially. This helped to continue the operation. The activities of the individual enzymes during the continuous operation are given in Figures 9 and 10.

The α-amylase and glucoamylase lost 40 and 60% of their original activities after the first day. These observations show agreement with the results obtained from the experiments done in the test tubes. The partition coefficients K_p for the enzymes was almost constant during the experiment. Glucoamylase had a K_p of 0.40–0.62 and α-amylase had a K_p of 0.60–0.74. During the 8 days of operation no bacterial or fungal contamination was observed.

When 30% (w/w) starch was used as substrate, the glucose concentration in the effluent reached its peak value after 30 h, and the productivity of glucose at this point was 39 g/L h (Fig. 11).

After 42 h, the substrate started to accumulate in the reactor. Furthermore, problems with clogging in the membrane unit were also encountered at this point. The membrane was cleaned and the experiment continued.

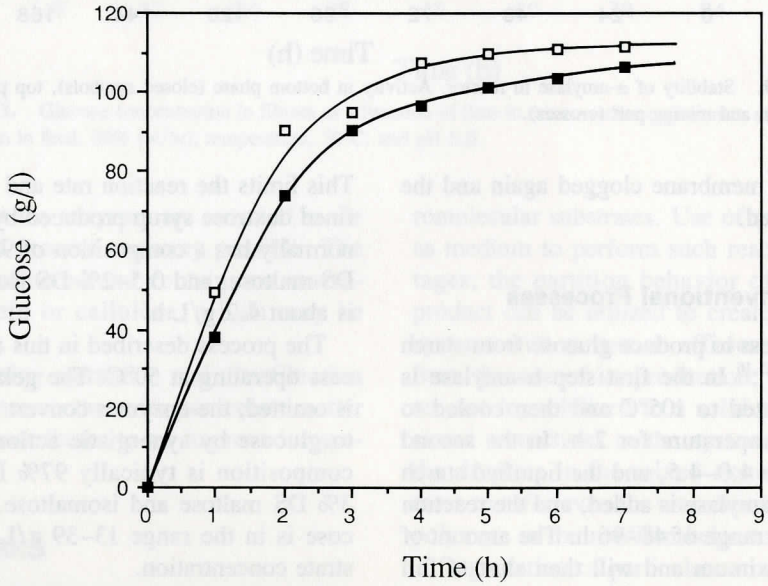

Figure 7. Glucose produced as a function of time in aqueous two-phase system (open symbols) and in buffer solution (closed symbols).

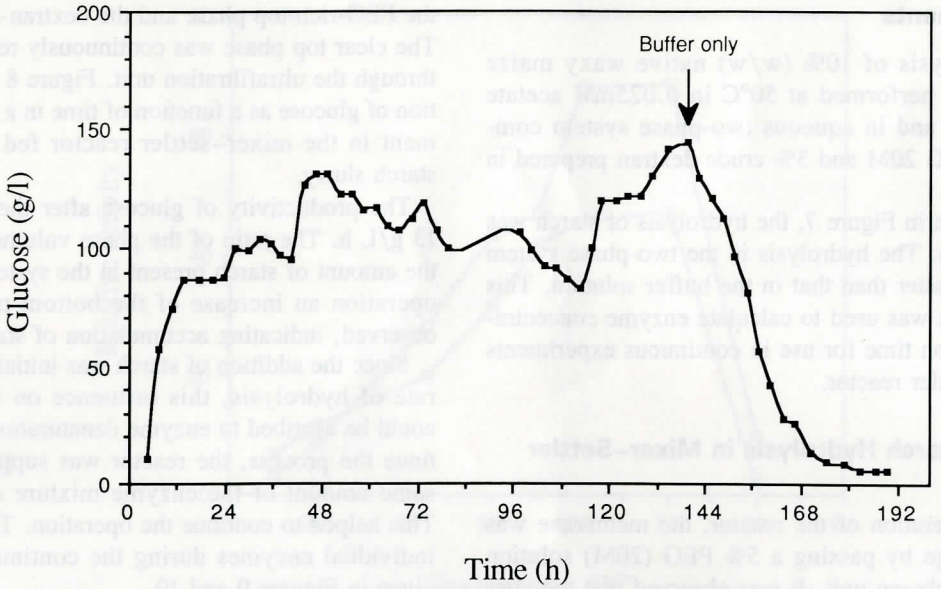

Figure 8. Glucose concentration in filtrate as a function of time in mixer–settler experiment: starch concentration in feed, 10% (w/w): temperature, 50°C; pH 5.0.

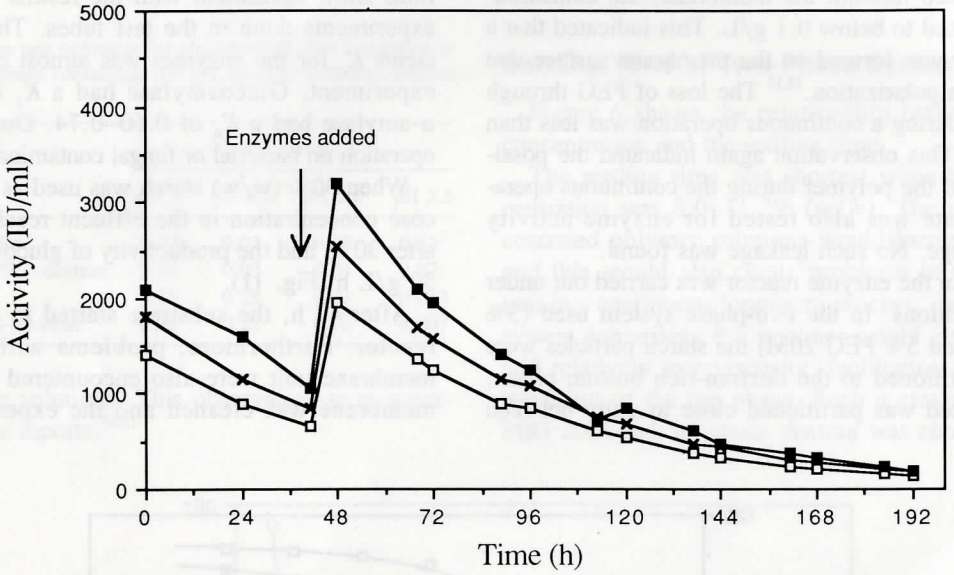

Figure 9. Stability of α-amylase in reactor. Activity in bottom phase (closed symbols), top phase (open symbols), and mixing part (crosses).

However, after 94 h the membrane clogged again and the experiment was terminated.

Comparison with Conventional Processes

The conventional process to produce glucose from starch is divided in two steps.[15,16] In the first step α-amylase is added. The slurry is heated to 105°C and then cooled to 95°C and kept at this temperature for 2 h. In the second step the pH is adjusted to 4.0–4.5, and the liquified starch is cooled to 60°C. Glucoamylase is added, and the reaction time in this step is in the range of 48–96 h. The amount of glucose will reach a maximum and will then start to fall due to condensation of glucose molecules to maltose and isomaltose. This reverse reaction will be more pronounced at high substrate concentrations or high enzyme levels. This limits the reaction rate and thus the productivity. Refined dextrose syrup produced by the conventional method normally has a composition of 95–96% DS glucose, 1-2% DS maltose, and 0.5–2% DS isomaltose. The productivity is about 4.2 g/L h.

The process described in this article is a continuous process operating at 50°C. The gelatinization of the substrate is omitted; the enzymes convert the solid substrate directly to glucose by synergistic action at pH 5.0. The product composition is typically 97% DS glucose and less than 3% DS maltose and isomaltose. The productivity of glucose is in the range 13–39 g/L h depending on the substrate concentration.

The process described here for starch hydrolysis eliminates the traditional gelatinization step, which is energy intensive. This results in reduced energy consumption and,

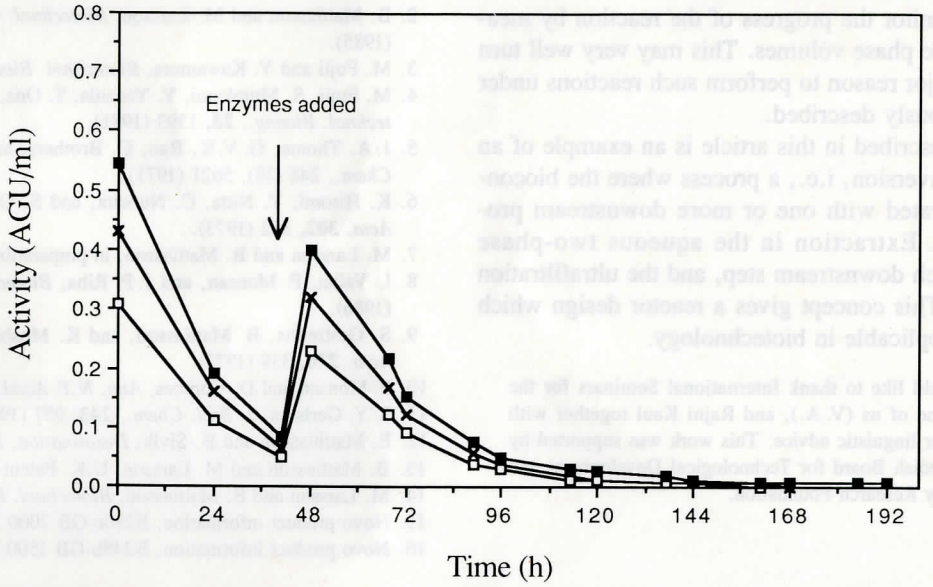

Figure 10. Stability of glucoamylase in reactor. Activity in bottom phase (closed symbols), top phase (open symbols), and mixing part (crosses).

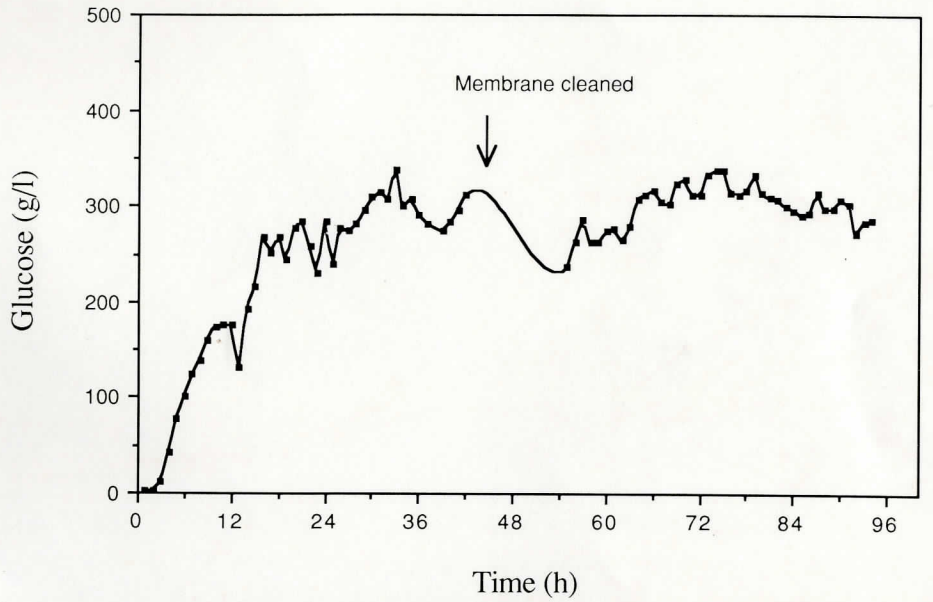

Figure 11. Glucose concentration in filtrate as a function of time in mixer–settler experiment: starch concentration in feed, 30% (w/w); temperature, 50°C; and pH 5.0.

together with the fact that the enzymes are recycled in the process, makes improved process economy possible. The produced glucose is ultrafiltered and contains no macromolecules such as protein or cellulose, which may be present in the substrate.

The concept of combining extraction and ultrafiltration protects the membrane from contact with particulate matter in the medium. Thus it contributes to an improved operational membrane life.

CONCLUDING REMARKS

The present study can also be seen as a model for reactions aimed at enzymatic degradation of particulate or macromolecular substrates. Use of aqueous two-phase systems as medium to perform such reactions offer certain advantages; the partition behavior of enzyme, substrate, and product can be utilized to create temporarily immobilized systems with at best an efficient extraction of the product from the site of its production; the fact that the enzymes are not immobilized to a solid phase results in low diffusional restrictions to the system; the small dimensions of the droplets in the mixed system facilitates an efficient mass transfer even for macromolecules; the high content of polymers in the medium helps to stabilize the enzymes.

Degradation of particulate structures is often a complex process that may be difficult to control by conventional means. The use of aqueous two-phase systems opens the

possibility to monitor the progress of the reaction by measuring the relative phase volumes. This may very well turn out to be one major reason to perform such reactions under conditions previously described.

The system described in this article is an example of an extractive bioconversion, i.e., a process where the bioconversion is integrated with one or more downstream processing step(s). Extraction in the aqueous two-phase system is one such downstream step, and the ultrafiltration is another one. This concept gives a reactor design which will be highly applicable in biotechnology.

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