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THE ETHANOL TOLERANCE OF *Pichia stipitis* Y-7124 STUDIED BY MICROCALORIMETRY *

L. Núñez Regueira[1], I. Gómez-Orellana[1], N. Barros Pena[1], R. Chamy[2] and J.M. Lema[2].

[1]Departamento de Física Aplicada. Universidad de Santiago de Compostela. Spain.

[2]Departamento de Ingeniería Química. Universidad de Santiago de Compostela. Spain.

SUMMARY

The inhibitory effects of ethanol on the fermentative activity of *Pichia stipitis* was studied by microcalorimetry. It was observed .that the specific growth rate decreases when increases the level of ethanol in the culture medium.

INTRODUCTION

Hemicellulose forms up to 35 % of the dry biomass of some lignocellulosic materials with D-xylose as the major constituent sugar [1,2]. Plant biomass can be used for the production of ethanol through the fermentation of pentose sugars, and more specifically D-xylose. D-xylose is converted to ethanol by different yeasts [3-7]. It is typical of xylose fermenting yeasts that xylitol accumulates in the fermentation broth. Xylitol production is not desirable. For this reason *P. stipitis* was chosen as the fermentation agent, because this yeast exhibits a high ethanol yield from xylose and, apparently, produces no xylitol [8].

In the production of ethanol by a fermentative process it is necessary to obtain a high final ethanol concentration, as the energy consumed for distillation is a "capital" factor, from the economic point of view. So, it is important to consider the ethanol tolerance of a yeast strain capable of significant ethanol production. The inhibitory action of both the ethanol produced during the fermentation and that externally added is very

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complex. There are different and separated effects on the growth rate, the viability and the ethanol production. One of the main difficulties in studing the ethanol tolerance of yeasts rests on the fact that there is no universally accepted technique to measure or define this tolerance.

Ethanol tolerance of *P. stipitis* was investigated using a technique based on measuring, by classical analytical methods, the ethanol production rate in batch cultures increasing the initial ethanol concentration in the culture medium [9]. Owing to the low specific ethanol production obtained on the pentose fermentation by yeasts (and even *P. stipitis* shows one of the higher production rates) the results show that this technique is not useful for short lengths of time because both the ethanol produced and the substrate consumed are not large enough to guarantee the accuracy of the analytical measures. For this reason we started a microcalorimetric study.

MATERIALS AND METHODS

Microcalorimetry

The microcalorimeter used was a 2277 Thermal Activity Monitor (Thermometric AB, Sweden), the performance and details of which have been previously described [10]. The instrument has the following characteristics: detection limit 0.15 μW, precision $\pm$ 0.2 % and time constant of 140 s. Baseline stability over 8 hours was $\pm$ 0.2 μW. A microcomputer was connected to the microcalorimeter for data collection and plot results. Experiments were performed in ampoule mode using 3 cm^3 glass vials as reaction vessels. Temperature of the water thermostat bath was 35.00 $\pm$ 0.02°C in all experiments.

Microorganism and culture

Pichia stipitis Y-7124 was supplied by the Northern Regional Research Center, United States Department of Agriculture, Peoria, USA. The culture was maintained on an agar slant of malt extract medium. A 250 cm^3 Erlenmeyer flask containing 100 cm^3 of activation medium (composition per litre: xylose 10 g; yeast

extract 3 g; malt extract 3 g; peptone 5 g; and salt solution 10 cm^3) was inoculated from the agar culture and incubated for 48 hours on an orbital shaker at 35°C. From this culture, 0.5 cm^3 were transferred to 100 cm^3 of culture medium (composition per litre: xylose 50g; yeast extract 3 g; $(NH_4)_2SO_4$ 3 g; $MgSO_4 \cdot 7H_2O$ 1.1 g; KH_2PO_4 2 g; salt solution 10 cm^3. The pH was maintained at 4.5 by citrate buffer) in a 250 cm^3 Erlenmeyer flask and incubated for 48 hours (125 r.p.m., 35°C). The suspension was then centrifugated and the cells were suspended in salt solution (compositon per litre: CaO 1.1 g; ZnO 0.4 g; $FeCl_2 \cdot 6H_2O$ 5.4 g; MgO 0.36 g; $CuSO_4 \cdot 5H_2O$ 0.25 g; $CoCl_2$ $6H_2O$ 0.24 g; H_2BO_2 0.06 g; HCl (con.) 13 cm^3). This last suspension was kept at 4°C and used as inocula.

Cultivations were conducted in 250 cm^3 cotton-wool stoppered Erlenmeyer flasks containing 100 cm^3 of culture medium. The initial cell density was 0.89 $g \cdot dm^{-3}$. Initial ethanol concentration was 0, 5, 10, 15, 20, 30, 40 $g \cdot dm^{-3}$. Ethanol was added to the culture medium before inoculing. Flasks were agitated on a rotary shaker (125 r.p.m.) at 35°C for 3 hours. Then 1 cm^3 of this suspension was enclosed in a 3 cm^3 glass vial and introduced in the calorimeter. The reference was the culture medium without cells. Vials were loaded in the equilibrium position in the calorimeter for 15 minutes and then lowered at the measuring position.

RESULTS AND DISCUSION

Figure 1 shows the power-time curve obtained for the growth culture without ethanol in the medium. After a lag phase, the microcalorimetric curve assumes an exponential shape. During this period, we obtained the specific growth rate constant value, μ, from the slope of the corresponding straight lines drawn by semilog conversion of the experimental power-time curves. Since in the exponential phase of growth the cell number and the culture time correspond to an exponential law,

$$N = N_o e^{\mu t} \qquad (1)$$

If p is the power output per cell during this phase,

$$N \cdot p = N_o \cdot p \cdot e^{\mu t} \qquad (2)$$

if $P = N \cdot p$ and $P_o = N_o \cdot p$ we can write,

$$P = P_o \cdot e^{\mu t} \qquad (3)$$

The thermogenesis curve of the exponential phase of growth should obey equation (3).

The values calculated for the specific growth rate constant from the power-time curves were 0.112 h^{-1} for microaerobic conditions and 0.056 h^{-1} for anaerobic conditions. These results were compared with those obtained by classical analytical methods [9] in the same operation conditions (μ = 0.19 h^{-1} for microaerobic growth and = 0.06 h^{-1} for anaerobic growth). The differences may be explained in terms of sedimentation problems of the yeast in the calorimetric vessel.

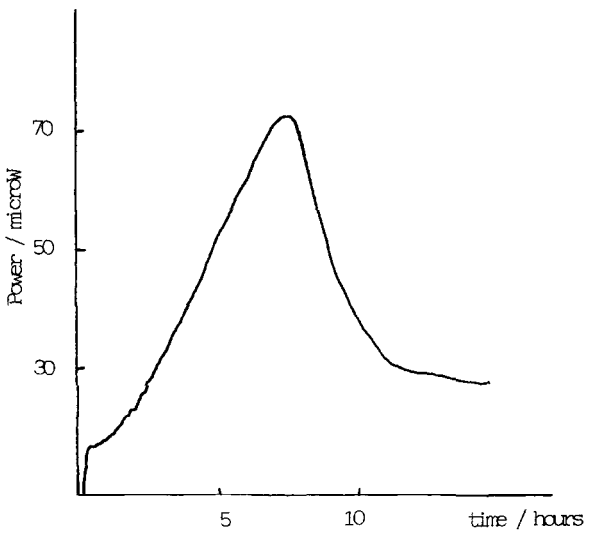

Fig. 1. Power-time curve of a growing *Pichia stipitis* culture on xylose medium, without ethanol.

Figure 2 shows the power-time curves obtained with cultures with initial ethanol concentration varying from 0 to 15 $g \cdot dm^{-3}$. Values of the specific growth rate constant calculated from the thermogenesis curve are shown in table 1.

TABLE 1

C_e initial ($g \cdot dm^{-3}$)	0	5	10	15
μ (h^{-1})	0.112	0.073	0.052	0.027

These data were correlated using the model proposed by Luong [11] to describe ethanol inhibition on the specific rates of growth and ethanol production,

$$\mu_i/\mu_o = 1 - (C_e/C_{em})^{\gamma} \qquad (4)$$

were C_e is the initial ethanol concentration; μ_i is the specific growth rate at $C_e > 0$; μ_o is the specific growth rate at $C_e = 0$; C_{em} is the highest ethanol concentration allowing growth; and γ is a dimensionless constant.

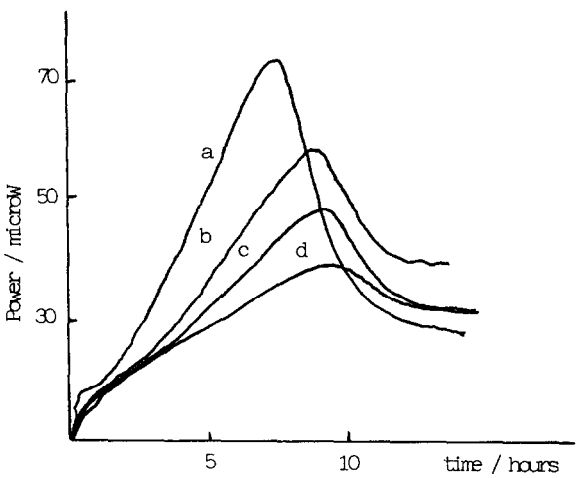

Fig. 2. Effect of the initial ethanol concentration in the culture medium on the thermogenesis curves for growth metabolism of *P. stipitis*. Ethanol concentration was: a)0, b)5, c)10, d)15 $g \cdot dm^{-3}$.

We obtained the following equation (correlation coeficient 0.996),

$$\mu_i/\mu_o = 1-(C_e/23.11)^{0.699}$$

The model indicates that the maximum ethanol concentration above which cells do not grow is 23.11 $g \cdot dm^{-3}$. The γ value is below 1, showing that ethanol inhibition was involved, with an increase of initial ethanol concentration a rapid initial drop in the growth, followed by a slow decrease to zero.

Power-time curves of cultures with initial ethanol concentration exceeding 20 $g \cdot dm^{-3}$ do not show an exponential increased heat production rate; rather they show a steady state which indicates a growth inhibition. The thermogenesis curve corresponds to a maintenance metabolism. Figure 3 shows that the heat flux corresponding to the steady state decreases at higher initial ethanol concentrations; the power-time curve corresponding to the maintenance metabolism of the yeast without ethanol was also recorded.

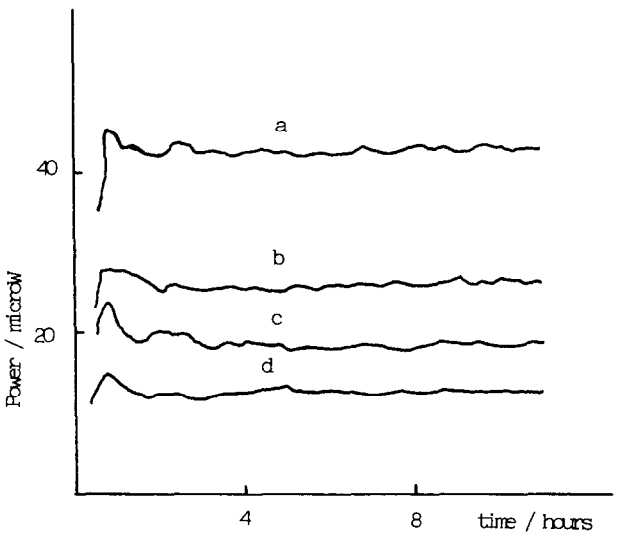

Fig. 3. Power-time curves of *P. stipitis* cultures at different initial ethanol concentration in the culture medium. a)20, b)30, c)40 $g \cdot dm^{-3}$ of ethanol; d)maintenance metabolism without ethanol.

Assuming that the power value at steady state is proportional to the specific ethanol production without cellular growth, it was intended to correlate these values according the Luong model; the equation obtained (correlation coeficient 0.997) was,

$$P'/P_o = 1 - (C_e/44.4)^{0.688}$$

where P'and P_o are the power values corresponding to the steady state of the thermogenesis curve at different initial ethanol level and without initial ethanol in the culture medium.

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