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Comparative Performance Trials with Yeast and *Zymomonas* for Fuel Alcohol Production from Corn

Scientific Note

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ABSTRACT

Z. mobilis (ATCC 29191) produced ethanol in significantly greater yield than *S. cerevisiae,* (0.49 vs 0.45 g ethanol/g glucose entering the system), in multistage continuous culture, on commercial feedstocks obtained from corn wet milling operations. In the same system, *Z. mobilis* showed higher volumetric productivity (4.14 vs 3.06 g/L/h) than *S. cerevisiae.* The approximately 9% increase improvement in ethanol production that should result from the improvement in yield makes the cost for retrofitting a plant for the *Z. mobilis* process minimal.

Index Entries: *Zymomonas;* yeast; ethanol; yield; productivity; corn.

INTRODUCTION

Biotechnology in the fuel alcohol industry relates to the various bioengineering strategies for improving the fermentation process with respect to the efficiency of substrate to product conversion (yield, Yp/s), final product concentration, and productivity (*8*). Fermentation is generally

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considered a low productivity and capital intensive approach to producing organic chemicals. Most fermentation ethanol is currently produced by traditional batch fermentations using yeast. The engineering approach to improving process productivity has involved operating the fermentors continuously at higher cell densities with systems employing cell retention or recycle. The biological approach to process improvement addresses the characteristics of the process organisms.

For the past 10 years we have been investigating the potential of various organisms to effectively replace yeast, and our attention has been focused on the ethanologenic bacterium *Z. mobilis*. Yeast and *Z. mobilis* are both equally sensitive to ethanol toxicity and produce similar final concentrations of ethanol, but, in contrast to yeast, *Z. mobilis* is an anaerobe and does not require any oxygen for growth. Several independent studies with this organism have now produced a consensus concerning the superiority of *Z. mobilis* both with respect to product yield and specific fermentation activity (*11,7*). In terms of cost, Flannery and Steinschneider (1983) estimated that a 5% improvement in ethanol yield could lead to a 2–3% reduction in the total cost of alcohol production from corn starch hydrolyzate. The specific rate of ethanol production (Qp) of *Z. mobilis* is at least 25 times faster than yeast under similar culture conditions.

In high cell density continuous flow systems (operating with cell recycle (*1*) or cell retention either as immobilized (*2,6*) or flocculated cultures (*3*), at equivalent cell loading to yeast), the superior kinetic properties of *Z. mobilis* can be exploited to effect about a fivefold improvement in volumetric productivity. The patented "Bio-Hol Processes" (US Patent Nos 4647534 and 4731329) for motor fuel alcohol production incorporate advanced biotechnologies and exploit the high-performance and ultra-efficiency characteristics of *Z. mobilis* in significant improvements of traditional yeast-based fermentations with respect to product yield, concentration, and productivity. On laboratory grade chemical feedstocks the Bio-Hol Process produces a beer with an ethanol concentration in excess of 10% (w/v) with a carbohydrate conversion efficiency of 97% (*1*).

In the present study, a direct side-by-side comparison of *Z. mobilis* with a commercial yeast strain on an industrial-grade medium was undertaken, to demonstrate the superiority of the bacterium for commercial purposes. The present study focuses on the potential for yield improvement using an industrial feedstock (specifically enzymatically-hydrolyzed corn starch) in which all essential growth nutrients are supplied exclusively by the process water (corn steep liquor) from a corn wet-milling operation. We hoped to demonstrate the benefits accruing to a current commercial operation through the simple replacement of yeast with *Z. mobilis*. However, because of certain limitations in the design configurations of the equipment employed (e.g., operated without biomass retention or cell recycle), there was no attempt to demonstrate the full extent of the kinetic advantages of *Z. mobilis* in improving productivity.

MATERIALS AND METHODS

Microorganisms

A culture of *Saccharomyces cerevisiae* was obtained from an American commercial fuel ethanol producer. *Z. mobilis* (ATCC 29191) was purchased from American Type Culture Collection, Rockville, MD.

Media

Microorganisms were maintained on 6% (w/v) glucose plus 0.6% (w/v) yeast extract (Difco Laboratories Ltd.), solidified with 1.5% (w/v) Bacto-Agar (Difco Laboratories Ltd.). This same medium without agar was used for starter cultures. Commercial feedstocks containing enzymatically hydrolyzed corn starch, corn steep liquor, and recycled stillage from two corn wet milling operations were used. This production medium was provided by a major North American ethanol producer.

Culture Conditions

Fermentations were conducted in comparable one- and two-stage continuous systems (*1*) (Fig. 1). The two-stage cascade system was constructed using a New Brunswick Scientific (NBS, Edison, NJ) "BioFlo" Model C-30 vessel for Stage 1 and a NBS "MultiGen" Model F-2000 vessel (modified to include an overflow weir) for Stage 2. The working volumes were approximately 0.35 L for Stage 1 and 1.5 L for Stage 2. Temperature was controlled at 30°C with an electrical heater and circulating cooling water. The pH value was controlled at 5.0 for *Z. mobilis* and 4.2 for *S. cerevisiae* (natural pH of the feedstock as maintained in the supplier's commercial ethanol production process) by addition of 2.5 *M* NaOH. Sugar feed concentrations and other experimental details are given in the Table legends.

Impeller tip velocities were set at 46.2 m/min for Stage 1 and 61.6 m/min for Stage 2. The single CSTR system was comparable to Stage 1 of the two-stage system.

Inoculum size was 10% (v/v), taken from an overnight starter culture. Culture medium flows were initiated at the end of batch growth. The flowrates were set at the maximum flows attainable while still maintaining minimal efflux of sugar from the two systems. Samples for analysis were removed from culture vessels after three medium volume changes, over a period of 50 h. No appreciable oscillating behaviour was apparent over this time.

Analyses

Glucose was determined using a YSI Industrial Analyzer, Model 27 (Yellow Springs Instrument Co. Inc., OH). Ethanol was determined by a standardized U.V. assay using alcohol dehydrogenase (Boehringer Mann-

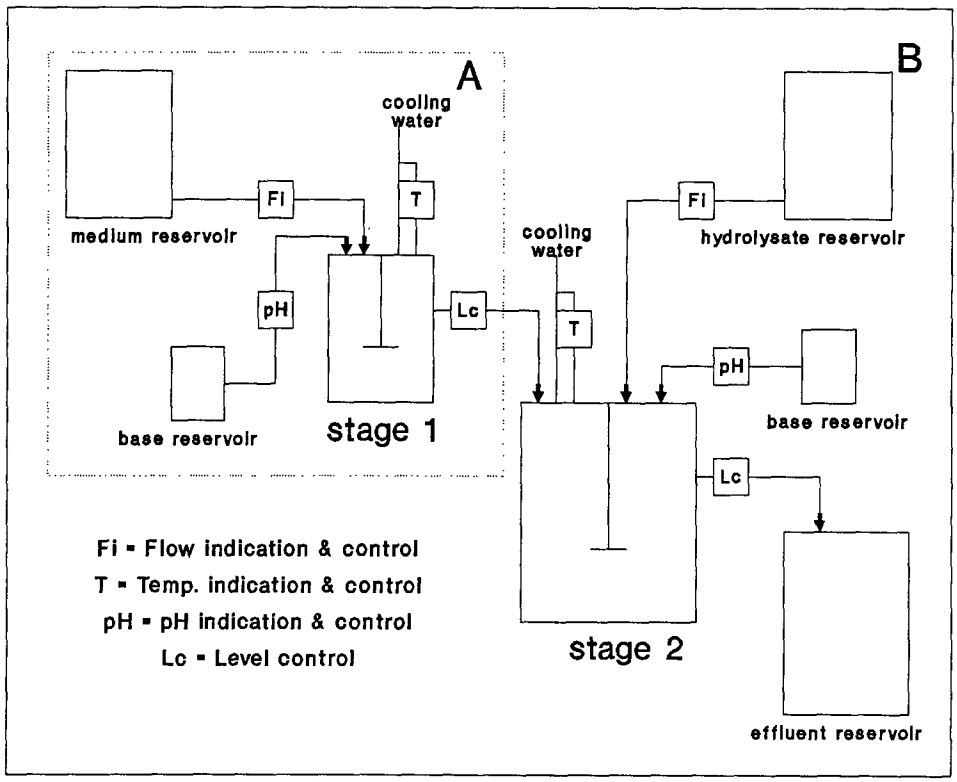

Fig. 1. Schematic diagram of a one and two-stage CSTR system. (A) One-Stage System; (B) Two-Stage Cascade System.

heim, Quebec, Canada). The values calculated for ethanol yields were analyzed using a *t*-test for the comparison of the means of two samples. The minimum level of significance was $P<0.05$. Reproducible biomass concentrations in the culture vessels could not be obtained because of the heavy load of particulate matter in the medium.

RESULTS

In the one-stage system, *Z. mobilis* and *S. cerevisiae* were compared using media formulated in the laboratory from commercial feedstocks. In the two-stage cascade system, the feed sugar concentrations for *S. cerevisiae* were those used in the actual commercial process.

In these experiments, *Z. mobilis* could not be operated under the same conditions as *S. cerevisiae* with respect to feed sugar concentration. The use of a feed glucose concentration of 159 g/L led to cycles of growth and washout of the *Z. mobilis* culture. Similar observations were reported by Lee et al. (1979) for *Z. mobilis* ATCC 10988 grown on a laboratory medium.

Table 1
Comparison of Parameters for *Z. mobilis* ATCC 29191 and *S. cerevisiae* in One-Stage Continuous Culture[a]

Parameter	*Z. mobilis*	*S. cerevisiae*
Volume (L)	0.36	0.35
Dilution rate (L/h)	0.10	0.10
[Ethanol] (g/L)	50.7	55.1
Glucose in (g/h)	4.1	4.2
Glucose used (g/h)	4.1	4.2
Ethanol produced (g/h)	1.8	1.9
Vol. productivity (g/L/h)	0.51	0.55
Ethanol yield (g/g)	0.44	0.46
Residual glucose (g/L)	0.08	0.00

[a]The glucose concentration of the commercial feed medium was 114 g/L for *Z. mobilis* and 120 g/L for *S. cerevisiae*. Temperature = 30°C; pH = 5.0 for *Z. mobilis* and 4.2 for *S. cerevisiae*. Ethanol yield is expressed as g ethanol produced/g glucose used.

Table 2
Comparison of Parameters for *Z. mobilis* ATCC 29191 and *S. cerevisiae* in Stage 1 of a Two-Stage Continuous Culture[a]

Parameter	*Z. mobilis*	*S. cerevisiae*
Volume (L)	0.38	0.38
Dilution rate (L/h)	0.15	0.15
[Ethanol] (g/L)	53.7	49.4
Glucose in (g/h)	6.8	9.1
Glucose used (g/h)	6.6	6.2
Ethanol produced (g/h)	3.1	2.8
Ethanol yield (g/g)	0.46	0.44
Residual glucose (g/L)	2.7	49.8

[a]The glucose concentration of the commercial feed medium was 119 g/L for *Z. mobilis* and 159 g/L for *S. cerevisiae*. Temperature = 30°C; pH = 5.0 for *Z. mobilis* and 4.2 for *S. cerevisiae*. Ethanol yield is expressed as g ethanol produced/g glucose used.

These workers attributed the oscillations to product inhibition of growth at an ethanol concentration in excess of 50 g/L.

There was no significant difference between the ethanol yields for *S. cerevisiae* (0.46 g ethanol/g glucose used) and *Z. mobilis* (0.44 g ethanol/g glucose used) in one-stage continuous culture when the microorganisms were grown on similar feedstocks (Table 1). These values were not significantly different from those obtained for the first stage of the two-stage continuous culture (0.45 g/g for *S. cerevisiae* and 0.46 g/g for *Z. mobilis*) using a similar culture medium but from a different source, and operating at a higher dilution rate (Table 2).

Table 3
Comparison of Overall Kinetic Parameters for *Z. mobilis* ATCC 29191 and *S. cerevisiae* in Two-Stage Continuous Culture[a]

Parameter	*Z. mobilis*	*S. cerevisiae*
Volume (L)	1.88	1.77
Dilution rate (L/h)	0.05	0.04
Glucose in (g/h)	16.8	11.8
Glucose used (g/h)	16.1	11.7
Ethanol produced (g/h)	7.9	5.4
Ethanol yield (g/g)	0.49	0.46
Vol. productivity (g/L/h)	4.1	3.1

[a]The glucose concentration of the commercial feed medium was 119 g/L for *Z. mobilis* and 159 g/L for *S. cerevisiae* in Stage One and 298 g/L and 213 g/L, respectively, in Stage Two. Temperature = 30°C; pH = 5.0 for *Z. mobilis* and 4.2 for *S. cerevisiae*. Ethanol yield is expressed as g ethanol produced/g glucose used.

In two-stage continuous culture, *Z. mobilis* showed both a significantly higher overall yield (0.49 vs 0.46 g ethanol/g glucose used) and a higher volumetric productivity (4.14 vs 3.06 g/L/h) than *S. cerevisiae* (Table 3).

DISCUSSION

The ethanol yields and volumetric productivities for *Z. mobilis* obtained in this work using commercial feedstocks were similar to those obtained in our previous studies using laboratory culture media (*A,1*). The fact that similar yields were obtained for both *Z. mobilis* and *S. cerevisiae* in a single energy-requiring stage system is not surprising. The "energetic uncoupling" phenomenon (whereby energy supply is not regulated by the energy processes of the organism) shown by *Z. mobilis*, which leads to near theoretical conversion yields of sugar to ethanol, is achieved in a nongrowing environment, i.e., the second stage of a two-stage process. The ability of *Z. mobilis* to produce ethanol in a growing environment cannot, therefore, be expected to be vastly different from that of *S. cerevisiae* under similar conditions.

In the second stage of the two-stage system, *Z. mobilis* showed a significantly higher ethanol yield than *S. cerevisiae*, i.e., 0.51 vs 0.46 g ethanol/g glucose used, respectively, a clear demonstration of the more efficient conversion of glucose to ethanol by energetically-uncoupled nongrowing cells of *Z. mobilis*. The value for *Z. mobilis*, obtained using a laboratory culture medium, agreed well with published data (*1*). Although resting yeast cultures show a high ethanol yield, their rate of ethanol production is slow. Under such conditions, high cell densities are required to achieve an acceptable rate of fermentation (*9*).

Table 4
Comparison of Kinetic Parameters for Z. *mobilis* ATCC 29191 and *S. cerevisiae* in Stage 2 of Two-Stage Continuous Culture[a]

Parameter	*Z. mobilis*	*S. cerevisiae*
Volume (L)	1.51	1.40
Dilution rate (L/h)	0.06	0.05
Glucose in (g/h)	10.16	5.61
Glucose used (g/h)	9.46	5.51
Ethanol prod. (g/h)	4.81	2.54
[Ethanol] (g/L)	86.9	76.6
Ethanol yield (g/g)	0.51	0.46
Residual glucose (g/L)	7.7	1.4

[a]The glucose concentration of the commercial feed medium was 298 g/L for Z. *mobilis* and 213 g/L for *S. cerevisiae*. Ethanol yield is expressed as g ethanol produced/g glucose used.

Table 4 shows that the ethanol concentration in Stage 2 of the two-stage continuous culture was 76.6 g/L for *S. cerevisiae* and 86.9 g/L for Z. *mobilis*. By increasing the sugar feed concentration to Stage two under conditions permitting total sugar utilization (e.g., cell recycle or increased hydraulic retention time), it is possible to attain higher final ethanol concentrations (*1*). Our experiments were designed to simulate an existing commercial yeast-based ethanol fermentation process in a corn wet-milling operation. As such, the maximum sugar feedstock concentration that could be economically produced in the plant was about 300 g glucose/L.

The overall process yield (g ethanol/g glucose entering the system) was 0.49 for Z. *mobilis* and 0.45 for *S. cerevisiae*. The improvement of yield should lead to approximately a 9% increase in ethanol production for the same rate of sugar consumption. The realizable profits from such an increase make the additional costs for retrofitting a plant for the Z. *mobilis* process minimal.

The results provide clear evidence that Z. *mobilis* has the capacity to improve the product yield in a multi-staged continuous process using an industrial fermentation feedstock. The two-staged cascade design employed in this study permitted Z. *mobilis* to properly express its capacity for yield improvement since, under conditions of nongrowth, substrate carbon is directed almost exclusively to ethanol and carbon dioxide. However, this design did not allow for a similar demonstration of advantage with respect to volumetric productivity. This is because there was no attempt to retain the biomass within the second bioreactor and operate the Z. *mobilis* and yeast processes at equivalent biomass loadings. It is known that under conditions which promote rapid growth, Z. *mobilis* exhibits a specific rate of ethanol production about 2–5 times faster than yeast, whereas a resting culture (whereby alterations in the physicochemical factors yield an environment not conducive to growth) will produce etha-

nol about 25–30 times faster than yeast (*7,11*). Hence systems involving biomass retention (either by cell recycling or cell immobilization or use of a flocculent mutant) express the kinetic advantage of *Z. mobilis* with a concomitant improvement in both yield and volumetric improvement.

Bio-Hol Developments is committed to transferring its *Z. mobilis* related technologies to the fuel alcohol industry and is currently directing efforts to reactor designs that will be compatible with efficient heat transfer.

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