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Optimization of Seed Production for a Simultaneous Saccharification Cofermentation Biomass-to-Ethanol Process Using Recombinant *Zymomonas*

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ABSTRACT

The five-carbon sugar D-xylose is a major component of hemicellulose and accounts for roughly one-third of the carbohydrate content of many lignocellulosic materials. The efficient fermentation of xylose-rich hemicellulose hydrolyzates (prehydrolyzates) represents an opportunity to improve significantly the economics of large-scale fuel ethanol production from lignocellulosic feedstocks. The National Renewable Energy Laboratory (NREL) is currently investigating a simultaneous saccharification and cofermentation (SSCF) process for ethanol production from biomass that uses a dilute-acid pretreatment and a metabolically engineered strain of *Zymomonas mobilis* that can coferment glucose and xylose. The objective of this study was to establish optimal conditions for cost-effective seed production that are compatible with the SSCF process design.

Two-level and three-level full factorial experimental designs were employed to characterize efficiently the growth performance of recombinant *Z. mobilis* CP4:pZB5 as a function of nutrient level, pH, and acetic acid concentration using a synthetic hardwood hemicellulose hydrolyzate containing 4% (w/v) xylose and 0.8% (w/v) glucose. Fermentations were run batchwise and were pH-controlled at low levels

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of clarified corn steep liquor (cCSL, 1–2% v/v), which were used as the sole source of nutrients. For the purpose of assessing comparative fermentation performance, seed production was also carried out using a "benchmark" yeast extract-based laboratory medium. Analysis of variance (ANOVA) of experimental results was performed to determine the main effects and possible interactive effects of nutrient (cCSL) level, pH, and acetic acid concentration on the rate of xylose utilization and the extent of cell mass production. Results indicate that the concentration of acetic acid is the most significant limiting factor for the xylose utilization rate and the extent of cell mass production; nutrient level and pH exerted weaker, but statistically significant effects. At pH 6.0, in the absence of acetic acid, the final cell mass concentration was 1.4 g dry cell mass/L (g DCM/L), but decreased to 0.92 and 0.64 g DCM/L in the presence of 0.5 and 1.0% (w/v) acetic acid, respectively. At concentrations of acetic acid of 0.75 (w/v) or lower, fermentation was complete within 1.5 d. In contrast, in the presence of 1.0% (w/v) acetic acid, 25% of the xylose remained after 2 d. At a volumetric supplementation level of 1.5–2.0% (v/v), cCSL proved to be a cost-effective single-source nutritional adjunct that can support growth and fermentation performance at levels comparable to those achieved using the expensive yeast extract-based laboratory reference medium.

Index Entries: Recombinant *Zymomonas*, seed production via co-fermentation of glucose and xylose, corn steep liquor, pH, acetic acid, synthetic hemicellulose hydrolyzate.

INTRODUCTION

Fermentation ethanol is currently produced from six-carbon hexose sugars derived either from starch or sucrose; however, the value of these carbohydrates as potential food (feed) resources seriously restricts fermentation ethanol from cost-effective competition in the alternative transportation fuels market *(1–3)*. Therefore, cost reduction is the driving force for R&D directed toward the use of alternative fermentation feedstocks. Lignocellulosic biomass (including short rotation energy crops, agricultural, forestry, and municipal wastes) is considered an excellent alternative fermentation feedstock, because it is inexpensive, plentiful, and renewable *(4,5)*.

Although *Saccharomyces* yeast currently enjoys a monopoly as the fermentation process biocatalyst in the fuel ethanol industry *(6)*, it is not the only ethanol-producing microorganism. Furthermore, the yeasts currently used in starch and sucrose-based fermentations cannot ferment pentose sugars *(7)*. One biological aspect of engineering a process that uses alternative fermentation feedstocks involves the use of an alternative process organism (fermentation biocatalyst) that has been either selected for, or tailored to, the specific requirements of the biomass-to-ethanol

process. Research in this area has produced a variety of pentose-utilizing ethanologenic organisms, including yeasts, molds, and bacteria. Some of these are natural isolates; others represent genetically engineered recombinant variants *(8–12)*.

The National Renewable Energy Laboratory (NREL) is considering a variety of bioconversion processes for converting lignocellulosic biomass to ethanol on an industrial scale. In the general process design, feedstock comminution is followed by a dilute-acid pretreatment process. Economic sensitivity analysis of several process designs has demonstrated the substantial cost reduction that accompanies modifying the design from one of sequential hydrolysis and fermentation (SHF) to a simultaneous saccharification and fermentation (SSF) process *(1,11–13)*. Furthermore, there is potential for additional cost reduction (capital and operating costs) by combining into a single-unit operation the pentose fermentation and cellulose conversion (SSF) unit operations of the process *(14)*. However, such a design would require a biocatalyst that can coferment xylose and glucose. NREL has surveyed numerous potential industrial ethanol fermentation biocatalysts in a comprehensive study that compared known metabolic characteristics to a weighted list of fermentation performance criteria (including yield, ethanol tolerance, specific productivity, generally recognized as safe (GRAS) status, and sensitivity to inhibitory compounds typically present in biomass hydrolyzates) *(15)*. Using a nutrient-supplemented, dilute-acid hardwood prehydrolyzate as a screening medium, several strains of *Zymomonas* were selected as targets for improvement by metabolic engineering *(16)*.

The anaerobic growth characteristics of *Saccharomyces* yeast and the ethanologenic bacterium *Zymomonas mobilis* are similar, and the ethanol tolerance of both organisms is comparable *(17–19)*. Several comprehensive reviews in the literature pertain to the biology and physiology of *Zymomonas (20,21)* as well as biochemical and bioengineering aspects relating to its fermentation performance, and its potential as a process organism for producing fuel ethanol both in batch and continuous processes *(17,18,22)*.

By virtue of its demonstrated superior fermentation performance characteristics, *Zymomonas* offers an opportunity for process improvement with respect to both conversion efficiency (yield) and productivity *(17,19,22–24)*. It has the potential to revolutionize the fuel ethanol industry, and although not currently used commercially (for fermentations trials at industrial scale, *see* ref. *25*; for pilot-scale trials, *see* review by Doelle et al., ref. *22*), laboratory- and pilot-scale operations indicate it can generate near-theoretical maximum yields from several feedstocks, including sugar cane *(26)*, molasses *(27)*, saccharified starch from corn *(28)*, wheat *(25)*, cassava and sago *(29)*, and an enzymatic hydrolyzate of wood-derived cellulose *(30,31)*.

A serious limitation to the potential of *Zymomonas* as a universal biocatalyst in the fuel ethanol industry is its capacity to utilize only glucose,

fructose, or sucrose. It lacks a complete pentose metabolism pathway necessary for xylose fermentation. Previous attempts to engineer metabolically *Zymomonas* for xylose fermentation were not entirely successful, as evidenced by the failure of the recombinants to grow on xylose as a sole carbon source *(32,33)*. In addition to a marker gene for tetracycline resistance, the plasmid constructed by NREL (designated as pZB5) carries genes, cloned from *Escherichia coli*, for four enzymes (xylose isomerase, xylulose kinase, transketolase, and transaldolase) that were required to create a functional xylose metabolism pathway *(34)*. *Z. mobilis* CP4, transformed with pZB5, grows on xylose as sole carbon source, and coferments xylose and glucose to ethanol in high yield *(34)*.

One of several biomass-to-ethanol processes currently under investigation by NREL is the simultaneous saccharification and cofermentation (SSCF) process that is based on the use of an NREL-proprietary genetically engineered strain of *Z. mobilis* CP4:pZB5 *(14)*. Encouraging cofermentation performance data (yield and productivity) have been obtained for this organism using laboratory media *(14,34)*. However, prior to the present investigation, the potential to achieve high ethanol yield on cost-effective media formulations and to perform efficiently in synthetic prehydrolyzate media that contain inhibitory substances, such as acetic acid, had not been demonstrated. Fermentation nutrient costs, for both seed production and SSF, can be a significant contributor to the overall cost of producing ethanol *(35)*.

The metabolic engineering of *Zymomonas* to ferment xylose to ethanol in high yield represents a major step forward in the economic production of fuel ethanol from biomass, but other parameters, such as the nutritional and physical environment to which the recombinant will be exposed, could significantly alter both yield and productivity. Culture media used to screen microorganisms for process-related characteristics seldom become incorporated into the industrial process simply because they include economically unattractive nutrients. Several reports in the literature concern the use of defined and minimal media formulations that are commensurate with high-performance fermentation by *Zymomonas (23,36–40)*; however, the influence of specific medium components is not fully understood. Furthermore, previous work in this area has been largely restricted to wild-type cultures, and little is known concerning the nutritional requirements of xylose-fermenting recombinant *Zymomonas*.

The objectives of this investigation were to gain insight into the relative importance of three key process variables—nutrient level, pH, and acetic acid concentration—anticipated to affect growth of recombinant *Zymomonas* and to identify any interactions among these variables that significantly affect cell mass production. This information is prerequisite to optimizing seed production (i.e., maximizing the cell mass yield on substrate and minimizing nutrient costs) in the context of the proposed SSCF process design. Statistical experimental design concepts were applied to

enable us to characterize efficiently the dependence of seed production on these variables. Both two-level and three-level full-factorial designs with duplicate centerpoints were employed. A series of batch pH-controlled fermentations was carried out to complete each design. The fermentation medium consisted of a synthetic hardwood hemicellulose hydrolyzate (prehydrolyzate) with a xylose-to-glucose mass ratio of approximately 5:1. Analysis of variance (ANOVA) was used to determine the main and interactive variable effects significantly affecting cell mass concentration and the rate of xylose utilization.

MATERIALS AND METHODS

Organism

The recombinant *Z. mobilis* strain CP4 carrying the plasmid pZB5 (designated as Zm CP4:pZB5) (*34*) was received from M. Zhang (NREL, Golden, CO).

Long-Term Storage and Maintenance of Organism

Plasmid-bearing cultures, grown from single-colony isolates on selective agar medium (xylose + tetracycline), were stored at –70°C in RM medium supplemented with antifreeze (glycerol at 15 mL/dL). The phenotypic characteristics of the recombinant culture were related to growth in the presence of tetracycline and the production of ethanol from D-xylose. Cultures were generally revived in RM medium that contained 2% (w/v) glucose and 2% (w/v) xylose.

Fermentation Equipment

Batch fermentations were conducted in 1- or 2-L stirred-tank bioreactors (STR). pH-stat STR batch fermentations were conducted in a volume of 500 or 1500 mL in MultiGen (New Brunswick Scientific, Edison, NJ) bioreactors fitted with agitation, pH, and temperature control (30°C). The pH was monitored using a sterilizable combination pH electrode (Ingold) and was controlled by adding 4*N* KOH (NBS model pH-40 controller).

Methods of Preculture and Inoculation Procedures

A 1-mL aliquot of a glycerol-preserved culture was removed from cold storage (freezer) and transferred to about 100 mL of complex medium (RM), containing about 2% (w/v) xylose and 2% (w/v) glucose supplemented with tetracycline (Tc) (20 mg/L) in 125 mL screw-cap Erlenmeyer flasks, and grown overnight at 30°C. Alternatively, the inoculum was prepared by transferring an aliquot of a glycerol-preserved culture to a clarified corn steep liquor (cCSL) medium containing 2% (w/v) xylose, 2% (w/v) glucose, 0.2% (w/v) KH_2PO_4, and 20 mg/L tetracycline.

STR batch fermentations were inoculated by transferring approx 10% (v/v) of the overnight flask culture directly to the medium in the bioreactor. For STR fermentations, the initial cell density was monitored spectrophotometrically to give an OD_{600} in the range 0.1–0.2 corresponding to 30–50 mg dry wt cells/L.

Fermentation Media

For comparative purposes, the "benchmark" or reference medium for fermentation performance testing was the nutrient-rich, complex culture medium designated as RM (*36*). RM medium consists of 1% (w/v) Difco yeast extract (YE) (Difco Laboratories, Detroit, MI), and 0.2% (w/v) KH_2PO_4/distilled water. A CSL medium, which consisted of autoclaved tap water (TW) supplemented with centrifugally clarified CSL (range 1–2% v/v), was added at the time of inoculation.

A synthetic "biomass prehydrolyzate" (BPH) was formulated to model the sugar concentration in the NREL hardwood dilute-acid prehydrolyzate. The synthetic BPH medium was made with tap water, and contained 4% (w/v) xylose and 0.8% (w/v) glucose; it was supplemented with either 1% (w/v) YE and 0.2% (w/v) KH_2PO_4 or cCSL. All media contained 20 mg/L Tc.

Analytical Procedures

Growth was measured turbidometrically at 600 nm (1 cm lightpath) (Unicam spectrophotometer, model SP1800). In all cases, the blank cuvet contained distilled water. Dry cell mass (DCM) was determined by microfiltration of an aliquot of culture followed by washing and drying of the filter to constant weight under an infrared heat lamp. Fermentation media and cell-free spent media were compositionally analyzed by HPLC with a refractive index monitor and computer-interfaced controller/integrator (Bio-Rad Labs, Richmond, CA). Separations were performed at 65°C using an HPX-87H column (300 × 7.8 mm) (Bio-Rad Labs).

Statistical Analysis

ANOVA on results of the 2^3 full-factorial design was performed using an estimate for standard error based on a pooled standard deviation based on the two sets of duplicate centerpoints. ANOVA on the results of the 3^2 design was performed using Design-Expert™ version 4.0.5c software (Stat-Ease, Inc., Minneapolis, MN).

Determining Growth and Fermentation Parameters

The ethanol yield ($Y_{p/s}$) was calculated as the mass of ethanol produced (final concentration) per mass of sugar added to the medium, and was not corrected either for the dilution caused by adding alkali during the fermentation or for the contribution from fermentable components other

than xylose and glucose. The average volumetric rate of xylose utilization ($_{av}Q_s$xyl) was determined by dividing the initial sugar concentration by the total time required to deplete sugar from the medium completely.

RESULTS AND DISCUSSION

Growth and Fermentation Characteristics of Recombinant ZmCP4:pZB5 in a Synthetic Hardwood Prehydrolyzate

The pioneering work of Zhang and her colleagues at NREL with recombinant Zm CP4:pZB5 involved the use of a nutrient-rich laboratory medium (RM), and experiments were conducted in the absence of pH control *(34)*. All batch fermentations in our investigation were conducted in bench-scale bioreactors fitted with controllers for agitation, pH, and temperature. For comparative fermentation performance testing, the RM medium *(36)* used in this study as the benchmark or reference medium was the same as that used previously *(34)*.

Figure 1 shows a typical growth and fermentation time-course using the recombinant culture pZB5 in the nutrient-rich RM medium. With the pH controlled at 6.0, the specific maximum growth rate (μ_{max}) in 4% (w/v) xylose was 0.23/h (Fig. 1), whereas in the absence of pH control, it was previously much slower (0.057/h) *(34)*. In another experiment (results not shown), we observed that use of a seed culture that had been grown in xylose as sole carbon (energy) source led to very slow growth (μ_{max} = 0.032/h) following subculturing into fresh medium of the same composition. Therefore, for seed production, a medium containing both glucose and xylose is preferred. With 4% (w/v) xylose as sole carbon and energy source, the ethanol yield was 0.48 g/g, which represents a conversion efficiency of 94%, compared to 86% without pH control (*34*). Without pH control, the recombinant bacterium exhibited an average volumetric productivity of 0.23 g/L/h *(34)*, but with the pH controlled at 6.0, the productivity is increased almost threefold to 0.62 g/L/h (Fig. 1).

Addition of 0.8% (w/v) glucose to the 4% xylose-RM medium profoundly enhanced performance of recombinant Zm CP4:pZB5 (Fig. 1). These concentrations were selected to mimic the composition of a dilute-acid hardwood hemicellulose hydrolyzate *(14)*, and as such, this medium represented a synthetic BPH medium. Apart from phosphate, the sole source of nutrients was provided by 1% (w/v) Difco YE. In this medium at pH 6.0, both the specific growth rate and final cell mass concentration were increased about twofold to 0.43/h and 1.46 g DCM/L, respectively (Fig. 1 and Table 1). At 0.52 g/g, the ethanol yield is greater than the theoretical maximum of 0.51, which probably reflects the potential for some minor contribution from noncarbohydrate elements in the complex medium (Table 1). Compared to the medium with 4% xylose as the sole carbon source, the rate of xylose utilization in the synthetic BPH medium is

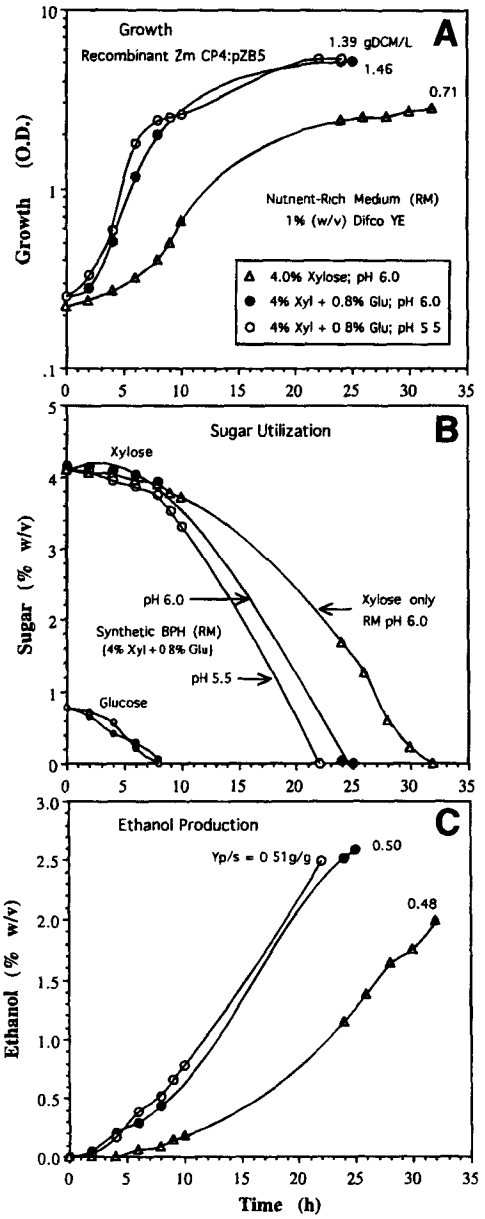

Fig. 1. Time-course of pH-stat batch fermentations with recombinant *Z. mobilis* CP4:pZB5 in yeast extract medium. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The medium was RM, and was supplemented with either 4% (w/v) xylose or a combination of 4% (w/v) xylose and 0.8% (w/v) glucose. The temperature was kept constant at 30°C. The pH-control set point was either 5.5 or 6.0. In panel A, the final DCM concentration (as determined directly by ultrafiltration) is indicated, and the ethanol yield ($Y_{p/s}$) is shown in panel C.

increased from 1.28–1.71 g/L/h (Table 1). The pH optimum for both growth rate and cell mass yield for wild-type *Z. mobilis* is close to 6.0 *(24,41)*. In the SSCF process design proposed by NREL *(14)*, the saccharifying enzymes operate at higher efficiency at pH <6.0 Figure 1 shows little effect

Table 1
Summary of Growth and Fermentation Parameters
for Recombinant Zm CP4:pZB5

Medium composition°	pH	Cell Mass (g DCM/L)	$Y_{p/s}$ (g/g)	av Q_s (xyl) (g Xyl/L.h)
RM (4% xylose)	6.0	0.71	0.48	1.28
RM (4% xyl + 0.8% glu)	6.0	1.46	0.52	1.71
RM (4% xyl + 0.8% glu)	5.5	1.39	0.51	1.86
TW+ 1.0% cCSL	6.0	0.81	0.48	1.25
TW+ 2.0% cCSL	6.0	1.40	0.50	1.56
TW + 2% cCSL + 0.5% Ac	6.0	0.92	0.46	1.24
TW + 2% cCSL + 1.5% Ac	6.0	0.43	0.49*	0.30*

°All media contain 20 μg/mL Tc and all fermentations were conducted at 30°C
*Based on sugar consumed at T = 54 h.
RM = rich medium; xyl = xylose (%w/v); glu = glucose (%w/v); TW = tap water; cCSL = clarified corn steep liquor (%v/v); Ac = acetic acid (%w/v).

on growth and fermentation performance of recombinant Zm CP4:pZB5 when the pH control set point was decreased from 6.0–5.5. At pH 5.5, the rate of xylose utilization increased from 1.71–1.86 g/L/h, representing a 4-h decrease in fermentation time from 26 to 22 h (Fig. 1B).

CSL has been shown to be a cost-effective means of nutrient supplementation in fermentations with ethanologenic recombinant *E. coli (42,43).* Lawford and Rousseau *(44)* have reported at this symposium that cCSL (i.e., with insolubles removed by centrifugation) cost-effectively satisfies the nutritional requirements of wild-type *Z. mobilis* CP4. Figure 2 shows the comparative growth and fermentation performance of recombinant Zm CP4:pZB5 in a synthetic BPH medium that consists of TW and 2% (v/v) cCSL. With the pH controlled at 6.0, the values for the final cell mass concentration and ethanol yield were very similar to those observed for supplementation with 1% YE (Table 1). The small decrease in the rate of xylose utilization probably reflects the proportional decrease in cell mass concentration (Table 1). A 50% reduction in the level of supplementation by cCSL produced a fermentation performance that was similar to that observed with the 4% xylose-RM medium (Fig. 2 and Table 1). At a cCSL supplementation rate of 1% (v/v), the final cell mass concentration was 0.81 g DCM/L, the ethanol yield was 0.48 g/g, and the rate of xylose utilization was 1.25 g/L/h (Fig. 2 and Table 1).

A well-known byproduct of dilute-acid pretreatment of lignocellulosic biomass is acetic acid *(45,46)* and, based on known structure and composition of the fermentation feedstock, the acetic acid concentration

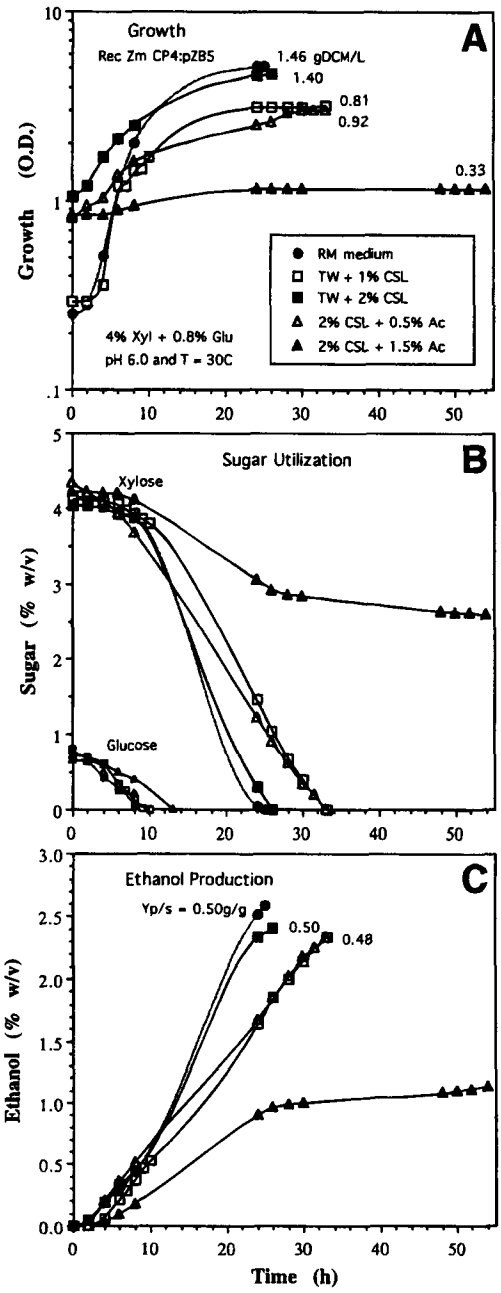

Fig. 2. Time-course of pH-stat batch fermentations with recombinant *Z. mobilis* CP4:pZB5 in CSL medium. **(A)** Growth, **(B)** glucose and xylose utilization, and **(C)** ethanol production. The medium consisted of TW supplemented with either 1 or 2% (v/v) cCSL. The triangle symbols represent fermentations with cCSL media supplemented with either 0.5 or 1.5% (w/v) acetic acid (Ac). The pH and temperature were controlled at 6.0 and 30°C, respectively. In all cases, the media contained 4% (w/v) xylose and 0.8% (w/v) glucose. The reference (control) medium was RM. In panel A, dry cell mass concentration is indicated, and the ethanol yield ($Y_{p/s}$) is shown in panel C.

can be predicted *(47)*. For hardwood biomass prehydrolyzate containing about 5% (w/v) sugars, the anticipated level of acetic acid is in the range 1.2–1.5% (w/v) *(47–49)*. The pH-dependent inhibitory effect of acetic acid on ethanologenic bacteria *(49,50)*, including *Zymomonas (51)*, is well documented. In terms of acetic acid toxicity, the undissociated protonated form of acetic acid is the causative agent, and the effect is potentiated at lower pH values *(50,51)*. The inhibitory effect of acetic acid is minimized by controlling the pH at 6.0 *(51)*. In a synthetic BPH medium with 2% (v/v) cCSL at pH 6.0, the presence of 0.5% (w/v) acetic acid results in growth and fermentation performance of Zm CP4:pZB5 that is very similar to the synthetic BPH medium in the absence of acetic acid and where the level of cCSL supplementation was only 1% (v/v) (Fig. 2 and Table 1). At a level of 1.5% (w/v) acetic acid, growth and fermentation kinetics are seriously compromised (Fig. 2). Although the ethanol yield (based on sugar consumed) remains high, about two-thirds of the xylose remains unfermented after 2 d (Fig. 2). From the perspective of seed production, the effect of acetic acid is most dramatic, and at the upper limit of 1.5% (w/v), the cell mass concentration decreases to 0.43 g DCM/L (Table 1).

Statistical Approach to Optimization

Optimization by the traditional method involves changing one independent variable while holding all others constant. This "one factor at a time" approach can be effective, but it is laborious and poorly suited for identifying interactions among multiple factors. A better approach is to use statistical experimental designs that enable the significance of multiple variables (or factors) to be assessed in an efficient manner *(52–55)*. Two-level full-factorial designs can be run, for example, to identify the most important process factors (variables) and factor–factor interactions (i.e., those that have the largest effect on the process). Then, once the most important variables are identified, higher-level designs—the simplest being a three-level factorial design—can be used to characterize better the response of the process within the experimental design domain.

Our earlier studies showed that batch seed growth was influenced by nutrient (cCSL) level, pH, and exogenous acetic acid concentration. Therefore, we designed a 2^3 full-factorial experiment (with duplicate centerpoints) to characterize efficiently the significance of these three factors on seed production performance and to determine whether there were any significant interactions among them. Their influence on cell production from glucose–xylose mixtures was examined using sugar loadings similar to those expected in hardwood hemicellulose hydrolyzates, (i.e., 4% w/v glucose + 0.8% w/v xylose). The overall design is depicted in Fig. 3A. which shows that the nutrient supplementation level varied from a low level of 1% (v/v) to a high level of 2% (v/v); pH ranged from a low level of 5.5 to a high level of 6.0; and acetic acid level varied from 0.5% (w/v) to 1.5% (w/v).

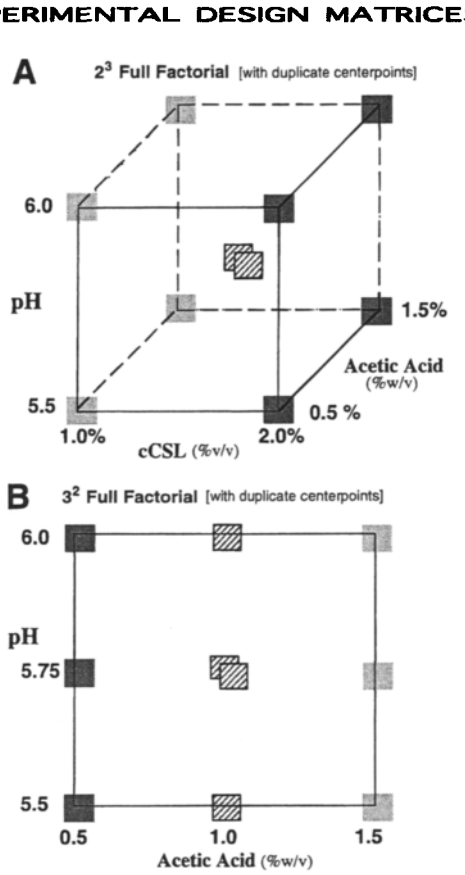

Fig. 3. Experimental design matrices. **(A)** 2^3 Full-factorial with duplicate center points and **(B)** 3^2 full-factorial with duplicate center points.

Results of the 2^3 full-factorial experiment are depicted in Fig. 4 and listed in Table 2, which shows that final cell mass concentration ranged from a low of 0.17 g DCM/L (at 1% [v/v]) cCSL, pH 5.5, and 1.5% [w/v] acetic acid to a high of 0.92 g DCM/L (at 2% [v/v] cCSL, pH 6.0, and 0.5% [w/v] acetic acid). Figure 4B shows that the average volumetric rate of xylose utilization behaved similarly, ranging from a low of 0.178 g/L/h (at 1% [v/v] cCSL, pH 5.5, and 1.5% [w/v] acetic acid) to a high of 1.24 g/L/h (at 2% [v/v] cCSL, pH 6.0, and 0.5% [w/v] acetic acid). Although more variable, the response in terms of cell mass yield as a function of factor levels also showed the same general trend (results not shown graphically, but values listed in Table 2). Figure 4 shows that cell mass production and xylose utilization exhibit similar behavior with respect to changes in pH and acetic acid concentration, but the influence of nutrient (cCSL) is different. Cell mass production shows a smaller dependence on the level of cCSL than the rate of xylose consumption, particularly at high pH. Figure 4B suggests that interactions may exist between the variables

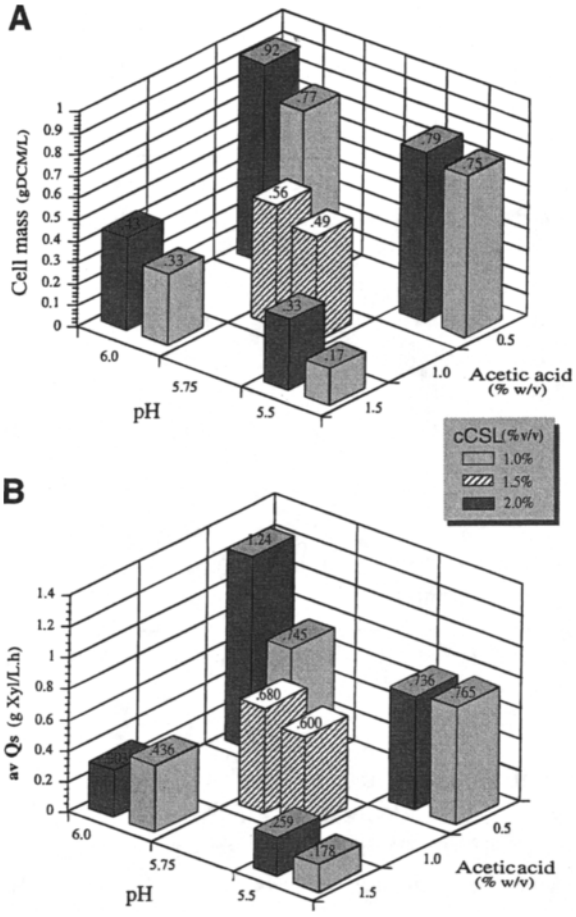

Fig. 4. Response profiles for 2^3 full-factorial study with Zm CP4:pZB5 using a synthetic biomass prehydrolyzate. **(A)** Cell mass concentration and **(B)** average volumetric rate of xylose utilization. The experimental design matrix is illustrated in Fig. 3A. The center bars in the 3D plot represent the duplicate center points. The base medium contained 4% xylose and 0.8% glucose. The three variables (levels in brackets) were pH (5.5 and 6.0), acetic acid (0.5 and 1.5% w/v), and cCSL (1 and 2% v/v).

with respect to xylose utilization. For example, at high pH and low acetic acid level, increasing cCSL level increases the utilization rate. Conversely, at high pH and high acetic acid level, increasing cCSL level results in a decrease.

ANOVA was performed to assess the statistical significance of these apparent effects. Results of the ANOVA on cell mass production show that at the 90% confidence level, all three factors significantly affects final cell mass concentration; the effects of acetic acid and nutrient are higher than the 95% confidence level (CL > 95%). Acetic acid concentration exerts the largest effect and negatively affects cell mass production, with nutrient level and pH exerting smaller, but positive effects on seed production. No

Table 2
Summary of Conditions and Responses for a 2^3 Factorial Study with Recombinant pZB5 and a Synthetic BPH Medium[a]

Exp. #	ID #	Conditions			Responses		
		cCSL (% v/v)	pH	Acetic acid (% w/v)	Cell mass (g DCM/L)	Growth Yield (gDCM /g S used)	av Qs (Xyl) (g Xyl/L.h)
1	37a	1.0	5.50	0.5	0.75	0.016	0.765
2	37c	2.0	5.50	0.5	0.79	0.017	0.736
3	37b	1.0	6.00	0.5	0.77	0.016	0.745
4	37d	2.0	6.00	0.5	0.92	0.018	1.238
5	44c	1.0	5.50	1.5	0.17	0.011	0.178
6	44e	2.0	5.50	1.5	0.33	0.018	0.259
7	44d	1.0	6.00	1.5	0.33	0.016	0.436
8	36b	2.0	6.00	1.5	0.43	0.023	0.303
9	39e	1.5	5.75	1.0	0.56	0.021	0.680
10	44f	1.5	5.75	1.0	0.49	0.018	0.600

[a]For description of experimental design matrix, *see* Fig. 3A. For graphical representation of the responses, *see* Fig. 4.

significant interactions are evident. Results of the ANOVA on xylose utilization rate (avQ_s) also indicate that all three factors significantly affect ${}_{av}Q_s$ (CL > 90%); the effects of acetic acid and pH (rather than nutrient) are higher than the 95% confidence level (CL > 95%). As with cell mass production, acetic acid concentration has the largest effect on avQ_s, and it is a negative effect, with the average rate of xylose consumption decreasing with increasing acetic acid concentration. pH again has a small, but statistically significant positive effect, with avQ_s increasing when seed growth is carried out at higher pH. Nutrient (cCSL) level also has an overall positive effect, although it is less pronounced than the effect of pH. ANOVA of avQ_s results also indicates that significant factor–factor interactions are present, with the nutrient × pH two-way interaction significant at above the 80% confidence level (CL > 80%) and the nutrient × acetic acid interaction significant at the 95% confidence level (CL > 95%). The three-way nutrient × pH × acetic acid interaction also appears to be highly significant (CL > 95%). Surprisingly, a significant interaction is not observed between pH and acetic acid for avQ_s.

The results of the 2^3 design conclusively demonstrate that low levels of inexpensive cCSL (1.5–2% v/v) cost-effectively supply nutrients for seed growth on synthetic hardwood hemicellulose hydrolyzates. The results of the 2^3 experiment also show that at acetic acid concentrations above 0.5% (w/v), seed production must be carried out at a pH above 5.5 to achieve robust cell growth. This finding contrasts with cell mass

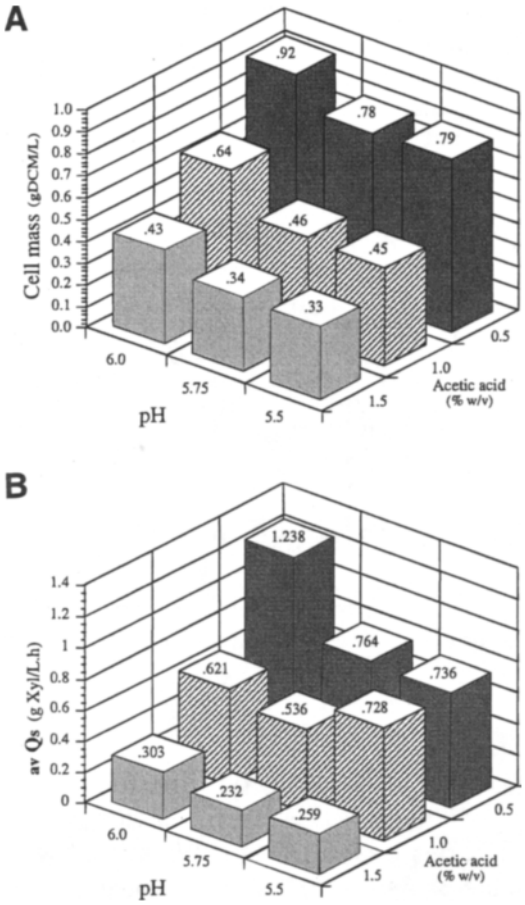

Fig. 5. Response profiles for 3^2 full-factorial study with Zm CP4:pZB5 using a synthetic biomass prehydrolyzate. **(A)** Cell mass concentration, and **(B)** average volumetric rate of xylose utilization. The experimental design matrix is illustrated in Fig. 3B. The center bar in the 3D plot represents the average of the duplicate center points. The two variables (levels in brackets) were pH (5.5, 5.75, and 6.0), acetic acid (0.5, 1.0, and 1.5% w/v). The medium contained 4% xylose and 0.8% glucose, and was supplemented with 2% (v/v) cCSL.

production in the absence of acetic acid, which exhibits no dependence on pH within the pH range of 5.5–6.0 (Fig. 1).

A follow-up 3^2 full-factorial experiment (with duplicate centerpoints) was designed to probe further for an anticipated pH × acetic acid interaction by characterizing, at a preliminary level, the response surfaces of cell mass production (as final cell mass concentration) and rate of xylose utilization (as avQ_s) in the acetic acid-pH design space. The overall experimental design is graphically depicted in Fig. 3B. Nutrient level was fixed at 2% (v/v) cCSL in this experiment to minimize the likelihood of nutrient limitation; sugar loading was the same as in the 2^3 experiment. Thus, an additional six fermen-

Table 3
Summary of Conditions and Responses for a 3^2 Factorial Study with Recombinant pZB5 and a Synthetic BPH medium[a]

Exp. #	ID #	Conditions		Responses		
		Acetic acid (% w/v)	pH	Cell mass (g DCM/L)	Growth Yield (gDCM /g S used)	av Q_s (Xyl) (g Xyl/L.h)
1	37c	0.5	5.50	0.79	0.017	0.736
2	50a	1.0	5.50	0.45	0.017	0.728
3	44e	1.5	5.50	0.33	0.018	0.259
4	37d	0.5	6.00	0.92	0.018	1.238
5	50f	1.0	6.00	0.64	0.028	0.621
6	36b	1.5	6.00	0.43	0.023	0.303
7	50b	0.5	5.75	0.78	0.027	0.764
8	50c	1.0	5.75	0.45	0.021	0.550
9	50d	1.0	5.75	0.47	0.026	0.522
10	50e	1.5	5.75	0.34	0.021	0.232

[a]For description of experimental design matrix, *see* Fig. 3B. For graphical representation of the responses, *see* Fig. 5.

tations at the 2% (v/v) cCSL level beyond the four completed in the 2^3 design had to be run to complete this 3^2 full-factorial design.

Results of the 3^2 response surface study are shown in Fig. 5 and values are listed in Table 3. As Fig. 5A shows, there is not much curvature evident in the final cell mass concentration response. Dependence on acetic acid level is pronounced compared to dependence on pH. Values are highest at the lowest acid acid level regardless of pH, although at any particular acetic acid level, cell growth is highest at pH 6.0. The avQ_s response surface is more complicated and exhibits more curvature. The rate of xylose utilization (avQ_s) at the lowest acetic acid concentration and highest pH is significantly greater than at any other condition.

ANOVA calculations performed on the 3^2 results indicate that the final cell mass concentration response exhibits curvature with respect to both acetic acid level and pH (CL > 95%), but that no interaction between acetic acid and pH is present. In contrast, ANOVA for avQ_s indicates that the response of average xylose utilization rate has significant curvature with respect to pH (CL > 85%), but is linear with respect to acetic acid concentration (CL > 95%). However, the response of avQ_s exhibits a significant pH x acetic acid interaction (CL > 90%). These results indicate that the pH x acetic acid two-way interaction primarily influences the rate of xylose utilization. Thus, knowledge of this two-way interaction is necessary to maximize the rate of xylose utilization, but not to maximize the extent of cell mass production.

The results shown in Fig. 5 can be used to determine the approximate optimum pH to maximize seed production for a given (known) concentration of acetic acid. This is useful because acetic acid levels in hemicellulose hydrolyzates vary for different feedstocks. Alternatively, the results shown in Fig. 5 can be used to motivate the need to maintain acetic acid concentrations below 1.0% (w/v) to achieve robust cell production.

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REFERENCES

1. Wright, J. D. (1988), *Chem. Eng. Prog.* **84,** 62–74.
2. Wright, J. D., Wyman, C. E., and Grohman, K. (1988), *Appl. Biochem. Biotechnol.* **18,** 75–90.
3. Wyman, C. E. and Hinman, N. D. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 735–753.
4. Lynd, L. R. (1989), *Adv. Biochem. Eng. Biotechnol.* **38,** 1–52.
5. Lynd, L. R., Cushman, J. H., Nichols, R. J. and Wyman, C. E. (1991), *Science* **251,** 1318–1323.
6. Keim, C. R. (1983), *Enzyme Microbiol. Technol.* **5,** 103–114.
7. Jeffries, T. W. (1981), *Biotechnol. Bioeng., Symp.* **11,** 315–324.
8. Jeffries, T. W. (1983), *Adv. Biochem. Eng. Biotechnol.* **27,** 1–32.
9. Jeffries, T. W. (1990), in *Yeast: Biotechnology and Biocatalysis,* Verachtert, H. and De Mot, R., eds., Marcel Dekker, New York, pp. 349–394.
10. Hahn-Hägerdahl, B., Hallborn, J., Jeppson, H., Olsson, L., Skoog, K., and Walfridson, M. (1993), in *Bioconversion of Forest and Agricultural Plant Residues* Saddler, J. N., ed., C.A.B. International, Wallingford, UK, pp. 231–290.
11. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
12. Hinman, N. D., Schell, D. J, Riley, C. J., Bergeron, P. W., and Walter, P. J. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 639–649.
13. Lynd, L. R. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 695–719.
14. Picataggio, S. K., Eddy, C., Deanda, K., Franden, M. A., Finkelstein, M., and Zhang, M. (1996), *Seventeenth Symposium on Biotechnology for Fuels & Chemicals* (Paper #9).
15. Picataggio, S. K., Zhang, M., and Finkelstein, M. (1994), in: *Enzymatic Conversion of Biomass for Fuels Production,* Himmel, M. E., Baker, J. O., and Overend, R. A., eds., American Chemical Society, Washington, DC, *ACS Symposium Series* 566, pp. 342–362.
16. Zhang, M., Franden, M. A., Newman, M., McMillan, J., Finkelstein, M., and Picataggio, S. K. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 527–536.
17. Rogers, P. L., Lee, K. J., Skotnicki, M. L., and Tribe, D. E. (1982), *Adv. Biochem. Eng.* **23,** 37–84.
18. Lawford, G. R., Lavers, B. H., Good, D., Charley, R., Fein, J., and Lawford, H. G. (1982), in *International Symposium on Ethanol from Biomass,* Duckworth, H. E. and Thompson, E. A., eds., Royal Society of Canada, Winnipeg, Canada, pp. 482–507.

19. Lawford, H. G. (1988), *Proc. VIII Int'l Symp. on Alcohol Fuels*, New Energy Development Organization, Tokyo, pp. 21–27.
20. Swings, J. and DeLey, J. (1977), *Bacteriol. Rev.* **41,** 1–46.
21. Montenecourt, B. S. (1985), in *Biology of Industrial Microorganisms*, Demain, A. L. and Simon, N. A., eds., Benjamin/Cummings, Meno Park, CA, pp. 216–287.
22. Doelle, H. W., Kirk, L., Crittenden, R., Toh, H., and Doelle, M. (1993), *Crit. Rev. Biotechnol.* **13,** 57–98.
23. Lawford, H. G. (1988), *Appl. Biochem. Biotechnol.* **17,** 203–219.
24. Lawford, H. G. and Ruggiero, A. (1990), *Biotechnol. Appl. Biochem.* **12,** 206–211.
25. Bringer, S., Sahm, H., and Swyzen, W. (1984), *Biotechnol. Bioeng.* **Symp. No. 14,** 311–319.
26. Rodriguez, E. and Callieri, D. A. S. (1986), *Biotechnol. Lett.* **8,** 745–748.
27. Doelle, M. B., Greenfield, P. F., and Doelle, H. W. (1990), *Proc. Biochem.* **25,** 151–156.
28. Beavan, M., Zawadzki, B., Droiniuk, R., Fein, J. E., and Lawford, H. G. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 319–326.
29. Lee, G. M., Kim, C. H., Lee, K. J., Zainal Abidin Mohd, Y., Han, M. H., and Rhee, S. K. (1986), *J. Ferment Technol.* **64,** 293–297.
30. Parekh, S. R., Parekh, R. S., and Wayman, M. (1989), *Proc. Biochem.* **24,** 85–91.
31. Park, S. C., Kademi, A., and Baratti, J. C. (1993), *Biotechnol. Lett.* **15(11),** 1179–1184.
32. Liu, C.-Q., Goodman, A. E., and Dunn, N. W. (1988), *J. Biotechnol.* **7,** 61.
33. Feldman, S. D., Sahm, H., and Sprenger, G. A. (1992), *Appl. Microbiol. Biotechnol.* **38,** 354.
34. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. K. (1995), *Science* **267,** 240–243.
35. Lynd, L. R., Elander, R. T., and Wyman, C. E. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 641–661.
36. Goodman, A. E., Rogers, P. L., and Skotnicki, M. L. (1982), *Appl. Environ. Microbiol.* **44(2),** 496–498.
37. Fein, J. E., Charley, R. C., Hopkins, K. A., Lavers, B., and Lawford, H. G. (1983), *Biotechnol Lett.* **5,** 1–6.
38. Nipkow, A., Beyeler, W., and Feichter, A. (1984), *Appl. Microbiol. Biotechnol.* **19,** 237–240.
39. Galani, I., Drainas, C., and Typas, M. A. (1985), *Biotechnol. Lett.* **7,** 673–678.
40. Baratti, J., Varma, R., and Bu'Lock, J. D. (1986), *Biotechnol. Lett.* **8,** 175–180.
41. Lawford, H. G., Holloway, P., and Ruggiero, A. (1988), *Biotechnol. Lett.* **10,** 809–814.
42. Lawford, H. G. and Rousseau, J. D. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 307–326.
43. Asghari, A., Bothast, R. J., Doran, J. B., and Ingram, L. O. (1996), *J. Ind. Microbiol.* **16,** 42–47.
44. Lawford, H. G. and Rousseau, J. D. (1997), *Appl. Biochem. Biotechnol.* (18th Symp.), **63–65,** 287.
45. Grohman, K., Himmel, M., Rivard, C., Tucker, M., Baker, T., Torget, R., and Graboski, M. (1984), *Biotechnol. Bioeng. Symp.* **14,** 139–157.
46. Kong, F., Engler, C. R., and Soltes, E. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 23–35.
47. Timell, T. E. (1964), *Adv. Carbohydrate Chem.* **19,** 247–302.
48. Lawford, H. G. and Rousseau, J. D. (1993), in *Energy from Biomass and Wastes XVI* (March 1992), Klass, D. L., ed., Institute of Gas Technology, Chicago, IL, pp. 559–597.
49. McMillan, J. D. (1994), in *Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M. E., Baker, J. O., and Overend, R. A., eds., American Chemical Society, Washington, DC, *ACS Symposium Series* **566,** pp. 411–437.
50. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 301–322.
51. Lawford, H. G. and Rousseau, J. D. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 437–448.
52. Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978), in: *Statistics for Experimenters* Wiley, New York.
53. Davies, O. L. (1967), in *Design and Analysis of Industrial Experiments*, Hafneri, New York.
54. Maddox, I. S. and Richert, S. H. (1977), *J. Appl. Bacteriol.* **43,** 197–204.
55. Myers, R. H. (1971), in *Response Surface Methodology*, Allyn and Bacon, Boston.