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The Effect of Lactic Acid on Fuel Ethanol Production by *Zymomonas*

HUGH G. LAWFORD* AND JOYCE D. ROUSSEAU

Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

ABSTRACT

Lactic acid bacteria have a profoundly negative influence on the fermentation performance of *Zymomonas mobilis*. Lactic acid bacteria are an important part of the steeping process in starch wet-milling. Their prevalence and acid pH optimum for growth make these organisms opportunistic contaminants of continuous ethanol fermentations, where the feedstock is enzymatic starch hydrolysate. At concentrations of lactic acid in the range of 3–10 g/L, cell density, product yield, and productivity are reduced. However, under similar operating conditions, a chemostat culture fed a synthetic glucose medium with added DL-lactic acid (range 0–13 g/L) did not exhibit a decrease in either yield or productivity. In pH-stat (5.0) batch cultures, with about 11 g/L lactic acid added to the medium, growth and conversion efficiency were only very slightly inhibited. At pH 4.5, this amount of lactic acid caused a 23% decrease in growth yield, but only a 3% reduction in ethanol yield. Acetic acid is a minor metabolic byproduct of lactic cultures, and at pH 5, the growth rate and growth yield of *Z. mobilis* were inhibited 17 and 23% respectively, by the addition of 3.37 g/L lactic acid. Ethanol yield was decreased only 3.5% by this amount of acetic acid.

It is concluded that lactic acid *per se* is not the causative inhibitory agent. Also, although a possible role for acetic acid and unidentified natural antibiotic substances is implied, the mechanism whereby lactic acid bacteria negatively affect *Zymomonas* remains unresolved.

Index Entries: *Zymomonas;* lactic acid; acetic acid; fuel ethanol; lactic acid bacteria; starch hydrolysate.

*Author to whom all correspondence and reprint requests should be addressed.

NOMENCLATURE

D	dilution rate (h^{-1})
LA	lactic acid
HAc	acetic acid
μ	sp. growth rate (h^{-1})
EFT	elapsed fermentation time (h)

INTRODUCTION

Fermentation fuel ethanol is produced primarily from sugar cane in Brazil, but in the United States, it is made from starch crops, mostly corn (*1,2*). Last year in the US about 800 million gallons of fermentation fuel ethanol were produced from 320 million bushels of corn (*3*). The economics of fuel ethanol is dominated by the feedstock cost (*4,5*). Consequently, the bioconversion process parameter of highest economic sensitivity is the yield (i.e., the sugar-to-ethanol conversion efficiency) (*4,6*). If there is to be a reduction in production costs, the industry must utilize more inexpensive sources of renewable raw materials (*3,7,8*). Product concentration and productivity are ranked second and third in order of technoeconomic importance (*4,6*). Whereas the engineering approach to improving the fermentation process has generally been directed to increasing productivity through the use of high-cell-density, continuous-flow systems, the biological approach has focused on process yield and has involved the use of alternative biocatalysts that are tailored to the requirements dictated by the expanding variety of feedstocks (*9*). Although *Saccharomyces* yeast currently enjoys a monopoly in the fuel alcohol industry (*1,2*), it is not the only ethanol-producing microorganism. The bacterium *Zymomonas mobilis* is generally recognized as being superior to *Saccharomyces* yeast with respect to both conversion efficiency (yield) and productivity (*9–13*). Furthermore, *Zymomonas* is just as tolerant to ethanol as yeast (*14*). Our research on *Zymomonas* fermentations has spanned over a decade and is the subject of several patented processes (for review, *see 11*). Although *Zymomonas* is not currently being used industrially, both laboratory and pilot-scale operations have shown that it is capable of generating near theoretical maximum yields from several different feedstocks, including sugar cane (*15*), molasses (*16*), saccharified starch from corn (*17*), wheat (*18*), cassava and sago (*19*), as well as an enzymatic hydrolysate of wood-derived cellulose (*20*).

Lactic acid bacteria are an important part of the steeping process in starch wet-milling operations (*11*). Their abundance and their acid pH optimum for growth permit these organisms to become opportunistic contaminants of continuous ethanol fermentations, where the feedstock is saccharified wet-milled starch (*11*). The possibility for infection is particularly acute in a process where saccharification precedes fermentation

in a separate unit operation. The permissive conditions within the "sacc tanks" promote proliferation of lactic acid bacteria (*11*). It has been our experience (*11,17*) and that of others (*18*) that infection by lactic cultures has a profoundly antagonistic effect on the fermentation performance of *Zymomonas* in continuous starch hydrolysate systems. Pfeifer-Langen (Germany) once operated the only industrial-scale ethanol facility that used *Zymomonas,* but the company was forced to revert to using yeast because of problems of instability caused by recurring infections by lactic cultures (*11*). The 50 m^3 continuous fermentors (D = 0.08/h) were supplied with saccharified wheat B-starch (approx 12% glucose) with a reported conversion efficiency in the range 92–96% (theoretical maximum), but "problems are caused by accumulation of 5–10 g/L lactic acid during continuous operation; this is frequently followed by an increase in the glucose concentration in the fermentor effluent" (*18*). Further, it was shown that the isolated infecting lactobacilli were capable of producing 1.6 g/L of lactic acid, in batch culture, from the spent *Zymomonas* medium (with < 1 g/L glucose) (*18*). Compositional analyses determined that the lactic acid was derived from the pentose sugars, xylose and arabinose, which were present in the spent medium (*18*). It was concluded that "more work is needed to prevent contamination by lactic acid bacteria" (*18*).

The literature is silent with respect to the effect of lactic acid on *Zymomonas.* Acetic acid is only a minor byproduct of lactobacilli glucose metabolism; however, acetic acid is known to be an effective antimicrobial agent (*21*), and since the active form is the protonated species, it is particularly antagonistic at acid pH (*22*). The effect of acetic acid on *Zymomonas* has not been previously described. This report is part of our continuing series on *Zymomonas*-based ethanol fermentations (*23–30*). In this study, we have examined batch and continuous fermentations using a synthetic glucose/mineral salts medium, and exogenous organic acids, to assess quantitatively the effect of lactic acid (and acetic acid) on the neotype strain of *Zymomonas mobilis* (ATCC 29191).

MATERIALS AND METHODS

Organism

The neotype strain of *Zymomonas mobilis* ATCC 29191 was obtained from the American Type Culture Collection (Rockville, MD). Stock cultures and inocula were prepared as described previously (*23*).

Fermentation Media and Equipment

The synthetic glucose mineral salts medium contained 1.5 g/L yeast extract (Difco) and ammonium chloride (30 m*M*) as sources of assimilable nitrogen. The composition with respect to inorganic salts and vitamins was as previously described by Lawford and Ruggiero (*30*). Glucose was

autoclaved separately. The industrial, saccharified, corn starch medium used in the continuous fermentations was prepared from "process streams" (corn steep liquor and stillage) obtained from corn wet-milling operations and was formulated according to the recipe of Beavan et al. (*17*). The batch and continuous fermentations were conducted in bench-top stirred-tank bioreactors with pH and temperature control, as described previously (*11*).

Analytical Procedures

Growth was followed turbidometrically at 550 nm (1-cm lightpath), and culture dry wt was measured by microfiltration—washing and drying the filter (0.45 μ) to constant weight under an infrared heat lamp. Compositional analyses (glucose and ethanol) of fermentation media and cell-free spent media were determined using an HPLC equipped with an RI monitor and computer-interfaced controller/integrator (Bio-Rad Labs, Richmond, CA). Separations were performed at 65°C on an HPX-87H column (Bio-Rad); the injection volume was 20 μL.

RESULTS AND DISCUSSION

As part of our continuing series of investigations concerning comparative physiology of alternative ethanologenic organisms with potential for industrial application, we recently published the results of a direct side-by-side comparison of *Z. mobilis* (ATCC 29191) and a yeast strain currently being used commercially in a large-scale (70*M* gal/yr) fuel ethanol plant (*17*). The results emphasized the potential for yield improvement through the substitution of *Zymomonas* for yeast using an industrial feedstock (saccharified corn starch) in which all essential growth nutrients were supplied exclusively by the process water (corn steep liquor) from a corn wet-milling operation (*11,17*). Although it is not included in our reports (*11,17*), we occasionally observed instability in the *Zymomonas* continuous fermentation, indicated by the appearance of glucose in the effluent and coinciding with the accumulation of lactic acid. Figure 1 illustrates the typical pattern of changes in process parameters of a continuous *Zymomonas* fermentation, which occurs as a consequence of infection by lactic acid bacteria. Similar observations have been reported by Bringer et al. (*18*) with continuous *Zymomonas* fermentations based on hydrolyzed wheat starch from a wet-milling operation.

Since *Zymomonas* is gram-negative (*31*), culture contamination by gram-positive organisms is relatively easy to detect by microscopic examination of a gram-stained preparation. The contaminating culture was isolated from agar-solidified media, and the identity deduced as lactobacilli with the use of a commercial diagnostic kit. A confirmatory feature was the accumulation of lactic acid in the effluent.

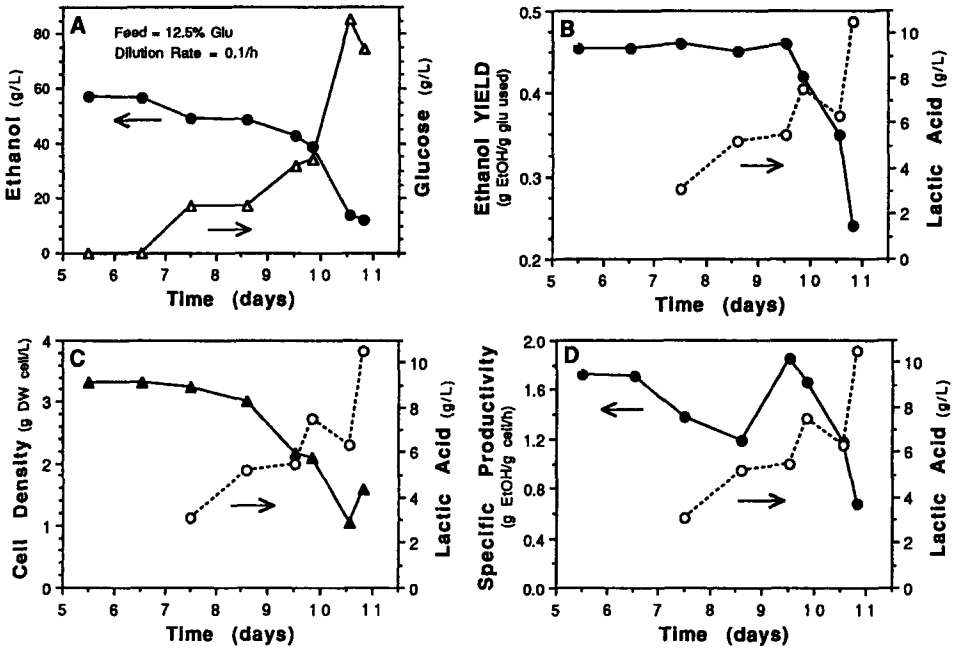

Fig. 1. The effect of contamination of a *Zymomonas* corn starch hydrolysate continuous fermentation by lactic acid bacteria. The medium was an enzymatically hydrolyzed corn starch in which all essential nutrients for *Zymomonas* are supplied exclusively by corn steep liquor as described previously (*17*). The continuous-flow bioreactor was operated at a dilution rate of 0.1/h, with the pH controlled at 5.0 and temperature at 30°C. Glucose in the feed was 125 g/L. (A) Steady-state concentrations of effluent ethanol and glucose; (B) ethanol yield; (C) steady-state biomass concentration; (D) specific productivity; and (B–D) effluent lactic acid (open circles).

With reference to the results shown in Fig. 1, lactic acid (3 g/L) was first detected in the effluent after 7.5 d of continuous fermentation at a dilution rate of 0.1/h, and the concentration increased steadily thereafter. Coincident with the appearance of lactic acid was the increasing accumulation of glucose in the effluent (Fig. 1A), and because the amount of glucose utilized decreased proportionately, the process yield (determined as the mass ratio of ethanol produced to glucose in the feed) decreased (not shown). Interestingly, however, the product yield (or glucose-to-ethanol conversion efficiency) remained relatively constant until the lactic acid reached a concentration >5 g/L, at which point (9.5 d) it dropped precipitously from 0.46 to 0.24 g ethanol/g glucose consumed (Fig. 1B). At 9.5 d EFT, only 76% of the glucose feed (125 g/L) was being consumed, but by the time the fermentation was terminated (11 d), glucose utilization has decreased to only 40% (equivalent to 50 g/L). Over the last 36 h of operation, the lactic acid concentration increased by about 5.5 g/L (Fig 1B).

To synthesize that amount of lactic acid from glucose would require an equal mass of glucose. If the product yield that was observed at the end of the experiment were to be corrected for lactate production, it would only increase from 0.24 to 0.27 g ethanol/g glucose. Therefore, the decrease in product yield (from 0.46 to 0.24 g/g) cannot be accounted for simply by the amount of lactic acid produced.

We observed that coincident with the appearance of lactic acid in the effluent there was a gradual decrease in the steady-state cell density (Fig. 1C). There is not, however, a constant proportionality between the biomass concentration and the amount of glucose utilized over the entire time-course (growth yield not shown), and the effect attributable to lactic acid is complicated by operation with high-glucose concentrations, since under similar operating conditions, the growth yield (determined as mass dry wt cells/equivalent mass of glucose consumed) is known to increase in response to a decrease in sugar utilization (*23*). The growth yield (0.026 g/g) is the same at 6.5 d as at 10.5 d of elapsed fermentation time, when the cell density was 3.3 and 1.1 g/L, respectively (Fig. 1C). It is not known to what extent an increase in growth yield, owing to the utilization of less sugar, is opposed by an opposite effect resulting from lactic acid or the presence of the contaminating lactic culture. For this reason, even an apparent effect of lactic acid on growth yield is equivocal under these operating conditions. A similar, related problem arises in interpreting the pattern of changes in the specific productivity, which might account for the seemingly erratic nature of the response (Fig. 1D).

Our approach to examining the effect of lactic acid *per se* on *Zymomonas* was to conduct pH-controlled fermentations with a synthetic laboratory medium to which DL-lactic acid was added over the same concentration range observed in fermentations with industrial feedstocks contaminated with lactobacilli. Exogenous lactic acid (11.2 g/L equivalent to 124 m*M*) did not appreciably inhibit growth of *Z. mobilis* in batch culture in a synthetic salts medium, which contained glucose at 30 g/L, and where the pH was controlled at 5.0 (Fig. 2A). Under these conditions, lactic acid caused only a 3% reduction in the specific growth rate (Fig. 2A) and had no effect on the growth yield (0.038 g/g) (Fig. 3). Similarly, under these conditions, lactic acid did not appreciably affect the rate of fermentation (Fig. 2B); the conversion efficiency (as percent of theoretical maximum) was only slightly decreased, from 98 to 96.7% (Fig. 4). The effect of this same concentration of lactic acid on the growth yield is more pronounced when the pH was controlled at 4.5 (decrease of 23%) (Fig. 3). However, at pH 4.5, lactic acid causes only a 3% reduction in conversion efficiency, from 95.7 to 92.7% (Fig. 4).

Acetic acid is a minor byproduct of glucose metabolism by lactobacilli and was not detected at significant levels in infected fermentations. Nevertheless, acetic acid is used in the food industry as a preservative (*21*), and its efficacy is based on its antimicrobial activity. The inhibitory effect of

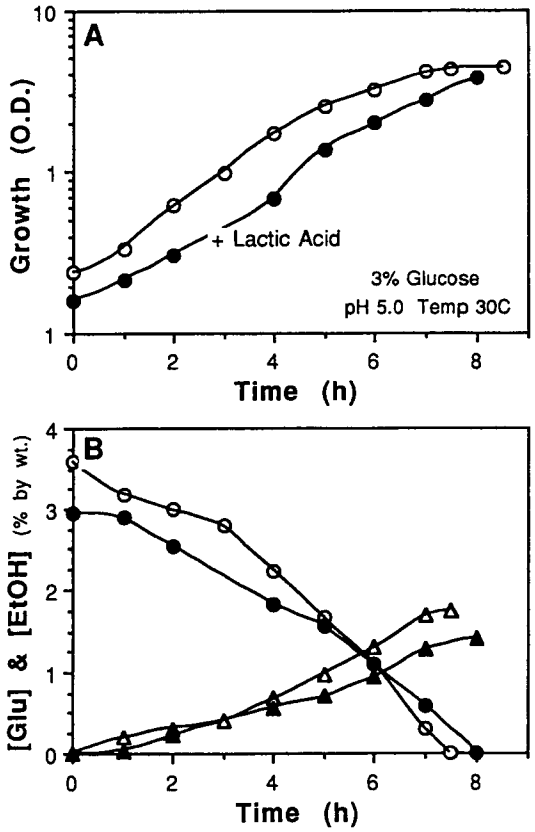

Fig. 2. The effect of exogenous lactic acid on batch fermentation by *Zymomonas*. The synthetic medium contained 30 g/L glucose. The fermentation was conducted in a stirred bioreactor (100 rpm) with pH control (5.0) and temperature at 30°C. (A) Growth: (open circles) control; (filled circles) with 11.2 g/L of DL-lactic acid; (B) glucose utilization (circles) and ethanol production (triangles) —filled symbols are for medium with added lactic acid.

acetate is potentiated at acid pH (22). By virtue of its ability to traverse the cell membrane freely, the protonated species (i.e., undissociated acid) acts as a protonophore and causes its inhibitory effect by bringing about the acidification of the cytoplasm, thereby collapsing the transmembrane pH gradient and destroying the homeostasis with respect to the intracellular pH (32–34). For example, it has been reported that the growth of *E. coli* K12 is completely inhibited by 0.2 m*M* of the protonated form of acetic acid (equivalent to 35 m*M* acetate at pH 7) (33). However, there is no report in the literature on the effect of acetic acid on *Zymomonas*.

The effect of adding acetate to the synthetic glucose/salts medium on *Zymomonas* is shown in Figs. 3, 4, and 5. The amount of acetate (56 m*M*) is about 10-fold higher than might be expected to be produced by an infecting lactic culture (Lawford and Rousseau, unpublished observations).

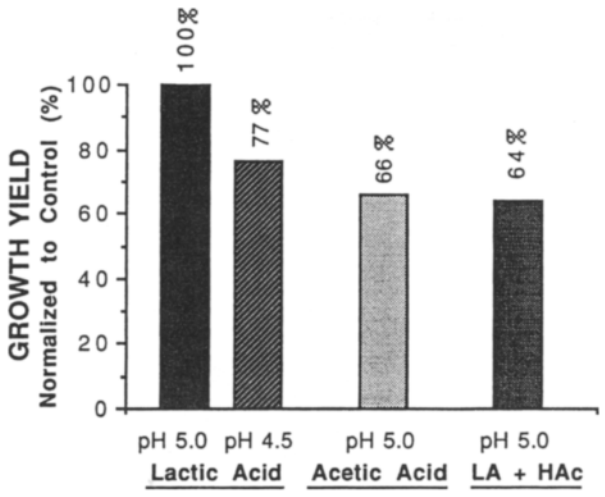

Fig. 3. Effect of lactic acid and acetic acid on the growth yield of *Zymomonas* in batch fermentations. The concentrations of DL-lactic acid and acetic acid (HAc) were 11.2 and 3.37 g/L, respectively. The growth yield (g dry wt cell/g glucose) at pH 5.0 and 4.5 was normalized to the "control" condition in the absence of added organic acid(s).

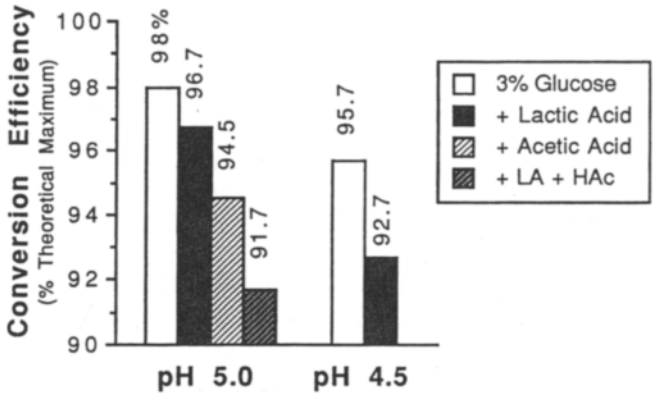

Fig. 4. Effect of lactic acid and acetic acid on glucose-to-ethanol conversion efficiency by *Zymomonas* in batch fermentations at pH 5.0 and pH 4.5. The concentrations of DL-lactic acid and acetic acid (HAc) were 11.2 and 3.37 g/L, respectively.

Using the Henderson-Hasselbalch relationship ($pK_a = 4.75$), it can be calculated that, at pH 5, the concentration of the protonated form of acetic acid is 20 m*M*; this is an amount that is 100-fold higher than the concentration reported to cause complete inhibition of *E. coli* (33). The effect of this concentration of acetic acid on glucose utilization and ethanol production by *Zymomonas* is shown in Fig. 5. The control for this experiment is shown in Fig. 2B, but in comparing the effects of lactic and acetic acids

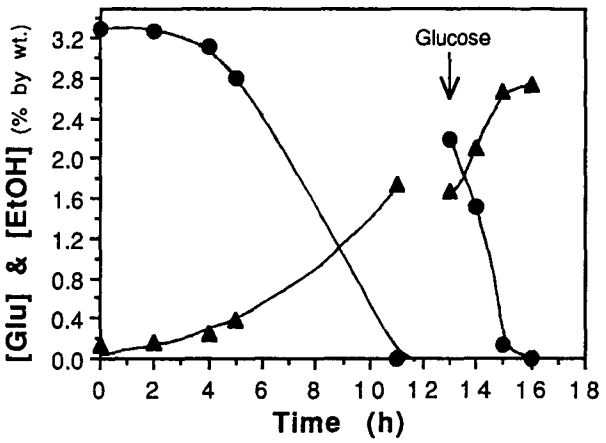

Fig. 5. The effect of acetic acid on *Zymomonas* performance in a fed-batch fermentation at pH 5.0. The amount of acetic acid added to the synthetic medium was 3.37 g/L. The arrow indicates the addition of glucose, 2.2% (by wt), at 13 h. Symbols: (circles), glucose; (triangles), ethanol. Few "control," compare to kinetic profile of Fig. 2B, but note difference in time scale.

(Figs. 2B and 5, respectively), it is important to note the difference with respect to the time scales in the two figures. At pH 5, this concentration of acetate causes a 50% reduction in volumetric productivity (Fig. 5); however, this is due primarily to the lag and 17% reduction in growth rate (results not shown) rather than an inhibition of metabolism, as illustrated by the noninhibited rate of glucose utilization following glucose feeding at 13 h (Fig. 5). Under the conditions employed, acetic acid causes a 34% reduction in the growth yield (Fig. 3). The conversion efficiency is affected to a lesser extent, and falls from 98 to 94.5% (Fig. 4). The effect on these parameters is not enhanced when both acids are added simultaneously (Figs. 3 and 4).

Figure 6 summarizes the results of a continuous fermentation designed to mimic the conditions of the experiment illustrated in Fig. 1. The experiment, represented in Fig. 6, was designed to assess the effect of exogenous lactic acid under steady-state conditions. The glucose concentration in the synthetic salts medium was 110 g/L, and the dilution rate was constant at 0.15/h with the pH controlled at 5.0. Under these conditions, the glucose was completely utilized, but neither the product yield nor the specific productivity appeared to be negatively affected by lactic acid over the range tested (0–13 g/L) (Fig. 6). These observations are at variance with those of Fig. 1, and it is concluded from the results of this investigation that lactic acid *per se* cannot be regarded as the causative agent with respect to the negative effect of lactic culture infection on the fermentation performance of *Z. mobilis*. The results do not rule out the possible involvement of acetic acid, but further research is needed to quantitate its effect on *Zymomonas*.

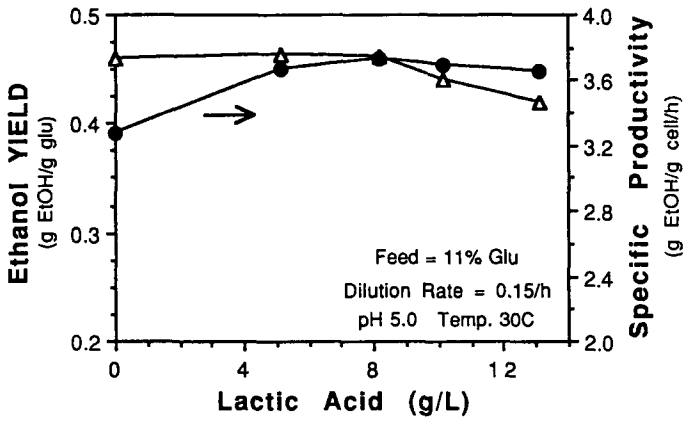

Fig. 6. Effect of exogenous lactic acid on performance of *Zymomonas* in continuous fermentation. The synthetic medium contained glucose at 110 g/L. The dilution rate was 0.15/h, and the pH and temperature were maintained at 5.0 and 30°C. Symbols: (circles), specific productivity; (triangles), product yield.

Although itself resistant to many antibiotics (*31*), *Zymomonas* is known to synthesize substances ("zymocins") that are antagonistic to the growth of certain pathogens (H. G. Lawford, G. R. Reid, and P. Holloway, unpublished observations), and this has been the basis for its use as a therapeutic agent (*31,35,36*). However, certain lactobacilli also synthesize antibiotic substances (*37,38*) although relatively little research has been conducted in this area, and they are not well characterized either with respect to chemical identity or their mode of action. Two examples are acidolin (*39*) and lactocidin (*40*). The possible role of such substances as specific *Zymomonas* antagonists cannot be discounted by the results of the present study. This will be the focus of the next phase of our research on this subject.

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