Extremophiles (2007) 11:445–452
DOI 10.1007/s00792-006-0054-x

ORIGINAL PAPER

In situ monitoring by quantitative Raman spectroscopy of alcoholic fermentation by *Saccharomyces cerevisiae* under high pressure

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Received: 2 October 2006 / Accepted: 24 November 2006 / Published online: 22 December 2006

Abstract We monitored alcoholic fermentation in *Saccharomyces cerevisiae* as a function of high hydrostatic pressure. Ethanol production from 0.15 M glucose was measured by Raman spectroscopy in situ in a diamond-anvil cell. At 10 MPa, fermentation proceeds three times faster than at ambient pressure and the fermentation yield is enhanced by 5% after 24 h. Above 20 MPa, the reaction kinetics slows down with increasing pressure. The pressure above which no more ethanol is produced is calculated to be 87 ± 7 MPa. These results indicate that the activity of one or several enzymes of the glycolytic pathway is enhanced at low pressure up to 10 MPa. At higher pressures, they become progressively repressed, and they are completely inhibited above 87 MPa. Although fermentation was predicted to stop at ca. 50 MPa, due to the loss of activity of phosphofructokinase, the present study demonstrates that there is still an activity of ca. 30% of that measured at ambient pressure at 65 MPa. This study also validates the use of Raman spectroscopy for monitoring the metabolism of living microorganisms.

Keywords High hydrostatic pressure · *Saccharomyces cerevisiae* · Alcoholic fermentation · In situ monitoring · Raman spectroscopy

Communicated by K. Horikoshi.

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Introduction

The yeast *Saccharomyces cerevisiae* is the best known unicellular eukaryote, thanks to the characterization of its genome (Goffeau et al. 1996) and physiology, and to the multiple industrial applications. The response of yeast to high hydrostatic pressure (HHP) has been extensively studied in order to improve the understanding of the survival strategies of deep-sea piezophiles, and was recently reviewed (Abe 2004; Fernandes 2005). HHP greater than 15 MPa considerably reduces the growth rate and a pressure of 50 MPa completely prevents cell division (Abe and Horikoshi 1995). However, yeast cell viability (measured as colony-forming ability) decreases at higher pressures, the effect being more pronounced above 150 MPa. The ultrastructure is disrupted and yeast cells are killed at around 220 MPa (Kobori et al. 1995). HHP causes a general stress response. On the one hand, the analysis of pressure-regulated gene expression in yeast shows that pressure induces the transcription of genes in the categories of energy, cell rescue and protein destination, and the damage is similar to that caused by detergents, oils and freezing/thawing (Iwahashi et al. 2003; Fernandes et al. 2004). On the other hand, Iwahashi et al. (1991) demonstrated that a heat-shock treatment increased the resistance to HHP and produced a high increase in viability at 150 MPa and showed that pressure affects membrane structure at inhibiting-growth pressure. Ethanol production from glucose has been shown to occur at 40 MPa by ex situ measurements, where it was shown to be 75% of that produced at ambient pressure (Abe and Horikoshi 1997). In the presence of fermentable sugars, intracellular pH becomes transiently more

acidic under high pressure (Abe and Horikoshi 1995, 1997). The low cytoplasmic pH could slow down the glycolysis, and in extend stop ethanol fermentation around 50 MPa (Abe and Horikoshi 1998; Abe 2004), because pH changes readily affect the activity of a key enzyme of the glycolytic pathway, phosphofructokinase (Hofmann and Kopperschlager 1982). The information available on the effects of pressure on yeast is already vast; however, no direct measurement of its metabolic activity under pressure has been obtained.

Thus, we decided to monitor in situ alcoholic fermentation as one aspect of energetic metabolism by the yeast *S. cerevisiae* under HHP. In this paper, we report the combined use of in situ quantitative Raman spectroscopy (QRS) and diamond-anvil cell (DAC) technology to get direct measurements of the concentration of ethanol during fermentation of glucose by yeast at 30°C from ambient pressure up to 100 MPa. Ethanol can be easily quantified in the medium by QRS in the DAC.

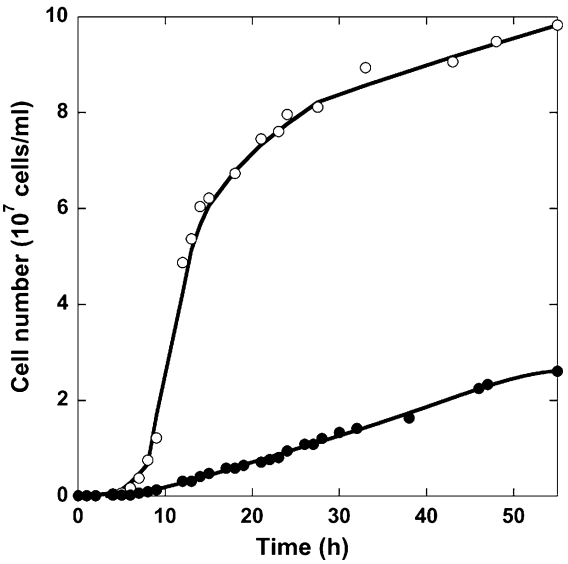

Fig. 1 Growth of *Saccharomyces cerevisiae* in YPD (*open circles*) and LFM (*closed circles*) media

Materials and methods

Organism, growth conditions and materials

An industrial strain of *S. cerevisiae* (Lesaffre™) was chosen due to its highly efficient ethanol production capabilities. Cells were grown at 30°C in YPD (yeast extract 10 g l^{-1}, bacto-peptone 20 g l^{-1} and glucose 20 g l^{-1}) with shaking at 180 rpm. Complex carbon sources present in YPD exhibit a strong fluorescence signal upon excitation at 514.5 nm, which hampers the Raman signal. Thus, a specific low-fluorescent medium (LFM) was developed for Raman spectroscopic measurements, omitting yeast extract and peptone. LFM contains only 2 g l^{-1} KH_2PO_4, 1 g l^{-1} $MgSO_4$, 2 g l^{-1} $(NH_4)_2SO_4$, 0.1 g l^{-1} NaCl and 2 mg l^{-1} $CaCl_2$. Glucose (0.15 M) was used as the sole carbon source. As shown in Fig. 1, yeast grows slower in LFM than in YPD with typical generation times of 12 and 1.5 h, respectively. It reaches lower cell concentrations of 2.6×10^7 and 9.8×10^7 cells ml^{-1}, respectively. Nevertheless, the morphology of the yeast cells is indistinguishable between those grown in YPD or LFM. All chemicals were from Sigma™. Yeast extract and peptone were from Difco®.

Preparation of cells for fermentation and budding monitoring

Yeast cells for fermentation monitoring were harvested at the beginning of the stationary phase (40 h in YPD at 30°C and 180 rpm) and washed twice in LFM medium. The cell concentration was then adjusted to 3×10^7 cells/ml in LFM, and the cell suspension was either loaded in the DAC or used in glass tubes for controls at ambient pressure. The remaining atmosphere in glass tubes was substituted by N_2 to reproduce the anaerobic conditions of the DAC. The glass tubes and the DAC were incubated at 30°C without shaking. Yeast cells to monitor the budding ability were harvested in early exponential phase after 6 h of culture in YPD at 30°C and 180 rpm. Cells were washed twice and adjusted at a concentration of 1×10^6 cells/ml in YPD medium, and then loaded in the DAC.

Determination of viability after pressure treatment

The viability of yeast cells was determined in the same conditions as those of the fermentation monitoring. The stationary-phase cells were treated for 24 h from ambient pressure to 70 MPa at 30°C, in LFM medium in high-pressure vessels (Top Industries S.A., Industrial zone ''Le plateau de la bière'', Dammarie-les-Lys, France). They were directly plated on YPD-agar after the release of pressure. The viability was measured as the difference between the log of colony-forming units (CFU) after pressure treatments and the log of CFU before pressure treatment.

High-pressure incubator

Incubations of yeast cells under pressure were performed in the low-pressure diamond-anvil cell (DAC),

which has been described in details elsewhere (Daniel et al. 2006). The incubation chamber consists of a cylinder of 500 μm diameter hole drilled in a 4 mm diameter and 300 μm thick nickel gasket, which implies a volume of ca. 60 nl. Pressure inside the incubator was adjusted by tuning the force on the diamond anvil. This could be achieved by gently inflating or deflating the steel ram above the piston of the DAC with helium gas. Pressure conditions inside the incubation chamber was estimated after the calibrated shift of the fluorescence emission of the 514 nm/535 nm FluoSpheres® pressure gauge loaded in the incubation chamber together with the sample (Picard et al. 2006). The error on pressure with this pressure gauge is as low as 6%. The fluorescence of FluoSpheres® was excited with the 514.5 nm line of the Ar^+ laser operating at 1 μW. The temperature inside the DAC is controlled at ±0.1°C by the use of a heating ring.

Raman spectrometer setup

Raman spectra were measured using a Labram HR 800 Raman spectrometer (Jobin Yvon®) in the backscattering geometry. It is a single monochromator spectrometer equipped with a CCD and a 1,800 grooves/mm grating. The 514.5 nm-line from an Ar^+ laser (Spectra Physics®) was used as the excitation source, with power of light on the sample evaluated at 40–50 mW. It was focused on the sample into the DAC with a Mitutoyo® 50× long-working distance microscope objective. Holographic hedge and notch filters reject the Rayleigh light and an interference filter rejects the plasma lines of the laser. Each Raman spectrum was acquired as ten accumulations of 20 and 60 s for experiments in glass tubes and in DAC, respectively.

Quantitative Raman analyses

We established a calibration of ethanol in the growth medium in order to be able to determine the concentration of ethanol in situ during the fermentation of glucose by yeast. As shown in Fig. 2a, the Raman spectrum of ethanol displays eight specific peaks in the frequency range investigated, among those, three were very intense peaks, corresponding to the (CH_2) and (CH_3) stretching modes of the molecule and located at 2,878, 2,929 and 2,972 cm^{-1}. Due to interference with the Raman spectrum of water in the 3,000 cm^{-1} region of the spectrum, the amount of ethanol in cultures was deduced from the less intense symmetric C–C stretching mode of ethanol at 883 cm^{-1} (Fig. 2a, b). Although the Raman signal intensity is directly linked to the solute concentration, a better precision is achieved by using an internal standard, which is measured in the same conditions as the peak of interest, but which does not participate in the reaction. Calculating the ratio of the intensity of the peak of interest to the intensity of the internal standard automatically corrects for all multiplicative distortions, e.g., laser intensity variations or absorbance of the laser light (Aarnoutse and Westerhuis 2005). In our experimental conditions, the sulfate S–O stretching mode at 980 cm^{-1} was selected.

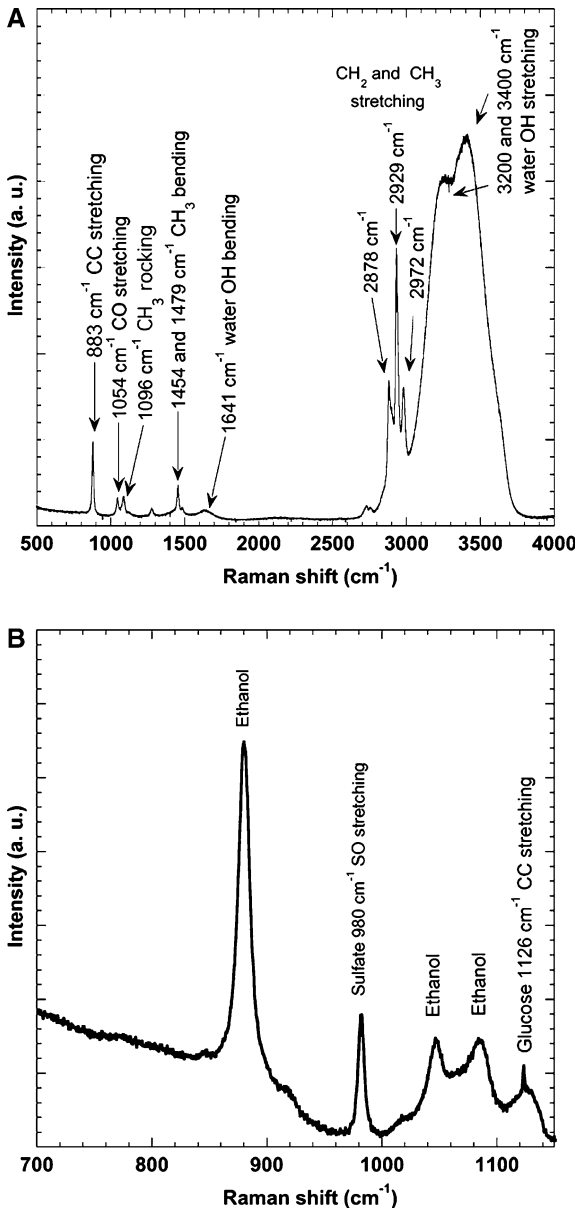

Fig. 2 **a** Raman spectrum of a 2 M aqueous solution of ethanol. **b** Details of the low frequency part of the Raman spectrum of LFM medium containing 0.3 M of ethanol. The evolution of the intensity of the CC stretching mode of ethanol at 883 cm^{-1} is used to evaluate the fermentation process

Indeed, sulfate is always added to growth media for synthesis of sulfur proteins. However, the amount of sulfate used is negligible compared to the initial concentration. Last but not the least, the S–O stretching mode can be acquired simultaneously as the C–C stretching mode of ethanol. The Raman spectra were analyzed using the commercially available software Peakfit® (version 4.11) and the two chosen peaks were fitted assuming a Pearson IV profile after subtraction of a linear baseline.

The calibration was determined on solutions of ethanol in LFM of known concentrations ranging from 0 to 0.4 M at ambient pressure, measured six times. A linear relationship could be obtained between the concentration of ethanol in solution and either the ratio of the ethanol and sulfate peak height (Fig. 3) or peak area (data not shown). The best accuracy of the calibration is obtained with the ratio of height of the Raman peaks. The concentration of ethanol in the medium is given by the following equation:

$$C = 0.218\ (\pm 0.002) \times I_{\text{ethanol}}/I_{\text{sulfate}},$$

with C being the concentration of ethanol in the medium (mol/l) and I_{ethanol} and I_{sulfate} the intensity of the Raman peak of ethanol at 883 cm^{-1} and sulfate at 980 cm^{-1}, respectively. This calibration is independent of pressure up to at least 200 MPa. It was used to estimate the concentration of ethanol in the growth medium of yeast at any pressures from ambient to 100 MPa. The stability of the experimental setup was estimated by repeated measurements of the same sample. This gives a standard deviation of 0.02. No deviation was associated with the fitting of the data. Therefore, the total deviation on the determination of the concentration of ethanol is ca. 1.7%.

Results and discussion

In situ monitoring of ethanol production by *Saccharomyces cerevisiae* at ambient pressure

At ambient pressure, two series of experiments were performed in glass tubes and in the DAC, in order to investigate the effect of the experimental volume on yeast activity. Indeed, the volume in the DAC is restricted to 60 nl, which might be small enough to inhibit yeast activity. Figure 4 displays selected Raman spectra recorded through time during a 24-h kinetics in glass tubes. The characteristic intense Raman band of ethanol, at 883 cm^{-1}, that is used in the present study to quantify the fermentation activity of yeast, is readily visible after 2 h, and progressively increases in intensity as a function of time. The appearance of ethanol in the growth medium is also qualitatively confirmed by the concomitant appearance of the less intense CO stretching and CH_3 rocking modes of ethanol at 1,054 and 1,096 cm^{-1}, respectively, and by the three ethanol C–H stretching modes in the shoulder of the Raman

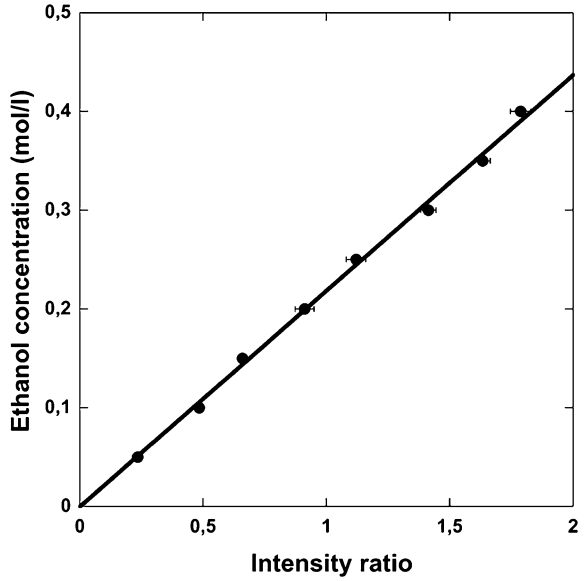

Fig. 3 Relationship between the concentration of ethanol in LFM medium and the intensity of the CC stretching mode of ethanol at 883 cm^{-1}, normalized to the intensity of the stretching mode of sulfate at 980 cm^{-1}

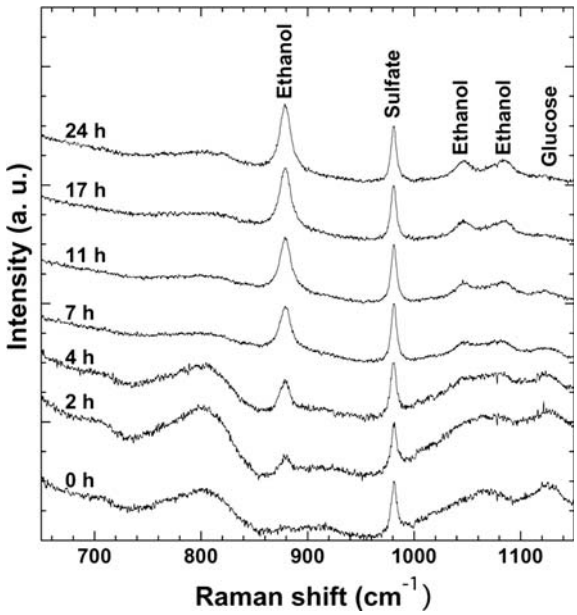

Fig. 4 Time-series of Raman spectra of a *S. cerevisiae* culture in LFM medium at 30°C, in a glass tube at ambient pressure

band of water around 2,950 cm^{-1} (not shown in Fig. 4). Simultaneously, the C–C stretching mode characteristic of glucose at 1,126 cm^{-1} slowly disappears (Fig. 4). This proves the alcoholic fermentation of glucose by yeast, since the experimental controls performed in the absence of yeast cells or with yeast cells and an inhibitor of glycolysis (10 mM of $CuSO_4$) (Shanmuganathan et al. 2004) do not show any ethanol Raman bands for 24 h at least. One also notices that the spectra look slightly different before and after ca. 6 h. Before 6 h, the spectra are noisy and display a broad band at ca. 800 cm^{-1}, both of which disappear later. Eye observations of the tubes indicate that yeast cells sediment slowly and that the medium becomes clear after 6 h. Consequently, we interpret the noise and the band at 800 cm^{-1} as the signal of yeast cells suspended in glass tubes. The kinetics of ethanol production by yeast at ambient pressure in the DAC is indistinguishable from that in tubes (data not shown). Moreover, microscopic observations in the DAC reveal a budding activity in YPD medium at ambient pressure and 30°C, without latency time (Oger et al. 2006). The estimated time for cellular cycle (97 ± 5 min) agrees well with the typical generation time of 1.5 h in YPD for this strain. This confirms that the metabolism of yeast is not affected by the limited space available in the DAC. The estimated final concentration of ethanol, 0.27 M after 24 h at ambient pressure is calculated as 90% of the theoretical maximum, 0.3 M that could be obtained from an initial concentration of 0.15 M of glucose. The yield attained in practical fermentations does not usually exceed 90–95% of theoretical. This is partly due to the requirement for some nutrient to be utilized in the synthesis of new biomass and other cell maintenance related reactions. Hence, the present experimental value is also consistent with our observations of limited budding in LFM during fermentation experiments. This subsequently confirms that the use of glucose was restricted to alcoholic fermentation within the present conditions and to maintenance. The data are characteristic of a first order kinetic reaction, as described in the following equation:

$$[\mathrm{Eth}] = 2 \times [\mathrm{Glc}] \times \left(1 - \mathrm{e}^{-kt}\right),$$

with [Eth] as the ethanol concentration expressed in mol l^{-1}, [Glc] as the glucose concentration used to produce ethanol, expressed in mol l^{-1} (the factor 2 comes from the general equation of fermentation, 1 mole of glucose giving 2 moles of ethanol), k as the reaction constant in h^{-1} and t the time in h. From this equation, we determined the parameters for ambient pressure kinetics, which are 0.27 M as the final ethanol concentration, and $k = 0.152\ h^{-1}$ as the reaction constant.

The present Raman spectroscopic results at ambient pressure should be compared with those recently obtained by infrared spectroscopy. The latter technique was tested to develop calibration models for the different parameters involved in alcoholic fermentation, in order to routinely optimize the biomass and the product synthesis (Blanco et al. 2004; Finn et al. 2006). The uncertainty of 1.70% in the determination of ethanol concentration in the present study is similar to that obtained by ex situ near-infrared (NIR) spectroscopy, that is 1.60% (Blanco et al. 2004) and 1.90% (Finn et al. 2006). We chose Raman instead of IR spectroscopy, since the cost of an IR-DAC with IIa nitrogen-free diamonds is significantly higher, and since the IR bands of ethanol are broader than the corresponding Raman peaks. Consequently, the characteristics of the Raman ethanol bands are obtained with a better accuracy, even if the Raman signal might be impaired by the fluorescence of compounds of the growth medium, like yeast extract or peptone. However, minimal growth media are currently developed for most microorganisms, and the development of a low-fluorescence medium for visible Raman spectroscopy is no more a significant constraint.

In situ monitoring of ethanol production by *Saccharomyces cerevisiae* at high pressure

Figure 5 displays kinetics of ethanol production by yeast in the DAC from ambient pressure at 100 MPa. This shows that yeast is able to produce ethanol up to 65 MPa at least, and that fermentation does not occur at 100 MPa. Up to 65 MPa, all data are characteristic of a first order kinetic reaction, as described above for ambient pressure. In all experiments, a plateau is reached. It seems that an increasing quantity of glucose is used for cell maintenance and thus less glucose is available for fermentation. The adjustment of the data to a first order kinetic law gives kinetic parameters, i.e., the final concentration of ethanol and the reaction constant k. These parameters are given in Table 1 as a function of pressure. Interestingly, as pressure increases up to 10 MPa, the speed of the reaction increases significantly and the ethanol production is slightly enhanced by ca. 3–4%. The maximal ethanol production, 0.288 M from 0.15 M of glucose, is already achieved at pressure as low as 5 MPa and corresponds to 96% of the theoretical maximum yield, i.e., to the maximum practical yield. However, the reaction speed increases progressively and reaches 10 MPa at a maximum value of 0.345 h^{-1}. This indicates that the reac-

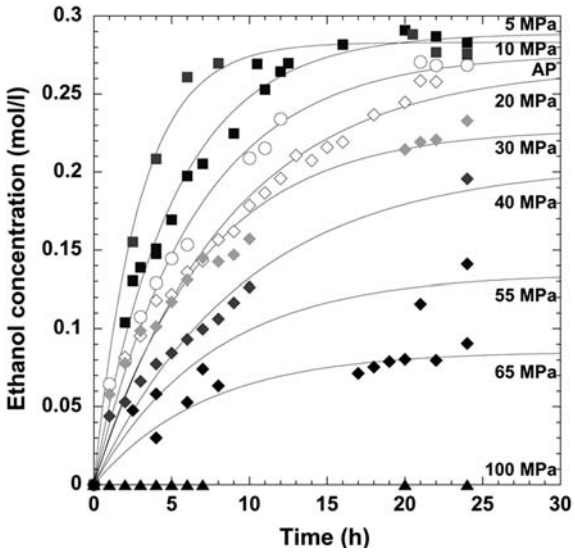

Fig. 5 Kinetics of ethanol production in the DAC as a function of pressure to 100 MPa

Table 1 Kinetic parameters of the alcohol fermentation by *Saccharomyces cerevisiae*, as a function of pressure

Pressure (MPa)	Final concentration of ethanol (mol/l)	k (h^{-1})	Yield (%)
0.1	0.276 ± 0.0047	0.152 ± 0.010	96
5	0.288 ± 0.0048	0.196 ± 0.009	100
10	0.284 ± 0.0048	0.345 ± 0.022	99
20	0.267 ± 0.0043	0.117 ± 0.009	93
30	0.228 ± 0.0038	0.143 ± 0.015	79
40	0.205 ± 0.0032	0.104 ± 0.016	71
55	0.130 ± 0.0022	0.132 ± 0.028	45
65	0.083 ± 0.0014	0.140 ± 0.021	29
100	0	0	0

tion is approximately three times faster at 10 MPa than at ambient pressure. Hence, 10 MPa and 30°C are probably close from the optimal conditions for ethanol fermentation with the present yeast strain. Indeed, at 20 MPa, the speed and the yield of fermentation are already lower than at ambient pressure, and they both further decrease at higher pressure. For instance, the final ethanol concentration at 40 MPa is approximately 74% of the ambient pressure production. This value is almost identical to the results obtained by Abe and Horikoshi at the same pressure, despite a different yeast strain (Abe and Horikoshi 1997). More generally, the yield of the reaction decreases linearly as a function of pressure between 20 and 65 MPa, with a rate of $2.0 \pm 0.1 \times 10^{-3}$ mol l^{-1} MPa^{-1}. The extrapolation of the linear relationship to zero yield indicates that fermentation stops at 87 ± 7 MPa. This is in good agreement with our measurements at 100 MPa, which do not reveal any ethanol production in the medium even after 24 h. This is certainly not due to massive cell death, since the number of viable cells decreases only of 1 log after 24 h in LFM at 70 MPa and 30°C, compared to ambient pressure.

The arrest of alcoholic fermentation at 87 Mpa, in the present study, occurs at a slightly higher pressure than previously predicted, that is 50 MPa (Abe and Horikoshi 1998; Abe 2004). Indeed, a key enzyme in glycolysis, phosphofructokinase is sensitive to pH (Hofmann and Kopperschlager 1982), and increasing pressure induces the acidification of both the cytoplasm and the vacuole of yeast in the presence of fermentable sugars. This starts at ca. 20 MPa and results in a decrease of the cytoplasmic pH of 0.3 at ca. 50 MPa (Abe and Horikoshi 1997). Subsequently, the pressure-induced acidification leads to the inactivation of phosphofructokinase, and subsequently to the arrest of alcoholic fermentation, which was predicted at the same pressure, i.e., 50 MPa. The difference between the presently observed and the predicted pressures of arrest of fermentation is probably due either to the use of different strains or to the various stress compensations in living cells under high hydrostatic pressure. The observation that metabolism occurs in a cell at pressures that are not permissive for cell division is not typical of yeast. In fact, it has already been shown that growth and metabolic activity are not strictly coupled. It is not surprising either, since energetic metabolism is only one among several pathways required for growth.

The yield and velocity of the glycolysis and/or fermentation are higher between ambient pressure and 10 MPa. Several phenomena could account for this increased activity under pressure. The uptake of glucose may be enhanced in yeast at 10 MPa. It has been shown that the sugar transport of a marine isolate is stimulated at high hydrostatic pressure, up to 60 MPa (DeLong and Yayanos 1987). In addition, the activity of one or several enzymes of the glycolysis and/or fermentation pathways might be enhanced. Since we do not observe a measurable lag phase, we may exclude a possible pressure-induced increase in protein synthesis. The pressure-increased activity is not limited to yeast. Indeed, the application of high pressure can enhance enzyme activity. The behavior of all systems, including biosystems, under high pressure is governed by the Le Chatelier's principle, which predicts that the application of pressure shifts an equilibrium toward the state that occupies a smaller volume, and accelerates processes that involve a transition state with a smaller volume than the ground state. This has been observed for the bovine pancreatic α-chymotrypsin, which activity in vitro is enhanced 6.5 times at 470 MPa compared to ambient pressure (Mozhaev et al. 1996), or for the

Lactococcus lactis peptidase PepC, which activity in a suspension of cells is 10.5 times higher at 700 MPa than at ambient pressure (Malone et al. 2003). Therefore, the fermentation pathway of yeast must contain at least one protein with a similar behavior. So far, such a pressure-enhanced activity of enzymes is more often observed for those of piezophile organisms than for those of mesophile organisms. For instance, some pressure-enhanced activity has been shown in the thermophilic methanogen archaeon *Methanococcus jannaschii*. Indeed, a higher methane formation rate is observed at 25 MPa in vivo in this microorganism (Miller et al. 1988). The activity of hydrogenase in cell extracts of *M. jannaschii* is three times higher at 25 MPa than at ambient pressure. A more recent study in this archaeon demonstrated for a newly isolated protease in vitro a pressure-enhanced activity, more than two times higher than at ambient pressure (Michels and Clark 1997). Pressure-increased metabolism has already been reported in subsurface systems. For example, sulfate reduction yields are up to 40 times higher in marine sediments at 45 MPa than at ambient pressure (Kallmeyer et al. 2003; Kallmeyer and Boetius 2004). The same observation was confirmed with isolated sulfate-reducing strains (Parkes et al. 1995; Bale et al. 1997). Finally, ethanol might be expelled from the cell more efficiently under pressure due to an increase of passive diffusion plasma. This would lead to a decreased intracellular ethanol concentration and to the reduction of the ethanol retroinhibition. This would shift the equilibrium toward ethanol production.

To conclude, the enhanced yield and rate of alcoholic fermentation evidenced in the present study for yeast at 10 MPa suggests that pressure-enhanced catabolic activity is not exclusive to piezophile and could be observed in other mesophiles. The present results might also be of interest for fermentation industries, since the rate of alcohol production and the amount of alcohol can be significantly improved by applying pressure on the order of 10 MPa, which are available at the industrial scale. Finally, this study proves the ability of Raman spectroscopy to provide in situ quantitative measurements in conditions of high pressure. Since this technique can detect a large range of organic molecules, it might allow monitoring various microbial metabolisms under high-pressure conditions.

Acknowledgments We thank Hervé Cardon and Jean-Claude Chervin for technical assistance in the diamond-anvil cell technology, and the two anonymous reviewers for their helpful comments. This work was supported by grants from the CNRS MRCT and from the CNRS interdisciplinary French program Geomex.

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