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Modelling the kinetics of growth of *Acetobacter aceti* in discontinuous culture: influence of the temperature of operation

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Abstract Acetic acid fermentation is the biochemical process by which, under strict conditions of aerobiosis, *Acetobacter aceti* oxidises the ethanol contained in alcoholic substrates into acetic acid. This paper studies the effect of temperature on the specific growth rate of the microorganisms (μ_C), in particular, the mathematical modelling of this process, with the aim of developing previous studies of the mathematical relationships between μ_C of *A. aceti* and the concentrations of substrate (ethanol), product (acetic acid) and dissolved oxygen. Until now this relationship has not been widely studied, and only a few studies have looked at the influence of temperature on growth kinetics of this bacteria. We have developed an extensive experimental system, to determine precisely the influence of temperature on the maximum specific growth rate.

Introduction

Acetic acid fermentation can be defined as an aerobic process of biological oxidation (thermodynamically favourable), during which a substrate with an ethanol concentration of 50–100 g/l is partially oxidised by the action of acetic acid bacteria to produce acetic acid and water. This oxidation takes place according to the following basic equation (Suárez-Lepe 1990):

$$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$$
$$(\Delta G^0 = -494.5 \text{ kJ mol}^{-1})$$

The result is a solution with a high acetic acid content and a small amount of residual unconverted ethanol, together with a good number of secondary products. The most influential variables in the metabolism of the acetic acid bacteria are ethanol and acetic acid concentrations (De Ley and Gillis 1984), dissolved oxygen concentration (Amerine et al. 1980), pH (De Ley and Gillis 1984), SO_2 concentration (Lafon-Lafourcade and Joyeux 1981), population density (Soo Park et al. 1989) and temperature of cultivation (Brock 1978).

Some of the most frequently studied factors in such metabolism are the effects of substrate and product concentrations. Although the ability to metabolise ethanol to acetic acid is the distinctive characteristic of the acetic acid bacteria, both have complicated influences on their growth. Different authors have reported the difficulties experienced when growing a strain of *Acetobacter aceti* in media containing over 15% ethanol (Drysdale and Fleet 1985; Joyeux et al. 1984) and the booster effect and high inhibition likely to appear if there are low (less than 10 g l^{-1}) or high (more than 30 g l^{-1}) acetic acid concentrations in the medium respectively (Bar et al. 1987).

The dissolved oxygen concentration is another metabolic parameter that has been thoroughly studied. When the O_2 concentration is too low, the process slows down and certain secondary reactions, such as the formation of ethyl acetate by esterification of ethanol and acetic acid, are favoured (Drysdale and Fleet 1988). Therefore, it is necessary to achieve a good oxygen transfer in the fermenter, usually by means of intensive aeration and suitable mixing of air and liquid phases.

There are several kinetic models that predict the effect of various parameters on the specific growth rate of acetic acid bacteria, either individually (Cho and Wang 1990; Nanba and Tamura 1984; Soo Park et al. 1991) or in conjunction, when the synergistic effect is considered (Romero et al. 1994). In the latter case, the most complete one, the resultant expression is a combination of three functions, one depending on the ethanol concentration, f(E), another on the acetic acid concentration, g(A), and the last depending on the dissolved oxygen concentration, h(O):

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$$\mu_c = \mu_{max} \cdot \underbrace{\frac{[E]}{[E] + K_{SE} + \left(\frac{[E]}{K_{IE}}\right)^2}}_{f(E)} \cdot \underbrace{\frac{1 + \left(\frac{[A]}{K_{SA}}\right)}{1 + \left(\frac{[A]}{K_{IA}}\right)^3}}_{g(A)} \cdot \underbrace{\frac{\frac{[O]}{K_{SO}}}{1 + \left(\frac{[O]}{K_{IO}}\right)^3}}_{h(O)} \quad (1)$$

It should be noticed that, even though this model is quite complex, it does not take account of the influence of the temperature. Until now, there has been only one report in the literature that uses a general equation taking account of the influence of temperature on the growth of microorganisms (Sinclair and Topiwala 1970):

$$\mu_{max} = A \cdot e^{-E_a/RT} - B \cdot e^{-E_b/RT}$$

where E_a and E_b are the activation energies for cellular multiplication and thermal denaturation processes respectively, and A and B are the exponential factors. As the energies activating the biochemical processes that cause the denaturation are higher than those for multiplication processes, such curves are asymmetrical in about the optimum, with a steeper slope on side nearer to the maximum temperature than at lower temperatures. This is because, when the temperature increases above the optimum, the decomposition reactions of cellular compounds, such as proteins and nucleic acids, are largely favoured and, at the same time, irreversible damage takes place in the plasma membrane, causing dispersion of the cellular components (Brock 1978), suddenly reducing the metabolic functions.

E_a and E_b values for different types of microorganisms have been reported (Boulton 1979; Caro et al. 1991); however, there are no such studies for *A. aceti* and only approximate ranges of viability are known. Thus, some authors (Lafon-Lafourcade and Joyeux 1981) place the optimum temperature between 30 °C and 35 °C, although a great capacity for adaptation to more severe conditions has been reported. There are no specific references to any minimum temperature for viability, but it is considered that below 10 °C growth is hardly possible (Joyeux et al. 1984). Similarly, there are no reliable data on the maximum temperature, but this is supposed to be around 35 °C.

Materials and methods

Microorganism strain and media

The microorganism used in all experiments was isolated from a vinegar produced in the Jerez area, and was classified as *Acetobacter aceti* ATCC15973 according to Bergey's Manual criteria (De Ley and Gillis 1984).

A complex synthetic medium was used prior to the fermentation, to grow and adapt the acetic acid bacteria to the fermentation conditions, and a complex natural medium was used as a basis for the experimentation. In both cases, appropriate sterile conditions were used.

Bacterial cultures were grown, before the experiments, in a complex synthetic medium (6% YEPE): 10 g l^{-1} yeast extract, 20 g l^{-1} peptone, and 60 g l^{-1} absolute ethanol. The ethanol concentration chosen was that offering the best relationship between growth rate and adaptation of the microorganism to the industrial fermentative medium, as recommended (Mesa 1996).

In all experiments, the cultures were inoculated into a young wine from the Jerez wine-making area (Spain) containing 14% v/v ethanol. Before each inoculation, this wine was sterilized by filtration with membrane filters (0.22 μm pore size), in order to avoid changes on the characteristics of the wine by using conventional thermal processes.

Reaction equipment

The reaction system used has the general characteristics of the equipment most commonly used on an industrial scale, though the experiments were carried out on a laboratory scale. Cylindrical glass bioreactors of 500 ml capacity were used, with a working volume of 450 ml. Bioreactors were provided with a gas inlet coupled to an air sparger of porous glass (average diameter of pore: 100 μm), ensuring the necessary mixing of the medium (Fig. 1).

In order to provide each bioreactor with the necessary oxygen, membrane aeration pumps, able to keep the volumetric flow at the desired values, were used. The temperature was controlled by using a cooling/heating bath, with an accuracy range of 0.1 °C.

Inoculation procedure and working programme

This consisted of two steps. In the first step, 200 μl *A. aceti* culture, conserved in glycerine and kept at −20 °C, was grown in 500 ml 6% YEPE, according to the protocol developed by Mesa (1996), in order to guarantee a high-population culture with (100–500) × 10^6 cells/ml in the exponential growth phase that can be used as an inoculum for the experiments.

In the second step, each bioreactor containing 405 ml natural medium was inoculated with 45 ml (10%) of the prepared inoculum described above, under conditions of maximum sterility. Once the bioreactors had been inoculated, an aeration rate of 0.2 vvm (air volume medium volume^{-1}minute^{-1}) was maintained in order to guarantee the oxygen supply, the agitation and culture homogeneity. Within the operation range, reaction experiments were carried out at 15, 20, 25, 30 and 35 °C, using quadruplicate measurements in each case in order to ensure the reproducibility of the data obtained.

Determination of ethanol, acetic acid and total biomass

Ethanol and acetic acid concentrations were determined by gas chromatography using a glass capillary column (25 m × 0.2 mm

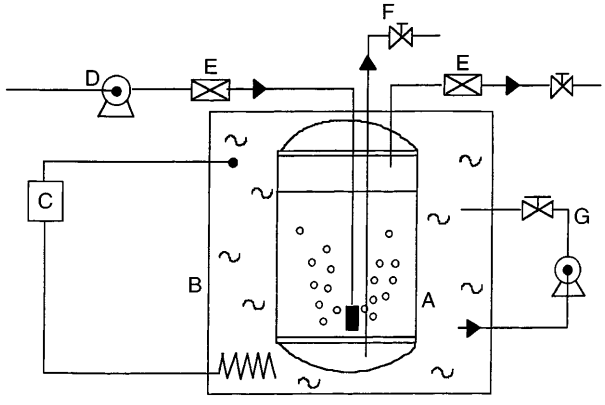

Fig. 1 Scheme of the experimental fermentation equipment. *A* Bioreactor of 500 ml, *B* thermostatic/cryostatic bath, C automatic control equipment, *D* aeration pump, *E* antimicrobial filters, *F* sampling, *G* water recirculation

inner diameter). The stationary phase comprised Carbowax 20 M over Chromosorb (0.2 μm), with flame ionisation detection. The operating conditions used were appropriate for this type of analysis: injector temperature 210 °C; detector temperature 250 °C; temperature programme: 75 °C for 3.6 minutes rising by 70 °C min^{-1} to 150 °C and followed by 3 min at 150 °C; carrier gas: hydrogen (40 kPa); flame ionisation detector: air (400 ml min^{-1}, 2.8 MPa), hydrogen (30 ml min^{-1}, 1 MPa) helium (20 ml min^{-1}, 4 MPa).

Total biomass was determined by counting in a Neubauer chamber. In order to maintain homogeneous dimensions, the total biomass was converted to mg dry weight l^{-1} according to conversion factors published previously for this purpose (Gómez et al. 1994). The dissolved oxygen concentration was determined by a commercial polarographic electrode (Oxi-92, Crison Instruments).

Results

Experimental data, plotting ethanol (g l^{-1}), acetic acid (g l^{-1}) and total biomass (mg dry weight l^{-1}) against time (h) for each temperature studied, are shown in Figs. 2–6. Although pH and dissolved oxygen concentrations have not been shown in the figures, these data were also recorded. The pH decreased with time, following the curve of ethanol concentration, and the average values for dissolved oxygen concentration in the medium during the exponential growth phase are shown in Table 1. It is necessary to point out that the reactions were not followed to exhaustion, because the region of greatest

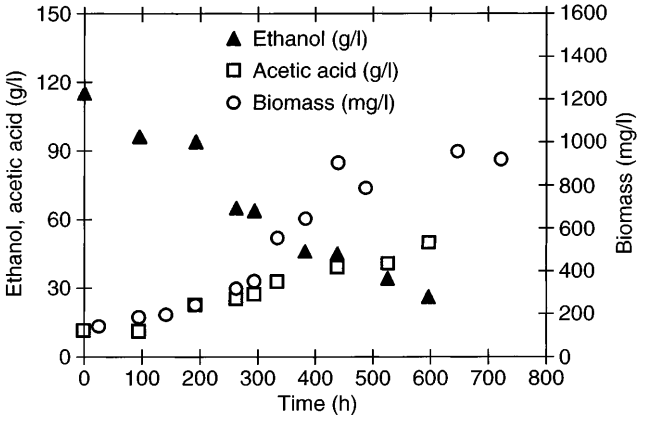

Fig. 2 Experimental data of ethanol, acetic acid and total biomass plotted against time at 15 °C

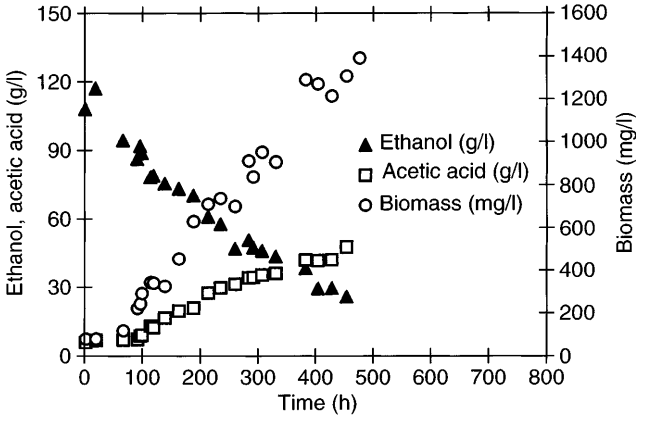

Fig. 3 Experimental data of ethanol, acetic acid and total biomass plotted against time at 20 °C

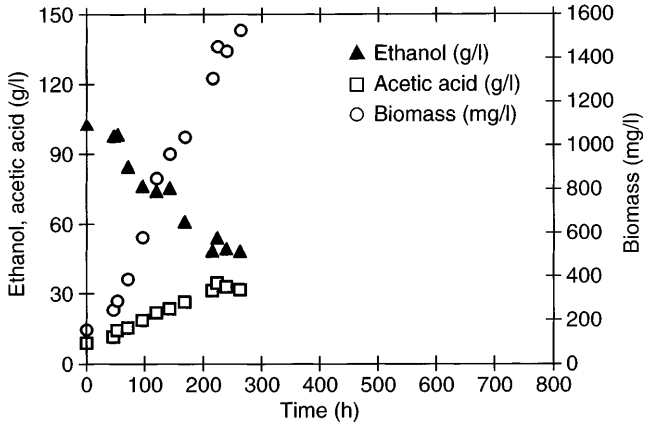

Fig. 4 Experimental data of ethanol, acetic acid and total biomass plotted against time at 25 °C

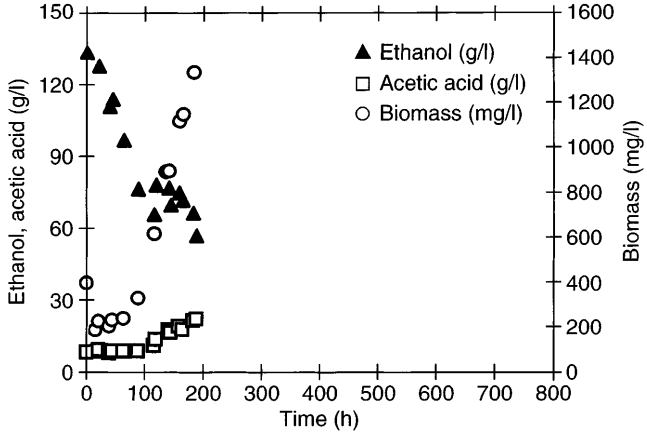

Fig. 5 Experimental data of ethanol, acetic acid and total biomass plotted against time at 30 °C

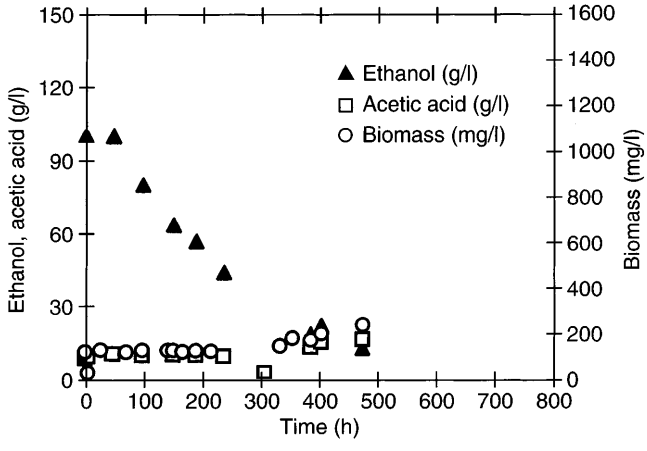

Fig. 6 Experimental data of ethanol, acetic acid and total biomass plotted against time at 35 °C

Table 1 Average value of dissolved oxygen concentration in the medium and maximum specific growth rate (μ_{max}) for each working temperature

T (°C)	Dissolved oxygen (mg l^{-1})	μ_{max} (h^{-1})
15	1.93	0.0262 ± 0.02
20	1.28	0.0375 ± 0.01
25	0.92	0.0584 ± 0.01
30	0.70	0.0944 ± 0.04
35	0.50	0.0106 ± 0.01

interest in our study was the exponential growth phase. In this phase, cells grow at a constant specific rate, for constant medium conditions and it is possible to estimate the specific growth rate (μ_c) accurately.

Maximum specific growth rate data (μ_{max}) were calculated from μ_c, on the basis of the known concentrations of ethanol, acetic acid and dissolved oxygen and the kinetic equation of Romero et al. (1994) (Eq. 1), in which $K_{SE} = 21.1 \pm 6.7$ g l^{-1}, $K_{IE} = 2.83 \pm 0.2$ g l^{-1}, $K_{SA} = 12.6 \pm 2.5$ g l^{-1}, $K_{IA} = 17.9 \pm 1.2$ g l^{-1}, $K_{SO} = 0.372 \pm 0.05$ ppm and $K_{IO} = 1.985 \pm 0.21$ ppm. Romero et al. (1994).

A study of the values obtained for μ_{max} suggested that a Sinclair-type equation (Eq. 2) was adequate to describe its behaviour. Maximum specific growth rate data are shown in Table 1.

In order to fit the experimental data (μ_{max}) obtained to the typical equation (Eq. 2), a non-linear regression method, based on the Marquardt algorithm (Marquardt 1963), was used. At low temperature, the cellular multiplication term prevails, while at temperatures above 30–40 °C the thermal deactivation term dominates. In a qualitative way, the starting iteration values of E_a and E_b can be evaluated from the average slopes of experimental μ_{max} data. After suitable calculations using the Marquardt algorithm, the following values of the temperature equation parameters were obtained: $A = 0.5$ h^{-1}, $B = 8.97 \times 10^7$ h^{-1}, $E_b = 5.974$ kJ mol^{-1}, $r^2 = 0.959$ with $R = 8.31$ J K^{-1} $E_a = 417.2$ J mol^{-1}.

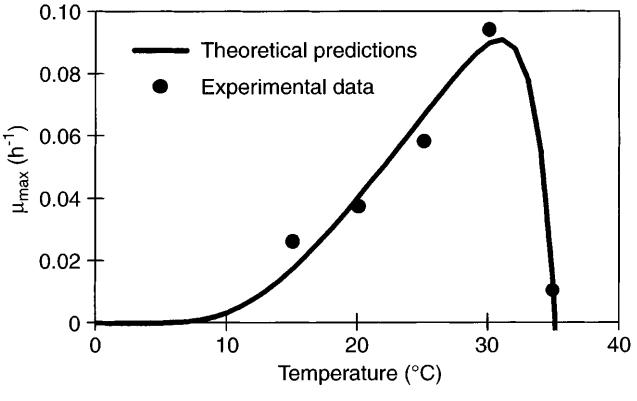

Fig. 7 Model fit and experimental data of maximum specific growth rate (h^{-1}) for *Acetobacter aceti* plotted against temperature (°C)

Figure 7 shows the model fit and experimental data of maximum specific growth rates (h^{-1}) for *A. aceti* plotted against temperature (°C).

Discussion

As can be seen, the plot of the maximum specific growth rate of *A. aceti* against the operating temperature is of a skewed Gauss type. At temperatures above the optimum, bacterial deactivation processes occur in which essential enzymes are denatured, membrane damage causes cellular constituents to scatter and the organism becomes increasingly sensitive to the toxic effect of acetic acid. The energy required for these processes is greater than that required for bacterial activation, which explains why the optimum temperature is closer to the maximum of the μ_{max} curve than to the minimum.

The temperature below which it was considered that there is no growth of *A. aceti* (less than 1% of the optimum μ_{max}) appears to be about 8 °C. The maximum temperature, beyond which the growth of the microorganisms is totally inhibited, is around 35 °C. Finally, according to the equation obtained, the optimum temperature that maximises the maximum specific growth rate is 30.9 °C.

The activation energy values calculated may be compared with those proposed in the literature for other microorganisms (Boulton 1979; Caro et al. 1991). For a particular type of yeast, energies associated with a multiplication factor of 42 kJ mol^{-1} were obtained, apparently much higher than those calculated for *A. aceti*. Further, the denaturation activation energies are also higher in yeasts (about 420 kJ mol^{-1}), suggesting that, in general, above the optimum, bacteria suffer the harmful effects of temperature less than yeasts do.

In short, the proposed equation for the behaviour of μ_{max} and the values of the energies (E_a and E_b) involved can be useful for when choosing a working temperature for acetic acid fermentations, making it possible to operate at the optimum conditions.

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