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A microcalorimetric study of the inhibitory effects of ethanol on the fermentation process of D-xylose by *Pichia stipitis*

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Abstract

Pichia stipitis is one of few yeasts capable of an efficient fermentation of pentoses to ethanol. This is a promising way to obtain ethanol from hemicellulose. In order to optimize the fermentation conditions for this process it is important to know the ethanol tolerance of the yeast. Here we report a microcalorimetric study of the inhibitory effects of ethanol on *P. stipitis*.

INTRODUCTION

Interest in the recovery of biomass as a renewable energy source has increased during recent years, due to both economic and ecological reasons, because it could consume large amounts of existing residue.

Lignocellulosic materials are perhaps the most used biomass source from the energetic point of view. They are composed of three main parts: cellulose, hemicellulose and lignin. The hemicellulose fraction mainly consists of pentoses and hexoses, D-xylose being the most important constituent [1]. Thus, the economic feasibility of a process of obtaining ethanol from hemicellulose hydrolysates depends on an efficient fermentation of pentoses.

Pachysolen tannophilus, Candida shehatae and *Pichia stipitis* are the most efficient of the few yeasts found capable of direct D-xylose fermentation to ethanol. Among them, *Pichia stipitis* seems to be the most promising for the production of ethanol from hemicellulose hydrolysates, because this yeast has the most efficient ethanol productivity without significant formation of xylitol, which is an undesirable product of this process. *P. stipitis* can also ferment to ethanol most of the sugars obtained by hydrolysis of agricultural and forest biomass: D-glucose, D-mannose,

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D-galactose, D-xylose and D-cellobiose [2,3]. The ethanol yield of fermentation of D-xylose by *P. stipitis* is similar under aerobic and microaerobic conditions, but it has been observed that oxygen stimulates the ethanol production rate [4–6].

From the economic point of view, it is important to obtain a high final ethanol concentration in the fermentation process; therefore it is necessary to know the ethanol tolerance of the yeast. The inhibitory effect of ethanol is complex; in different and separate ways, it affects the growth rate, the viability and the ethanol productivity. One of the main difficulties in the study of the ethanol tolerance of yeasts is that there is no universally accepted way of defining this tolerance, because it depends on the parameter to which it is referred [7–10].

Here we report the results of a microcalorimetric study of the inhibitory effects of ethanol on the D-xylose fermentation to ethanol by *P. stipitis*. As has been demonstrated, microcalorimetry allows continuous, fast and sensitive measurements of cell metabolism, and is a useful tool for monitoring fermentation processes and also for analysis of inhibitory and toxicity effects of different substances on microorganisms [11–19].

MATERIAL AND METHODS

The microcalorimeter used was the flow-through vessel of a 2277 Thermal Activity Minotor (ThermoMetric AB, Sweden) [20]. Measurements were performed at 35°C. The volumetric calibration constant of the instrument was determined by the procedure based on the hydrolysis of triacetin in imidazole buffer [21].

Pichia stipitis NRRL Y-7124 was supplied by Northern Regional Research Center of the United States Department of Agriculture, Peoria, Illinois.

The composition of the culture medium was: D-xylose ($5\,g\,l^{-1}$); yeast extract ($3\,g\,l^{-1}$); $(NH_4)_2SO_4$ ($3\,g\,l^{-1}$); $MgSO_4 \cdot 7H_2O$ ($1.1\,g\,l^{-1}$); KH_2PO_4 ($2\,g\,l^{-1}$) and $10\,ml\,l^{-1}$ of a salt solution. The pH was maintained at 4.5 by citrate buffer. D-xylose was 99% purity (Sigma Chemical Company); the other substances were from Merck. The salt solution composition was: CaO ($1.1\,g\,l^{-1}$); ZnO ($0.4\,g\,l^{-1}$); $FeCl_2 \cdot 6H_2O$ ($5.4\,g\,l^{-1}$); MgO ($0.36\,g\,l^{-1}$); $CuSO_4 \cdot 5H_2O$ ($0.25\,g\,l^{-1}$); $CoCl_2 \cdot 6H_2O$ ($0.24\,g\,l^{-1}$); H_3BO_3 ($0.06\,g\,l^{-1}$); and concentrated HCl ($13\,ml\,l^{-1}$).

The inoculum used for the experiments was taken from a dense inoculum suspension, stored in physiological serum at 4°C. This dense suspension was obtained by activation of a yeast strain maintained on agar slant of malt extract [22].

Batch cultures were conducted microaerobically in 250 ml cotton-wool-stoppered Erlenmeyer flasks. Flasks were agitated at $125\,rev\,min^{-1}$ on a rotary shaker, placed in a thermostatic bath at 35 ± 0.1°C.

Using a peristaltic pump (Masterflex model, Cole Parmer Instrument), part of the culture was continuously pumped through the microcalorimetric vessel and returned to the Erlenmeyer flask. The flow rate was 120 ml h^{-1} and the residence time of the culture in the flow line was 90 seconds; these conditions avoid yeast sedimentation inside the flow line. Before circulating the culture, the flow line was sterilized by flowing ethanol; then deionized and sterilized water was used to eliminate the ethanol.

Xylose, ethanol and cell mass determinations were made from samples taken from cultures performed simultaneously and under the same conditions as the culture pumped to the calorimeter. Xylose determinations were made by the dinitrosalicylic acid method with a Hitachi U-2000 spectrophotometer at 540 nm. Ethanol was measured by gas chromatography in a Perkin-Elmer Sigma 3B apparatus equipped with a 80/100 mesh Porapak Q column. Cell mass concentration was determined turbidimetrically at 640 nm with the above spectrophotometer.

RESULTS AND DISCUSSION

Figure 1 shows the power–time curve obtained for the fermentation process. After a lag phase, where the calorimetric signal is constant, there is an exponential increase in the thermal power evolved by the culture. This exponential increase is parallel to the cell mass increase, determined during the log phase. At the end of this phase, the calorimetric signal decreases sharply, when almost all the xylose has been consumed.

The linear relationship obtained by plotting the thermal power against cell mass concentration during the log phase (Fig. 2) indicates that for this fermentation process, the thermal power per unit of cell remains constant during the exponential growth phase; hence, the thermogenesis curve may be considered as the cell growth curve, and thus the specific growth rate μ may be determined from the slope of the straight line obtained by the

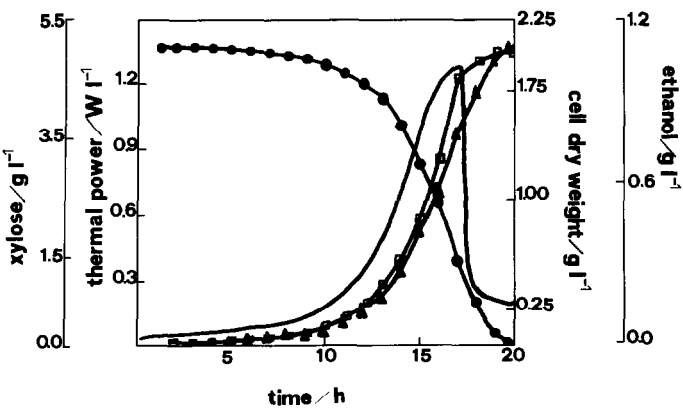

Fig. 1. Batch xylose fermentation by *P. stipitis*: ●, xylose; □, cell mass; ▲, ethanol; ——, thermal power.

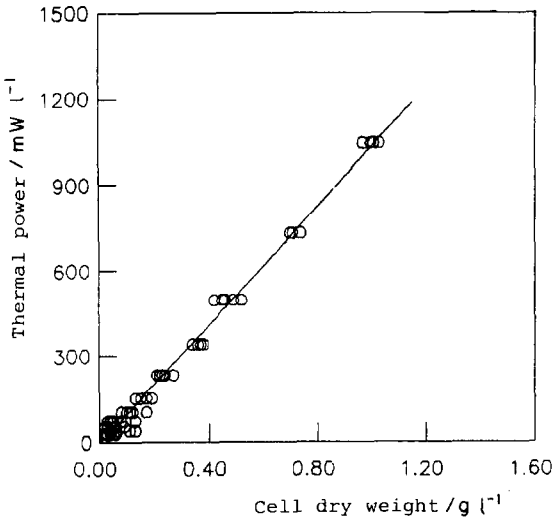

Fig. 2. Correlation between thermal power and cell mass during the exponential growth phase.

semi-logarithmic conversion of power–time curves during the log phase, because the equation that defines the cell mass increment with time may be expressed as

$$dm/dt = \mu m \quad (1)$$

where m is cell mass (dry weight of cells per unit of volume), t represents time and μ is the specific growth rate constant. By integration, this equation becomes

$$\ln(m/m_0) = \mu t \quad (2)$$

where m_0 is the cell mass when growth starts. If the power per unit of cell

TABLE 1

Specific growth rate μ determined from semi-logarithmic conversion of the thermogenesis curves

No. of experiment	μ (h^{-1})	r_{corr}
1	0.360	0.998
2	0.375	0.997
3	0.346	0.999
4	0.356	0.998
5	0.380	0.996
Mean ±SD	0.363 ± 0.013	

mass remains constant during exponential growth, then

$$\ln P = \ln P_0 + \mu t \tag{3}$$

where P and P_0 correspond to the heat production rate developed by the culture at any time during the log phase, and at the beginning of this phase, respectively.

Values for the specific growth rate constant calculated in this way for five calorimetric experiments, are shown in Table 1.

In order to analyze the toxicity effects of ethanol on this fermentation process, new cultures were performed using the same conditions as above. Eleven hours after inoculation, just when the exponential growth phase starts, ethanol was added to the culture at concentrations of 5, 10, 20, 30 and 40 $g\,l^{-1}$, in different experiments. It was observed (Fig. 3) that after ethanol addition the calorimetric signal suddenly decreased, and after some time, started to increase exponentially again. This last increase was not observed when the ethanol concentration was 40 $g\,l^{-1}$, indicating that growth was completely inhibited.

The specific growth rate for the exponential phase after ethanol addition was calculated from the slope of the plots of ln P versus time drawn for the experiments with different ethanol concentrations. These values are shown in Table 2.

The specific growth rate decreases exponentially with the increase of ethanol concentration in the culture medium. Plotting $\ln(\mu_i/\mu_0)$ versus

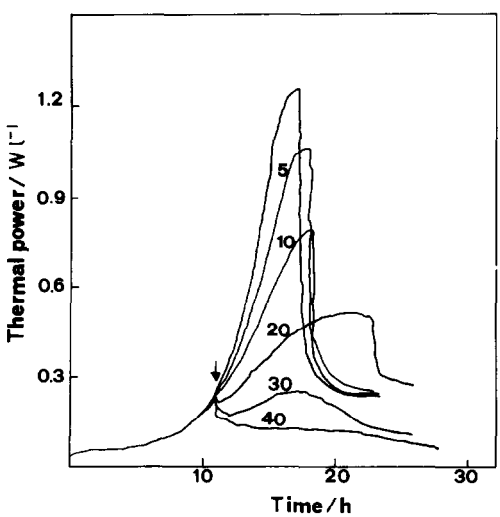

Fig. 3. Power–time curves for the cultures with ethanol concentrations of 5, 10, 20, 30 and 40 $g\,l^{-1}$. The arrow indicates when the ethanol was added.

TABLE 2

Specific growth rate constant μ_i (in h^{-1}) determined when the ethanol concentration in the culture medium was 5, 10, 20 and 30 $g\,l^{-1}$

No. of experiment	μ_5	μ_{10}	μ_{20}	μ_{30}
1	0.333	0.215	0.148	0.111
2	0.304	0.227	0.161	0.123
3	0.296	0.237	0.154	0.105
4	0.299	0.223	0.175	0.113
5	0.281	0.240	0.147	0.102
Mean	0.303	0.228	0.157	0.111
±SD	±0.019	±0.010	+0.012	±0.008

the ethanol concentration (Fig. 4), a linear relationship is obtained, from which it may be determined that the specific growth rate decreases to 50% when the ethanol concentration in the culture medium reaches the level of 21 $g\,l^{-1}$.

These results agree with those obtained in other studies on the inhibitory

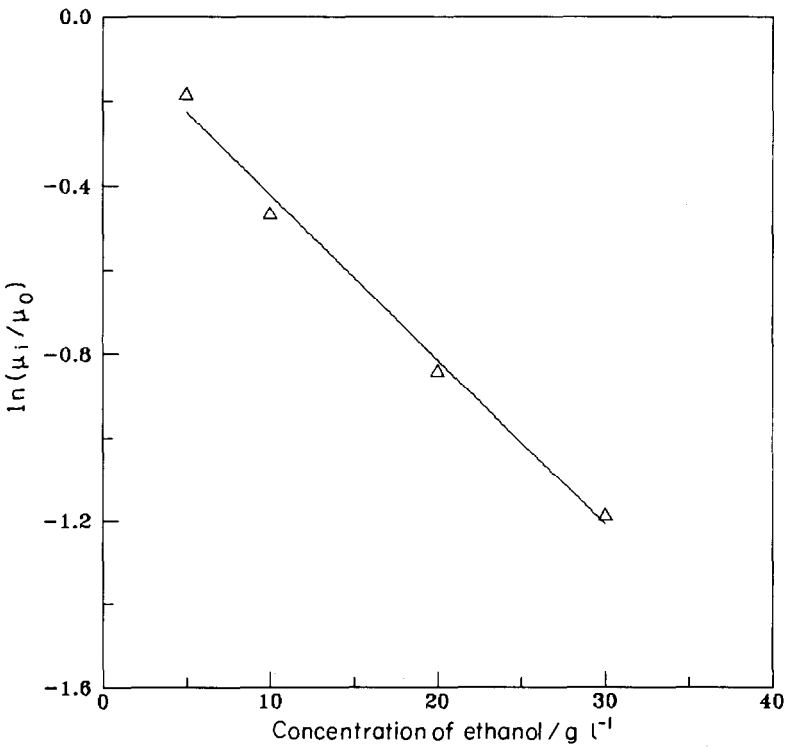

Fig. 4. Variation of the specific growth rate with ethanol concentration in the culture medium.

effects of ethanol on *P. stipitis*, based on measurements of the specific ethanol productivity rate [22,23], where an exponential decrease in the specific ethanol productivity rate with increasing ethanol concentration was also found: the ethanol productivity decreased sharply when the ethanol concentration was higher than $20\,g\,l^{-1}$, and the ethanol productivity fell close to zero with ethanol concentrations above $39\,g\,l^{-1}$. This confirms the validity of calorimetry as an alternative method for analysis and control of fermentation processes, with the advantages that calorimetry is a non-destructive, rapid and very sensitive technique.

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