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Conditions that Promote Production of Lactic Acid by *Zymomonas mobilis* in Batch and Continuous Culture

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ABSTRACT

This study documents the similar pH-dependent shift in pyruvate metabolism exhibited by *Zymomonas mobilis* ATCC 29191 and ATCC 39676 in response to controlled changes in their steady-state growth environment. The usual high degree of ethanol selectivity associated with glucose fermentation by *Z. mobilis* is associated with conditions that promote rapid and robust growth, with about 95% of the substrate (5% w/v glucose) being converted to ethanol and CO_2, and the remaining 5% being used for the synthesis of cell mass. Conditions that promote energetic uncoupling cause the conversion efficiency to increase to 98% as a result of the reduction in growth yield (cell mass production). Under conditions of glucose-limited growth in a chemostat, with the pH controlled at 6.0, the conversion efficiency was observed to decrease from 95% at a specific growth rate of 0.2/h to only 80% at 0.042/h. The decrease in ethanol yield was solely attributable to the pH-dependent shift in pyruvate metabolism, resulting in the production of lactic acid as a fermentation byproduct. At a dilution rate (D) of 0.042/h, decreasing from pH 6.0 to 5.5 resulted in a decrease in lactic acid from 10.8 to 7.5 g/L. Lactic acid synthesis depended on the presence of yeast extract (YE) or tryptone in the 5% (w/v) glucose–mineral salts medium. At D = 0.15/h, reduction in the level of YE from 3 to 1 g/L caused a threefold decrease in the steady-state concentration of lactic acid at pH 6. No lactic acid was produced with the same mineral salts medium, with ammonium chloride as the sole source of assimilable nitrogen. With the defined salts medium, the conversion efficiency was 98% of theoretical maximum. When chemostat cultures were used as seed for pH-stat batch fermentations, the amount of lactic acid produced correlated well with the activity of the chemostat culture; however, the mechanism of this prolonged induction

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effect is unknown. The levels of lactic acid produced by Z. *mobilis* in this study have not been previously reported. *Zymomonas* is Gram-negative, and at no time did microscopic inspection of lactic-acid-producing cultures indicate the presence of Gram-positive organisms. Although these observations are very preliminary in nature, they have implications for the regulation of glycolytic flux in *Zymomonas*, and demonstrate the possibility of an alternative fate for pyruvate previously presumed not to exist.

Index Entries: *Zymomonas*; lactic acid; lactic acid dehydrogenase; ethanol yield; continuous fermentation.

INTRODUCTION

Cost sensitivity analyses associated with the industrial-scale production of fermentation fuel ethanol have identified product yield as the most important factor affecting production costs *(1–3)*. The bacterium *Zymomonas mobilis* (for reviews, *see* refs. *4–6*) exhibits fermentation performance characteristics *(7)* that have placed it high on the candidacy list of ethanologenic biocatalysts currently being investigated for their bioconversion efficiency in proposed fermentation processes utilizing diverse feedstocks *(8)*. Given the economic importance of ethanol yield *(3)*, a particularly attractive feature of *Zymomonas* is its high degree of ethanol selectivity, with a sugar-to-ethanol conversion efficiency typically in the range of 94–98% of theoretical maximum *(7,9–10)*. The genes for both pyruvate decarboxylase and alcohol dehydrogenase II have been cloned from *Zymomonas* and used to transform bacteria such as *Escherichia coli (11–14)* and *Klebsiella oxytoca (15)* which are capable of utilizing the different hexose and pentose sugars found in lignocellulosic hydrolysates, into highly ethanologenic recombinants. In a survey conducted by the National Renewable Energy Laboratory (NREL) (Golden, CO), *Zymomonas* was selected as the most promising host for metabolic engineering directed to the utilization of pentose sugars *(8)*. NREL used a hardwood prehydrolysate medium to test the hardiness of several *Zymomonas* strains as part of its screening for superior hosts *(16)* for genetic transformation with their proprietary *(17)* xylose assimilation and utilization plasmid *(18)*.

The authors previously, reported on the fermentation performance characteristics of one of NREL's recombinant *Zymomonas (18)* in a study that focused on the optimization of seed production for a proposed simultaneous saccharification cofermentation biomass-to-ethanol process *(19)*. In extending the investigation from batch to continuous cofermentations, it was observed that certain operating conditions appeared to promote a decrease in the ethanol yield because of the production of lactic acid. In their review of the biology of *Zymomonas*, Swings and DeLey *(4)* point out the conflicting reports in the early literature pertaining to lactic acid production by *Zymomonas* (previously known as *Termobacterium mobile* and *Pseudomonas lindneri*). Swings and DeLey *(4)* stated that "One of the impor-

tant conclusions on the carbohydrate metabolism of *Zymomonas* is that, when growing in a complex medium, 98% of the glucose is converted to ethanol, CO_2, ATP, and heat, and only 2% is used as building material for the cell."

The concentrations of lactic acid that were observed in the author's work with recombinant *Zymomonas* were considerably greater than those reported by others for various fermentations using a variety of different *Z. mobilis* isolates (20–24) in which lactic acid production did not appreciably alter the ethanol yield. With respect to other fermentation byproducts, *Zymomonas* is known to produce mannitol, glycerol, and dihydroxyacetone from fructose *(21)*, and fructose, levan, and sorbitol from sucrose *(26–28)*. The production of these byproducts can cause the ethanol yield to decrease from 0.50 to 0.35 g/g *(29)*. Therefore, most of the reports in the literature relating to the effect of environmental conditions on ethanol yield in *Zymomonas* are based on sucrose fermentations *(23,24)*. The reduction of fructose to sorbitol is dependent on the dilution rate in continuous fermentations, with very little byproduct formation observed at low dilution rates *(23)*. In a study of nutritional effects on glucose conversion efficiency by chemostat cultures of *Z. mobilis*, Cromie and Doelle *(30)* concluded that "the conversion efficiency of the glucose taken up to ethanol is not affected at all by environmental conditions." Glucose is metabolized by the Entner-Doudoroff pathway in *Zymomonas*, and there is no synthesis of fructose *bis*-phosphate *(4–6)*, but the fate of glyceraldehyde-3-phosphate is the same as in the yeasts commonly used in ethanol fermentations, with the exception that in *Zymomonas* there are two isozymes of alcohol dehydrogenase *(31)*. The regulation of glycolytic flux in *Z. mobilis* has been investigated *(32,33)*, but there was no mention of lactic acid dehydrogenase. In the author's literature search concerning lactic acid and *Zymomonas* it was discovered that a segment of DNA that lies between the genes for phosphoglycerate mutase *(pgm)* and alcohol dehydrogenase I (*adhA*) has recently been assigned as the gene for a D-isomer-specific 2-hydroxyacid dehydrogenase (*ddh*) *(34)*. The designation of this gene as *ddh* was made on the basis of the correlation in both identity (31.5%) and similarity (55.4%) between its transcription product (331 amino acid polypetide with a NAD-binding domain) and the D-isomer lactic acid dehydrogenase from *Lactobacillus plantarum (34)*. However, the precise function of this gene in *Zymomonas* remains unknown *(34)*. Fructose *bis*-phosphate is known to play a regulatory role in lactic acid dehydrogenase activity in certain bacteria *(35)*, and, although it is not an intermediate of the glucose dissimilation pathway in *Zymomonas*, fructose-6-phosphate is produced during xylose metabolism as a product of the transketolase activity in recombinant *Z. mobilis (18)*.

The present study was undertaken with wild-type cultures of *Z. mobilis* to ascertain if the shift in metabolism, which was observed in continuous fermentations with recombinant *Z. mobilis* at relatively low dilution rates, was a response common to natural isolates, or a response that was

a consequence of the genetic transformation—specifically, the operation of the metabolically engineered pentose metabolism pathway, by virtue of the expression of the four plasmid-encoded xylose utilization genes.

MATERIALS AND METHODS

Organisms

Z. mobilis strains ATCC 29191 and ATCC 39676 were obtained from the American Type Culture Collection (Rockland, MD).

Fermentation Media, Equipment, and Operating Conditions

The composition of the different media used in this study are described in Table 1. Bacto Yeast Extract (YE) and Bacto Tryptone were obtained from Difco (Detroit, MI). Other chemicals were laboratory-grade purity. Glass-distilled water was used to prepare all media.

Batch fermentations were conducted in 2-L MultiGen stirred-tank bioreactors (Model F2000, New Brunswick Scientific, Edison, NJ) fitted with agitation (100 RPM), pH, and temperature control (30°C). The working volume was 1500 mL, and the pH was controlled by the addition of 4 *N* KOH (NBS model pH-40 controller). Continuous fermentations were conducted with 750 mL MultiGen bioreactors (Model F1000, New Brunswick Scientific), except that the glass vessel had an overflow outlet. The working volume of the chemostats was about 350 mL. The flow rate of the feed medium was determined by collecting the effluent into a graduated cylinder for a specified period of time. Sampling was effected in a similar fashion. Steady state was assumed only after a minimum of 3 vol had exchanged. In all fermentations, the amount of preculture (inoculum) added was sufficient to produce an OD_{600nm} reading (1-cm light path) of 0.1–0.2 (equivalent to an initial cell density of approx 30–60 mg dry cell mass/L).

Analytical Procedures

Growth was measured turbidometrically at 600nm (1-cm light path) (Unicam spectrophotometer, model SP1800). In all cases, the blank cuvet contained dH_2O. 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, Hercules, CA). Separations were performed at 65°C, using an HPX-87H column (300 × 7.8 mm) (Bio-Rad), as previously described *(19)*. The ethanol yield ($Y_{p/s}$) was calculated as the final concentration of ethanol divided by the concentration of glucose determined to be in the medium prior to inoculation. The $Y_{p/s}$ was not corrected for the dilution caused by

Table 1
Zymomonas Media Formulations

Ingredient (g/L)	Medium type and designation					
	Complex RM[a]	Semisynthetic ZM			Defined salts DS[b]	
Glucose	100	50	50	100	50	100
Yeast Extract (Difco)	10.0	3.0	–	3.0	–	–
Tryptone (Difco)	–	–	2.1	–	–	–
NH_4Cl	–	1.6	1.6	2.4	1.6	2.4
KH_2PO_4	2.0	3.48	3.48	3.48	3.48	3.48
$MgSO_4$	–	0.49	0.49	0.49	0.49	0.49
$FeSO_4 \cdot 7H_2O$	–	0.01	0.01	0.01	0.01	0.01
Citric acid	–	0.21	0.21	0.21	0.21	0.21
Ca Pantothenate (mg)	–	–	–	–	1.0	1.0
Biotin (mg)	–	–	–	–	1.0	1.0

[a] Goodman et al. (1982) *Appl. Environ. Microbiol.* **44**: 496–498.
[b] Ref. 43.

adding alkali during the fermentation. For the purpose of carbon balancing (% C recovery), the carbon content of the cell mass (48.7%) was considered to be constant. Carbon dioxide was not measured, but was assumed to be produced at a molar equivalent to ethanol.

RESULTS AND DISCUSSION

Z. mobilis ATCC 39676 is one of the strains selected by NREL for metabolic engineering directed to xylose fermentation, using their proprietary transformation vector *(17)* carrying *E. coli* genes for xylose isomerase, xylulose kinase, transaldolase, and transketolase *(18)*. Recombinant *Z. mobilis* 39676:pZB4L is the subject of ongoing research conducted in collaboration with NREL. The fermentation performance of this recombinant in continuous cofermentations is reported elsewhere *(36)*.

Z. mobilis ATCC 39676 is a patent strain *(37)* with claimed superior sucrose fermentation characteristics, which has been developed from the designated neotype strain *Z. mobilis* ATCC 29191 (NCIB 11199) by Doelle and his research colleagues at the University of Queensland *(25,29,38)*. The performance of strain 39676 has been tested in large-scale fermentations (17,000 gal) using both milo (sorghum) *(39)* and corn starch *(40)*. Jones and Doelle *(41)* used continuous cultures of strain 39676 in a laboratory investigation on the effects of pH and nutrient limitation on fermentation performance. Since *Z. mobilis* 39676 is a patent culture for which the physiological characteristics are largely unknown, were interested in comparing its fermentation characteristics to that of strain ATCC 29191, which has been the subject of extensive research in their laboratory over the past 18 years *(9,10,42–45)*.

Under conditions of glucose-limited growth in continuous culture, using a semisynthetic medium containing 50 g/L glucose and 3 g/L yeast extract (YE) (Table 1), and with the pH controlled at 6.0, both *Z. mobilis* 39676 and 29191 produce lactic acid as a fermentation byproduct at dilution rates (i.e., growth rates) <0.2/h (Fig. 1A). Over the range of dilution rates tested (0.048 to 0.2/h), the steady-state concentration of lactic acid increased as the dilution rate decreased, and the pattern exhibited by both cultures, as a function of dilution rate, appeared similar (Fig. 1A). With strain 39676 at D = 0.042/h, the steady-state level of lactic acid decreased from 10.8 to 7.5 g/L when the set-point for the pH controller was changed from 6.0 to 5.5 (Fig. 1A). There was good closure of the carbon balance (results not shown), indicating that, apart from cell mass and carbon dioxide, lactic acid, and ethanol were the only products of glucose fermentation.

Accordingly, the decrease in ethanol yield from 0.484 g ethanol/g glucose (i.e., 95% conversion efficiency) at D = 0.2/h to 0.41 g/g (80% conversion efficiency) at D = 0.042/h, was assumed to be a direct consequence of the redirection of pyruvate metabolism from ethanol to lactic acid (Fig. 1B). *Z. mobilis* is Gram-negative *(4)*, and at no time in this study did microscopic inspection of lactic acid-producing cultures reveal the presence of Gram-positive organisms such as lactobacilli. To the authors' knowledge, the synthesis of such high levels of lactic acid by *Z. mobilis* has not been previously reported in the literature. Viikari *(23,27)* reported that *Z. mobilis* strain VTT-2-78042 produced 0.2 g/L lactic acid (D- and L-isomers) from 15% (w/v) fructose, at pH 5.5. Schmidt and Schügerl *(21)* reported that *Z. mobilis* ATCC 29191 produced a maximum of 0.2g/L lactic acid from 10% (w/v) glucose in chemostat culture, at a dilution rate of about 0.04/h. What was particularly interesting about the observations of Schmidt and Schügerl *(21)* was the decrease in lactic acid concentration from a maximum at low dilution rates to a minimum at D = 0.2/h. Although the absolute concentrations were vastly different, the pattern of acidogenesis as a function of dilution rate was similar to the authors' results (Fig. 1A).

Figure 1 shows that replacing the YE-based semisynthetic medium in the chemostat reservoir with a defined salts (DS) medium, in which ammonium chloride is the sole source of assimilable nitrogen results in a virtual eradication of the lactic acid synthesis (with the possible exception of relatively low dilution rates [< 0.08/h]). Although Figure 1 shows only the results for *Z. mobilis* 29191, identical results were obtained with strain 39676. Even at the theoretical maximum ethanol yield (0.51 g/g), the ethanol in these fermentations is not expected to be at a growth-inhibitory concentration, and the value of the maximum dilution rate, with either the semisynthetic or defined salts medium, is known to be greater than 0.2/h *(45)*. The addition of YE to strain 29191 grown in DS feed medium (1g/L), when the chemostat was being operated at a dilution rate of 0.15/h, resulted in the steady-state lactic acid concentration of 1.6g/L (Fig. 1A) which was about one-third the level of lactic acid produced by the same medium

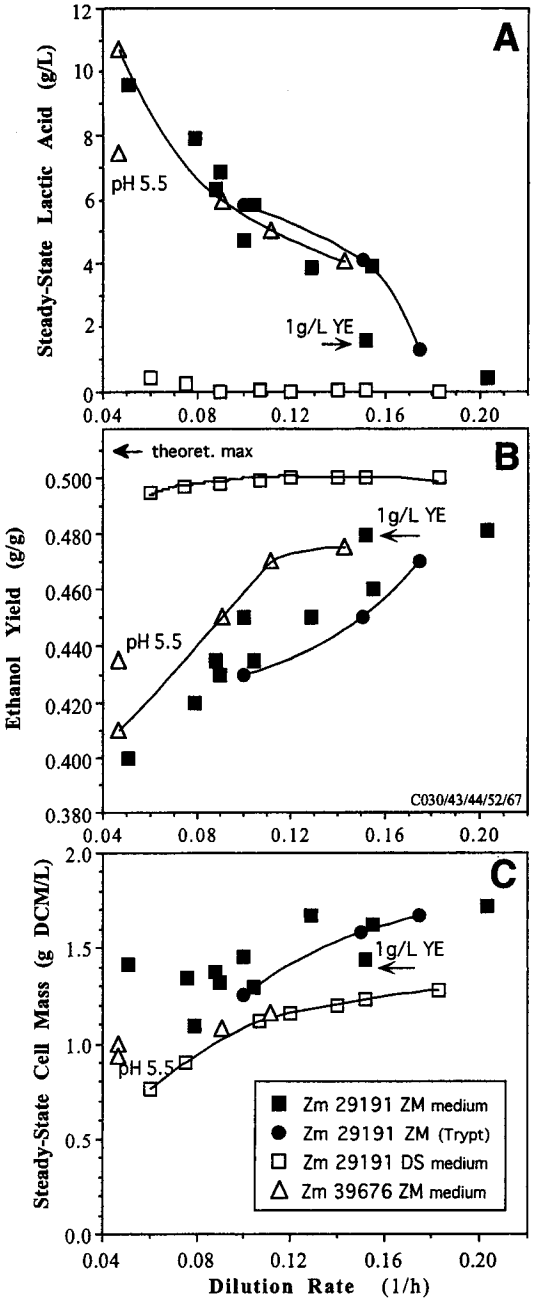

Fig. 1. Glucose-limited continuous culture of *Z. mobilis* 29191 and 39676. **(A)** Steady-state concentration of lactic acid, **(B)** ethanol yield (g ethanol/g glucose), and **(C)** steady-state DCM concentration, as a function of dilution (growth) rate. All media contained 5% (w/v) glucose, the temperature was maintained at 30°C, and the pH was controlled at 6.0 by the addition of 4 *N* KOH. For Zm 39676 in ZM medium, the pH was controlled at 5.75. Details of media composition are given in Table 1. Also shown are separate experiments with Zm 39676 in which the pH was 5.5, and with Zm 29191 in which the level of YE in the ZM medium was 1 g/L. The arrow in B indicates the theoretical maximum ethanol yield of 0.51 g/g.

containing 3× as much YE. Apart from the obvious stoichiometric correlation between the YE concentration of the medium and the level of lactic acid, this observation suggested that a component of the YE was the causative agent that was somehow responsible for lactic acid synthesis. Yeast extract is a complex nutritional adjunct, and is generally regarded as a source of inorganic elements, vitamins, and organic nitrogen. Bacto tryptone has a composition similar to Bacto yeast extract (YE), but it lacks the vitamins and growth factors that are present in YE. Replacing the YE (3 g/L) in the semisynthetic medium with an equivalent amount of Bacto Tryptone *(46)* (2.1 g/L, based on specifications provided by Difco with respect to the total nitrogen content of their products) (Table 1) had no appreciable effect on the pattern of lactic acid production and ethanol yield as a function of dilution rate (Fig. 1). Collectively, these observations with different media suggest that the causative agent might be an amino acid(s). In this context, it is interesting to note that in continuous fermentations with *Z. mobilis* ATCC 31821, using defined mineral salts media containing mixtures of different amino acids *(47)*, Beyeler et al. *(48)* did not observe any appreciable difference in ethanol yield relative to a complex YE-based medium; however, the relatively high dilution rate at which the glucose-limited chemostats were operated in the work of Beyeler et al. *(48)* compromises the significance of their observations in terms of the proposed effect of amino acids on *Zymomonas* metabolism at low growth rates. Work is ongoing in our laboratory to ascertain the chemical nature of the causative agent and the mechanism for the regulation of pyruvate metabolism.

Batch Fermentations

There are two probable reasons why lactic acid has not been commonly observed as a product of *Zymomonas* metabolism. First, lactic acid is not produced in batch fermentations, which the inoculum has been produced by the usual procedure of flask culture. Under these conditions, growth is near maximal. In order to maximize productivity, most work with *Zymomonas* with continuous fermentation systems has been performed at relative high dilution (growth) rates. In systems in which cell recycle or cell retention (immobilized) has been employed, and in which growth is likely to be less than maximal, the ethanol yield is often lower than in comparable cell-free systems *(49)*. Second, the majority of *Zymomonas* fermentations have been done at pH 5.0–5.5 *(7)*, and it is known that the ethanol yield is increased at pH <6.0 *(44,50)*

Figure 2 shows a typical time-course for two batch fermentations with *Z. mobilis* 29191 in a stirred-tank fermentor, with the pH controlled at 6.0. Two different media were used: DS medium, with 2.4 g/L NH_4Cl as sole source of assimilable nitrogen (open squares); and a semisynthetic medium (ZM) of identical composition, except that it was supplemented with 3 g/L YE (filled squares) (Table 1 and Fig. 2). For these batch fermentations, the inoculum was generated in a glucose-limited chemostat operating at D = 0.12/h, with a DS medium (Table 1). The observed specific growth rates

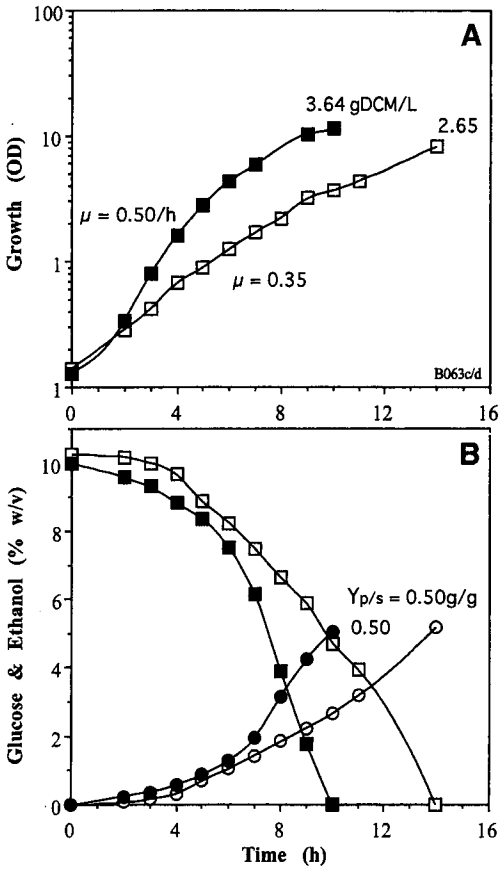

Fig. 2. Time-course of pH-stat batch fermentations with *Z. mobilis* 29191. **(A)** Growth, and **(B)** glucose utilization and ethanol production. (□) defined mineral salts medium (DS) with 10% (w/v) glucose; (■) DS medium with 3 g/L YE and 10% glucose. Details of the composition of the media are given in Table 1. The temperature was 30°C and the pH was 6.0. Values for both the specific growth rate (μ) and final cell mass (dry w-DCM) are given in A. The value of the ethanol yield ($Y_{p/s}$) is indicated in B.

with the DS and ZM media were 0.35/h and 0.50/h, respectively (Fig. 2A); however, with both media, the ethanol yield ($Y_{p/s}$) was 0.50 g ethanol/g glucose (i.e., 98% theoretical maximum conversion efficiency), and no lactic acid was detected (Fig. 2B). More typically, for batch fermentations, the inoculum (preculture or seed) is prepared as a flask culture using a nutrient-rich, complex medium, and a typical pH-stat batch fermentation would exhibit a growth and fermentation time-course very similar to the one shown in Figure 2. (results not shown).

Effect of pH and Inoculum History

Table 2 shows the results of a series of batch fermentations using four pH-stat batch fermentors containing RM medium with 10% (w/v) glucose (Table 1) were inoculated with *Z. mobilis* 29191 cultures produced in two

Table 2
Effect of Physiological History of Inoculum on Production of Lactic Acid by *Z. mobilis* 29191 in Batch Fermentation with pH Controlled at Either 5.5 or 6.0

Growth of inoculum		Batch fermentation parameters				
Chemostat feed medium	Dilution rate (1/h)	pH	Final cell mass (gDCM/L)	Lactic acid (g/L)	Ethanol yield (gEtOH/g Glu)	Conversion efficiency (% theoret. max)
ZM [DS + 3g/L YE]	0.08	5.5	3.34	6.79	0.472	92.4
		6.0	3.55	9.09	0.465	91.0
DS	0.08	5.5	3.21	1.77	0.50	97.8
		6.0	3.25	2.56	0.495	96.9

Note: The defined mineral salts medium (DS) contained 2.4 g/L NH_4Cl and the semisynthetic medium (ZM) was the same composition, except that it contained 3 g/L Difco YE. Both media contained 10% (w/v) glucose. The temperature of the pH-stat stirred-tank fermentors was maintained constant at 30°C.

glucose-limited chemostats operating at D = 0.08/h (Fig. 1) with either YE-based ZM or a DS (Table 1). The pH was controlled at either 5.5 or 6.0. The YE-based complex medium (RM) was selected for these experiments, because it has been used extensively by others as a reference medium in research with *Zymomonas* directed to comparative fermentation performance and nutritional requirements in formulating cost-effective media for use in industrial-scale operations *(46)*. At D = 0.08/h, the chemostat culture growing in a DS medium produced very little lactic acid (Fig. 1). However, when transferred to the batch fermentor, with the pH controlled at 6.0, it produced 2.56 g/L lactic acid, with an ethanol yield of 0.495 g/g (96.9% conversion efficiency) (Table 2). When the pH was controlled at 5.5, the final concentration of lactic acid produced from 10% (w/v) glucose decreased to 1.77 g/L, and the ethanol yield was correspondingly increased to 0.50 g/g (97.8%) (Table 2). At D = 0.08/h, the chemostat culture growing in YE-based ZM medium produced about 8 g/L lactic acid (Fig. 1), and when transferred to the batch fermentor, with the pH controlled at 6.0, it produced 9.09 g/L lactic acid, with an ethanol yield of 0.465 g/g (91%) (Table 2). When the pH was controlled at 5.5, the lactic acid level decreased to 6.79 g/L and the ethanol yield increased to 0.472 g/g (92.4%) (Table 2). These observations clearly demonstrate the beneficial effect of pH on ethanol yield whereby the synthesis of the coproduct is reduced by controlling the pH <6.0.

If lactic acid synthesis is dependent on a reduced glycolytic flux, as is achieved under a condition of glucose-limited growth in a chemostat operating at relatively low dilution rates, it is expected that lactic acid production would be minimized in batch fermentations, growth and glucose catabolism are maximal. In these experiments, growth in batch culture represents only a few generations (about five or six); however, the persistent nature of lactic acid production exhibited in pH-stat batch fermentations, in which the inoculum (seed) came from a lactic acid-producing chemostat, suggests a mechanism of enzyme induction rather than allosteric regulation, the latter being more instantaneous with respect to stopping acidogenesis.

These experiments are only exploratory in nature. Further research will be required to properly describe both the causative agent(s) and conditions that are responsible for promoting lactic acid synthesis by *Zymomonas*. Collectively these observations suggest that the characteristic high ethanol selectivity of *Zymomonas* can be compromised by the production of lactic acid. The mechanism of the metabolic shift from solventogenesis to acidogenesis appears to be exacerbated by elevated pH and a consequence of the low glycolytic flux achieved through glucose-limited growth in complex media containing organic nitrogen (amino acids). The results of this study are consistent with results of continuous fermentations using a xylose-utilizing recombinant *Z. mobilis*, in which the pH was controlled at 6.0 to minimize the acetic acid toxicity of the lignocellulosic hemicellulose hydrolysate medium *(36)*.

This study did not include an investigation into either strain specificity or the stereoisomeric specificity of lactic acid synthesis. This work has established the operating parameters for a systematic screening of our *Zymomonas* culture collection for strain specificity with respect to lactic acid production.

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REFERENCES

1. Wright, J. D. (1988), *Chem. Eng. Prog.* **84,** 62–74.
2. Hinman, N. D., Wright, J. D., Hoagland, W., and Wyman, C. E. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 391–401.
3. Wyman, C. E. and Hinman, N. D. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 735–753.
4. Swings, J. and DeLey, J. (1977), *Bacteriol. Rev.* **41,** 1–46.
5. Montenecourt, B. S. (1985), in *Biology of Industrial Microorganisms,* Demain, A. L. and Simon N. A., eds., Benjamin/Cummings, Meno Park, C, pp. 216–287.
6. Doelle, H. W., Kirk, L., Crittenden, R., Toh, H., and Doelle, M. (1993) *Crit. Rev. Biotechnol.* **13,** 57–98.
7. Rogers, P. L., Lee, K. J., Skotnicki, M. L., and Tribe, D. E. (1982), *Adv. Biochem. Eng.* **23,** 37–84.
8. 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.
9. Lawford, H. G. (1988), *Appl. Biochem. Biotechnol.* **17,** 203–219.
10. Beavan, M., Zawadzki, B., Droiniuk, R., Fein, J. E. and Lawford, H. G. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 319–326.
11. Alterthum, F. and Ingram, L. O. (1989), *Appl. Environ. Microbiol.* **54,** 397–404.
12. Ingram, L. O., Conway, T., and Alterthum, F. (1991), US Patent 5,000,000.
13. Ohta, K., Alterthum, F., and Ingram, L. O. (1990), *Appl. Environ. Microbiol.* **56,** 463–465.
14. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotechnol. Bioeng.* **38,** 296–303.
15. Doran, J. B., Aldrich, H. C., and Ingram, L. O. (1994), *Biotechnol. Bioeng.* **44,** 240–247.
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. Picataggio, S. K., Zhang, M., Eddy, C. K., Deanda, K. A., and Finkelstein, M. (1996), US Patent 5,514,583.
18. Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., and Picataggio, S. K. (1995), *Science,* **267,** 240–243.
19. Lawford, H. G. and Rousseau, J. D. (1997), *Appl. Biochem. Biotechnol.* **63–65,** 269–286.
20. Jöbses, I. M. L., Egberts, G. T. C., van Baalen, A., and Roels, J. A. (1985), *Biotechnol. Bioeng.* **27,** 984–995.
21. Schmidt, W. and Schügerl, K. (1987), *Chem. Eng. J.* **36,** B39–B48.
22. Wecker, M. S. A. and Zall, R. R. (1987), *Appl. Environ. Microbiol.* **53,** 2815–2822.
23. Viikari, L. (1988), *Crit. Rev. Biotechnol.* **7,** 237–261.
24. Johns, M. R., Greenfield, P. F., and Doelle, H. W. (1991), *Adv. Biochem. Eng. Biotechnol.* **44,** 97–121.
25. Doelle, H. W. and Greenfield, P. F. (1985), *Appl. Microbiol. Biotechnol.* **22,** 405–410.
26. Viikari, L. (1984), *Appl. Microbiol. Biotechnol.* **19,** 252–255.
27. Viikari, L. (1984), *Appl. Microbiol. Biotechnol.* **20,** 118–123.

28. Viikari, L. and Korhola, M. (1986), *Appl. Microbiol. Biotechnol.* **24,** 471–476.
29. Suntinanalert, P., Pemberton, J. P., and Doelle, H. W. (1986), *Biotechnol. Lett.* **8,** 351–356.
30. Cromie, S. and Doelle, H. W. (1982), *Eur. J. Appl. Microbiol.* **14,** 69–73.
31. Keshew, K. F., Yamano, L. P., An, H., and Ingram, L. O. (1990), *J. Bacteriol.* **172,** 2491–2497.
32. Osman, Y. A., Conway, T., Bonetti, S. J., and Ingram, L. O. (1987), *J. Bacteriol.* **169,** 3726–3736.
33. Algar, E. M. and Scopes, R. K. (1985), *J. Biotechnol.* **2,** 275–281.
34. Yomano, L. P., Scopes, R. K., and Ingram, L. O. (1993), *J. Bacteriol.* **175,** 3926–3933.
35. Garvie, E. I. (1980), *Microbiol. Rev.* **44,** 106–139.
36. Lawford, H. G., Rousseau, J. D., Mohagheghi, A., and McMillan, J. D. (1998), 19th Symp. *Appl. Biochem. Biotechnol.* **70–72.**
37. Doelle, H. W. (1989), US Patent 4, 797,360.
38. Doelle, M. B., Greenfield, P. F., and Doelle, H. W. (1990), *Process Biochem.* **25,** 151–156.
39. Millichip, R. J. and Doelle, H. W. (1989), *Process Biochem.* **24,** 141–145.
40. Doelle, M. B., Millichip, R. J., and Doelle, H. W. (1989), *Process Biochem.* **24,** 137–140.
41. Jones, C. W. and Doelle, H. W. (1991), *Appl. Microbiol. Biotechnol.* **35,** 4–9.
42. Fein, J. E., Lawford, H. G., Lawford, G. R., Zawadski, B. C., and Charley, R. C. (1983), *Biotechnol. Lett.* **5,** 19–24.
43. Lawford, H. G. and Stevnsborg, N. (1986), *Bioeng. Biotechnol. Symp.* **17,** 209–219.
44. Lawford, H. G. (1989), US Patent 4,840,902.
45. Fein, J. E., Charley, R. C., Hopkins, K., Lavers, B., and Lawford, H. G. (1983), *Biotechnol. Lett.* **5,** 1–6.
46. Lawford, H. G. and Rousseau, J. D. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 307–326.
47. Nipkow, A., Beyeler, W., and Fiechter, A. (1984), *Appl. Microbiol. Biotechnol.* **19,** 237–240.
48. Beyeler, W., Rogers, P. L., and Fiechter, A. (1984), *Appl. Microbiol. Biotechnol.* **19,** 277–280.
49. Prince, I. G. and Barford, J. P. (1982), *Biotechnol. Lett.* **8,** 525–530.
50. Lawford, H. G., Holloway, P., and Ruggiero, A. (1988), *Biotechnol. Lett.* **10,** 809–814.