NOTES

Viability Reduction of *Acetobacter aceti* Due to the Absence of Oxygen in Submerged Cultures

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In this work, the influence that an aeration interruption may have upon acetic acid fermentation processes is studied. As a result, a kinetic model is proposed that foretells the cellular specific death rate in situations lacking in oxygen. Such a model collects the combined influences of substrate (ethanol) and product (acetic acid).

Introduction

The acetic acid fermentation process is distinguished by its high oxygen consumption, so that this is one of the decisive factors in the process total rate in this type of fermentation. The acetic bacteria are extremely sensitive to situations lacking in oxygen, where they are not only limited to reducing their metabolism but also end up degrading themselves (Hromatka and Ebner, 1962). Therefore, the medium oxygenation must be continuous because interruptions longer than 30 s may be enough to inactivate part of the bacterial population (Drysdale and Fleet, 1989). Muraoka *et al.* (1982, 1983) also report that the damage in the acid production, due to a lack of oxygen, is greater the higher the acid percentage is.

Park *et al.* (1989, 1991), working in continuous and in repeated fed-batch cultures, propose two mathematic expressions that establish a direct relation between the specific death rate and the acetic acid concentration (range between 40 and 85 g/L) for optimum levels of dissolved oxygen and the ethanol concentration at about 30 g/L. These expressions include only the acetic acid influence since, normally, the influence of other variable factors during the process is considered less important. Namba *et al.* (1984) analyzed the synergistic effects of the ethanol substrate and acetic acid product on the growth of *Acetobacter* sp. in vinegar fermentation using a turbidostat culture, but they did not propose any combined equations.

In this work, a new expression that takes into account acetic acid, as well as ethanol, concentration influence is studied.

Materials and Methods

Microorganism. The acetic acid bacterium used in this study was classified as *Acetobacter aceti* UCA1 (criterion according to *Bergey's Manual of Systematic Bacteriology*, 9th ed.), which was isolated from vinegars industrially manufactured in the viticultural area of Jerez-Xeréz-Sherry, and it is properly stored in our department.

Culture Medium Composition. A complex medium was used as the fermentation substrate, formed by a young wine from the above-mentioned area: ethanol, 1−2 g/L; superior alcohols, 0.5−1.0 g/L; volatile esters, 1−5 mg/L; pH, 2.9−3.0; and SO_2, 60−70 mg/L.

Batch Culture. The fermentative process was carried out in a batch operation mode, using fermentors with a working volume of 450 mL. All of the experiments were developed at a constant shaking rate of 200 rpm, an aeration flow of 0.5 vvm, and a temperature of 30 °C.

To carry out the study on the effect of the temporary lack of oxygen, aeration shut-offs were done when the culture was in its exponential growing phase and the acetic acid and ethanol concentrations had reached fixed values. During the aeration interruption, with the aim of achieving total medium anoxia, the culture was gasified with a pure N_2 current, eliminating the residual oxygen and keeping an inert atmosphere inside the fermentor. After a certain time, the aeration was restored again. At the same time, control tests were carried out under identical temperature and shaking conditions and uninterrupted aeration.

Assays. Ethanol and acetic acid concentrations were determined by gas chromatography, total biomass concentration was determined by means of cell counting in a Neubauer chamber, and dissolved oxygen concentration was determined by using a polarographic sensor.

Results and Discussion

Growth and Production in Normal Aerated Submerged Cultures. The different phases of microbial growth can be clearly distinguished in the several experiments carried out, as can the typical production cycle of the strain used in this study, in batch culture, and under uninterrupted aeration conditions. The normalized evolution can be seen in Figure 1, followed by the different interesting variables along the whole process and for the overall data experimentally obtained.

A lag phase in acetic bacterium growth can be seen in Figure 1. Extension of this phase depends on the initial ethanol concentration and the SO_2 levels present in the medium. During this phase, the acetic acid concentration is a minimun; however, the ethanol concentration decreases progressively with respect to the initial maximum value due to leaks by evaporation present in the system. On the other hand, the bacterium population density is also a minimum, indicating minimum oxygen demand on the part of the culture. This results from the fact that the dissolved oxygen concentration, during this phase, is a maximum. After this adaptation period, the development of the fermentative process itself is started, and

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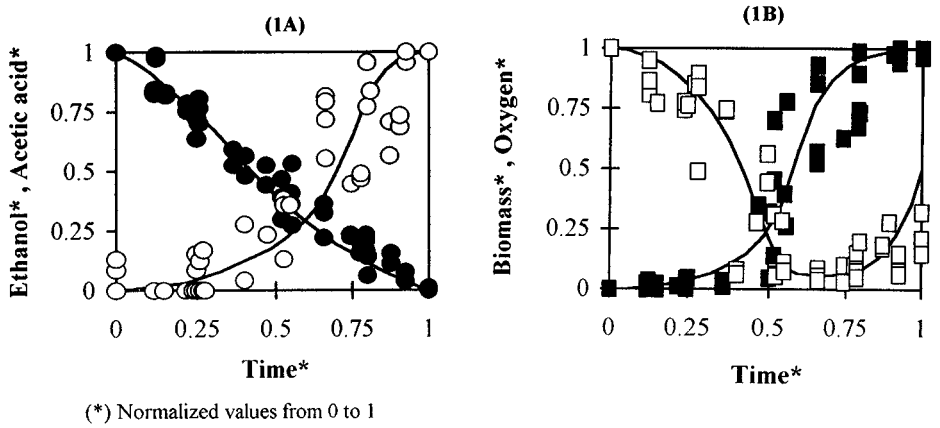


Figure 1. Normalized evolution for acetic fermentation in cultures treated without aeration interruption: (A) ● ethanol, ○ acetic acid; (B) ■ total biomass, □ dissolved oxygen.

we observe a population density increase, a dissolved oxygen concentration decrease (as a consequence of the higher demand), and an acetification rate increase. At the end of this phase the viability reduction starts, as a result of the high values reached by the acetic acid concentration and the low ethanol concentration. The result of this microbial death phase is an acetification rate decrease, which is sometimes imperceptible by simple comparison of the acetic acid concentration values, but clearly detectable by the dissolved oxygen concentration.

Aeration Interruption Effect on Aerated Submerged Cultures. In cultures treated with aeration interruption, the fermentative process phases observed differ significantly from those mentioned earlier for the standard cultures. In Figure 2, the different phases of microbial growth for the cultures that were under a shut-off in the oxygen supply are represented after normalization.

A value of 1 represents the values corresponding to the shut-off moment, and a value of 2 represents the final instant values. First of all, in this case we can see how, after the interruption and aeration restoration, a new delay period appears. This second lag phase is also extended in terms of the medium composition at the moment of the interruption and the duration of the shut-off. With regard to the dissolved oxygen variations in the experiments with aeration shut-off, the results obtained under different conditions are collected in Table 1.

The fact that an oxygen saturation recovery is detected in the medium for specific cases indicates an important loss of biomass viability existing in the medium and, therefore, the loss of oxygen consumption capacity (respiratory function). Moreover, when the oxygen concentration subsequent to restoration of the supply becomes stabilized at low values, the presence of a great quantity of viable biomass consuming the corresponding oxygen is reflected. The data in Table 2 confirm the preceding effect, in the sense that the higher the acetic acid concentration in the medium and the longer the interruption, the greater the biomass viability damage.

Calculation of Biomass Specific Death Rate in the Absence of Oxygen. The μ_d values can be calculated by integrating the following equation:

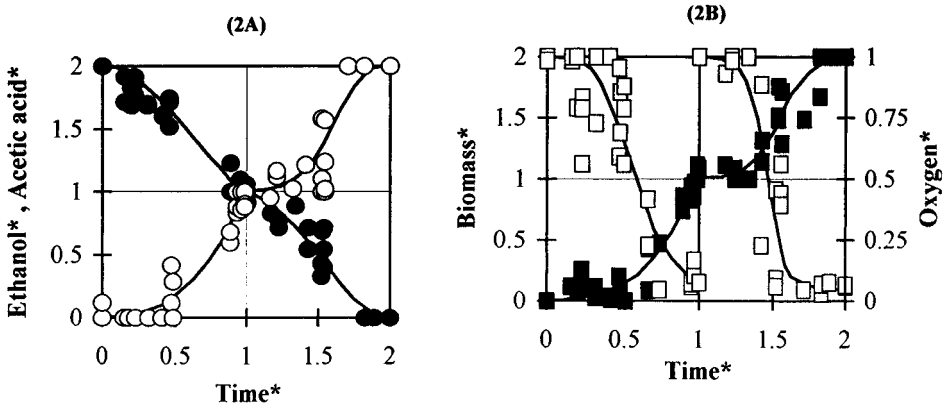
$$\mu_d = \frac{\ln(X_{vo}/X_v)}{t - t_o} \qquad (1)$$

where $t - t_o$ means the time passed from the aeration shut-off (absence of oxygen) to a given time; t, X_{vo} is the viable cell concentration at the initial time when the air supply is shut off, and X_v is the culture viable cell concentration lowered in the absence of oxygen at the moment t. Then, to calculate the culture viability remaining (X_v/X_{vo}) and, therefore, the specific death rate, it is necessary to know the viable biomass (X_v) at each moment (t). This purpose can be carried out by using viable microbial counts or indirect estimation of the viability based on total biomass and specific growth rate detected (Romero *et al.*, 1994). From the viable biomass data, before and after the aeration interruption, we directly obtained the viability reduction, and by knowing the aeration interruption time, we can obtain μ_d through eq 1.

In Table 2, the values of the specific death rate obtained are presented compared to the variables under study for the different experiments carried out. From such experimental data, together with other similar data collected from the literature (Hromatka and Ebner 1951a,b, 1962; Muraoka *et al.*, 1982), the influences of ethanol and acetic acid in the cellular death phenomenon due to aeration interruption can be studied.

Ethanol and Acetic Acid Influences on the Microbial Specific Death Rate. In Figure 3 are represented the experimental data obtained for the specific death rate versus the acetic acid concentration for different ethanol concentration ranges. In view of the data, we can establish that the cellular viability reduction due to the absence of oxygen is according to the acetic acid concentration and the ethanol concentration in the medium. Therefore, the kinetic equation proposed for the specific death rate must show the combined influences of substrate and product under anoxic conditionss, μ_d = f(ethanol, acetic acid).

Thus, in general, we can consider that, for an ethanol concentration range narrow enough, the specific death rate is a function of only the acetic acid concentration. Besides, it can be seen how as the ethanol concentration increases, the observed specific rate of death decreases. In this sense, we must indicate that there is no kinetic expression in the literature that reflects this tendency; therefore, in this work a new expression is included that considers the above-mentioned facts. Consequently, the global expression must show the combined influences of substrate and product on the cellular death rate under medium anoxic conditions. In this sense, the general equation proposed for the cellular specific death rate is the following:



(*) Normalized values from 0 to 1 (at the interruption time) and then to 2

Figure 2. Normalized evolution for acetic fermentation in cultures treated with aeration interruption: (A) ● ethanol, ○ acetic acid; (B) ■ total biomass, □ dissolved oxygen.

Table 1. Changes in the Dissolved Oxygen Concentration in Cultures with Aeration Interruption

concentration during the interruption			oxygen saturation %	
acetic acid (%)	biomass (10^6 cells/mL)	interruption (min)	before	after
1.7	362	30	10	17
2.5	497	30	10	67
3.6	564	60	5	89

Table 2. Data of Biomass Viability Reduction and Specific Death Rate (μ_d) for the Different Experiments

ethanol (g/L)	acetic acid (g/L)	interruption (min)	viability reduction (%)	μ_d (1/h)
11.8	17.7	30	54	1.6
32.3	17.3	60	84	1.8
17.2	17.2	60	82	1.7
32.9	17.3	20	49	1.7
32.9	17.3	36	64	1.8
28.8	24.7	60	91	2.4
11.8	25.0	30	89	4.4
10.4	36.4	60	99	4.5

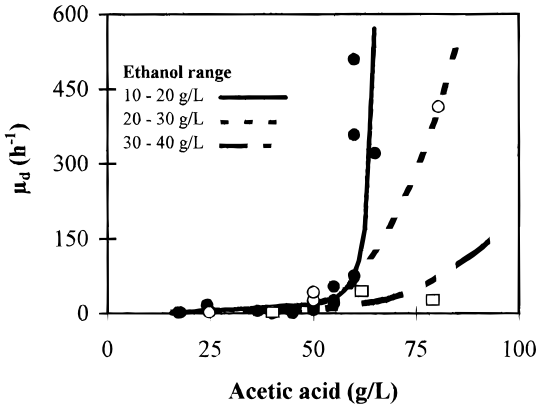


Figure 3. Graphic representation of specific death rate (μ_d) as a function of acetic acid concentration for different ethanol concentration ranges and a dissolved oxygen concentration of 0 ppm: ●, [ethanol] = 10−20 g/L; ○, [ethanol] = 20−30 g/L; □, [ethanol] = 30−40 g/L.

$$\mu_d = \frac{K_D A^a}{E^e + K_N} \qquad (2)$$

General Adjustment of the Cellular Death Equation Parameters. By applying the nonlinear regression analysis (Marquardt, 1963) to the whole set of experimental data, together with the literature data, the following values adjusted to the parameters were obtained: $a = 4$, $e = 3$, $K_D = 0.11$ L/g·h, and $K_N = 3.54 \times 10^3$ (g/L)^3, for the concentrations expressed in g/L and the specific rate in h^{-1}. With regard to model accuracy, it can be said that the calculations provide a good correlation coeffiecient ($r^2 = 0.96$), indicating that the dependence type of the selected variables gives a high confidence level. The Figure 4 corresponds to the plot of eq 2 for the preceding coefficient values.

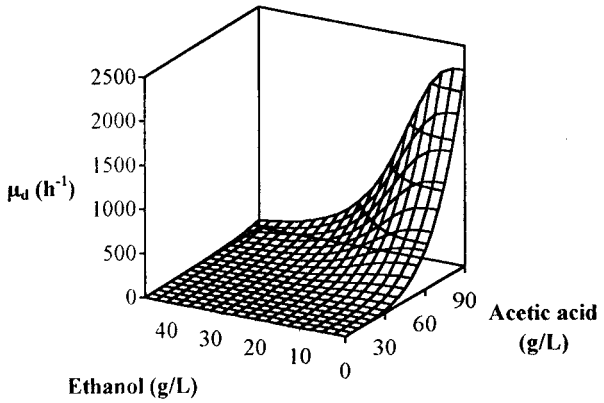


Figure 4. Graphic representation of the proposed equation. Combined dependence of ethanol substrate concentration and acetic acid product concentration for anoxic conditions is shown (DO = 0 ppm).

Conclusions

From different batch acetic acid fermentation experiments, carried out at the laboratory level, the biomass viability reduction is confirmed to be quickly damaged during aeration interruption and, therefore, the acetification rate is reduced.

It follows that the bacterium damage percentage is influenced by the acetic acid concentration, ethanol concentration, and aeration interruption time. With regard to ethanol, it is shown that for a constant ethanol concentration the degree of damage increases with increasing acetic acid concentration. Likewise, high acid concentrations (higher than 50 g/L) cause great viability reductions (more than 75%) in short periods of time (less than 60 s). On the other hand, it is observed that, for fixed acetic acid concentrations, cultures with higher ethanol concentrations after aeration interruption show a smaller lag phase and, therefore, the recovery time for the acetification rate decreases.

Such effects are collected with enough accuracy in the proposed specific death rate equation and the adjusted

values of the parameters introduced in the proposed equation to offer the possibility of establishing interesting valuations in the design and development of industrial acetic acid fermentation processes.

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