Continuous Bioconversion of Starch to Ethanol by Calcium-Alginate Immobilized Enzymes and Yeasts

J. E. McGHEE, M. E. CARR, and G. ST. JULIAN, Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture,[1] Peoria, IL 61604

ABSTRACT

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Continuous bioconversion of starch to ethanol by immobilized enzymes and yeasts was studied. Commercial cornstarch (10%, w/w) was first batch-liquefied with bacterial α-amylase. In continuous-flow systems, liquefied starch was then converted to glucose with calcium-alginate-entrapped fungal glucoamylase, and the resulting glucose was fermented to ethanol by calcium-alginate-entrapped Fleischmann's active dry yeast (FADY). The continuous-flow saccharification-fermentation processes were performed in either two-stage (sequential) or single-stage (simultaneous) operations. In the single-stage operation, immobilized glucoamylase produced glucose from liquefied starch continuously for a total of 11 days. In the simultaneous saccharification-fermentation technique using immobilized glucoamylase and FADY mixture in a single-stage column, ethanol production was 69% of theory for five days. In the two-stage operation, in which immobilized glucoamylase and FADY were contained in separate columns connected in tandem, ethanol production averaged 97% of theory for five days. We conclude that the overall alcohol production efficiency was significantly greater in the two-stage system than in the single-stage system.

Recent demands for alternative sources of energy and for production of essential or strategic materials have prompted increased interest in enzyme and immobilized-cell systems for conversion of biomass to fuels, chemical feedstocks, fibers, and feed (Detroy and St. Julian 1983). A variety of static, repeated batch, and continuous methods have been reported for modification, solubilization, and conversion of starch to glucose by enzymes attached to solid supports, immobilized as an insoluble matrix, or retained by a membrane that is permeable only to the hydrolysis products (Marshall and Whelan 1974, Madgavkar et al 1977, Boundy et al 1976, Hofreiter et al 1978, Bungay 1982, Carr et al 1982). Concomitantly, there has been increased interest in the use of immobilized cells to produce ethanol (Kierstan and Bucke 1977; Durand and Navarro 1978; Del Rosario et al 1979; Vieth and Venkatasubramanian 1979; Ghose and Bandyopadhyay 1980; Wada et al 1980, 1981; Chibata and Tosa 1981; McGhee et al 1982a).

Recently, we reported a continuous laboratory process for ethanol production by calcium-alginate immobilized *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, and *Zymomonas mobilis* (McGhee et al 1982b). This report discussed the effect of the combined use of the immobilized microbial cell and the continuous fermentor reactor (column) design on ethanol production from glucose. We now report the continuous conversion of starch to ethanol. Single- and two-stage systems were investigated. In the single-stage system, batch-liquefied cornstarch was converted to ethanol using a mixture of glucoamylase and yeast coencapsulated in beads of calcium-alginate. With the two-stage system, glucose and ethanol were produced sequentially, using glucoamylase and yeast separately encapsulated. Efficacy of these two continuous-flow systems for producing ethanol from starch are discussed.

MATERIALS AND METHODS

Enzymes and Yeasts

Two commercial enzymes purchased from the Enzyme Product Division of Miles Laboratory, Inc. were used. The enzymes were Taka-therm, a thermally stable bacterial α-amylase of *Bacillus licheniformis* origin, and Diazyme L-100D, a liquid glucoamylase derived from a selected strain of *Aspergillus niger*. Taka-therm is an endoamylase capable of randomly hydrolyzing the predominating α-D-1,4-glucosidic linkages of amylose and amylopectin to provide soluble dextrins and insignificant quantities of glucose and maltose. The Taka-therm activity (as packaged) is 170,000 Modified Wohlgemuth Units (MWU)/g. (One MWU is that amount of enzyme which will convert 1 mg of soluble starch to a definite size dextrin in 30 min.)

Diazyme L-100D is an exoglucosidase that hydrolyzes starch to single glucose units beginning at the nonreducing ends of the starch molecules. The Diazyme product is capable of hydrolyzing both the α-D-1,6-glucosidic and the predominating α-D-1,4-glucosidic linkages of starch. The Diazyme activity is 100 units/ml (one Diazyme unit is that activity which will produce 1 g of glucose in 1 hr). Hereafter, Taka-therm and Diazyme shall be referred to as α-amylase and glucoamylase, respectively.

Fleischmann's active dry yeast (FADY) Nabisco Brand (purchased from a local grocery store) was used to produce ethanol. To determine the number of yeast cells per gram of FADY, 1-g quantities were suspended in sterile distilled water, properly diluted, and the cells were counted with a Petroff-Houser bacterial counter using a Zeiss Phase-contrast microscope as previously described (McGhee et al 1982a). To determine the number of cells encapsulated within a specific bead, the bead was submerged in 1 ml of saturated sodium tripolyphosphate (100 mg/ml) and allowed to stand at room temperature until the alginate dissolved (approximately 20 min). The suspension then was shaken for several minutes with a vortex mixer, and samples were taken for total (microscopic) counts. Viable yeast cell counts were also determined at the beginning and end of each experiment, using a standard microbiological technique. Viability is defined here as the ability of the yeast to reproduce themselves. Direct microscopic counts showed an average of about 956×10^9 yeast cells per gram of FADY, and only about 20% of these cells were viable at the beginning of the experiments and about 3% were viable at the end of the experiments. Yeast cell viability is not required for the microorganism to ferment glucose to ethanol (McGhee 1982a).

Batch Liquefaction of Starch

Cornstarch was purchased from CPC International, Inc. (code 3005). Typical analyses were 99% starch, 0.06% fat, 0.1% ash, 0.05% nitrogen, 0.04% sulfur (dry starch basis), and 11–13% moisture. Starch content of the commercial starch product was determined by polarimetry (Standard Analytical Methods 1967).

For each experiment, cornstarch was liquefied as follows: to a wide-mouth 1-L Erlenmeyer flask was added 167 g of starch (150 g, dry basis), 433 g of distilled water, and 0.60 g of the α-amylase as shipped (0.4%, dry starch basis). The flask was placed in a 95°C water bath, and the contents were mechanically stirred with a

[1] Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

three-bladed marine-type propeller at about 1,000 rpm. After the mixture was heated for 30 min at 95°C, the temperature of the liquefied starch was raised to 100°C in about 15 sec (steam injection) and maintained at 100°C for 5 min. The liquefied starch was cooled to 35°C, buffered with 30 ml of 0.2M dibasic sodium phosphate and enough 0.1M citric acid (about 60 ml) to give pH 4.0. The buffered liquefied starch solution was then diluted to 1,500 g, (10% starch concentration, w/w) and used as the feed solution in the continuous-flow saccharification-fermentation experiments. The liquefied starch solution did not need clarifying for any of the experiments.

Batch Saccharification of Liquefied Starch

To 1,500 g of batch-liquefied starch (150 g of starch, dry basis), prepared as described above, was admixed 1.5 ml of glucoamylase solution as shipped. The mixture was swirled (200 rpm) at 40°C for 24 hr in a gyrotory water-bath shaker, cooled to 30°C, adjusted in concentration for water evaporation, and used as a feed solution for continuous alcohol production with immobilized FADY.

Immobilization Technique

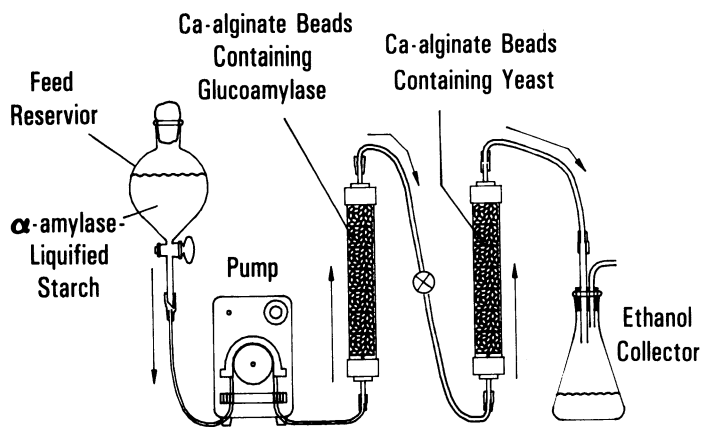For the two-stage saccharification-fermentation system, 1 g of

Fig. 1. Flow-sheet diagram depicting the continuous-flow saccharification and fermentation: Two-stage system.
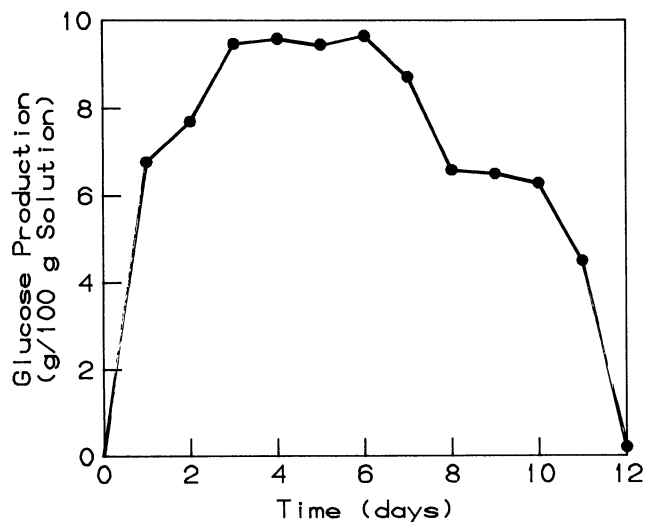

Fig. 2. Continuous saccharification (at 35°C) of liquefied starch to glucose via calcium-alginate immobilized glucoamylase. Flow rate was 3 ml/hr (dilution rate, 0.026 hr^{-1}). Starch concentration was 10% (w/w basis). Percent conversion = (grams of glucose produced / 11.1) × 100, where 11.1 = 10 g of original starch per 100 g of starch solution × 180/162. Average molecular weight of starch anhydroglucose unit = 162 and molecular weight of glucose = 180.

FADY (about 956 × 10^9 yeast cells) and 4 ml (400 units) of glucoamylase were separately immobilized. Each was blended with 1 g of sodium alginate and 98 or 95 g of sterile distilled water for 5 min at high speed in a Waring Blendor. For the single-stage, 1 g of sodium alginate and 1 g of FADY plus 4 ml of glucoamylase were blended in 94 ml of sterile distilled water. The resulting homogeneous mixtures were extruded drop by drop through a 10-ml serological pipette into 100-g portions of liquefied starch feed solutions containing 2.0 g of dissolved calcium chloride. A calcium-alginate bead was formed instantly from each drop of the alginate mixture entering the calcium chloride fortified feed solution. Diameter of the beads ranged from about 4 to 5 mm. Each bead contained an average of about 74 × 10^7 FADY cells (range of 58 × 10^7 to 81 × 10^7).

Two-Stage Continuous Saccharification-Fermentation

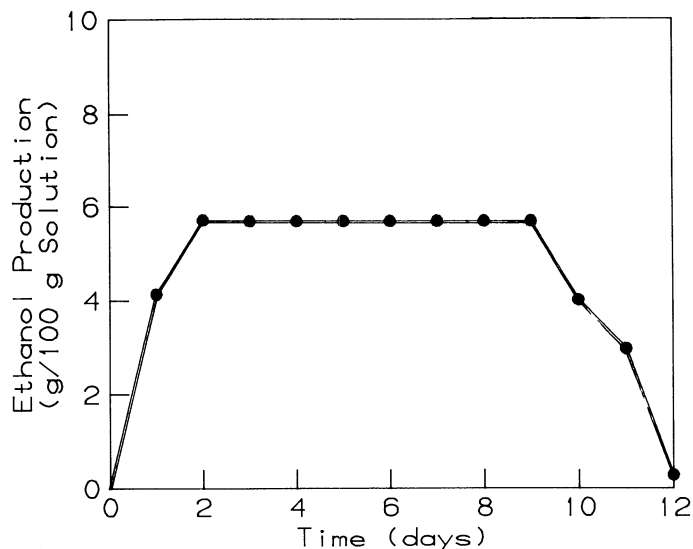Equipment previously described (McGhee et al 1982b) was used

Fig. 3. Continuous single-stage fermentation (at 35°C) of batch saccharified starch (10%, w/w) to ethanol via active dry yeast immobilized in calcium-alginate gel. Flow rate was 3 ml/hr (dilution rate, 0.026 hr^{-1}). Percent starch converted to ethanol = grams of ethanol produced / (0.51 × 11.1 g) × 100, where 11.1 g is the theoretical amount of glucose from 10 g starch and 0.51 is the ratio of ethanol to glucose for fermentation reaction.
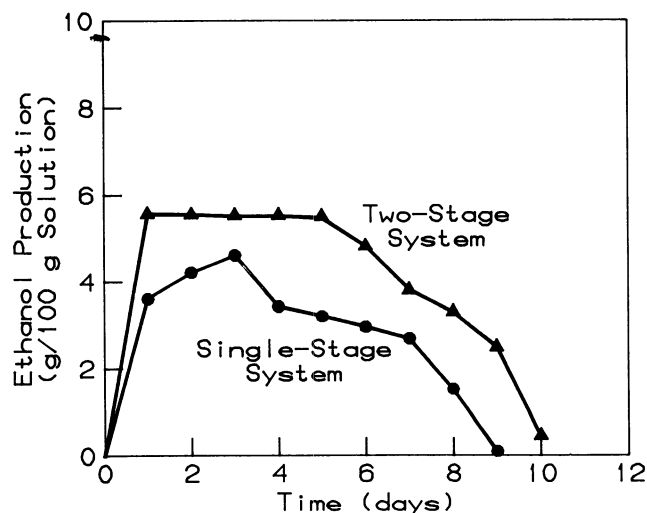

Fig. 4. Continuous two-stage and single-stage saccharification-fermentation (at 35°C) of liquefied starch to ethanol using glucoamylase and active dry yeast immobilized in calcium-alginate gel. Flow rate was 3 ml/hr (dilution rate, 0.026 hr^{-1}). Liquefied starch concentration was 10% (w/w basis).

to perform the two-stage continuous saccharification-fermentation process, except that two glass columns in tandem were used (Fig. 1). The first-stage column (saccharification) was filled with 1,200 beads of calcium-alginate immobilized glucoamylase, and the second-stage column (fermentation) was filled with 1,200 beads of immobilized yeast evenly distributed on the disks within the glass columns. The liquefied starch feed solution was pumped from the reservoir at 3.0 ml/hr (dilution rate, 0.026 hr^{-1}) upward into the bottom of the first column. When the liquefied starch exits the first column, it has been saccharified to glucose. This solution continues into the bottom of the second column upward through the bed of immobilized yeast cells and exits the column as a beer into the receiving flask. Samples were collected daily from the receiving flask and centrifuged at 6,000 × g. The supernatants of these samples were filtered through 0.45 μm Millipore filter paper for subsequent analyses for ethanol and residual glucose.

Single-Stage Simultaneous Saccharification-Fermentation

The single-stage continuous process involved one glass column filled with 1,200 beads prepared from a mixture of FADY and glucoamylase immobilized by the technique previously described. Liquefied starch (10%, w/w) was pumped at 3.0 ml/hr from the reservoir through the bottom of the column and upward through the beads. The beer was collected in the receiving flask and analyzed for ethanol and residual glucose.

All data presented are average values calculated from three to five separate experiments for each condition. A conversion temperature of 35°C was used in all experiments.

Analyses

Ethanol and glucose concentrations were determined by high-pressure liquid chromatography using a Bio-Rad (Richmond, CA) HPX-42 size-exclusion column with water as the mobile phase.

RESULTS AND DISCUSSION

Figure 2 shows results of continuously saccharifying batch-liquefied starch (10%, w/w) at 35°C with calcium-alginate-encapsulated glucoamylase in a single-column experiment. Glucose production occurred for a total of 11 days, with an average of 86% of the starch converted to glucose during days three through six. Glucose production was 78% on day 7; an average of 60% for days 8 through 10; 41% on day 11; and 3% on day 12.

Figure 3 demonstrates results from the continuous single-stage fermentation of batch liquefied-saccharified starch. When batch-saccharified starch was fed continuously through a column of calcium-alginate immobilized FADY, ethanol production was 72% of theory within 24 hr. By day 2 and through day 9, 100% of the available glucose was fermented to ethanol (Fig. 3).

Continuous ethanol production data for both the two-stage and the single-stage systems are shown in Fig. 4. Efficiency of liquefied starch conversion to ethanol was superior in the two-stage system. Maximum ethanol production was about 97% of theory from day 1 through day 5. Substantially lower amounts of ethanol were produced via the single-stage system containing an immobilized glucoamylase-FADY mixture. In this system, a maximum ethanol production of 81% of theory occurred, and then only for one day of the fermentation. Although the single-stage system eliminates the need for two separate reactors, the overall alcohol production efficiency was greater for the two-stage system.

Also, the two-stage system becomes a slightly more desirable choice over the single-stage process shown in Fig. 3 because of the undesirable added time and expense needed to batch-saccharify the liquefied starch. Previous studies (Cheetham et al 1979) significantly influenced our choice of alginate concentration for bead preparation. The use of 1 g of sodium alginate per 99 g of water should permit sugar to diffuse readily into the calcium-alginate gel beads and into the entrapped yeast cells. Previous studies (McGhee et al 1982a, 1982b) showed that no diffusion problems existed with glucose as the feed solution, using other yeast cells in calcium-alginate. The flow rate (3 ml/hr) and the alginate-pellet distribution within the column for present work was

previously shown to be ideal for maximum conversion of glucose to ethanol (McGhee et al 1982a). In present experiments with glucoamylase and FADY coencapsulated, analysis of the effluent showed no residual glucose, indicating that FADY was utilizing all of the glucose produced by the glucoamylase. In coencapsulation, FADY could cause the glucoamylase to be physically arranged into the calcium-alginate matrix in a manner that mechanically reduces the chance of the dextrins interacting with all of the glucoamylase. Another contributing factor for the lower values obtained in the single-stage system (Fig. 4) as compared to the two-stage system may be indicated from the work of Lee and Woodward regarding properties and application of immobilized β-D-glucosidase (Lee and Woodward 1983). Their data suggest that a significant concentration gradient could exist between "bulk" liquefied starch (feed solution) and the starch dextrin within the calcium-alginate bead so that rate of glucose produced would be less than optimum.

These and previously reported data from our laboratory demonstrate the potential usefulness of continuous-flow systems for the production of glucose and alcohol and show the efficacy of calcium-alginate gel as a viable matrix for the entrapment of yeast cells and enzymes. The two-stage saccharification-fermentation system seems to have an excellent potential for inexpensive, low-energy alcohol production. We believe that continuous-flow columns containing immobilized enzymes and microorganisms show promise for continuous alcohol production.

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