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Ethanol Production Using *Zymomonas mobilis* in a Cross-Linked Immobilized Cell Reactor

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ABSTRACT

The bacterium *Zymomonas mobilis* may be utilized to produce ethanol from glucose in a cross-linked immobilized cell reactor. Reactor startup is much more rapid with cross-linked *Zymomonas* than with the yeast *Saccharomyces cerevisiae*. Volumetric ethanol productivities (based on liquid holdup) three times those obtained with cross-linked yeast, and comparable to those obtained with *Zymomonas* immobilized by other methods, are possible.

Index Entries: *Zymomonas mobilis*; ethanol; cross-linked; immobilized cell reactor; *Saccharomyces cerevisiae*.

INTRODUCTION

Much research in recent years has been directed toward the development of improved methods for the continuous production of ethanol from renewable resources. One approach that has been extensively studied in this regard is the use of column-type immobilized cell reactors (ICRs). High cell densities can be attained in such reactors without significant washout even at high dilution rates, resulting in increased ethanol productivity. To the extent that plug flow is approximated in the column, the problem of product inhibition is minimized. The highest ethanol concentrations will occur only near the column outlet. Higher conversions of substrate with accompanying higher ethanol concentrations in the reac-

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tor effluent are possible with minimal reactor volume. Also, ICR systems are simpler to operate than cell recycle systems.

A number of different immobilization techniques have been applied in reactors for the continuous fermentation of ethanol. Some of this work has been done with various strains of yeast as the biocatalyst. More recently, the bacterium *Zymomonas mobilis* has been used (*1–5*). Rogers, et al. have summarized the advantageous characteristics of *Zymomonas* compared with yeast (*6*). These include specific rates of sugar uptake and ethanol production three to four times faster than those of yeasts; higher ethanol yields and lower biomass yields; and, ethanol tolerance comparable to, if not better than, that of yeasts.

Among the immobilization techniques that have been used in conjunction with *Zymomonas* are entrapment within calcium alginate (*7–10*) and K-carrageenan gels (*7,11,12*), and adsorption on glass fiber pads (*13*), ion exchange resins (*14*), and vermiculite (*15*). Absent from the literature is any mention of the use of covalent bonding or chemical cross-linking with *Zymomonas*.

The yeast *Saccharomyces cerevisiae* has been cross-linked onto the surfaces of gelatin-coated glass beads via the bifunctional reagent glutaraldehyde in the University of Arkansas laboratories. Factors affecting the performance of a column-type reactor containing yeast cells immobilized in this manner, including substrate concentration and flow rate, have been thoroughly studied (*16*). This cross-linking method offers a major advantage over the more widely-utilized entrapment techniques, in that it exposes cells to direct contact with the nutrient medium. The gel matrix used to entrap cells, on the other hand, presents a diffusional resistance to mass transfer between the cells and the medium, and also may deteriorate over time. Cross-linking provides simple and rapid reactor startup as well.

Having already demonstrated stable, high-productivity operation of a cross-linked ICR using yeast, (*16*), it was decided to further explore the potential of the cross-linking technique by investigating the use of *Zymomonas* as the biocatalyst in place of yeast. The purpose of this paper is to evaluate the feasibility of using *Zymomonas mobilis* in a cross-linked ICR for continuous ethanol fermentation, and to identify the advantages and disadvantages of such a system relative to cross-linked yeast, as well as to *Zymomonas* immobilized by other methods. Before the ICR experiments were undertaken, batch fermentations with *Zymomonas* were carried out in order to validate the experimental technique, and to evaluate the performance of the strain relative to published data.

EXPERIMENTAL PROCEDURES

Microorganism

Zymomonas mobilis ATCC 31821 was used as the bacterium in this study. Early attempts to grow the organism in nutrient medium con-

taining a high sugar concentration (200 g/L glucose or greater) were unsuccessful. Two remedial measures were taken. First, the medium was prepared in two parts, with the glucose sterilized separately from the other medium ingredients, instead of together in one solution, as was done originally. Apparently, when high sugar concentrations are used, the problem of glucose degradation through chemical reaction during autoclaving is more serious than when the sugar concentration is low (100 g/L or less). Second, a procedure was carried out to adapt the organism to higher sugar concentrations in a gradual manner. A series of tubes was prepared containing 5 mL portions of nutrient medium with 10 g/L yeast extract (Difco), and with glucose ranging from 100 to 250 g/L in 25 g/L increments. The initial pH of each culture was adjusted to 5.0, and incubations were carried out at 30°C. Once substantial growth was obtained at a given glucose concentration, the culture was inoculated into the medium containing the next highest concentration. After achievement of good growth in the 250 g/L glucose medium, the bacterium was subsequently stored at 4°C in a medium of 250 g/L glucose and 10 g/L yeast extract, with an initial pH of 5.0. This stock culture was transferred to 15 mL of fresh medium every three to four weeks.

Analytical Methods

Cell concentration, expressed in terms of dry weight, was determined by comparing the optical density of the solution at 580 nm with a prepared standard curve. A portion of uninoculated medium was used as the blank for zero absorbance. Glucose concentrations were measured using a YSI Model 27 industrial analyzer. Ethanol was determined by gas-solid chromatography. A .125 × 48 in. Chromosorb 102, 60/80-mesh column along with a HWD detector system were employed. The oven temperature was maintained at 125°C, the detector and injector temperatures were both 175°C, and the carrier gas was helium at a flow rate of 40 mL/min. Ratios of peak areas were related to previously determined calibration curves.

Batch Fermentation Studies

Simple batch reactors were constructed from 500 mL flasks to provide a working volume of 300 mL. Moderate agitation was provided through the use of magnetic stirrers. Experiments were carried out with initial glucose concentrations (S_o) of 90, 135, and 160 g/L. In each case seed cultures were prepared for inoculation to the batch culture with corresponding initial glucose concentrations. Other components of the medium were: yeast extract, 10 g/L; KH_2PO_4, 1 g/L; $(NH_4)_2\,SO_4$, 1 g/L; and $MgSO_4 \cdot 7H_2O$, .5 g/L. The medium was always prepared in two parts, with the glucose being sterilized separately from the other ingredients. After autoclaving at 15 psig for 20 min, the separate solutions were allowed to cool and then aseptically mixed. To begin each experiment, 3

mL of stock culture were transferred aseptically to 27 mL of seed culture medium. After incubation for 12–18 h at 30°C, the entire seed culture was inoculated to 270 mL of medium in a reactor flask, which in turn was also incubated at 30°C. All experiments were run with the initial pH adjusted to 5.0. For the S_o = 90 g/L and S_o = 135 g/L experiments, no attempt was made to control pH during the course of the fermentations. In the case of the S_o = 160 g/L experiment, sterile 1 N KOH solution was periodically added to the broth in amounts ranging from .25–1.0 mL during the first nine h of the fermentation.

Immobilized Cell Reactor Studies

The immobilized cell reactor consisted of a plug flow tubular column constructed of 3.18 cm id plexiglass tubing of .32 cm wall thickness, 47.0 cm long. Five samples ports were installed along the reactor length. Spherical glass beads of 4 mm nominal size were used as the packing material. These beads were first dip-coated in 25% gelatin solution, dried, soaked in a 3% glutaraldehyde solution, and then allowed to dry again before being poured randomly into the reactor. The reactor was sterilized using propylene oxide. After attachment of cotton plugs at each end of the column, the reactor was flushed for five min with the gas, sealed, and allowed to stand for 48 h. The system was then purged by attachment to an aspirator.

A seed culture for inoculation of the ICR was prepared as follows: 1 mL of stock culture was used to inoculate 100 mL of 100 g/L glucose medium. After incubation at 30°C for about 12 hours, this entire 100 mL culture was transferred to 900 mL of a seed culture medium with initial glucose concentration equal to 100 g/L. Other constituents of this seed culture and their compositions were identical to those used in the batch studies. Following a five h incubation, the seed culture was pumped into the column and allowed to stand for eight h. Feed medium having a glucose concentration of 150 g/L was then pumped into the reactor. Throughout the next 26 d the feed flow rate was gradually stepped up commensurate with the increasing amount of biomass in the reactor. At all times, an attempt was made to maintain greater than 90% conversion of feed glucose. This conversion was determined by withdrawing samples from the ports at the very bottom and very top of the column, and measuring the glucose consumption and ethanol production occurring between them.

Having obtained a very high concentration of cells in the reactor, experiments were carried out to generate values of the volumetric ethanol productivity as a function of the retention time. Beginning with a feed flow rate resulting in greater than 95% conversion of substrate, the flow rate was periodically increased until a conversion of only 50% or less was obtained. After each flow rate adjustment, at least three hydraulic retention times were allowed before sampling. All work with the immobilized

cell reactor was carried out in an environmental room maintained at 30°C.

RESULTS AND DISCUSSION

Batch Fermentation Studies

Figures 1 and 2 show plots of specific growth rate and specific ethanol production rate, respectively, as a function of extracellular ethanol concentration. Data from batch experiments with *Zymomonas* using two different initial substrate concentrations (S_o = 90 g/L and S_o = 135 g/L) are shown. Also depicted on the same figures are data from experiments using the yeast *Saccharomyces cerevisiae*, ATCC 24860 (*17*). From these figures it is clear that substantially higher specific rates of growth and production can indeed be achieved with *Zymomonas* over a wide range of extracellular ethanol concentrations.

Although the results obtained with *Zymomonas* were favorable when compared with the performance of the yeast, they were not as satisfac-

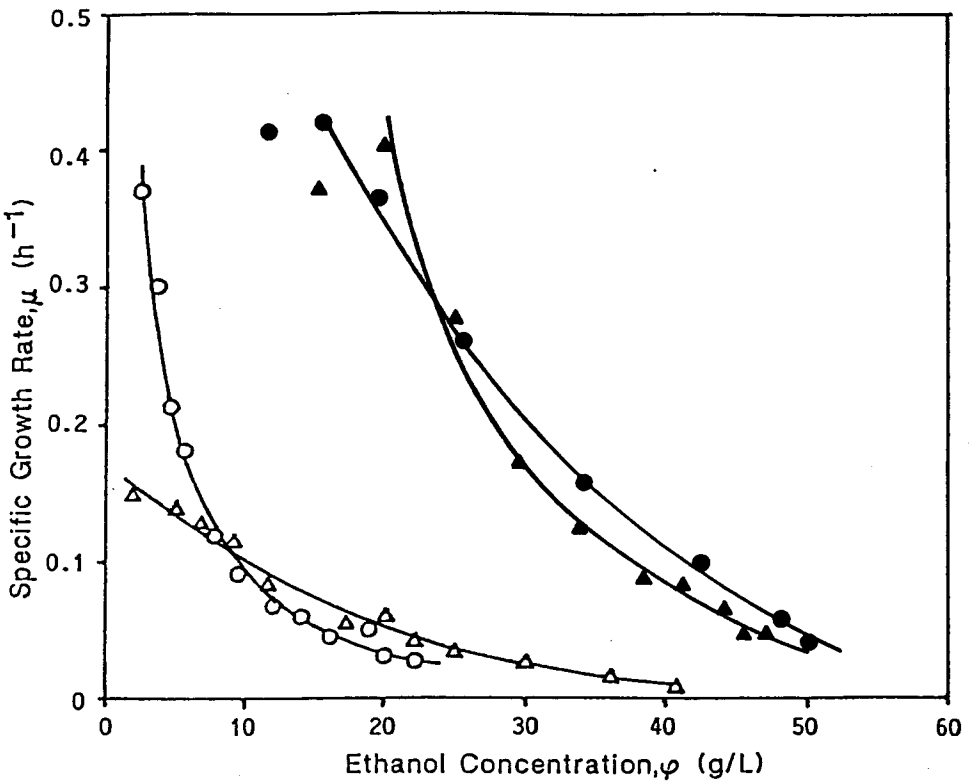

Fig. 1. Specific growth rates of *S. cerevisiae* and *Z. mobilis* in batch [*S. cerevisiae:* (○) X_o = .18 g/L, S_o = 100 g/L; (△) X_o = 1.32 g/L, S_o = 100 g/L; *Z. mobilis*: (●) X_o = .11 g/L, S_o = 90 g/L; (▲) X_o = .22 g/L, S_o = 135 g/L].

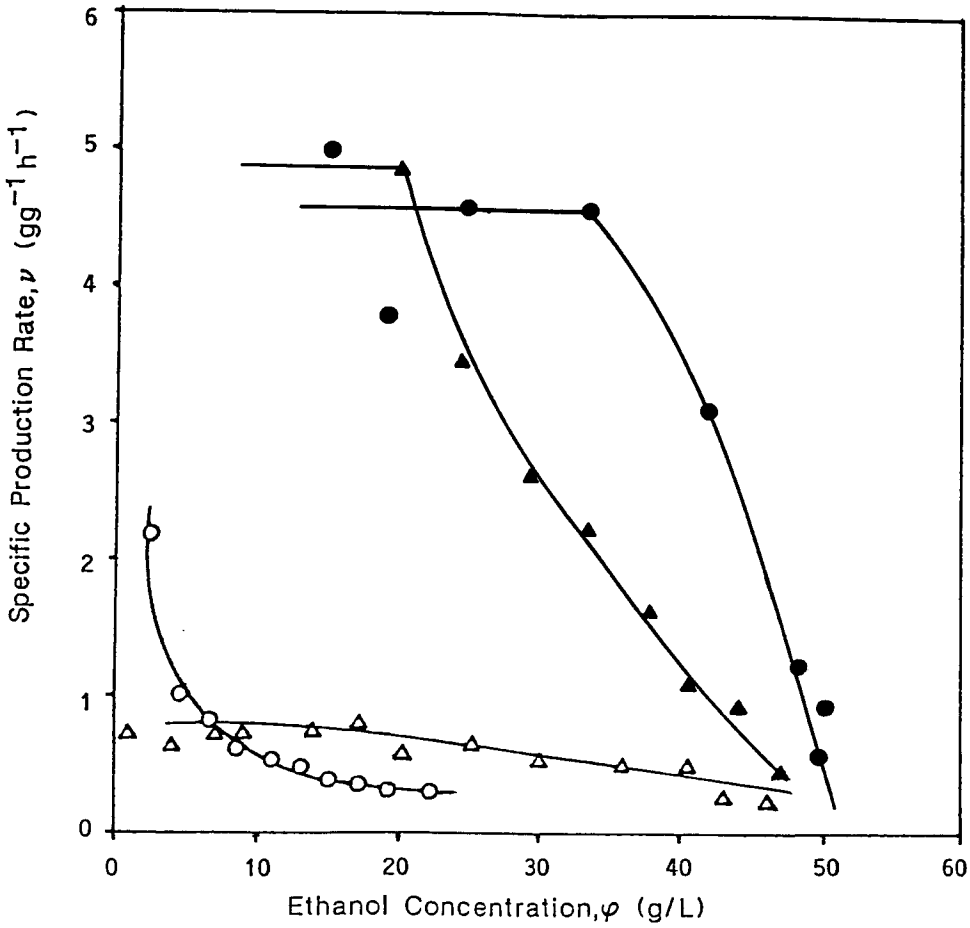

Fig. 2. Specific production rates of *S. cerevisiae* and *Z. mobilis* in batch [*S. cerevisiae:* (○) X_o = .18 g/L, S_o = 100 g/L; (△) X_o = 1.32 g/L, S_o = 100 g/L; *Z. mobilis*: (●) X_o = .11 g/L, S_o = 90 g/L; (▲) X_o = .22 g/L, S_o = 135 g/L].

tory when compared with the batch data for the same strain of the bacterium, as reported in the literature by Lee, et al. (*18*). When the S_o = 90 g/L experiment is compared with Lee's data for S_o = 100 g/L, the discrepancy is not great. In both cases almost complete conversion of the initial substrate took place within 10 h of inoculation. A lower biomass yield was obtained in our experiment (.022 g/g vs .032 g/g in the literature), but ethanol yields were comparable (.49 g/g vs .48 g/g in the literature). In the case of the S_o = 135 g/L experiment, however, only a 74% conversion of the initial substrate had occurred after 20 h of incubation. This is compared with nearly complete conversion of initial substrate within 14 h, as reported by Lee for S_o = 150 g/L. Biomass and ethanol yields were .026 g/g and .45 g/g, respectively, vs yields of .03 g/g and .47 g/g reported in the literature.

There was one potentially significant difference between Lee's experimental procedure and our own. In Lee's work, the pH of the fermen-

tation broth was automatically controlled to maintain its value at the optimum pH of 5.0, whereas no pH control was applied to our batch experiments. As a result, the pH would typically drop during a fermentation from an initial value of 5.0 to about 4.0, before increasing slightly during the stationary phase.

The original decision to omit pH control from the batch studies was based on the fact that pH is difficult to regulate in a packed-bed ICR, and no such control was planned for the subsequent ICR study. Batch data without pH control would thus provide a baseline for comparison with data from the ICR.

In order to test whether the discrepancy between Lee's results and our own was indeed a result of pH effects, a batch fermentation was carried out in which small volumes of a sterile, 1 N solution of KOH were added periodically to the broth during the first nine h from the time of inoculation. Although the pH could not be maintained at a set value in this way, it was kept from dropping as far and as fast as when no base was added. The initial glucose concentration used was 160 g/L. The results of this experiment are shown in Figs. 3 and 4. As in Figs. 1 and 2, specific rates of growth and production are plotted as a function of extracellular ethanol concentration. Data from the S_o = 135 g/L experiment, in which no base was added, are plotted on the same figures to serve as a basis for comparison.

The plots indicate that for ethanol concentrations greater than 15 g/L, much higher specific rates of growth and production can be achieved when base is added than when it is not. Furthermore, when base is added, higher ethanol concentrations can be reached before growth and production are totally inhibited (greater than 80 g/L vs 50–60 g/L). The S_o = 160 g/L experiment with base added also matches well with Lee's data for S_o = 150 g/L and automatic pH control. Essentially, complete conversion of initial substrate resulted within 16 h of inoculation. The biomass yield was .031 g/g and the ethanol yield was .50 g/g. (Again, Lee obtained almost complete conversion within 14 h, a biomass yield of .03 g/g, and an ethanol yield of .47 g/g.) It is evident that pH control is an important parameter in maximizing the productivity that can be obtained with *Zymomonas*.

Immobilized Cell Reactor Studies

During startup of the reactor, growth was observed on the surfaces of the beads used to pack the column, particularly where the packing came into contact with the column wall. Whether or not cells were actually immobilized through formation of covalent bonds with glutaraldehyde, as presumed in theory, was not demonstrated. It may well be that the "stickiness" of the gelatin coating on the beads was primarily responsible for the observed attachment of biomass. Nevertheless, it was clear that the treatment of the glass beads, described earlier in the experimental section, did result in the establishment of a bacterial film on the packing material, as intended.

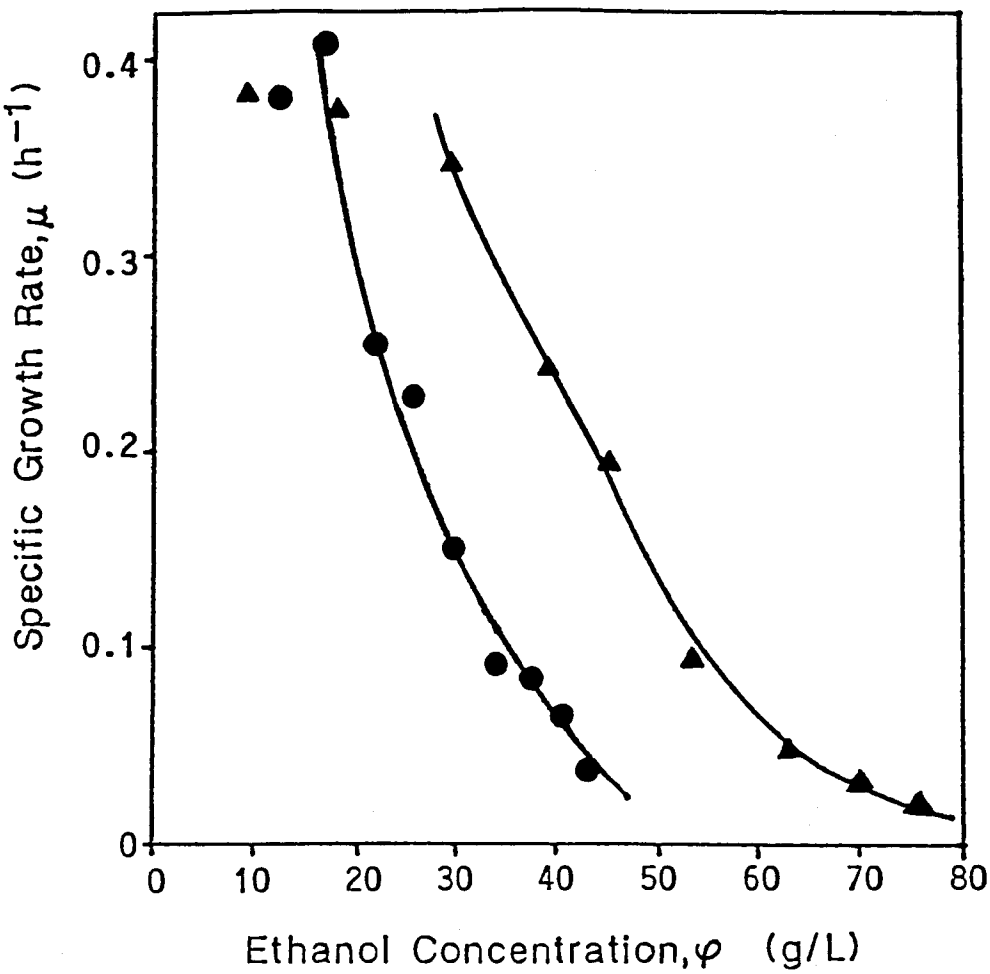

Fig. 3. Effect of the addition of aqueous base on the specific growth rate of Z. *mobilis* [(●) S_o = 135 g/L, no base added; (▲) S_o = 160 g/L, 1 N KOH added to broth during fermentation].

Within two d of inoculation, microscopic examination of samples from the reactor revealed the development of elongated, filamentous cells. These elongated cells clumped together to form flocs. The interstitial spaces of the reactor were gradually filled in by way of such floc formation, beginning at the base of the column. It should be noted that this filling in of interstices was by no means solely the result of floc sedimentation. On the contrary, a significant contribution was apparently made by attachment of flocs to expanding layers of cells surrounding the glass beads, which could be seen to increase in thickness from day to day. It is believed that the establishment of an initial bacterial film in the packed bed thereby enhanced the process of biomass loading by the flocs.

Overall startup of the reactor was rapid. After 10 d 90% conversion of the 150 g/L glucose feed was achieved with a volumetric ethanol pro-

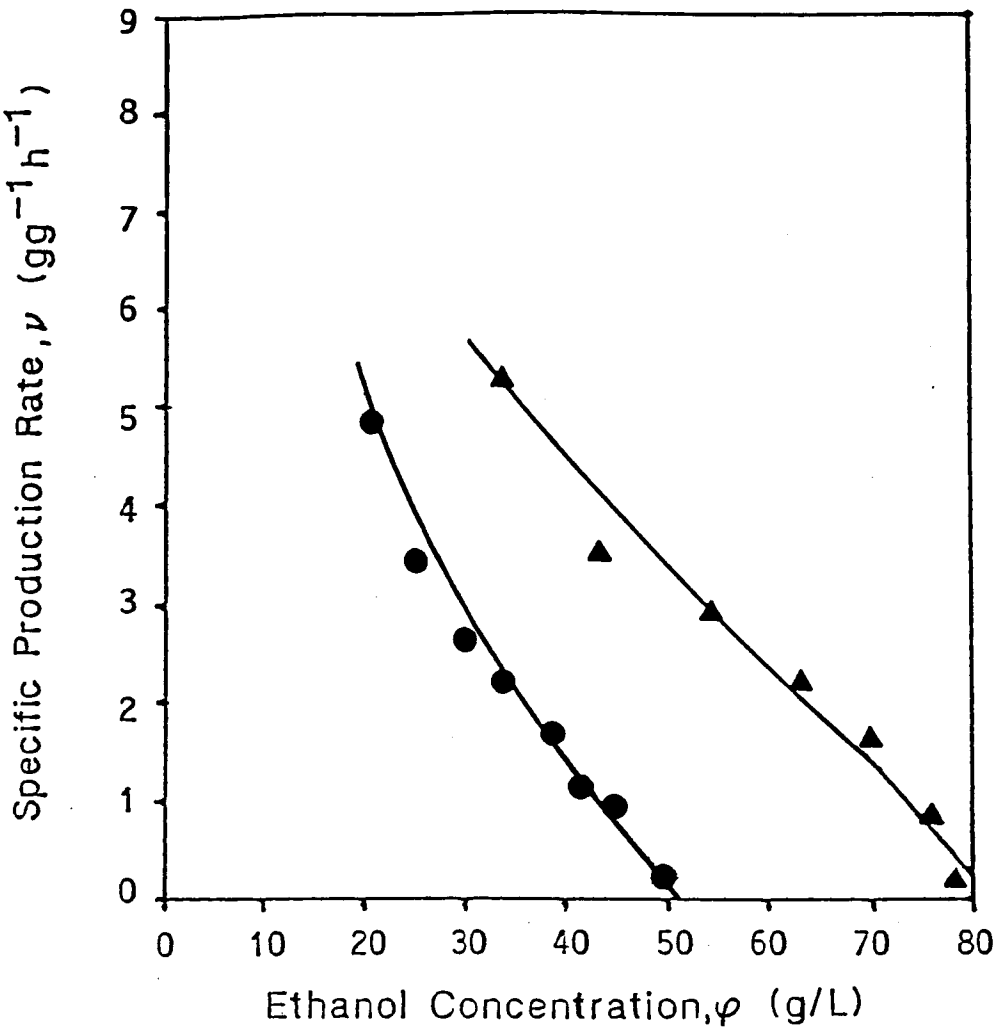

Fig. 4. Effect of the addition of aqueous base on the specific productivity of Z. *mobilis* [(●) S_0 = 135 g/L, no base added; (▲) S_0 = 160 g/L, 1 N KOH added to broth during fermentation].

ductivity (based on liquid holdup) of 60 g/L·h. Figure 5 presents a comparison of biomass loading of the ICR by *Zymomonas* and the yeast *Saccharomyces cerevisiae*, with data for the yeast compiled from earlier work in our laboratory (*19*). Data are shown beginning with the eighth day following inoculation, which was the first day on which the *Zymomonas* ICR was drained for measurements of void volume and liquid holdup. By that time the fraction of the initial void volume remaining free of cells in the case of *Zymomonas* had dropped to .63, whereas in the case of yeast the value was still .90. After 24 d from inoculation, the fraction of initial void volume remaining open in the *Zymomonas* ICR was only .26. The corresponding value in the yeast ICR had dropped only to a level of .73.

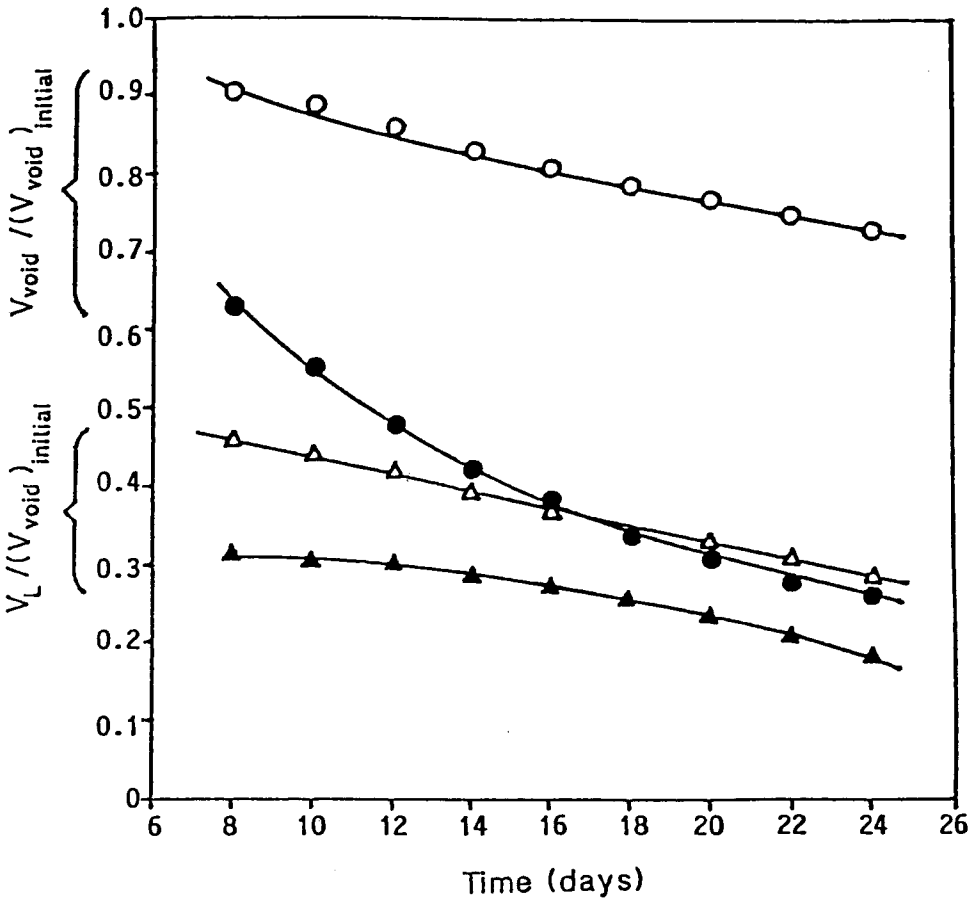

Fig. 5. ICR biomass loading [$V_{void}/(V_{void})_{initial}$ = fraction of initial void volume remaining for (●) *Z. mobilis* and (○) *S. cerevisiae*; $V_L/(V_{void})_{initial}$ = liquid holdup as a fraction of initial void volume over time for (▲) *Z. mobilis* and (△) *S. cerevisiae*].

Along with the much more rapid growth of *Zymomonas*, there was a proportionately higher rate of CO_2 evolution. Again referring to Fig. 5, it can be seen that at all times lower values of liquid holdup expressed as a fraction of the initial void volume were obtained with *Zymomonas* than with yeast. In fact, once the lower half of the *Zymomonas* ICR had completely filled in with cells, a buildup of gas pressure occurred near the column base. Gas was being formed more rapidly than it could escape through the bed of the reactor.

The increase in volumetric ethanol productivity, which accompanied the growth of cells in the reactor during the first few weeks of its operation, is traced in Figs. 6 and 7. In the case of Fig. 6, productivities were calculated on the basis of empty column volume, and were found to increase exponentially with time, eventually reaching a plateau after about 20 d. The productivities plotted in Fig. 7 were based on liquid holdup,

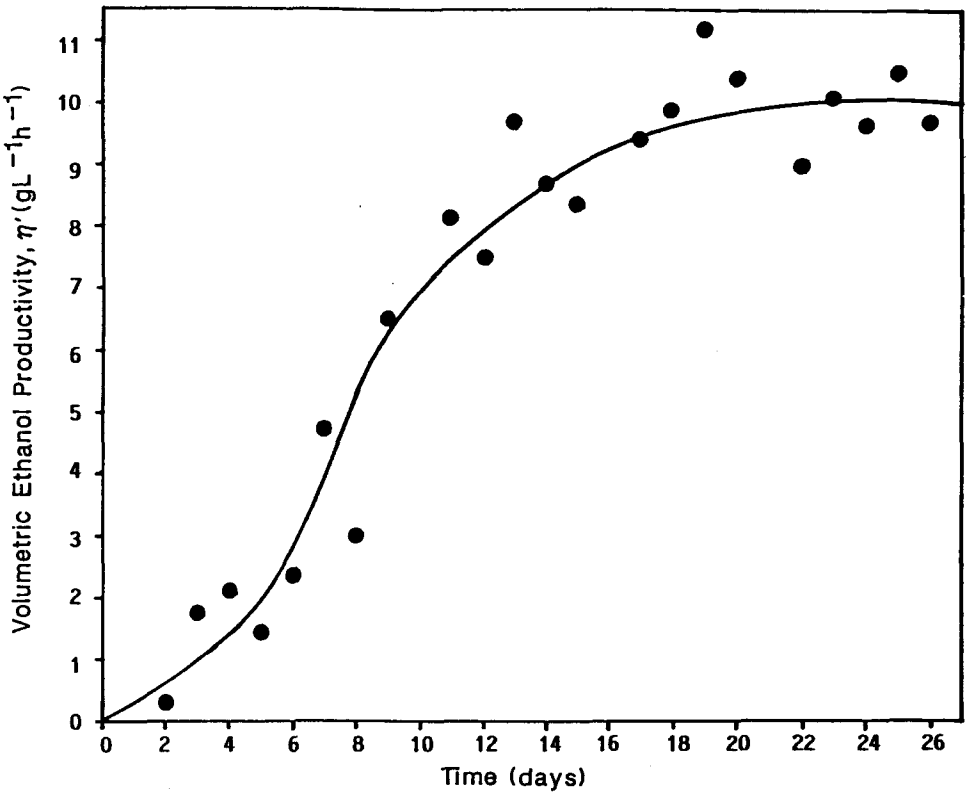

Fig. 6. Volumetric productivity in the ICR for Z. *mobilis* (based on empty column volume).

and were found to increase more or less linearly with time. A leveling of productivity is absent in the latter case because of continuous growth in the reactor, causing a decrease in the liquid holdup volume with each new measurement. Together, these plots demonstrate that there is an optimal biomass loading for the ICR. Increased productivities based on liquid holdup can be obtained with increased biomass, but beyond a certain point the specific productivity, i.e., the ethanol produced per unit cell mass per unit time, deteriorates. One factor that contributes to such deterioration is channeling, which becomes a more serious problem as the porosity in the reactor declines. Low porosity also hinders gas escape, a problem already alluded to above.

Clearly, the overall performance of the ICR would be substantially improved if biomass loading could be maintained near the optimum level, which in this experiment was reached within 20 d of inoculation. One very direct method for preventing overgrowth is to purge the reactor with N_2 at 30 psig using gas pressure to flush out a portion of the accumulated biomass. This technique was employed with the *Zymomonas* ICR, but only after 30 d of operation, when cell density had become large enough to make maintenance of flow through the reactor a problem of

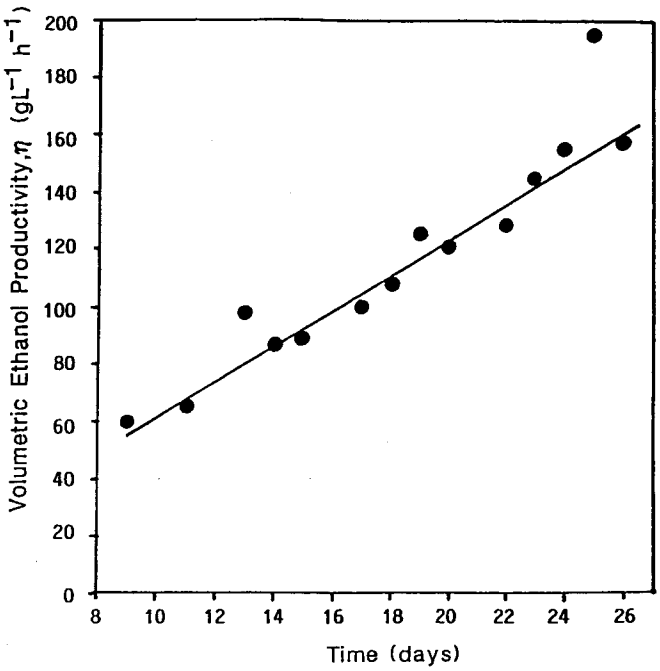

Fig. 7. Volumetric productivity in the ICR for *Z. mobilis* (based on liquid holdup volume).

constant concern. Possibly, if regular N_2 purging had been undertaken a week earlier, more stable long-term operation of the ICR could have been achieved.

It may also be possible to ameliorate the overgrowth problem through use of a more suitable packing or support material in the reactor. Daugulis and Krug have studied continuous fermentation with *Zymomonas* adsorbed onto an ion exchange resin within a column-type reactor (*20*). Although very high ethanol productivities were obtained, the reactor had to be shut down within a week of initiation because of excessive buildup of pressure in the column. Just as in the present investigation, filamentous cells of the bacterium were obtained. These were able to bridge the relatively small spaces between the resin beads, so that plugging of the reactor followed rapidly. Our own use of spherical glass beads as the support, on the other hand, provided over a month of operation before plugging problems emerged, instead of just one week. Of course, the spaces between such glass beads are much larger than those between the beads of an ion exchange resin. It may be that the optimal support is one that provides an even larger void space. An attempt was made, in fact, to operate a cross-linked ICR with Intalox saddles as the support in place of the glass beads. It turned out to be very difficult to obtain a good coating of gelatin on the saddles, which were made of a ceramic material. Consequently, very poor attachment of a monolayer of cells to the packing was obtained following inoculation, and the approach was not a success.

One other strategy for controlling overgrowth, which has been discussed in the literature, is the manipulation of medium composition to promote uncoupling of growth and production in *Zymomonas* (*21*). For example, Swings and De Ley have suggested that such uncoupling might be accomplished by limiting the concentration of pantothenate in the medium (*22*). As yet, little progress has been reported along these lines. However, in our work with cross-linked *Saccharomyces cerevisiae* in an ICR, it has been shown that the addition of short-chain fatty acids to the feed medium, once satisfactory production levels have been reached, prolongs catalytic life by reducing the growth rate. The fatty acids in the medium act to increase the maintenance coefficient of the yeast, so that as growth is reduced, ethanol productivity is increased (*23*). In light of these results, an investigation of the effect of short-chain fatty acids on ethanol fermentation with *Zymomonas mobilis* is planned.

As was noted once before, growth in the *Zymomonas* ICR proceeded essentially unchecked during the first month of operation. Toward the end of this period, 28 d after inoculation, quasi-steady state values for the volumetric ethanol productivity were measured over a flow rate range of 40–170 mL/h. The results are shown in Fig. 8, in which ethanol productivity based on liquid holdup is plotted against the retention time in minutes. Data from earlier work with cross-linked yeast are shown on the same plot for comparison. For both sets of data, the concentration of glucose in the feed medium was 150 g/L, and the porosity (liquid holdup volume divided by empty-column volume) was .05. In short, the superiority of *Zymomonas* over yeast, previously demonstrated in batch culture, is also borne out in the ICR. Much higher productivities and shorter retention times are possible with *Zymomonas*. With 97% conversion of substrate, and a retention time of 87 min, the yeast ICR had a productivity of 44 g/L·h. By contrast, the *Zymomonas* ICR yielded a productivity of 131 g/L·h, three times that of the yeast ICR, with 99% conversion of substrate and a retention time of only 28 min.

Perhaps of more interest than the relative performance of cross-linked *Zymomonas* and cross-linked yeast, is that of cross-linked *Zymomonas* and *Zymomonas* immobilized by other methods. Margaritis et al., using *Zymomonas* entrapped in calcium alginate beads, obtained a volumetric ethanol productivity of 71 g/L·h with 100 g/L glucose in the feed (*24*). Klein and Kressdorf reported a value of 98.5 g/L·h using the same immobilization technique, and with 150 g/L glucose in the feed (*25*). With *Zymomonas* adsorbed on an ion exchange resin, and feeding 100 g/L glucose, Daugulis and Krug measured a productivity of 89.8 g/L·h (*20*). In all these cases, productivities reported are based on liquid volume, and the conversions of substrate were equal to or exceeded 97%. Placed alongside these systems, the cross-linked ICR appears to offer some improvement in productivity.

The question that arises at this point is to what extent the productivity of cross-linked *Zymomonas* could be improved by implementing some means of pH control, as it suggested by the results of the batch studies.

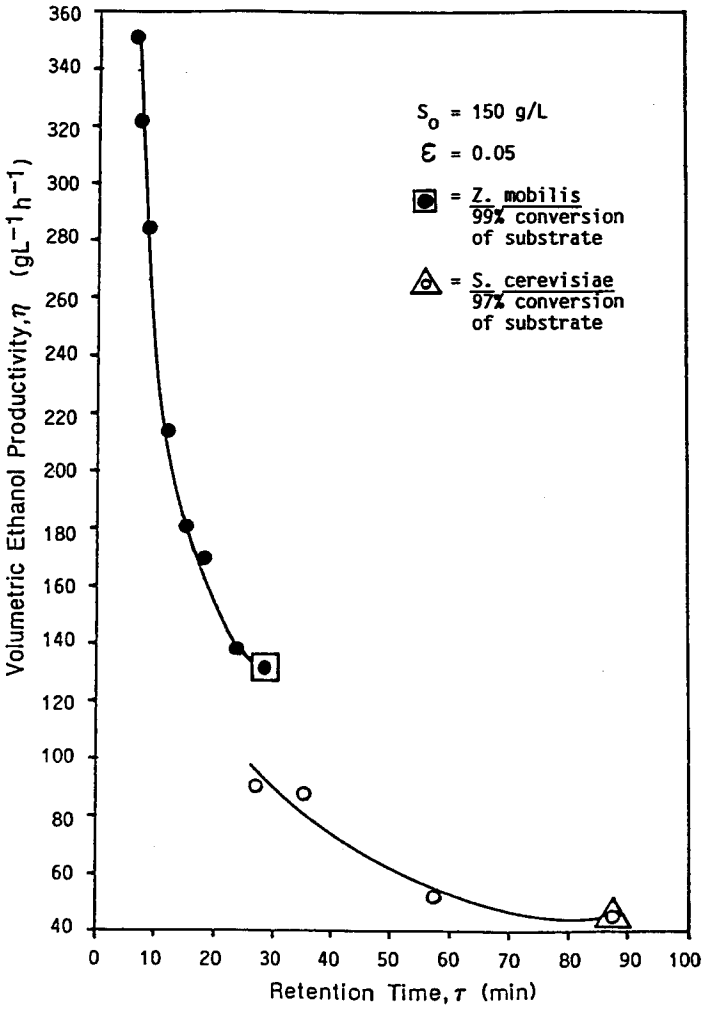

Fig. 8. Overall reactor productivity for (○) *S. cerevisiae* and (●) *Z. mobilis* (based on liquid holdup).

In our own experiments, pH varied downward along the length of the ICR from the inlet to the outlet, except at very high flow rates. Scott, who exploited floc formation for cell retention in a fluidized-bed bioreactor, observed an apparent optimum pH range of 5.0–6.0, with appreciable activity at a pH greater than 4.0. When no pH control was applied, a gradient was established up the column starting at about pH 5.0 and decreasing rapidly to a constant value of about 3.7. Ethanol production was essentially zero at this lowest pH level. When pH was maintained above 4.0 by addition of .1 N KOH at the first entry port up the column, there was significant ethanol production even at the upper part of the column (*28*). (Likewise, Prince and Barford applied a simple method of pH control in conjunction with their upflow floc tower fermentor. Base was added through a single port about one-third of the way from the bottom

of the column.) (*29*). Scott's article indicates that single-port injection of base into a column-type reactor can significantly improve ethanol productivity even without the elimination of pH gradients, at least when a fluidized bed is employed.

Less detailed information is provided by those who have applied pH control to packed-bed ICRs. Jain et al., in their study of continuous fermentation by *Zymomonas* cells entrapped in K-carrageenan, state that pH was controlled at 5.0 by addition of 1*M* KOH, and that pH was found constant from the bottom to the top of the reactor (*26*). Similarly, Melick, et al. describe using the value of pH measured at the top of an ICR, packed with *Zymomonas* cells entrapped in calcium alginate beads, to automatically control the addition of 2 N NaOH at the bottom of the column (*27*). This approach to pH control seems incompatible with the expectation that something approximating plug flow will occur in the packed columns. Daugulis and Krug describe using an elevated concentration of KH_2PO_4 in their fermentation medium in order to improve its buffering capacity, but do not follow up with information about the resulting pH profile in their reactor (*20*). Aside from these examples, no other references to pH control in column-type ICRs employing *Zymomonas mobilis* were found.

CONCLUSIONS

Control of pH is an important factor determining the results that can be achieved with *Zymomonas mobilis*, at least for initial substrate concentrations >100 g/L. If base is added during a batch fermentation, conversion of initial substrate is improved, higher specific rates of growth and production are obtained, and ethanol inhibition to growth and production appears to be mitigated.

Continuous ethanol fermentation using *Zymomonas* in a cross-linked ICR has considerable potential. Reactor startup is much more rapid with cross-linked *Zymomonas* than with yeast. Volumetric ethanol productivities (based on liquid holdup) three times those obtained with cross-linked yeast, and comparable to those obtained with *Zymomonas* immobilized by other methods, are possible. Further work needs to be directed toward development of effective pH control in the ICR; a means to uncouple growth and production in *Zymomonas*, or to otherwise restrict overgrowth; and a means to vent CO_2 from the reactor at intermediate points along its length, instead of through the top of the column only.

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