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Process Engineering of High-Ethanol-Tolerance Yeast for the Manufacture of Ethanol

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ABSTRACT

Inhibitory effects of ethanol and glucose on a high-ethanol-tolerance yeast strain (fusion product of *Saccharomyces diastaticus* and *Saccharomyces uvarum*) having high osmotic and ethanol tolerance were studied in batch cultures. A model incorporating both substrate and product inhibition was developed that represented the experimental data quite well. By performing fed-batch fermentation, an ethanol concentration of 13.3% (w/v) was obtained. The maximum allowable ethanol concentration for cell growth was predicted to be 129.9 g/L and ethanol-producing capability of cells was found to be completely inhibited at 136.4 g/L. On-line monitoring of the fermentation was performed using an ion trap mass spectrometer and a triple quadrupole mass spectrometer. Preliminary results are reported.

Index Entries: Ethanol; substrate inhibition; product inhibition; batch fermentation; membrane introduction mass spectrometry; flow injection analysis.

Nomenclature: K_S, Monod constant, for growth (g/L); K_S', Monod constant, for product formation (g/L); K_i, Inhibition constant, for growth (g/L); K_i', Inhibition constant, for production (g/L); P, ethanol concentration (g/L); P_m, ethanol concentration above which cells do not grow (g/L); P_m', ethanol concentration above which cells

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do not produce ethanol (g/L); S, substrate concentration (g/L); $Y_{X/S}$, cell yield constant (dimensionless); $Y_{P/S}$, product yield constant (dimensionless).

Greek Letters: α, constant defined in Eq. (3) (dimensionless); α', constant defined in Eq. (4) (dimensionless); μ, specific growth rate in the presence of ethanol (l/h); μ_m, specific growth at zero ethanol concentration (l/h); ν, specific rate of product formation at ethanol concentration P (l/h); ν_m, specific rate of product formation at zero ethanol concentration (l/h).

INTRODUCTION

In the past few years, there have been significant developments in the manufacture of ethanol from cellulosic biomass. Ethanol is a potential fuel that can be used in today's transportation sector. It has desirable properties, such as a clean burning, high octane fuel (*1*).

An interesting area of research in alcoholic fermentation is the study and use of new yeast strains that are more osmo- and ethanol-tolerant than the conventional yeast strains used. The ability of the yeast to perform fermentations at high temperatures (>30°C) is highly desirable, since it would reduce the operating costs of the fermentation.

In this work, alcoholic fermentation was studied using a high-ethanol-tolerant yeast (fusion product of *Saccharomyces uvarum* and *Saccharomyces diastaticus*) utilizing glucose as a substrate. Batch fermentations with initial glucose concentrations up to 35% were carried out. The data presented here clearly show substrate inhibition at higher concentrations. To reduce the inhibitory effect of substrate, fed-batch fermentations have been carried out, and an ethanol concentration of 13.3% (w/v) has been achieved. Various workers have proposed mathematical models to explain the effect of substrate and/or product inhibition on ethanol fermentation using a variety of yeast strains. In this research, a model has been proposed for the growth and fermentation by the yeast (having a high ethanol tolerance) incorporating the effect of both substrate and product inhibition.

For purposes of monitoring and control, on-line product analysis is not very well developed and has not proven its reliability to industry. We have monitored the fermentation on-line using a mass spectrometric system with flow injection analysis, and some preliminary results are presented here.

MATERIALS AND METHODS

Microorganism

Yeast strain 1400 was used in the experiments. It was provided by Labatt Breweries, Ontario, Canada. The yeast was maintained on agar

plates at 4°C. A sterile loop was used to transfer the cells from the plate to the medium. Each plate was kept for 8–10 wk and then replicated.

Medium

The medium used was similar to that of D'Amore and Panchal (2). The composition per liter of distilled water is as follows: bacto-peptone, 3.5 g; yeast extract, 3.0 g; KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; glucose (according to desired concentration).

The amounts of nutrients were doubled (2X media) in fermentations with glucose concentration of 100 g/L and above. Sterilization of the medium was accomplished by autoclaving at 121°C for 20 min. The nutrient media and the glucose solution were autoclaved separately.

Inoculum

The medium for the preparation of the inoculum was the yeast extract–peptone medium with 50 g/L glucose. The procedure began by aseptically transferring 100 mL of sterilized medium into a 250-mL Erlenmeyer flask. A sterile loop was used to transfer the yeast cells from the agar plate to the medium. The preculture was incubated at 30°C with agitation at 200 rpm in a floor shaker. The preculture was kept for 12–14 h (when the cells were in the late exponential phase) before being used to inoculate the fermentation medium.

Shaker-Flask Experiments

The batch cell-culture experiments were carried out in 250-mL Erlenmeyer flasks in a floor shaker. The temperature and agitation speed are the same as those for the inoculum. The amount of inoculum was kept constant at 1% (v/v). Each flask contained 100 mL sterilized medium with varying glucose concentrations (2–30%).

Cell Mass

A spectrophotometer (Coleman model 55, Perkin-Elmer, Maywood, IL) was used to measure the absorbance of the samples at a wavelength of 600 nm, which is in the visible region. Samples were diluted as required to assure absorbences of < 0.5. In this region, the calibration curve was linear with a slope of 0.65 g dry wt/U absorbance. Cell viabilities were determined by staining with methylene blue (3).

Analytical Methods

HPLC

Hitachi HPLC (Hitachi Ltd., Tokyo, Japan) with RI detector was used to analyze the concentrations of glucose, ethanol, and glycerol. A Bio-Rad HPX-87H ion-exclusion column was used. The mobile phase was 0.005*M* H_2SO_4 at a flow rate of 0.8 mL/min.

Glucose Analyzer

YSI 2700 Select Biochemistry Analyzer (YSI Inc., Yellow Springs, OH) equipped with glucose membrane was used for rapid analysis of glucose concentration in the fermentation media.

SYSTEM FOR ON-LINE MONITORING

Fermentor

Fermentations were carried out in a 2-L fermentor (New Brunswick Scientific, Edison, NJ). The agitation was maintained at 750 rpm. Temperature was controlled by a Versa-Therm electronic temperature controller, and fermentation pH was measured by an autoclavable pH probe (Ingold, Andover, MA). In order to control foaming during the course of the fermentation, a solution of antifoam agent (Antifoam 289 Mixed, Sigma Chemical Co., St. Louis, MO) was added as needed. The total amount of antifoam agent added during the course of the fermentation was about 1 mL.

Membrane Introduction Mass Spectrometry (MIMS)

The use of membranes for sample introduction was first reported by Hoch and Kok (*4*). Since then, MIMS has developed into a generally accepted technique for environmental and bioreactor monitoring. This is not surprising since MIMS offers many advantages: simplicity, ease of automation, fast response times, linear dynamic range over a few orders of magnitude, adaptability to various sample introduction systems (FIA) and detector systems (triple quadrupole, ion trap, sector instruments), and so forth. In addition, MIMS can be highly specific when used with a triple quadrupole mass spectrometer.

The analysis of organic solutes in water is one of the principal applications of MIMS. There are a variety of interface configurations that are in use. In some cases, the membrane is located inside the ion source of the mass spectrometer (*5*). In others, vacuum transport of the analyte or pneumatic transport of the analyte of the ion source is employed. Recently, a membrane/jet separator combination has been developed for two-stage enrichment of the analyte (*5*).

Ion Trap Instrument

The mass spectrometer used was a Finnigan ITS 40 GC/MS (San Jose, CA) quadrupole ion trap. The ion trap was fitted with a membrane/jet separator system (*5*). A microporous PTFE (2-μm pore size, 1 mm id × 1.8 mm od) membrane was encased in a stainless-steel tube. The reverse-flow geometry mode was used where the sample flows in one direction inside the membrane, while the helium gas flows in the opposite direction on the outside. The whole membrane assembly was connected to a

quartz jet separator. The temperature of the membrane/jet separator assembly was maintained at 70 and 150°C, respectively, and the ionization time was 1 ms.

Triple Quadrupole Instrument

The triple quadrupole mass spectrometer used in the experiments was a Finnigan TSQ 4500 (San Jose, CA) with the standard electron-multiplier detector. The TSQ 4500 uses the INCOS operating system. Ethanol, the major product of fermentation, was monitored in the selected ion-monitoring mode by using methane chemical ionization at a source pressure of 0.30 torr. A membrane probe was used to transport the analyte to the ion source of the mass spectrometer where it passed over the surface of the silastic sheet membrane.

Data System for TSQ 4500

A Zenith 248/12 personal computer (IBM PC/AT compatible) was used for the automation of bioreactor monitoring. The serial communication between the mass spectrometer and the personal computer is achieved by a two-way RS 232-C connection. The Zenith 248/12 personal computer is also connected to a direct memory access module, KEITHLEY 500A. For the automation of bioreactor monitoring, a DI01 card was used to control the FIA. The Program for automatic on-line monitoring was developed in C, with a Borland Compiler. LabWindows was used to provide a graphical user interface to carry out the on-line bioreactor monitoring experiments. Complete details of this system are given elsewhere (*6*).

Flow Injection System

A flow injection system was used to deliver the fermentation broth samples to the mass spectrometer via the membrane probe. An overview of the system is presented in Fig. 1 (*7*). Fermentation broth is circulated to a tangential flow filter by a Masterflex pump at a rate between 500 and 1000 mL/min. The filter is a Millipore 0.45-μm pore diameter Durapore filter housed in a stainless-steel housing as part of a Waters Filter Acquisition Module (FAM) (Waters, Milford, MA). A 250-μL loop is filled with filtered sample broth or standard, controlled by a TTL-controllable switching valve (Biochem Valve Corporation, East Hanover, NJ) and then injected into a constantly flowing water stream by which the plug is transported to the membrane probe. The water carrier stream is supplied by a Ismatec multichannel peristaltic pump at a rate between 1.0 and 1.2 mL/min. The supply rate of the filtered fermentation broth is maintained above 1 mL/min by controlling the broth recycling rate with the help of a small valve on the downstream side of the recirculating path. Filtered fermentation broth that is not utilized as sample is returned to the fermentor to maintain constant volume. All signals to the FAM are controlled manually or automatically by a custom-built FIA Module.

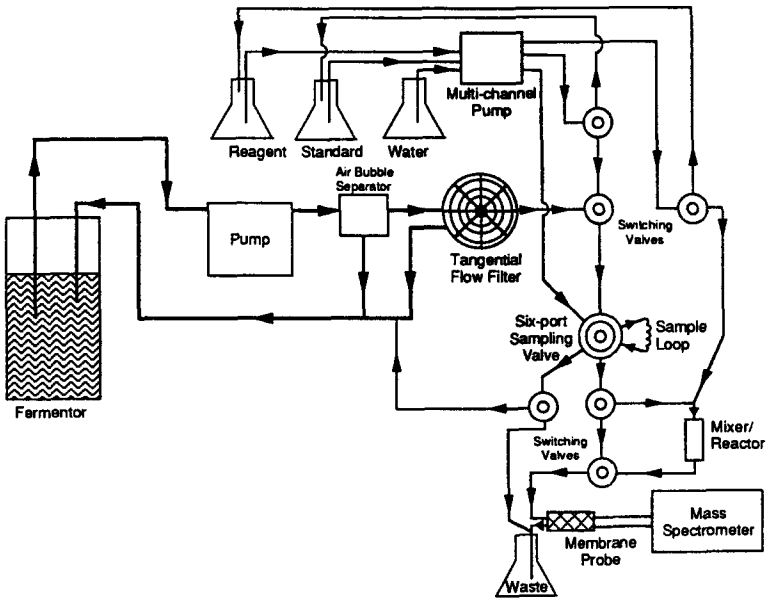

Fig. 1. Schematic of system for on-line monitoring of fermentation.

MODEL DEVELOPMENT

Substrate Inhibition

High substrate concentrations inhibit yeast growth and fermentation as a result of high osmotic pressure and low water activity (*8*). It has been reported that substrate inhibition becomes significant in the range of 15–25% (w/v) sugar and that there is complete substrate inhibition of fermentation at 40% (w/v) glucose in batch fermentations. It should be noted that substrate effects on growth rate begin at much lower sugar concentrations than for inhibition of fermentative activity.

In this work, the following expressions are used to account for the effect of substrate on cell growth and product formation:

$$\mu = (1 / X)(dX / dt) = \mu_m S / (K_s + S + S^2 / K_i) \quad (1)$$

$$\nu = (1 / X)(dP / dt) = \nu_m S / (K_s' + S + S^2 / K_i') \quad (2)$$

Product Inhibition

The effects of ethanol on the kinetics of yeast growth and fermentation have been studied for many years. Various correlations for product inhibition have been proposed in the literature, such as exponential (*9*), linear (*10,11*), hyperbolic (*12*) and parabolic (*13,14*).

In this work, the two constant model of Luong (*14*) is used to fit the experimental data:

$$\mu/\mu_m = 1 - (P/P_m)^{\alpha} \quad (3)$$

$$\nu/\nu_m = 1 - (P/P'_m)^{\alpha'} \quad (4)$$

The magnitude of the constant α determines the relation between μ and P; the magnitude of α' determines the relation between ν and P.

Overall Model of Growth and Fermentation Incorporating the Effect of Substrate and Product Inhibition

$$\mu = (1/X)(dX/dt) = \mu_m S/(K_s + S + S^2/K_i)[1 - (P/P_m)^{\alpha}] \quad (5)$$

$$\nu = (1/X)(dP/dt) = \nu_m S/(K'_s + S + S^2/K'_i)[1 - (P/P'_m)^{\alpha'}] \quad (6)$$

$$-(dS/dt) = (1/Y_{X/S})(dX/dt) = (1/Y_{P/S})(dP/dt) \quad (7)$$

RESULTS AND DISCUSSION

Fermentation Batch Experiments

Experimental data for cell growth are shown in Fig. 2A and B for initial glucose concentrations varying between 1.98 and 31.3% (w/v). Figure 3A and B illustrates the glucose concentration and ethanol production with time, respectively. From Fig. 2A and B, it can be seen that increasing glucose concentrations gave rise to increasing lag phases and decreasing specific growth rates. It can be inferred that there is no substrate inhibition until approx 10% (w/v) initial glucose and that substrate inhibition sets in significantly at about 20% (w/v) initial glucose. Doubling the nutrients in the media gave rise to higher cell densities than the earlier case where there was no nutrient supplementation.

The glucose concentration profiles indicate that there is a positive effect of increasing the media composition on the glucose consumption rate. The corresponding ethanol concentration profiles in Fig. 3B reveal an improvement in the ethanol production with nutrient supplementation. A maximum ethanol concentration of 12.5% (w/v) was obtained by the batch fermentation of 28.9% (w/v) glucose in the presence of excess nutrients. Thus, from the above data, it can be inferred that nutrient limitation has a significant impact on ethanol production. When the nutritional requirements are met, the inhibitory effects of high osmotic pressure appear to be reduced.

Modeling of Yeast Growth and Ethanol Production

Figure 4A depicts the variation of the initial specific growth rate μ and initial specific production rate ν with increasing initial glucose concentrations for the substrate inhibition study. It was observed that when there was no substrate inhibition, μ increased with glucose concentration until

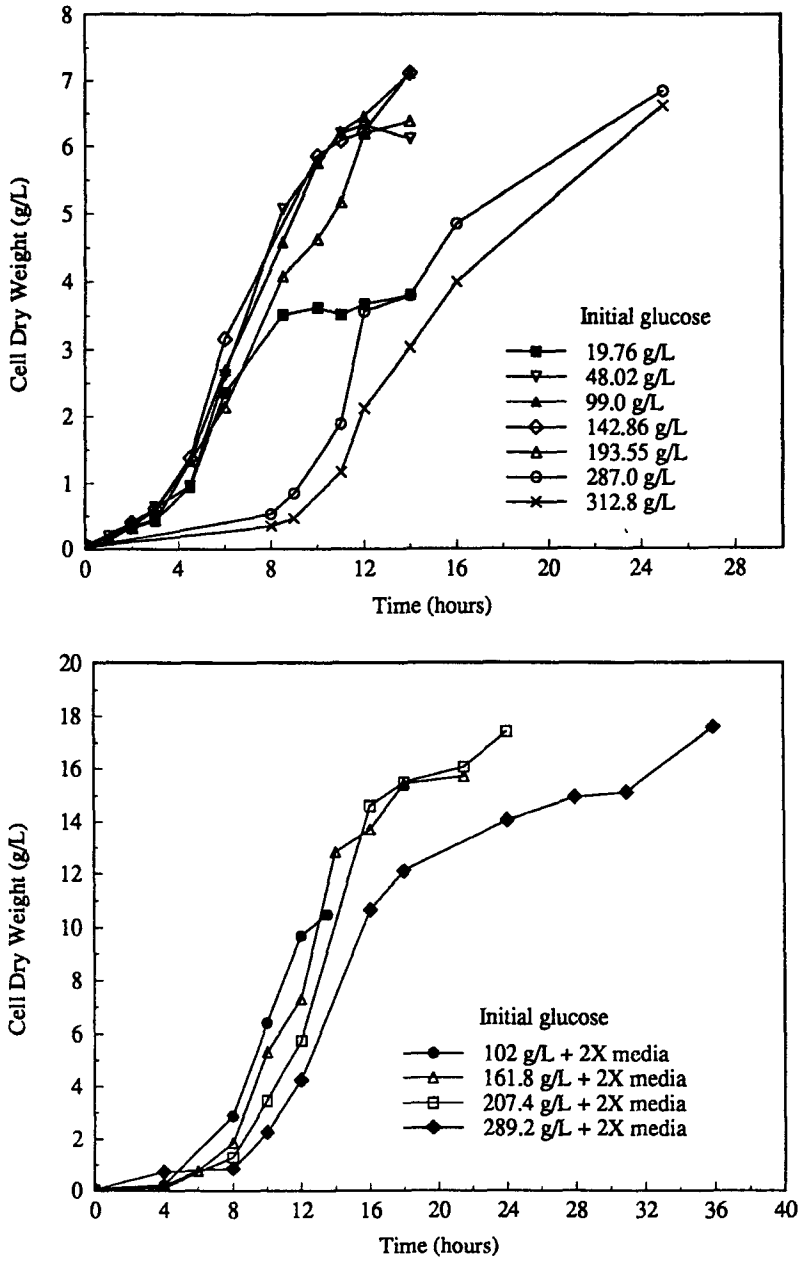

Fig. 2. Experimental profiles of cell density under different initial glucose concentrations.

about 4.8% (w/v) initial glucose and then began to drop gradually with increasing glucose. Similar observations were obtained for ν also. For the substrate-limiting case, the parameters of the Monod equation were estimated by plotting $1/\mu$ vs $1/S$. The data points followed the model quite well, and the parameters were estimated as follows: K_S = 0.572 g/L; μ_m = 0.63 h^{-1}. For fermentations carried out with high glucose concentrations

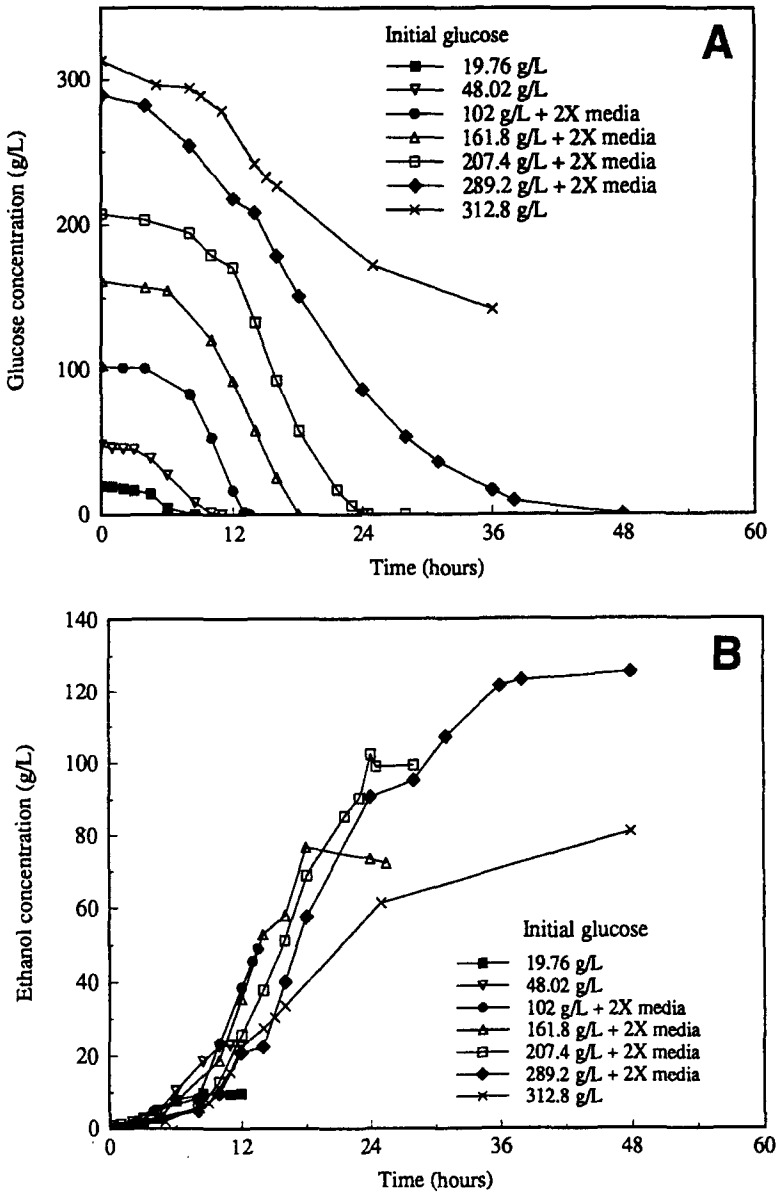

Fig. 3. Experimental profiles of (**A**) glucose and (**B**) ethanol under different initial glucose concentrations.

($S >> K_S$), and parameter K_S is negligible and an estimate for K_i can be obtained by plotting $1/\mu$ vs S. K_i was estimated to be 915.15 g/L.

In order to determine the effect of product inhibition, ethanol was added externally to the fermentation medium at the beginning of the fermentation in amounts varying from 0 to 13.6%. The data were fitted to Luong's model (Fig. 4B), and the parameters were obtained as follows:

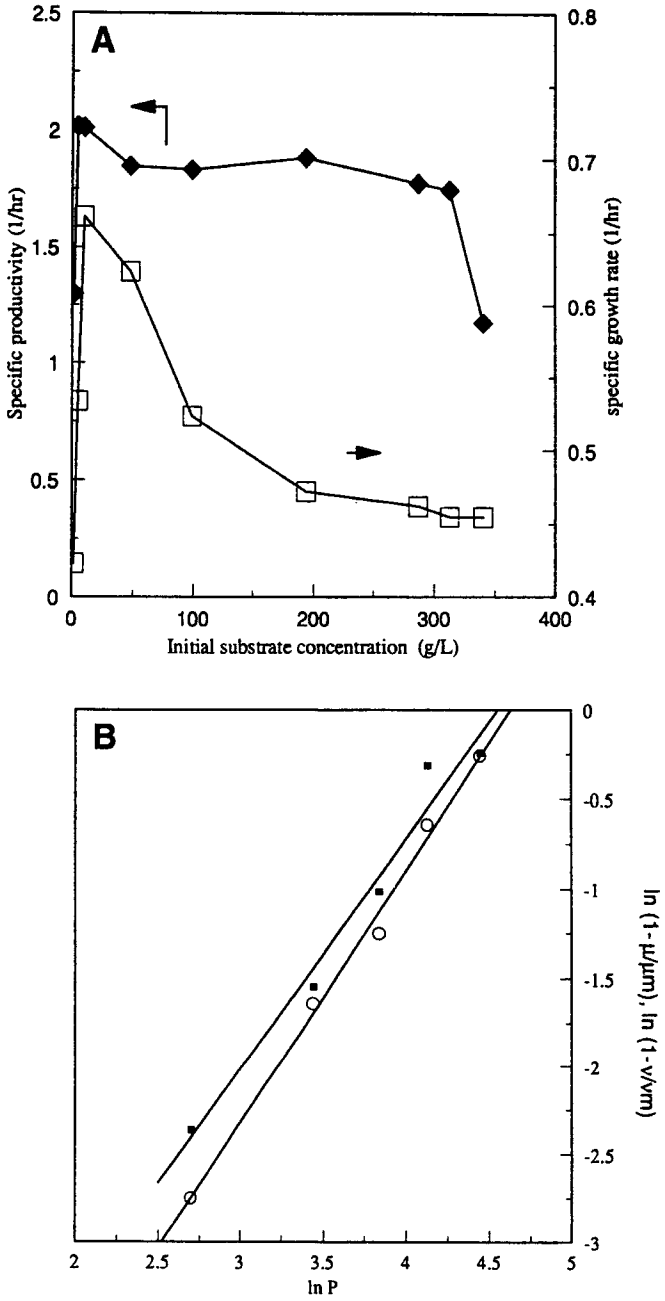

Fig. 4. (**A**) Inhibitory effect of substrate on initial specific growth rate (□) and initial specific production rate (◆). (**B**) ln $(1 - \mu/\mu_m)$ (■) and ln $(1 - \nu/\nu_m)$ (○) vs ln P for the experimental data.

$$\mu/\mu_m = [1 - (P/95.4)^{1.29}] \quad \text{for } P \leq 95.4 \text{ g/L}$$

$$\mu/\mu_m = [1 - (P/129.9)^{0.25}] \quad \text{for } 95.4 \leq P \leq 129.9 \text{ g/L}$$

$$\mu = 0 \quad \text{for } P > 129.9 \text{ g/L}$$

A similar approach to the above was followed for the estimation of the corresponding parameters for ethanol production. The estimated parameters are as follows:

$\nu_m = 2.008\ h^{-1}$; $K_S' = 1.34$ g/L; $K_i' = 5109$ g/L
$\nu/\nu_m = [1 - (P/103.03)^{1.42}]$ for $P \leq 103.03$ g/L
$0 < \nu < 0.255$ for $103.03 \leq P \leq 136.4$ g/L
$\nu = 0$ for $P > 136.4$ g/L

Estimation of Yield Coefficients

Yield coefficients for biomass and ethanol were estimated by taking the differences between the initial and final concentrations. The yield coefficient for ethanol ($Y_{P/S}$) was found to be 0.466 ± 0.027. This is similar to the results reported by other investigators (*10*). For the substrate-limiting case, the yield coefficient for biomass ($Y_{X/S}$) varies from 0.10 to 0.20. However, when both glucose and ethanol concentrations increase in the media, $Y_{X/S}$ decreases from 0.10 to 0.050. Depending on the initial glucose concentration, the corresponding value of $Y_{X/S}$ was used.

Cell Viability

Samples taken from batch fermentations were examined for viability using the methylene blue staining technique (*3*). It was observed that for fermentations with initial glucose <150 g/L, the viability remained above 95% throughout the fermentation. For higher glucose concentrations with no nutrient supplementation, the viabilities steadily dropped to about 30% at the end of the fermentation. However, with nutrient supplementation, viabilities were found to remain above 95% throughout.

Batch Fermentation Simulation Using SPEEDUP

For better estimation of model parameters, a comprehensive plant modeling package called SPEEDUP (licensed by Aspen Tech, Inc., Cambridge, MA) was used. The estimated model parameters as derived earlier were used as initial guess values. Given the model and experimental measurements, the parameter estimation feature of SPEEDUP seeks to solve the error-minimization problem. As an example, the estimated model parameters for batch fermentation of 10.2% glucose are given below:

$K_S = 0.572$ g/L	$\mu_m = 0.524\ h^{-1}$	$P_m = 95.4$ g/L
$K_S' = 1.34$ g/L	$\nu_m = 1.997\ h^{-1}$	$P_m' = 103.03$ g/L
$K_i = 1127.8$ g/L	$Y_{X/S} = 0.114$	
$K_i' = 4882.8$ g/L	$Y_{P/S} = 0.46$	

Figure 5 shows the simulation results for this fermentation, which closely follow the experimental results.

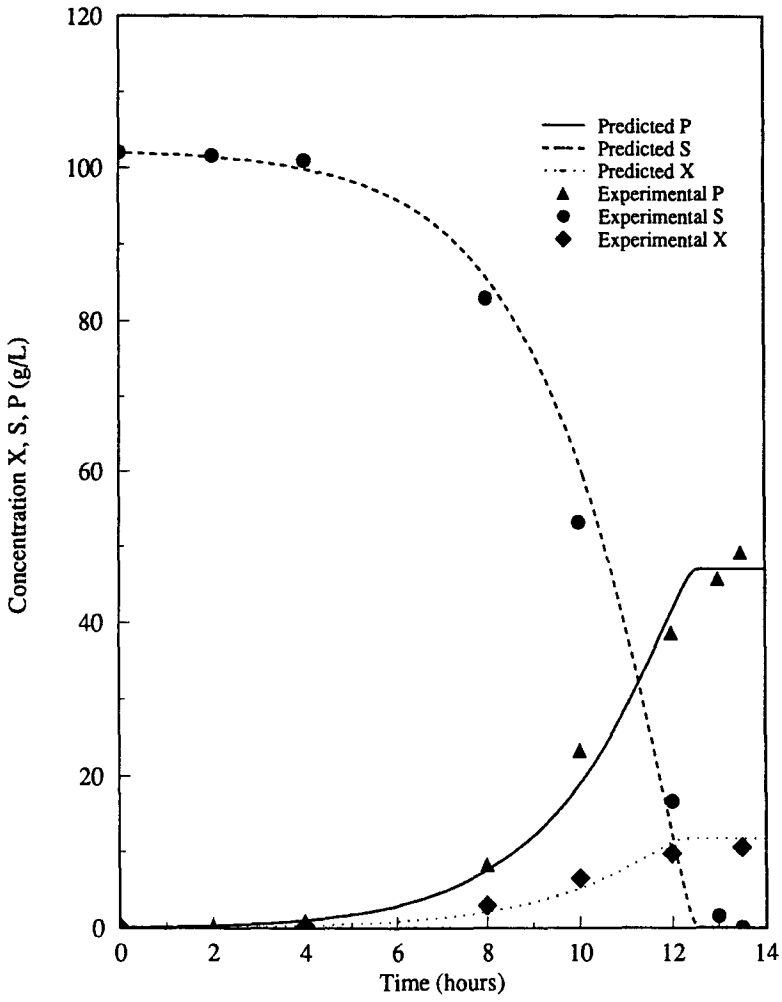

Fig. 5. Simulation of batch fermentation showing experimental and predicted profiles for substrate (*S*), product (*P*), and biomass (*X*).

Fed-Batch Fermentation

Figure 6A and B represents the ethanol and glucose concentration profiles during fed-batch fermentation, which is carried out to reduce the detrimental effects of substrate inhibition. The fermentation was begun with an initial glucose concentration of 10.6% (w/v). As and when the glucose concentration dropped to levels of 0–0.5%, fresh glucose solution with nutrients was added. Every injection of fresh glucose solution was also accompanied by the 2X peptone–yeast extract media as mentioned earlier. The total amounts of nutrients added during the course of the fed-batch fermentation are as follows: bacto-peptone, 1.575 g; yeast extract, 1.35 g; KH_2PO_4, 0.90 g; $(NH_4)_2SO_4$, 0.45 g; $MgSO_4 \cdot 7H_2O$, 0.45 g. The final volume of the fermentation broth was 225 mL. At the end of 67 h, a very high ethanol concentration of 13.3% (w/v) was obtained. The arrows in the figure indicate the times at which glucose with nutrients was added.

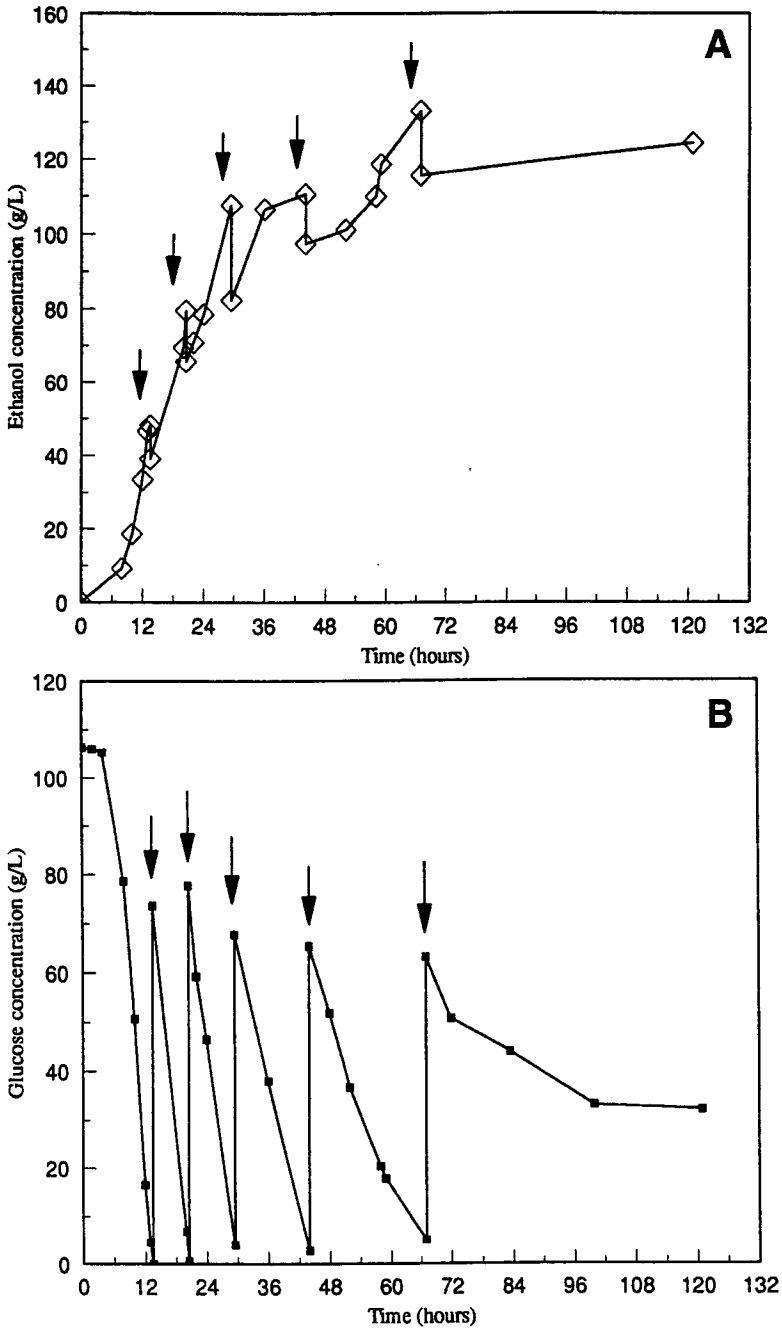

Fig. 6. (**A**) Ethanol and (**B**) glucose concentration profiles during fed-batch fermentation.

Data Obtained by On-Line FIA-MIMS

Figure 7A and 7B shows the results obtained by using the MIMS technique in combination with flow injection sampling for on-line monitoring of the fermentation. Figure 7A shows the concentration profile of ethanol during the fermentation as a function of time by using ITS40 ion trap quadrupole instrument. Ethanol was monitored as the protonated species

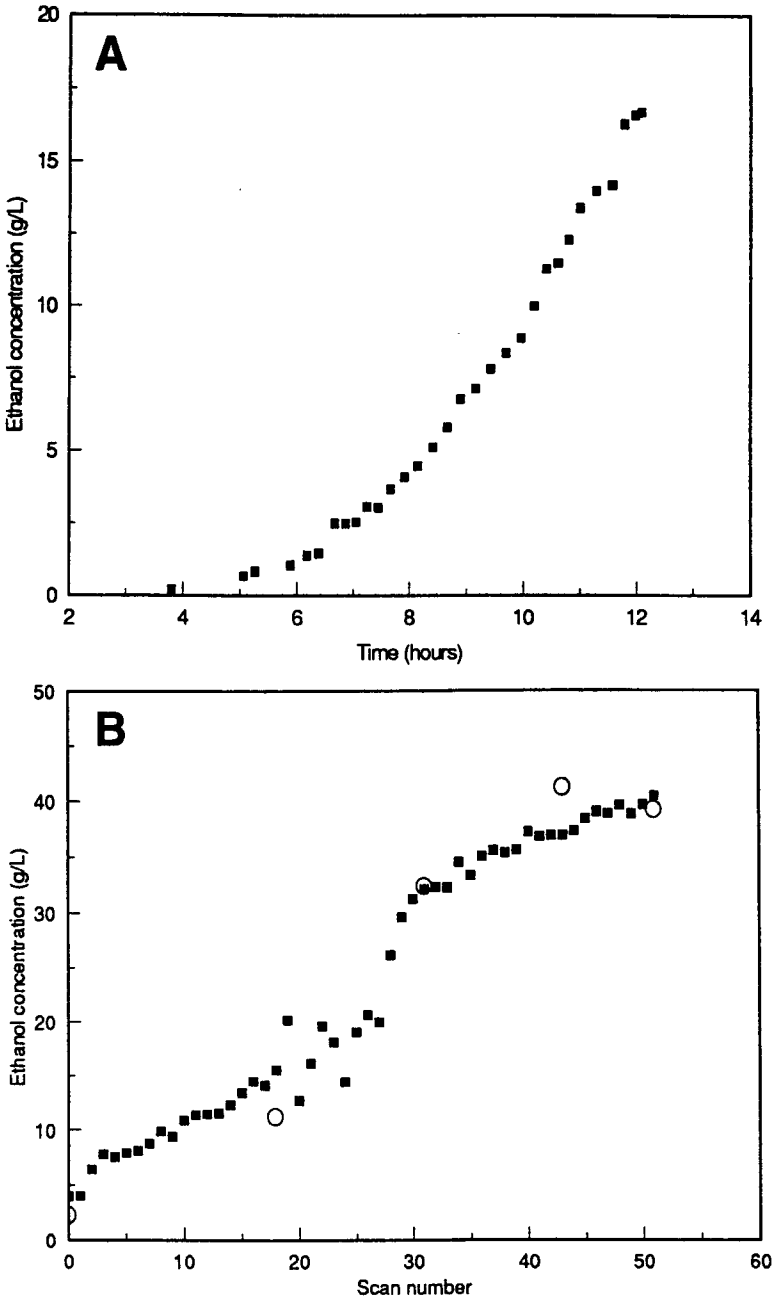

Fig. 7. Ethanol concentration from on-line fermentation by: (A) ion trap mass spectrometer (■) and (B) triple quadrupole mass spectrometer (■) and comparison with off-line HPLC analysis (○).

(m/z = 47). Figure 7B shows the concentration profile of ethanol during the fermentation as a function of scan number by using TSQ 4500 triple quadrupole instrument. This was done with the aid of the data system described previously. Each scan took around 7 min for alternate injections of broth sample and standard solutions. Also, an off-line HPLC measure-

ment of ethanol concentration was made to verify the concentration profile obtained, and this is shown in Fig. 7B. The results are found to be in good agreement with the on-line MIMS data.

CONCLUSIONS

The batch fermentation tests clearly show the existence of an inhibitory effect owing to initial glucose and ethanol concentration in the fermentation medium. Using the equations of best fit for the experimental data, a model was formulated for the growth and ethanol production. Preliminary results from on-line fermentation show MIMS technique as a potential tool for on-line monitoring and control.

ACKNOWLEDGMENTS

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