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A New Approach to Improving the Performance of *Zymomonas* in Continuous Ethanol Fermentations

HUGH G. LAWFORD

Departments of Biochemistry and Microbiology, University of Toronto, Toronto M5S 1A8, Ontario, Canada

ABSTRACT

Although most fermentation ethanol is currently produced in traditional batch processes with yeast, the ethanologenic bacterium *Zymomonas mobilis* is recognized as an alternative process organism for fuel alcohol production. Different strategies for improving the productivity of ethanol fermentations are reviewed. In batch and open-type continuous fermentations the advantage of replacing yeast by *Zymomonas* relates principally to the 10% higher fermentation efficiency (product yield), whereas in high cell density, closed-type continuous systems (operating with cell recycle or retention) the superior kinetic properties of *Zymomonas* can be exploited to affect about a five-fold improvement in volumetric productivity. Unlike yeast, the rate of energy supply (conversion of glucose to ethanol) in *Zymomonas* is not strictly regulated by the energy demand and a nongrowing culture exhibits a maintenance energy coefficient that is at least 25 times higher than yeast. As an alternative to process improvement through genetic engineering of the process organism this investigation has taken a biochemical and physiological approach to increasing the kinetic performance of *Z. mobilis* through manipulation and control of the chemical environment. Energetically "uncoupled" phenotypes with markedly increased specific rates of ethanol production were generated under conditions of nutritional limitation (nitrogen, phosphate, or potassium) in steady-state continuous culture. The pH was shown to influence energy coupling in *Zymomonas* affecting the maintenance coefficient (m_e) rather than the max growth yield coefficient ($Y_{x/s}^{max}$). Whereas the pH for optimal growth of *Z. mobilis* (ATCC 29191) in a complex medium was 6.0–6.5, the specific rate of ethanol production in continuous fermentations was maximal in the range 4.0–4.5. Fermentation conditions are specified for maximizing the specific pro-

ductivity of a *Zymomonas*-based continuous ethanol fermentation where the potential exists for improving the volumetric productivity in dense culture fermentations with an associated 35–40% reduction in capital costs of fermentation equipment and an estimated savings of 10–15% on cost of product recovery (distillation), and 3–7% on overall production costs based on the projected use of inexpensive feedstocks.

Index Entries: *Zymomonas mobilis*; continuous ethanol fermentations; energetic uncoupling; maintenance energy coefficient; growth & product yields; specific productivity; nutrient-limitation; pH control.

INTRODUCTION

Fermentation Ethanol—A Historical Perspective

The art of producing alcoholic beverages has been practiced by craftsmen for literally thousands of years with trade secrets being passed from one generation of brewers, vintners, or distillers to the next. Apart from the size of the operation and the more recent introduction of pure culture and asceptic techniques, the craft of fermentation has remained relatively unchanged over the centuries. In fact, it was only about 100 years ago that the causative agent for the process (fermentation) whereby sugar is converted to alcohol (specifically ethanol) and carbon dioxide, was identified by Pasteur as a living microorganism, namely yeast. Even today the economics associated with the production of potable alcohol is sufficiently insensitive to cost that there is little incentive in the industry for change or process improvement.

Ethanol is used in a variety of industries both as a solvent and as a chemical feedstock, and this so-called "industrial alcohol" is principally derived from petroleum. The productivity of the process for producing industrial alcohol from ethylene is orders of magnitude greater than the traditional batch fermentation process. The meteoric rise in the costs of fossil fuels and the so-called energy crisis of the 1970s gave rise to the American gasohol program with substantial government subsidies associated with denatured fermentation alcohol destined for use as a gasoline extender in 10% blends. In attempting to become energy self-sufficient, Brazil has implemented an ambitious program aimed at replacing a major portion of their gasoline requirements by fermentation alcohol. The idea of using ethanol as an automotive fuel is not new. Gasoline/ethanol blends were commonly used in Europe prior to World War II when the cost of petroleum favored the economics of fermentation ethanol production. Today, largely because of environmental concerns related to the use of lead in gasoline, the interest in fuel ethanol remains despite the return to cheaper petroleum from the major oil-producing countries. Now ethanol is marketed as the "octane booster" in lead-free gasoline. In 1981 it

was estimated that three-quarters of the world's production of 3.2 billion L of fermentation ethanol was produced by the same batch processing techniques that were employed prior to World War II (*1*). Cost analyses (*2,3*) made when the costs for fossil fuels were relatively high, indicating that fermentation ethanol might compete with ethylene-derived ethanol, are no longer valid and the present economic environment has provided the necessary incentive for developing new and more efficient fermentation processes.

The Engineering Approach to Process Improvement

Compared to chemical processes, fermentation is generally considered a low productivity and capital intensive approach to producing organic chemicals. The productivity (*VP*) of the fermentation process is defined as the product of the specific rate of ethanol production (*Qp*) and the cell density (measured as dry biomass). One of the first approaches taken in improving the yeast ethanol fermentation process involved operating the fermenters in a continuous mode rather than the more conventional batch mode and thereby increasing the productivity about threefold from about 2 to 6 g EtOH/L/h (*4*). Operating continuously at higher cell densities using continuous cell recycle reactors was another effective means of greatly increasing the productivity. A single-stage CSTR with cell recycle operating at high biomass loading (50–80 g yeast/L) has an ethanol productivity of 30–40 g EtOH/L/h (*5–7*). Several different technologies related to cell recycle (*8*) or cell retention have been investigated at lab or pilot scale, including tower (*9*), dialysis (*10*), hollow fiber (*11*), and immobilized cell reactors (*12,13*).

The Biological Approach to Process Improvement

Since the specific productivity (*Qp*) is a characteristic of the process organism (the biocatalyst), another approach to process improvement has been to replace the more traditional alcohol producing organism, namely yeast, with a more efficient and productive species. The gram-negative bacterium *Zymomonas mobilis* (*14*) has attracted considerable attention in this regard by virtue of its marked superior kinetic and yield characteristics (for reviews see (*15–17*). Furthermore, unlike yeast, *Zymomonas* does not require small amounts of oxygen for growth and is, therefore, a more suitable candidate for continuous fermentations of the type required for economic production on an industrial scale (*18*). However, *Zymomonas* is no more alcohol tolerant than yeast and, therefore, the principle improvement to be derived through the substitution of *Zymomonas* for yeast in either a batch or continuous process (operating without either cell recycle or cell retention), is by virtue of the approximate 10% improvement in product yield or fermentation efficiency (*19,20*) and this is significant because the feedstock represents the major portion of the cost of production. On the other hand, the productivity of a CSTR oper-

ating with either cell recycle or cell retention is directly proportional to the *Qp*, and for an equal cell mass in the reactor, the *Zymomonas*-based process surpasses the productivity of the yeast-based process of similar design by about fivefold. This has been confirmed in several independent lab trials on which productivities in the range 120–200 g EtOH/L/h have been reported (*17,21–23*).

The Genetic Approach to Process Improvement

The genetic approach to increasing process productivity attempts to improve the performance of the process organism by attempting to correct a recognized weakness or deficiency as, for example, the alcohol tolerance. End-product or ethanol toxicity limits the final product concentration to about 10% (w/v) in batch fermentations and 5% in single-stage continuous processes (*24*). This limitation fuels the constant search for more alcohol tolerant ethanologenic organisms. Claims have been made regarding the improved performance of a putative alcohol-tolerant mutant of *Z. mobilis* (strain ZM481) (*17,25–27*), although these have been recently disputed (*28,29*).

Unlike yeasts which are known to be capable of producing ethanol from a fairly wide spectrum of commercial raw materials (*30*), *Zymomonas* is very restricted principally to glucose, fructose, and sucrose as fermentable feedstocks (*14–17*) and presently this deficiency probably poses the greatest threat to the use of *Zymomonas* for large-scale commercial production. Since cellulosic materials represent the most abundant and inexpensive source of renewable carbon feedstock (*30*), the ultimate objective of the modern genetic engineer is the construction or creation of a "super-strain" of *Zymomonas* capable of efficient cellulose and hemicellulose digestion and fermentation (*15*). As an initial approach to transposon mutagenesis, several independent investigations (*31,33*) have been undertaken to establish a suitable, stable plasmid vector with which to insert the desired genetic information and certain success in this approach has been recently reported (*34,35*).

The Physiological Approach to Process Improvement

As an alternative to genetic manipulation, we have adopted a physiological approach. This approach derives from an appreciation that the parameters involved in process productivity, namely, the specific rate of ethanol production and the growth yield coefficient ($Y_{x/s}$), are affected by environmental factors such as the chemical composition of the fermentation medium—the concentration of essential nutrients or inhibitory substances, as well as the pH and temperature. This has not been an area which has received much attention in the past with only a few reports of the effects of nutritional manipulations on the performance of *Zymomonas* in continuous culture (*15,17,36–40*).

The objective of the present study was to exploit the controlled growth environment of the chemostat to determine the effect of various nutritional limitations and pH on the bioenergetics and fermentation performance of a representative type-strain of *Zymomonas mobilis* (ATCC 29191) under steady-state growth conditions.

MATERIALS AND METHODS

Organism

The type strain *Zymomonas mobilis* ATCC 29191 was obtained as a lyophilized culture from the American Type Culture Collection (Rockville, MD, USA).

Growth Media

All media were prepared with laboratory grade reagents in distilled water. The defined salts medium of Fein et al. (*38*) was modified to a "minimal" salts medium and contained the following in 1 L: KH_2PO_4, 3.48 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; citric acid, 0.21 g; calcium pantothenate, 0.001 g; biotin, 0.001 g and NH_4Cl, 1.6 g. The source of assimilable nitrogen in the complex medium was Difco yeast extract (in the range 0.3–0.5%) either with or without supplemental amounts of NH_4CL (see text for details). Glucose was added in the amount specified and was autoclaved separately. For potassium limitation, sodium phosphate was substituted for the potassium salt and KCl was added as specified as the sole source of potassium (NaOH replaced KOH as the titrant). PPG 2025 was added at 0.1 ml/L as antifoam.

Fermentation Equipment, Culture Conditions, and Analytical Procedures

Batch fermentations were conducted with 1 and 2 L bench-top stirred fermenters fitted with pH control (New Brunswick Scientific MultiGen® type fermenters, models F1000 and F2000). Continuous cultures were conducted in bench-top chemostats (Bioflo® C30, New Brunswick Scientific Co., Edison, NJ). The volume (V) was constant at approximately 350 mL and sterile medium was introduced at a controlled rate (F) by means of a peristaltic pump (dilution rate, $D = F/V\ h^{-1}$). The temperature was maintained at 30°C and pH was controlled by the addition of 3N KOH at 5.5, and the agitation rate as 200 RPM. When the cultures were sparged with nitrogen gas the rate was 0.3 v/v/m. Analytical procedures were as previously described (*29*).

RESULTS AND DISCUSSION

Comparative steady-state data for yeast and *Zymomonas* continuous fermentations (based on 10% glucose) are shown in Table 1. The maintenance energy coefficient (m_e) was derived by extrapolation as the y-axis

Table 1
Comparative Steady-state Fermentation Kinetics
for Yeast and *Zymomonas*

Physiological State sp. growth rate, μ (hr^{-1})	Specific Rate of Glucose Utilization[a] (gm glu/gm cell (DW)/hr)	
	Saccharomyces	*Zymomonas*
Nongrowing (m_e) $\mu = 0$	0.06[b]	1.5
Growing (Q_s)		
(a) Slowly $\mu = 0.06$	0.8[b]	3.0
(b) Fast (μ_{max}) $\mu = 0.4$ (approx)	1.7–2.1[c]	8.1–9.7

[a]The specific rate of glucose utilization (Q_s) was determined in steady-state chemostat culture as a function of the specific growth rate (μ) equivalent to the dilution rate and the value for the maintenance energy coefficient (m_e) was derived by extrapolation to $\mu = 0$ in plots of Q_s versus D.

[b]Ref: Searle, B., and Kirsop, B. H. (1979).

[c]Ref: Rogers, P. L., et al. (1979), and Lavers, B. H., et al. (1981).

intercept in plots of Qs versus the dilution rate (D) according to the relationship derived by Pirt (*41,42*), whereby

$$Q_s = \frac{D}{Y_{x/s}^{max}} + m_e$$

The slope of this plot yields the reciprocal of the maximum growth yield ($Y_{x/s}^{max}$) corrected for the maintenance energy. The corresponding value for the specific rate of ethanol production is a function of the product yield ($Y_{p/s}$) or the efficiency with which the fermentable carbon substrate is converted to ethanol since $Q_p = Q_s (Y_{p/s})$. The product yields associated with yeast and *Zymomonas* fermentations are 0.43 and 0.47 g EtOH/g glu, respectively (*19,20*).

Ethanol production by yeast has been shown to be almost completely a growth related process (*7*), the specific rate being approximately 35 times faster in the case of fast growing yeast compared to nongrowing yeast (*43*) (Table 1). Maintenance metabolism by nongrowing yeast accounts for some alcohol production, but the rate is comparatively slow (Table 1). Ethanol and CO_2 are byproducts of energy metabolism and they are produced in response to the energy demands of the various biosynthetic processes (including growth) of the yeast cell. Thus, in yeast, there exists a fairly tight energetic coupling between the anabolic and catabolic processes. For practical purposes, this well-regulated or conservative type of glucose metabolism means that high cell densities are required in order to achieve an acceptable rate of fermentation by nonproliferating yeast.

The situation that exists in yeast is in direct contrast to that with *Zymomonas* where energy metabolism is much less controlled or regu-

lated and energy supply is not obligatorily coupled to the energy demands of the biosynthetic or anabolic processes (*44–46*). Senez (*47*) introduced the term *uncoupled growth* to denote that under some conditions the growth yield is much less than expected on the basis of the energy yield (Y_{ATP}) which, according to Bauchop and Elsden (*48*), is a constant for anaerobic growth, being 10.9 g dry biomass/mol (net) ATP. Whereas in batch culture, uncoupled growth is recognized as a decrease in μ_{max} independent of Q_s, in continuous culture, where D (equivalent to μ) is fixed at a value less than μ_{max}, one observes a decrease in $Y_{x/s}$ and an increase in Q_s with an increase in the maintenance energy coefficient. The growth studies of Belaich and his associates (*49*) (summarized in Table 2) served as a background for this present work and provided evidence that the nutritional composition of the fermentation medium could influence the degree of energetic uncoupling in *Zymomonas*. The data shown in Table 2 suggest that growth of *Zymomonas* in a nutrient-rich, complex medium promotes maximal energy conservation or "coupling," as manifested by the maximum value of $Y_{x/s}$ whereas growth in either a synthetic or defined salts medium (with ammonium chloride as sole source of assimilable nitrogen) or a medium deficient with respect to the essential growth factor, pantothenic acid (*46*), appears to promote energetic uncoupling as evidenced by the lower values for $Y_{x/s}$ under these growth conditions.

In defining their strategy for improving the performance of *Zymomonas* in ethanol fermentations, Cromie and Doelle (*50*) incorrectly argued that conditions should be engineered so that *"as little as possible of the carbon and energy source should be diverted into growth and cell maintenance."* These authors failed to appreciate the advantages to be gained by increasing the nongrowth-associated rate of ethanol production. Therefore, having concluded that it would be of great advantage to be able to manipulate and control the fermentation conditions so as to promote energetic uncoupling thereby increasing the maintenance energy coefficient, our objective was to describe environmental fermentation conditions which promote energy uncoupling in *Zymomonas*. Whereas the studies of Belaich et al. (*46,49*) were conducted in batch culture at very low glucose concentrations (0.2%) under nonsteady-state growth conditions, our investigations have exploited the controlled environment afforded by the chemostat with steady-state growth conditions where it has been possible (by extrapolation) to determine the effect of various conditions on the maintenance energy coefficient. In a previous study we reported that, contrary to the findings of Belaich et al. (*49*), pantothenate-limited growth did not induce energetic uncoupling of *Zymomonas* in a chemostat culture (*51*).

In the present study *Z. mobilis* ATCC 29191 was grown in chemostat culture with the temperature controlled at 30°C and the pH at 5.5 in both a complex culture medium containing yeast extract and a defined salts minimal medium containing ammonium salts as the nitrogen source. In

Table 2
Effect of the Nutritional Composition of the Medium on Growth and Fermentation Characteristics of *Z. mobilis* ATCC 10988 in Batch Culture[a]

Type of medium	(Ca-panto) (mg/L)	Y_g (molar) (g cell/mol)	$Y_{x/s}$ (g cell/g glu)	μ_{max} (1/h)	Q_s^{max}[b] (g glu/g cell/h)
Complex (YE + tryptone), without buffer	—	8.3	0.046	0.40	8.67
Complex, with tris, pH 6.8	—	6.5	0.036	0.35	9.55
Synthetic (defined), excess panto	5.0	6.5	0.036	0.39	10.82
limit-panto	1×10^{-4}	3.0	0.017	0.20	12.17
Defined (with NH_4 Cl), excess panto	5.0	4.7	0.026	0.30	11.48
limit-panto	5×10^{-4}	2.5	0.014	0.15	11.02

[a]Data summarized from work reported by Belaich et al. (1972) (ref. 49).

[b]Q_s was measured thermometrically; (glucose) = 0.2% w/v; (calcium pantothenate) was as specified. The *synthetic* medium contained a mixture of different amino acids and vitamins. The *complex* medium contained 0.2% yeast extract (YE) and 0.2% tryptone.

one series of experiments the glucose was kept in the range 2–3% (w/v) in order to ensure that the ethanol concentration remained well below the inhibitory threshold. The growth parameters derived from plots of Q_s versus D are summarized in Table 3. Because glucose is metabolized via the Entner-Doudoroff pathway in *Z. mobilis* (*52*) with a net molar yield of 1 ATP/glu, the molar growth yield with respect to carbon (Y_g) is the same as Y_{ATP}. The results (Table 3) show that under certain conditions *Zymomonas* is capable of coupled growth, since the value for Y_g (Y_{ATP}) closely approaches that of the Bauchop and Elsden constant (*48*) of 10.9 g cell/mol ATP. Fieschko and Humphrey (*53*) reported values for Y_g^{max} and m_e of 2.7 g cell/mol glu and 0.5 g glu/g cell/hr for carbon-limited growth of *Z. mobilis* ATCC 10988 in continuous culture (at pH 6 and for $D < 0.1$/h) in a defined salts medium containing 2% glucose and that these parameters were, respectively, 2.0 g cell/mol glu and 2.2 g glu/g cell/hr for growth in the same medium with 6% glucose. They concluded that *Zymomonas* grew in an uncoupled fashion at specific growth rates faster than 0.1 h^{-1}. We observed that increasing the concentration of glucose from 2% to 11% (in a complex medium) resulted in a decrease in Y_g^{max} from 11.2 to 6.7 g cell/mol glu (Table 3), and it would appear that ethanol has an influence on Y_g^{max} in *Zymomonas*.

Belaich et al. (*49*) (see Table 2) also observed a decrease in the molar growth yield (Y_g = 4.7 g cell/mol glu) associated with growth in a defined salts medium, and collectively (*49,53*) these observations suggest that growth in a defined medium induces energy uncoupling in *Zymomonas*. However, the results of the present investigation suggest a contrary conclusion, since the observed value for Y_g^{max} (9.0 g cell/mol glu) was only slightly less than for growth in the complex medium, with m_e being relatively unaffected (Table 3).

The cellular level of ATP has been used as a means of detecting energetic uncoupling in *Zymomonas* and whereas it was demonstrated that ATP accumulates under a condition of pantothenate deficiency in batch culture as well as during growth in a defined medium, it does not similarly accumulate under a condition of nitrogen deficiency, suggesting that, by this criterion (*45*), nitrogen limitation does not induce "uncoupled growth" of *Zymomonas*. In an earlier study (*29*) we demonstrated that a shift from nitrogen excess to nitrogen deficiency with carbon-limited continuous cultures of *Z. mobilis* (ATCC 29191) operating at a fixed dilution rate of 0.16 h^{-1}, resulted in a decrease in growth yield ($Y_{x/s}$) from 0.029 to 0.018 g cell/g glu with an increase in Q_p from 2.6 to 4.3 g EtOH/g cell/h. In the present study we examined the effect of nitrogen deficiency as a function of the growth rate (dilution rate) and the results (Table 3) corroborate our previous conclusion that the condition of nitrogen-deficient growth induces energetic uncoupling as evidenced by the observed values for Yg^{max} (3.8–4.3 g cell/mol glu). Since, in these experiments, the relationship between Q_s and D was made largely over a range of D in which there was no glucose in the chemostat effluent, the culture could

Table 3
Effect of the Nutritional Composition of the Medium on the Steady-state Growth and Fermentation Characteristics of *Zymomonas mobilis* ATCC 29191[a]

	(Glu)	(YE)	(NH_4Cl)	Y_g^{max}	$Y_{x/s}^{max}$	m_e	Q_s^{max}
Type of Medium	(g/L)			(g cell/mol)	(g cell/g glu)	(g glu/g cell/h)	
Low sugar/nitrogen excess							
complex	22	1.5	1.0	11.2	0.062	1.5	8.1
defined	30	—	1.0	9.0	0.050	1.7	9.7
Low sugar/nitrogen deficient							
complex	22	1.0	—	4.3	0.024	1.0	9.0
defined	28	—	0.27	3.9	0.022	1.7	9.3
High sugar/nitrogen excess							
complex	110	5.0	1.62	6.7	0.037	1.5	8.3
High sugar/nitrogen deficient							
complex	120	4.5	—	3.8	0.021	1.4	9.5

[a]Values for maximum growth yield coefficients (Y_g^{max} and $Y_{x/s}^{max}$) were derived as the reciprocal of the slope in plots of Q_s versus D and m_e was extrapolated as the y-axis intercept at zero growth rate. Temperature and pH were controlled at 30°C and 5.5.

be considered to be carbon-limited but nitrogen-deficient. This condition is reflected in the value extrapolated for the maintenance energy coefficient (Table 3), which is similar to that of a carbon-limited culture and does not approach a value of Q_s^{max} as would be expected in the case of true nitrogen-limited growth.

This work was intended to extend our investigation of the effect of nutrient limitation on the performance of *Zymomonas* in chemostat culture, and to this end, the chemostat was used to ascertain the relationship between the steady-state biomass concentration and the amount of growth limiting nutrient in the fermenter feed. The growth yield coefficient with respect to the particular limiting nutrient was determined as the slope in a plot of biomass versus the concentration of the entering limiting nutrient. The experimentally determined growth yields (g dry biomass/g-atom limiting nutrient) for both phosphorus and potassium are 44 and 33, respectively, and correspond to 10 g cell/g KH_2PO_4 and 17 g cell/g KCl for *Z. mobilis* (ATCC 29191) (Table 4). The kinetic parameters Q_s and Q_p for glucose-, nitrogen-, phosphorus-, and potassium-limited cultures are compared in Table 5. The chemostat was operated at a constant dilution rate of 0.15 h^{-1} and the amount of limiting nutrient required to achieve complete utilization of the glucose (60 g/L) in the feed is also indicated in Table 5. Under the condition of nutrient deficiency, be it nitrogen, phosphorus, or potassium, it takes less biomass to accomplish complete fermentation and the fermenter operates at a higher specific productivity. It is evident from the results shown in Table 5 that each of these nutrient limitations caused energetic uncoupling, as manifested by the decrease in growth yield and increase in the specific fermentation activity of the biomass. The constant level of product (28 g EtOH/L) observed under all growth limiting conditions indicates that the product yield ($Y_{p/s}$) is not adversely affected by the condition of nutrient deficiency. In fact, with less carbon being diverted for biomass synthesis (growth), there exists the potential for an increase in $Y_{p/s}$. Belaich and Senez (*46*) reported that growth of *Z. mobilis* (ATCC 10988) in batch culture in a phosphate deficient medium resulted in an inhibition of cellular activity (amounting to a 20% reduction in Q_s), whereas this study reveals that, to the contrary, phosphate-limited growth produces a marked stim-

Table 4
Experimentally Determined Values of the Growth Yield (Y_N) with Respect to Different Nutrients for *Z. mobilis* ATCC 29191[a]

	Growth yield, Y_N	Composition of biomass
Type of limiting nutrient	(g biomass/g atom)	% (w/w)
Nitrogen	7.1	14
Phosphorus	44	2.3
Potassium	33	3.0

[a]Experimental procedures are described in the text.

Table 5
Performance of Nutritionally Generated Phenotypic Variants of *Z. mobilis* ATCC 29191 in Continuous Ethanol Fermentations: Energetic Uncoupling Induced by Nitrogen, Phosphorus, or Potassium Deficiency[a,b]

Culture condition	Effluent (Biomass) (g/L)	Effluent (Glucose) (g/L)	Effluent (EtOH) (g/L)	Q_s (g/g cell/h)	Q_p (g/g cell/h)	$Y_{x/s}$ (g/g)	Amount limiting nutrient (g/L)
Limiting nutrient							
carbon (excess nutrients)							
glucose	1.73	0	28	5.2	2.5	0.029	60.0
Carbon and							
(a) nitrogen							
YE (Difco)	1.10	0	28	8.3	3.9	0.018	2.4
NH_4Cl	—	—	—	—	—	—	0.59
$(NH_4)_2SO_4$	—	—	—	—	—	—	0.72
(b) phosphorus							
KH_2PO_4	1.25	0	28	7.2	3.4	0.021	0.13
(c) potassium							
KCL	1.20	0	28	7.5	3.6	0.020	0.071

[a]Chemostat was operated at constant dilution rate of $0.15h^{-1}$ at a controlled pH of 5.5 and temperature of 30°C. The culture was not sparged with nitrogen gas. The defined, minimal salts medium was based on that of Fein et al. (38) with added biotin and calcium pantothenate at 1 mg/L. The glucose concentration in the feed was 60 g/L.

[b]In all cases, the product yield was 92% max. theoretical. Because fermentation of the substrate was 100%, the cultures were carbon-limited but nutrient deficient.

ulation of both Q_s and Q_p. Rogers et al. (*17*) examined the effect of potassium and phosphate on the performance of *Z. mobilis* (strain ZM4) in continuous culture but, since their experiments were not conducted at growth limiting concentrations, energetic uncoupling was not observed and they concluded that a decrease in the amount of phosphate did not appreciably alter either the growth yield nor the specific rate of ethanol production.

Although the use of such nutritionally generated phenotypes of *Zymomonas* would increase the specific productivity, they would not effect an improvement in respect of the total productivity of an open-type CSTR operating without cell recycle or cell retention simply because, although Q_p increases, the cell density decreases and the productivity of the fermenter remains unaltered. However, the potential for improvement would be realized to best advantage in the operation of a CSTR with either cell recycle or cell retention where one of the major constraints to increasing the volumetric productivity is related to the capacity of the system to handle high cell densities. Nevertheless the utility of such a procedure in a large-scale industrial process is perhaps somewhat questionable because of the anticipated difficulty in attempting to ensure precise control with respect to the chemical composition of the fermentation feedstock. It was therefore particularly gratifying to discover, in the course of our investigation into factors influencing energy metabolism of *Zymomonas,* that the pH of the medium affects the growth yield ($Y_{x/s}$), maintenance energy coefficient and, consequently, the specific rate of ethanol production. Compared to generating energetically uncoupled phenotypes of *Zymomonas* by means of nutritional manipulation, pH control is relatively facile and certainly would provide a much more industrially feasible approach to increasing process productivity.

A search of the *Zymomonas* literature revealed that the majority of *Zymomonas* fermentations have been controlled at pH 5.0 (for example, see Rogers' review (*17*)), whereas in all our investigations into the performance of *Zymomonas* in batch and continuous fermentations the pH was routinely controlled at 5.5. The only investigation specifically involving an assessment of the effect of pH on the kinetics of *Zymomonas* ethanol fermentations was conducted by King and Houssain (*54*) using *Z. mobilis* ATCC 10988 in small batch fermentations (at 37°C) with a complex medium containing 0.6% yeast extract (no added phosphate) and 10% glucose. Their study showed that both Q_p and $Y_{x/s}$ remained relatively constant over the pH range 5.0–7.5, but decreased significantly at pH 8.0. In another independent investigation (*55*) of the effect of pH on the performance of *Z. mobilis* (ATCC 10988) in continuous fermentations, it was concluded that alginate-immobilized cells have a broader range for optimal fermentation activity (4.4–6.0) compared to a pH optimum of 5.0 for free cells. Apart from these studies, no systematic investigation into the effect of pH on *Zymomonas* ethanol fermentation has been reported to date.

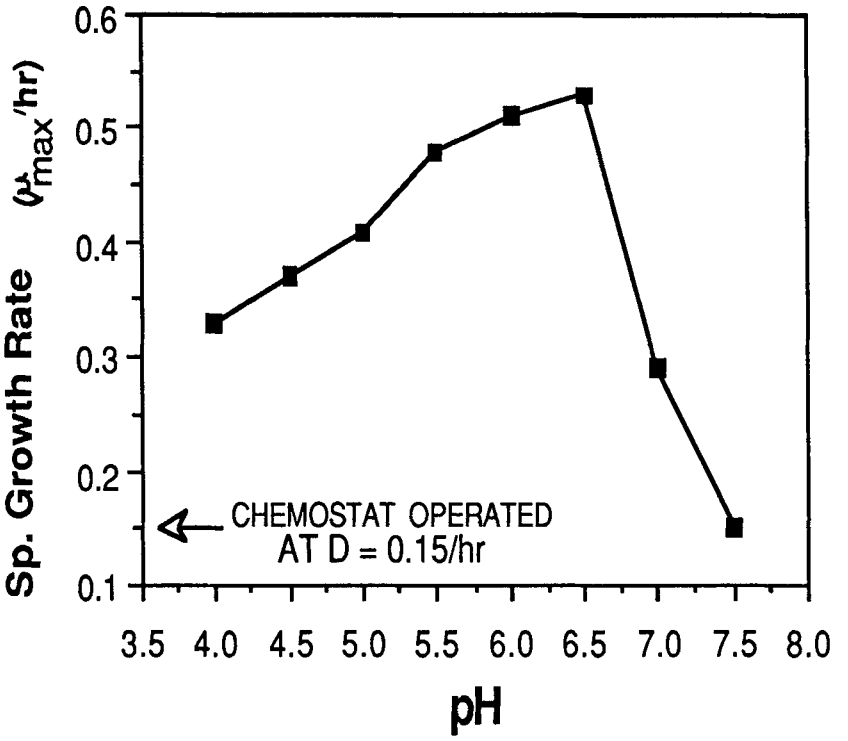

Fig. 1. The effect of pH on the maximum specific growth rate (μ_{max}) of *Zymomonas*. Z. *mobilis* ATCC 29191 was grown in a stirred-tank reactor in a complex growth medium containing mineral salts as described in the *Materials and Methods* section with 3 g/L yeast extract (Difco), 0.8 g/L NH_4Cl, and 50 g/L glucose. The glucose was autoclaved separately. The temperature was controlled at 30°C and the culture was sparged with N_2 gas at the rate of 0.3 V/V/M with the agitation at 200 rpm.

The effect of pH on μ_{max} was determined during exponential growth of Z. *mobilis* ATCC 29191 in batch cultures conducted in stirred fermenters fitted with pH and temperature (30°C) control. The complex medium contained 0.3% yeast extract, 15 m*M* NH_4Cl, and 5% glucose. The results are shown in Fig. 1. Under the conditions specified, the pH range which produced the fastest growth rate was 5.5–6.5 with an apparent optimum at pH 6.0 (Fig. 1). King and Houssain (*54*) concluded that the pH range for the most rapid growth was 6.0–7.5 with the optimum being pH 7.0. Their study indicated that the growth yield ($Y_{x/s}$) was relatively unaffected by pH over the range 5.0–7.5, being 0.016–0.012 g cell/g glu at the respective pH limits. It should be noted that these experiments were conducted at a supraoptimal temperature (37°C) for growth of *Zymomonas* (*56*) which might explain why the values determined for $Y_{x/s}$ are lower than other literature values where the growth temperature was 30°C.

The results of our experiment designed to examine the effect of pH on the performance of *Zymomonas* under steady-state growth conditions (D = 0.15 h^{-1}, T = 30°C, the complex medium contained 5% glucose

with 30 m*M* NH_4Cl) are summarized in Table 6. There was complete utilization of the glucose over the pH range from 4.0–6.5 but the culture washed out of the chemostat when the pH was decreased below 3.8. Since culture response to step changes in pH are often slow (55), a minimum of 6 turnovers (volume changes) were permitted before assuming steady-state growth. As evidenced by the value of the steady-state growth yield ($Y_{x/s}$), energy conservation appears to be maximal at pH 6.0, but as the pH decreases from 6.0–4.0 there is an apparent increasing degree of energetic uncoupling. In a separate series of growth experiments (results not shown), the effect of pH on Q_s was assessed as a function of the dilution rate (growth rate), and from an extrapolation of the linear relationship between Q_s and D, it was concluded that the pH affects m_e and not Y_g^{max}. A similar conclusion (53) was reached in connection with the effect of temperature on *Zymomonas* where the value of m_e increased from 0.5–2.5 g glu/g cell/hr with increasing temperature from 30–35°C, whereas Y_g^{max} was unaffected.

In conclusion, this study has revealed a strategy for improving the performance of *Zymomonas* in continuous fermentation processes. Through control of the chemical environment, whether it be an essential growth element such as nitrogen or phosphate, or a nongrowth related element such as in the case of the K^+ or H^+ concentration, the specific productivity of *Zymomonas* can be significantly increased. This physiological approach to process improvement was based on an understanding of energy metabolism and an appreciation of the influence of environmental factors on biological performance.

This approach is presently being evaluated at pilot scale (Bio-Hol Developments, Toronto, Canada), and it is estimated that replacement of yeast by *Zymomonas* for the purpose of fuel-alcohol production could result in a 35–40% reduction in capital costs of fermentation equipment

Table 6
The Effect of pH on Fermentation Kinetics and Growth Yield of *Z. mobilis* ATCC 29191 in Continuous Culture[a]

	(EtOH)	Qs	Qp	$Y_{x/s}$
pH	(g/L)	(g glu/g cell/h)	(g EtOH/g cell/h)	(g cell/g glu)
4.0	21.8	8.35	3.36	0.020
4.5	21.8	7.90	3.0	0.022
5.0	21.8	6.45	2.65	0.025
5.5	21.6	5.20	2.20	0.032
6.0	21.2	4.75	1.90	0.033
6.5	21.4	5.00	2.12	0.031
7.0[b]	16.0	—	—	—

[a]Chemostat was operated at constant dilution rate of $0.15h^{-1}$ and temperature, 30°C. The complex medium contained mineral salts, 3 g/L yeast extract, and 1.62 g/L NH_4Cl. Steady-state was assumed after a minimum of 6 volume changes.

[b]Biomass flocculated

with a savings of 10–15% on costs of product recovery (steam for distillation), and 3–7% on overall production costs based on the projected use of inexpensive feedstocks.

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