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EFFECT OF pH ON GROWTH AND ETHANOL PRODUCTION BY *Zymomonas*

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SUMMARY

In a mineral salts medium containing yeast extract, NH_4Cl and glucose (50g/L), the pH range producing the fastest growth of *Z. mobilis* was 5.5-6.5 with an apparent optimum at 6.5. At constant growth rate of $0.15hr^{-1}$, the specific rates of glucose utilization (q_s) and ethanol production (q_p) were relatively unaffected by pH over the range 7.0-5.5 but increased sharply as the pH was further decreased below 5.5 to 4.0. Under these conditions the ethanol yield was unaffected by pH over the range 4.0-6.5 but decreased markedly at pH of 7.

INTRODUCTION

Biotechnology in the fuel alcohol industry relates to the various bioengineering strategies for improving productivity and increasing product concentration and yield. Although yeast is the more traditional process organism in ethanol fermentations, the ethanologenic bacterium *Zymomonas* is recognized as a superior alternative in high performance continuous fermentations for the production of motor fuel alcohol (Lawford, 1988; Baratti and Bu'Lock, 1986; Rogers *et al.*, 1982). Improvements to be derived through the substitution of yeast by *Zymomonas* relate principally to about a 2-5 fold increase in specific productivity together with a 5-10% increase in product yield (Lavers *et al.*, 1981; Rogers *et al.*, 1982). Recent head to head comparative performance trials with yeast and *Zymomonas* using an industrial feedstock (corn starch hydrolysate), showed the potential for yield improvement with *Zymomonas* in a commercial process (Beavan *et al.*,1988). As an alternative to process improvement through genetic engineering, we have pioneered a physiological approach to increasing

the performance of *Zymomonas* through manipulation and control of the chemical environment. Energetically "uncoupled" phenotypes with markedly increased specific rates of ethanol production were generated under conditions of certain nutritional limitations in continuous fermentations (Lawford & Stevnsborg, 1986a; Lawford, 1988).

An objective of this study was to extend our previous investigations on the effect of the chemical environment on the behaviour of *Zymomonas* to include pH. Surprisingly, the literature contains very few references to systematic studies on the effect of pH on *Zymomonas* (King & Houssain, 1982; Bajpai & Margaritis, 1986; Borrego *et al.*, 1988) and one is left to infer a physiological response to changes in pH from comparative data gathered from the reported observations of several independent investigators. The significant deficiency in this approach is that the majority of studies have been conducted at a single pH value (namely 5.0) with others at higher pH values of 5.5 (Lavers, *et al.*, 1981; Charley, *et al.*, 1983; Lawford & Stevnsborg, 1986; Lawford, 1988) or 6.0 (Fieschko & Humphrey, 1983). Comparative response to pH is also complicated by the diversity in growth conditions in these investigations.

The present study examines the growth and ethanol yield characteristics of *Z. mobilis* as a function of pH over the range 4.0 to 7.0 under conditions of steady-state, carbon (energy)-limitation in chemostat culture.

MATERIALS and METHODS

The type strain *Zymomonas mobilis* ATCC 29191 was obtained as a lyophilized culture from the American Type Culture Collection (Rockville, MD, USA). All media were prepared with laboratory grade reagents in distilled water. The minimal salts medium was as previously described (Lawford, 1988a) and contained 3g/L yeast extract (Difco), 0.81g/L ammonium chloride and 50g/L glucose. The three fractions were autoclaved separately at 121°C for approximately 40 minutes. Batch and continuous fermentations were conducted in BioFlo® C30 bench-top chemostats (New Brunswick Scientific Co., Edison, NJ, USA) with working volumes of 1300ml and 350ml respectively. The fermenters were run at constant temperature (30°C) and constant agitation (200rpm). The pH was automatically controlled by the addition of 2N KOH.

In the batch experiments, a 7.7% innoculum, or 100ml innoculum/1300ml working volume, was used. It was prepared by transferring a streak of colonies from a plate to a flask containing the above mentioned medium, and allowed to incubate statically overnight. The fermenter vessel received no N_2-sparging. The chemostat experiments were initially innoculated at a 10%(v/v) level. They received N_2-sparging at a flow rate of 0.175ml/350ml min and were operated at a dilution rate of 0.150+/-0.006 /hr.

The samples optical density was measured in a 1cm cell with an Unicam SP1800 UV spectrophotometer. The biomass was determined by filtering 10ml through pre-weighed 0.45µ Millipore filters. The filters were subsequently washed

three times with approximately 10ml of distilled water. The filters were dried under an infrared heat lamp (Fisher Infra-Rediator) for at least 4 hours before being weighed on a Mettler H30 balance. Some of the sample was centrifuged at 15 000rpm for 10 minutes in a Sorvall RC2-B centrifuge. The supernatnant was then analysed for glucose using an YSI Industrial Analyser (Yellow Springs Instruments Co.). Ethanol concentration was determined by HPLC (Waters Associates Inc.) using an Aminex Ion Exclusion HPX-87H 300mm X 78mm column. The mobile solvent, 0.0066M H_3P0_4, was pumped through the HPLC at 0.7 ml/min.

RESULTS and DISCUSSION

Apart from a batch culture study by King and Houssain (1982) and one with alginate-immobilized *Z. mobilis* ATCC 10988 by Bajpai and Margaritis (1986) and another by Borrego *et al.* (1988) with passively immobilized *Z. mobilis* ZM4, there has been no systematic study on the effect of pH on *Zymomonas*. For the most part *Zymomonas* fermentations reported in the literature have been controlled at pH 5 following the early publications from P.L. Rogers' lab in Australia (Rogers *et al.*, 1980), although in our studies on the performance of *Zymomonas* in batch and continuous fermentations, the pH was routinely controlled at 5.5 (Lavers, *et al.*, 1981; Charley, *et al.*, 1983; Lawford & Stevnsborg, 1986; Lawford, 1988).

In examining the effect of pH on *Zymomonas* we restricted our investigation to a single type culture, namely *Z. mobilis* ATCC 29191. In order to avoid complications due to the possible inhibitory effect of ethanol (Jobses & Roels, 1985), the glucose concentration was kept low at 50g/L.

The effect of pH on μ_{max} was determined during exponential growth in batch culture conducted in stirred fermentors fitted with pH and temperature (30°C) control. 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 (1982) concluded that the pH range for most rapid growth was 6.0-7.5 with the optimum being at pH 7.0. When making comparisons with respect to the absolute values for μ between our results and those of King and Houssain (1982), it should be remembered that there were noteable differences in operating conditions with respect to temperature (37°C), chemical composition of the medium and the strain of *Z. mobilis* (ATCC 10988).

The changing conditions of a batch culture make interpretation of kinetic parameters difficult and we decided to conduct our investigation under conditions of steady-state growth in a chemostat. Figures 2, 3 and 4 summarize the results of our investigation into the effect of pH on the performance of *Zymomonas* under a condition of carbon and energy-limitation. The chemostat was operated at a fixed dilution rate of $0.15hr^{-1}$ which was significantly less than μ_{max} over the pH range of

4.0-6.5 (Fig. 1). Washout of the chemostat occurred when the pH was lowered to less than 3.8. Since culture response to step changes in pH are often slow (Bajpai & Margaritis, 1986), a minimum of 6 turnovers (volume changes) were permitted before assuming steady-state growth. Figure 2 shows that the specific activity of the culture, both with respect to glucose consumption (q_s, g glu/g cell·hr) and ethanol production (q_p, g ethanol/g cell·hr), appears relatively constant over the pH range 7.0-5.5 but increases dramatically as the pH is lowered below 5.5 to 4.0. Figure 3 shows that the ethanol concentration and yield (since no glucose appeared in the effluent) remains relatively constant and is unaffected by pH over the range 4.0-6.5.

The steady-state biomass (cell) concentration and hence growth yield ($Y_{x/s}$, g cell/g glu) is maximal over the pH range 5.5-6.5 (Fig. 4) and reflects the same pattern observed for the effect of pH on growth (Fig. 1). King and Houssain (1982) reported that $Y_{x/s}$ was relatively unaffected by pH over the range 5.0-7.5.

However, the absolute values reported by these investigators are only about one-half the value observed in this study (results not shown but may be determined from values of biomass, Fig. 4) but this may be due in part to the fact that growth was conducted at 37°C. Elevation in temperature >30°C results in an increase in maintenance metabolism which would be reflected in a decrease in $Y_{x/s}$ (Fieschko & Humphrey, 1983). Further comparisons between our results and those of King and Houssain (1982) are difficult because of other significant differences in growth conditions that might compromise interpretation. Such differences include the higher ethanol concentration (medium contained 10% glucose), use of a different strain of *Z. mobilis* (ATCC 10988) but most significantly, because their study was conducted in the batch mode, measurements were non steady-state in nature.

Energy conservation in *Zymomonas* appears to be maximal at around pH 6, but as the pH decreases from 6 - 4, there is an apparent increasing degree of energetic uncoupling similar to the effect caused by certain nutritional limitations (Lawford & Stevnsborg, 1986a). However, this study does not permit us to discriminate between an effect of pH on growth related metabolism and maintenance metabolism and further chemostat experiments are in progress to examine the effect of pH on q_s and q_p as a function of growth rate.

Regarding the application of these observations to the practice of producing fermentation ethanol, it is important to know that *Zymomonas* will perform efficiently within the same pH range (4.0-4.5) as is currently employed in most commercial, yeast-based, fuel alcohol plants.

Acknowledgements

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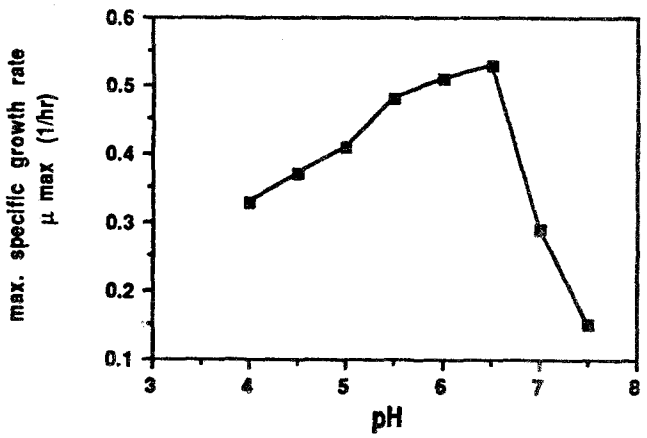

Figure 1 The effect of pH on the maximum specific growth rate (μ_{max}).
Batch fermentations of *Z. mobilis* ATCC 29191 were conducted in STR fitted with pH control (using 3N KOH). The minerals salts medium contained yeast extract, ammonium chloride and glucose (50g/L). The temperature was 30°C. The value for μ_{max} was determined as the slope of the straight line in a semi-log plot of OD_{620} versus EFT during mid-exponential phase of growth.

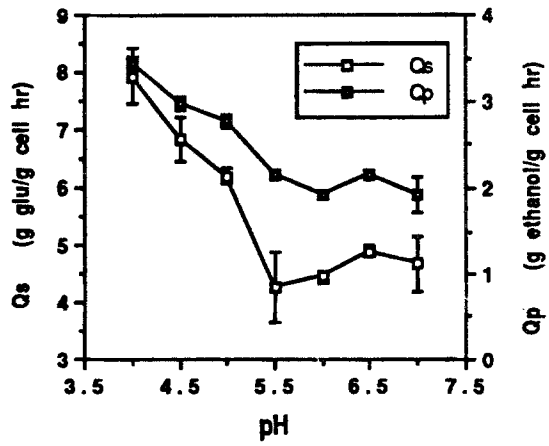

Figure 2 The effect of pH on specific rates of glucose utilization (q_s) and ethanol production (q_p) for *Z. mobilis* ATCC 29191 in carbon(energy)-limited chemostat culture at D=0.15/hr.
The minerals salts medium contained yeast extract, ammonium chloride and glucose (50g/L). Temp. was 30°C.

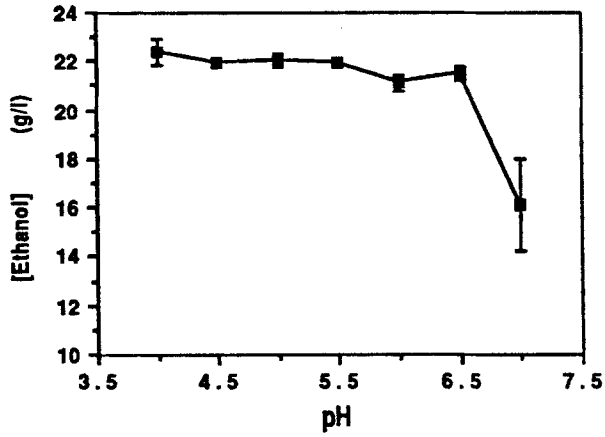

Figure 3 The effect of pH on ethanol concentration and yield for *Z. mobilis* ATCC 29191 in carbon(energy)-limited chemostat culture at D=0.15/hr.
The minerals salts medium was as in Fig. 2. Temp. was 30°C. Culture had a tendency to flocculate at pH > 6.5.

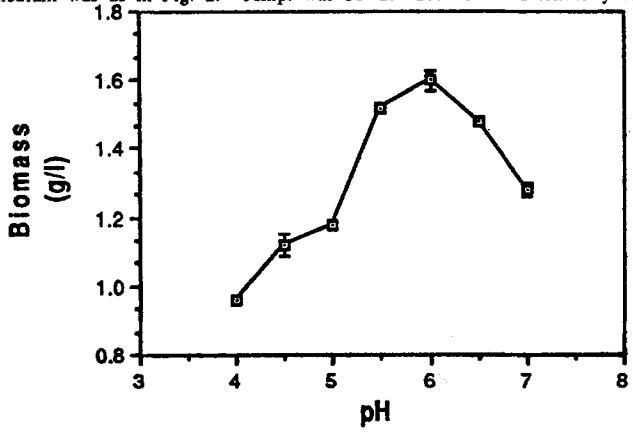

Figure 4 The effect of pH on biomass concentration and yield for *Z. mobilis* ATCC 29191 in carbon(energy)-limited chemostat culture at D=0.15/hr.
The minerals salts medium was as in Fig. 2. Temp. was 30°C. Culture had a tendency to flocculate at pH > 6.5.