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Intracellular acidification as a mechanism for the inhibition by acid hydrolysis-derived inhibitors of xylose fermentation by yeasts

EM Lohmeier-Vogel[1], CR Sopher[2] and H Lee[2]

[1]*Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4;* [2]*Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

The main degradation products (furfural, hydroxymethylfurfural, acetate) derived from acid hydrolysis of hemicellulosic materials inhibit growth on and fermentation of xylose by *Pachysolen tannophilus* and *Pichia stipitis*, with the latter yeast being the more sensitive. The inhibitory effect was more severe when the inhibitors were present together in the medium. Agarose immobilization partially protected the yeasts from the deleterious effects of these compounds. Intracellular de-energization and acidification may be the mechanism by which these compounds exert their toxic effect on the yeast cells.

Keywords: acetate; alcohol; furfural; immobilization; inhibitors; NMR; pentose; xylose; yeast

Introduction

Lignocellulosic materials found in wood and agricultural residues are an abundant and renewable source of cellulose and hemicellulose which can potentially serve as substrates for fuel alcohol production. Bioconversion of these polymeric materials requires a two-step process: acid or enzymatic hydrolysis of the polymers into monosaccharides, followed by bioconversion of the monomers into ethanol. Cellulose is a homogeneous polymer of glucose, and once hydrolyzed, the glucose can be readily fermented. Hemicellulose is a heterogeneous polymer of pentoses (xylose, arabinose), hexoses (glucose, mannose, galactose) and acids (acetic and glucuronic acids) [4]. The predominant sugar in hemicellulose is the aldopentose xylose which may comprise 20–40% of the total carbohydrates in some agricultural residues [3,4].

For a lignocellulose-to-ethanol process to be economically viable, microorganisms selected for such a bioconversion must efficiently convert both the hexoses and pentoses into ethanol [3]. In this connection, the discovery of pentose-fermenting yeasts in the early 1980s by Canadian and US researchers [20,21] was a significant milestone. Numerous studies in the past 15 years have been carried out on various aspects of xylose bioconversion by pentose-fermenting yeasts [see Refs 10, 18 for reviews]. The three yeasts used extensively in these studies and recognized to be relatively good xylose fermenters are *Pachysolen tannophilus*, *Pichia stipitis* and *Candida shehatae*.

Yeasts that ferment xylose efficiently in defined media generally perform poorly in acid hydrolysates of lignocellulosic materials. One problem associated with fermentation of such substrates is the presence of hydrolysis-derived inhibitors which adversely affect microbial growth and fermentation. These include furfural and hydroxymethylfurfural (degradation products of hemicellulose), phenolic and other aromatic compounds (degradation products of lignin), acetic acid (liberated from some hemicelluloses) and heavy metals such as chromium, copper, iron and nickel (from hydrolysis equipment) [22,24]. Several inhibitors can be present in a single hydrolysate depending on the source of the raw materials and hydrolysis conditions.

For efficient industrial-scale fermentation of acid hydrolysates of lignocellulosic materials, it is important that yeasts be resistant to inhibitors generated by the acid pretreatment process. Several studies have compared the ability of pentose-fermenting yeasts to convert xylose-rich hydrolysates into ethanol. The results showed that *Pichia stipitis* performs the best on spent sulfite liquor (SSL) [5] and corn-cob hydrolysate [8], whereas *Pachysolen tannophilus* performs best on hardwood hemicellulose hydrolysate [17]. In the latter study, mild acid pretreatment under high pressure generated three chemicals which were toxic to the fermenting yeast: acetic acid, furfural and hydroxymethylfurfural at concentrations of 10.9, 0.3 and 0.9 g L^{-1}, respectively. These values are considerably higher than the 3.0 g L^{-1} acetic acid and 0.2 g L^{-1} furfural levels found in SSL [5] and might explain the poor performance of *P. stipitis* on hardwood hydrolysates.

The present study was undertaken to assess how some inhibitors (furfural, hydroxymethylfurfural and acetate), either alone or in combination, affect the capacity of *P. stipitis* and *P. tannophilus* to ferment xylose. Although both strains require oxygen for growth [8,21], they can also ferment xylose anaerobically [12,23]. In our study, fermentation kinetics were monitored by HPLC in batch cultures with cell suspensions and by carbon-13 nuclear magnetic resonance (NMR) spectroscopy for perfusions using agarose-immobilized cells. We also performed phosphorus-31 NMR studies to monitor levels of intracellular phosphorylated intermediates (ATP, inorganic phosphate, etc) in order

Correspondence: Dr EM Lohmeier-Vogel, Department of Biological Sciences, University of Calgary, 2500 University Dr, NW, Calgary, AB, Canada T2N 1N4
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to obtain information on the effects of these inhibitors on cell energy status. The results suggest that intracellular de-energization may be one of the mechanisms by which some of these inhibitors exert their toxic effect.

Materials and methods

Culture conditions

Lyophilized cultures of *Pachysolen tannophilus* NRRL Y2460 and *Pichia stipitis* CBS 6054 were suspended in sterilized water and a loopful of the suspension streaked onto YEPD plates containing (per L): 10 g yeast extract, 20 g peptone, 20 g glucose and 15 g agar and incubated for 42 h at 30°C [10]. Cultures were subsequently maintained on YEPD plates at 4°C [10]. To prepare inocula, a loopful of cells was transferred to each of several 250-ml Erlenmeyer flasks, each containing 50 ml of YNB-xylose medium (7.1 g L^{-1} Yeast Nitrogen Base (Difco Laboratories, Detroit, MI, USA) without amino acids plus 50 g L^{-1} xylose). Flasks were placed on a New Brunswick shaker (Fisher Scientific, Toronto, ON, Canada) at 30°C and 200 rpm for 48 h. The following media were prepared: medium 1 which contained 20 g L^{-1} xylose plus 3.5 g L^{-1} YNB without amino acids in 50 mM MES (Sigma Chemicals, St Louis, MO, USA), pH 6.0; medium 2 which was medium 1 supplemented with 0.3 g L^{-1} furfural; medium 3 which was medium 1 supplemented with 0.9 g L^{-1} hydroxymethylfurfural; medium 4 which was medium 1 supplemented with 10.9 g L^{-1} sodium acetate; and medium 5 which was medium 1 supplemented with 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.9 g L^{-1} sodium acetate. Inocula (2 ml) were placed in 100 ml of each of the media contained in 250-ml Erlenmeyer flasks. Media 1–5 were also prepared with inhibitors whose final concentrations were double those listed above, and are referred to as media 1D to 5D. All flasks were incubated as described above. At various time intervals, 1-ml samples were removed for optical density measurements (A_{620}) and analyses for xylose, xylitol, ethanol and acetate concentrations by HPLC (Waters Corp, Mississauga, ON, Canada) equipped with a refractive index detector (Waters) and an HPX-87H column (Bio-Rad Laboratories, Mississauga, ON, Canada). Quantification was based on ratios between the peak height of the test compound and 10 g L^{-1} glycerol added as an internal standard [11].

Preparation of immobilized cells for NMR work

Whole cell immobilization using low-temperature-gelling agarose (Sigma Type VII) was performed as previously described [14]. For NMR experiments, 10 g (drained weight) agarose beads containing 1 g (wet weight) immobilized yeast were perfused in a 15-mm NMR tube with 100 ml medium 1 which contained 15 g L^{-1} xylose rather than the 20 g L^{-1} normally utilized in the batch culture studies. Typically, we perfused cells for one day in the absence of inhibitors to clearly establish rates of sugar utilization and product formation. Fresh medium containing 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.9 g L^{-1} sodium acetate was then perfused through the sample and data accumulated again. The rate of recycling of medium was typically 5 ml min^{-1}.

NMR spectroscopy

NMR spectra were obtained at 25°C on an AM-400 wide-bore spectrometer (Bruker Spectrospin, Milton, ON, Canada) using a ^{13}C-^{31}P switchable dual-tuned probe. Fully relaxed spectra were obtained and processed as previously described [14,15].

Results

Batch suspension culture studies

In the absence of inhibitors, 20 g L^{-1} xylose was completely consumed by *P. tannophilus* in 60 h and the final cell density of the cultures reached approximately 12.0 A_{620} units (equivalent to 2.1 mg dry cell weight) per ml of culture (Figure 1a). On average, 0.6 g L^{-1} ethanol and 3.0 g L^{-1} xylitol were produced. Assuming that 3 moles of xylose yield a maximum of 5 moles each of ethanol and CO_2, 1 g xylose should theoretically give rise to 0.51 g ethanol [21]. Thus, on this medium under batch growth conditions, *P. tannophilus* converted, on a weight by weight basis, only 3% of the available xylose to ethanol, 15% to xylitol and the rest to biomass or CO_2. These values changed very little in the presence of 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural or 10.9 g L^{-1} acetate when each of these inhibitors was present individually in the cultures (results not shown). However, when all three inhibitors were added to the medium, the rate of xylose consumption decreased such that at 60 h, about 50% of the initial xylose remained in the medium (Figure 1b). When the inhibitors were present in the cultures individually, but at double their original concentrations, fermentation kinetics with *P. tannophilus* were essentially the same as in Figure 1b (results not shown). Doubling the concentration of acetate alone or having all three inhibitors present at the 2-fold higher level resulted in a slight decrease in biomass production and a slight increase in xylitol levels (Figure 1c). Ethanol levels and the rate of xylose consumption were similar to those observed in Figure 1b.

Pichia stipitis proved to be more sensitive to inhibitors than *P. tannophilus*. In control cultures without inhibitors, *P. stipitis* consumed 20 g xylose in approximately 35 h (Figure 2a), grew to an average A_{620} of 14 (equivalent to 2.7 mg dry cell weight) per ml of culture and produced little detectable xylitol but 10 times the amount of ethanol (6 g L^{-1}) compared to the *P. tannophilus* cultures. Inhibitors added individually at the lower concentration did not affect fermentation parameters significantly. However, when all three inhibitors were present at the same time, the effect was dramatic; cell growth was inhibited, ethanol production decreased drastically and xylose consumption was extremely slow (Figure 2b). When furfural, hydroxymethylfurfural or acetate were added individually to cultures at double the original concentration, cell growth and ethanol production were severely inhibited as in Figure 2b (results not shown). The combination of all three inhibitors at the higher level led to a complete cessation of growth and fermentative activity (Figure 2c).

^{13}C-NMR perfusion studies

Natural abundance carbon-13 NMR can be used to monitor the progress of microbial fermentations [13]. By following

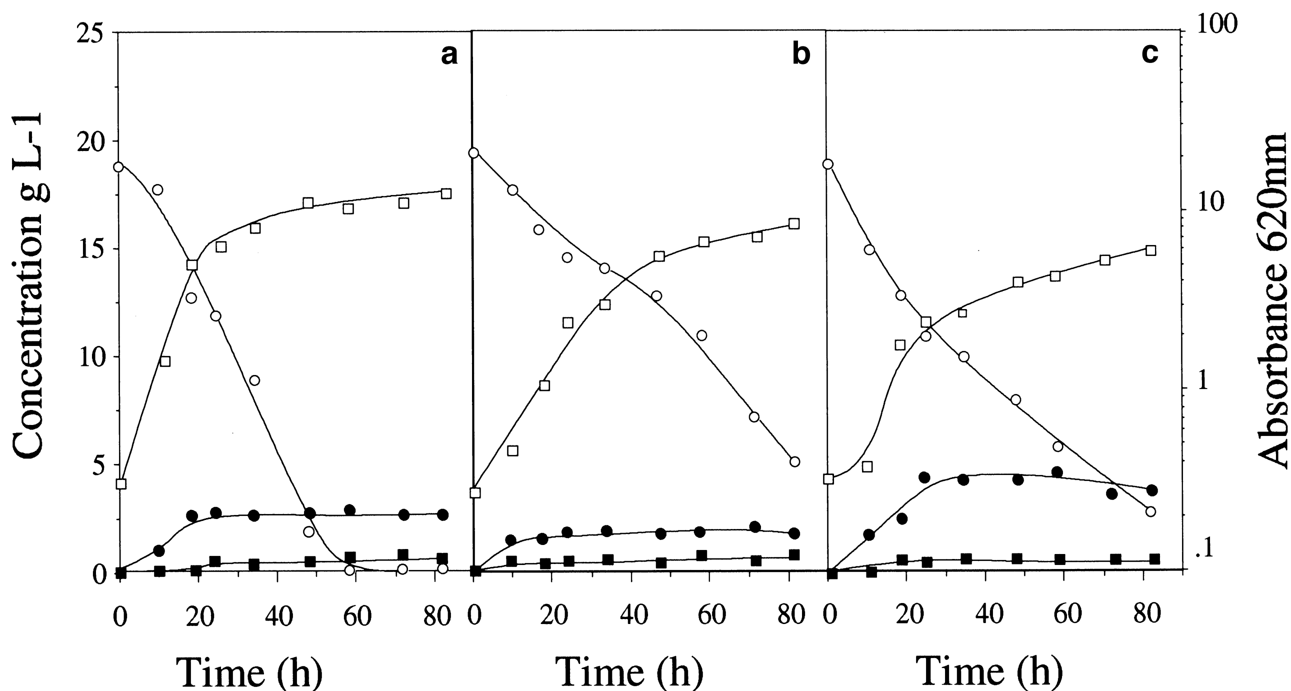

Figure 1 Batch cultures of *Pachysolen tannophilus* NRRL Y2460 grown in: (a) medium 1; (b) medium 1 plus 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.9 g L^{-1} sodium acetate; (c) as in (b), but with double the inhibitor concentrations. (○) = xylose; (□) = A_{620}; (●) = xylitol; (■) = ethanol. One A_{620} unit = 0.17 mg dry weight of cells.

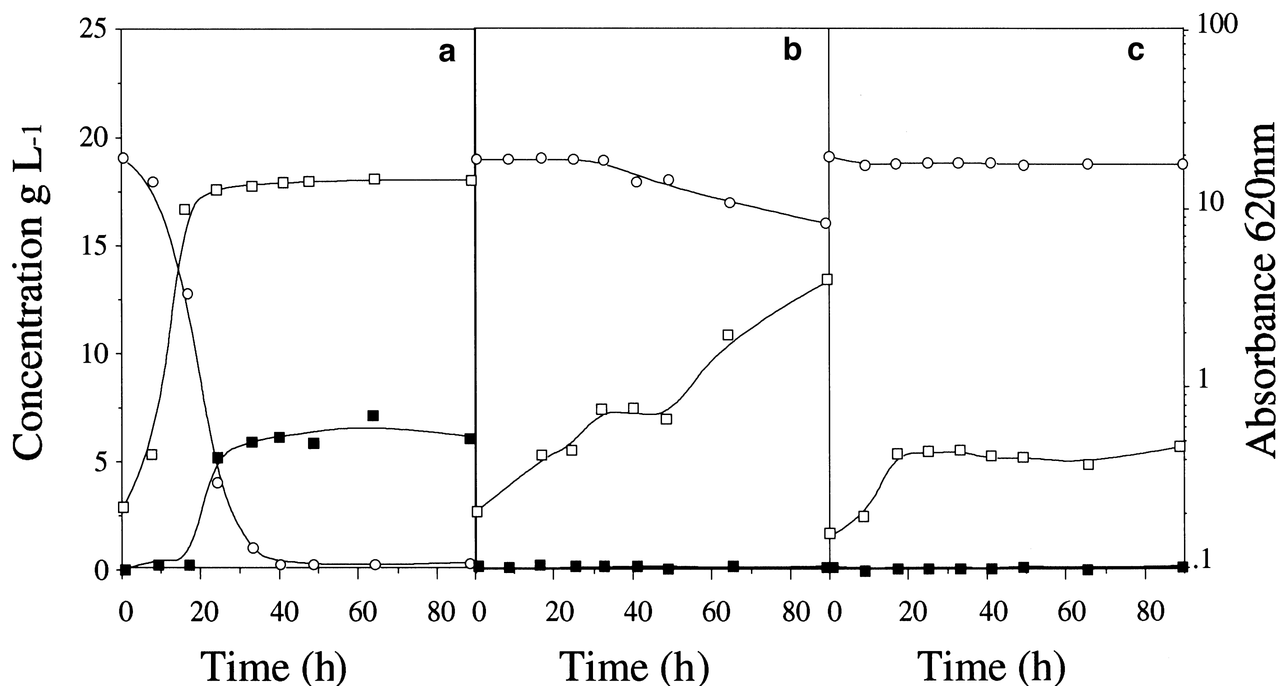

Figure 2 Batch cultures of *Pichia stipitis* CBS 6054 grown in: (a) medium 1; (b) medium 1 plus 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.9 g L^{-1} sodium acetate; (c) as in (b), but with double the inhibitor concentrations. (○) = xylose; (□) = A_{620}; (■) = ethanol. One A_{620} unit = 0.19 mg dry weight of cells.

the decrease in the areas of the C-1α and β anomeric carbon peaks of xylose, and the increase in xylitol C-3 and ethanol C-2 peaks in consecutive fully-relaxed ^{13}C natural abundance NMR spectra, the xylose fermentation kinetics of agarose-immobilized *P. tannophilus* cells were obtained. The results, plotted in Figure 3, show that *P. tannophilus* was capable of assimilating slightly over one third (0.56 g) of the 1.5 g xylose initially present in the medium, to yield 0.24 g xylitol and varying amounts of ethanol. In a total of 10 perfusion experiments with immobilized *P. tannophilus*,

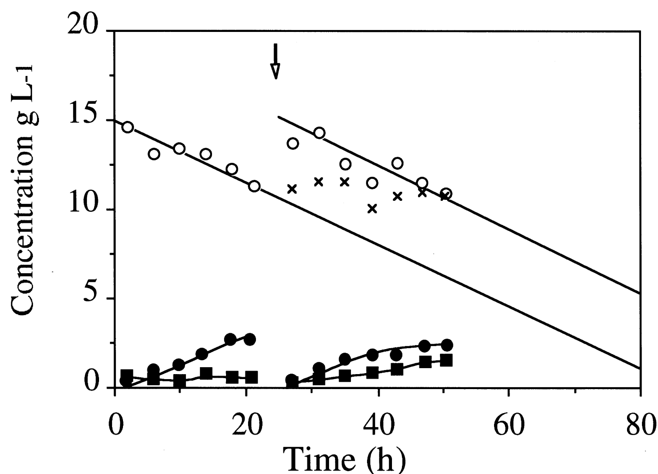

Figure 3 Xylose utilization and product formation by immobilized *P. tannophilus* as determined by ^{13}C-NMR. Cells were perfused with 100 ml of recycled, oxygenated medium containing 1.5 g of xylose (see Methods). The data in the first 24 h were obtained in the absence of inhibitors. At the point indicated by the arrow, the medium was changed to contain 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.9 g L^{-1} sodium acetate. (○) = xylose; (●) = xylitol; (■) = ethanol; (x) = acetate.

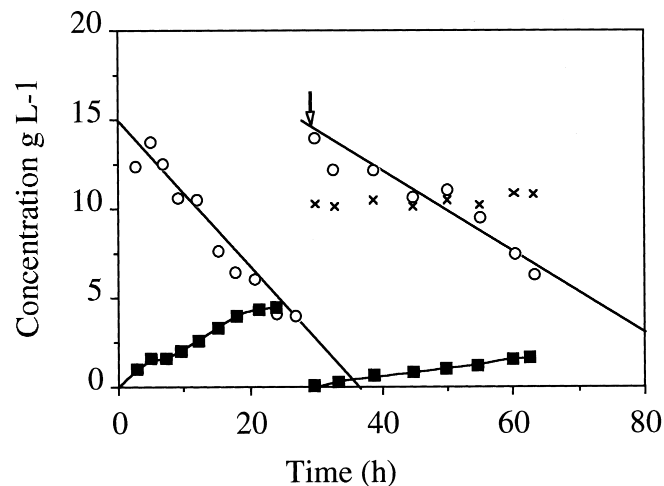

Figure 4 Xylose utilization and product formation in immobilized *P. stipitis* as determined by ^{13}C-NMR. Cells were perfused with 100 ml of recycled, oxygenated medium containing 1.5 g of xylose (see Methods). The data in the first 24 h were obtained in the absence of inhibitors. At the point indicated by the arrow, the medium was changed to contain 0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.9 g L^{-1} sodium acetate. (○) = xylose; (■) = ethanol; (x) = acetate.

significant ethanol peaks were only observed twice, and their appearance could not be correlated either to the rate of medium recycling (which varied from 0.5 to 10 ml min^{-1}) or the age of the culture used to prepare the immobilized cells (harvested at mid-log or late-log phases of growth).

The rates of xylose consumption and xylitol production were not greatly affected when fresh medium containing all three inhibitors was perfused through the beads. In the two perfusions where ethanol was observed, the presence of inhibitors slightly stimulated the production relative to rates observed in the absence of inhibitors. Acetate peaks, which could also be observed by ^{13}C-NMR, did not change significantly in size during the experiments, suggesting that acetate was not consumed by the cells.

In contrast to *P. tannophilus*, significant ethanol production by immobilized *P. stipitis* was consistently observed (Figure 4). As with batch cultures, xylitol production was not observed. From the 1.2 g xylose consumed in 24 h in the absence of inhibitors, 0.42 g ethanol was produced by immobilized *P. stipitis*. The presence of all three inhibitors decreased the xylose fermentation rate by 50%. Acetate concentrations did not significantly change during the course of these experiments.

^{31}P-NMR perfusion studies

Figure 5 shows the phosphorus-31 NMR spectra obtained with immobilized *P. tannophilus*. Because these cells are constrained within the bead matrix, the resolution of these spectra was not as good as was previously obtained with cell suspensions [14,15], but useful information about the cellular energy state was still obtained.

The chemical shift (δ) of the intracellular Pi resonance ($Pi_{(int)}$) is pH-sensitive and can be used to determine intracellular pH values in living cells non-invasively [7]. The $Pi_{(int)}$ resonance at 1.94 ppm in Figure 5(a) (dotted line, left-hand side) gives an estimate of 6.5 for the intracellular pH of immobilized *P. tannophilus* in the absence of inhibitors (see [14] for details). This acidic $pH_{(int)}$ indicates that the cells were unable to maintain a large transmembrane pH gradient and were de-energized in the immobilized state (the $pH_{(ext)}$ was 6.0). The absence of NMR-visible peaks for polyphosphate (PP), nucleoside triphosphates (NTP), nicotinamide adenine-dinucleotide(phosphate) (NAD(P)) or uridine diphosphoglucose (UDPG) gives further evidence that the cells were not highly energized, although they were able to metabolize xylose (Figure 3).

In the presence of inhibitors, the chemical shift of the $Pi_{(int)}$ resonance moved slightly upfield to 1.87 ppm (dotted line, right-hand side), which translates into a $pH_{(int)}$ of 6.45 units. This small intracellular acidification did not affect the rate of xylose metabolism significantly, as shown in Figure 3.

Figure 6 illustrates the phosphorus-31 NMR spectra obtained with immobilized *P. stipitis*. In the absence of inhibitors (Figure 6a), *P. stipitis* had an intracellular pH of 6.8 (δ $Pi_{(int)}$ = 2.38 ppm; dotted line, left-hand side). Some intracellular polyphosphate and NAD(P) was observed, along with low levels of NTP and UDPG. This information suggests that *P. stipitis* was more energized when encapsulated in agarose beads than *P. tannophilus*. Indeed, comparing the data in Figures 3 and 4 shows that *P. stipitis* metabolized xylose at a faster rate than *P. tannophilus*.

When inhibitors were added to the perfusion medium (Figure 6b), the intracellular pH of *P. stipitis* fell to 6.12 (δ $Pi_{(int)}$ = 1.69 ppm; dotted line, right-hand side), confirming that this strain is more sensitive to inhibitors. This large intracellular acidification in the presence of inhibitors may explain the significant decrease in xylose metabolism seen in Figure 4.

Phosphorus-31 NMR spectra can also be used to monitor the growth of immobilized cells non-invasively [14,15]. The large phosphodiester (PDE) resonances in the ^{31}P-NMR spectra in Figures 5 and 6 correspond to cell wall

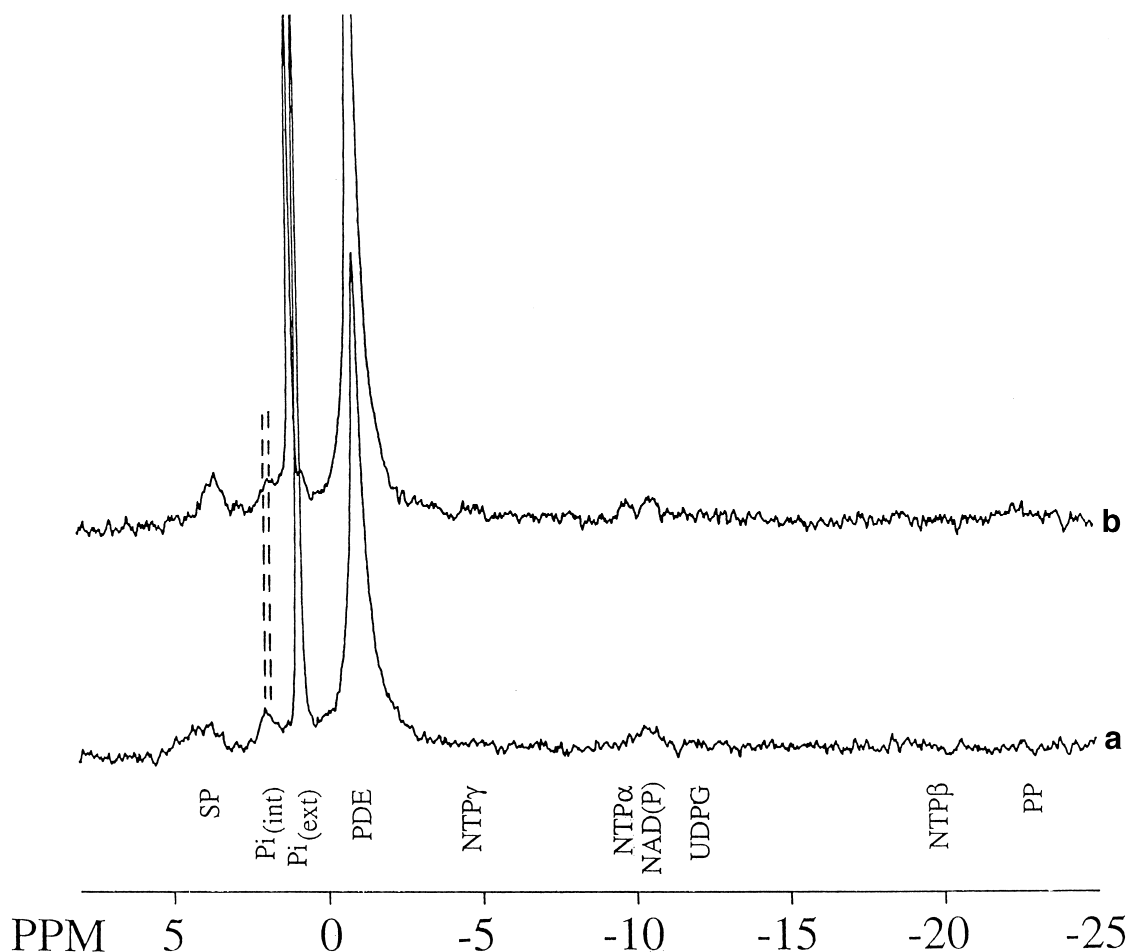

Figure 5 Fully relaxed, 1 h ^{31}P-NMR spectra of immobilized *P. tannophilis* metabolizing xylose. SP, sugarphosphomonoesters; $Pi_{(int)}$, intracellular Pi; $Pi_{(ext)}$, extracellular Pi; PDE, cell wall phosphodiesters; NTP(γ,α,β), nucleoside triphosphates (note: nucleoside diphosphate α and β resonances fall under the NTP γ and α peaks, respectively, but are not labelled as such); NAD(P), nicotinamide adeninedinucleotide(phosphate); UDPG, uridine diphosphoglucose; PP, polyphosphate. Peaks were assigned as previously discussed [7,14]. (a) Spectrum obtained in the absence of inhibitors; (b) spectrum obtained in the presence of inhibitors (0.3 g L^{-1} furfural, 0.9 g L^{-1} hydroxymethylfurfural and 10.0 g L^{-1} sodium acetate).

phosphomannans. By comparing the integrated areas of the PDE peaks in sequential ^{31}P-NMR spectra, cell growth within the beads could be monitored. Our results show that neither strain grew significantly within the beads during these experiments. *P. tannophilus* cell mass increased only by a factor of 27% and *P. stipitis* by a factor of 42% between 0 and 36 h.

Discussion

The effects of inhibitors were tested at concentrations found when hardwood hemicellulose is hydrolyzed with dilute sulphuric acid under high pressure [16]. Our results with batch cultures suggest that *P. tannophilus* is fairly resistant to furfural and hydroxymethylfurfural at these concentrations, but displays some sensitivity to acetate. Ethanol production was not significantly affected while xylitol production fluctuated inconsistently in the presence of inhibitors. *P. stipitis*, on the other hand, seemed to be equally sensitive to all three inhibitors at the higher levels (when present individually) and also to their cumulative effects when each was present at the lower concentration. Ethanol was not produced and cell growth was severely diminished in the presence of all three inhibitors. The synergistic actions of the inhibitors on both yeasts are not surprising. In a previous study, various short chain acids (acetate, propionate, butyrate) were shown to exacerbate the inhibitory effect of their corresponding short chain alcohols on yeast growth and fermentation, and this inhibitory effect became more severe at higher temperatures [1].

^{13}C NMR studies with immobilized cells showed that the rate of xylose metabolism with agarose-encapsulated *P. tannophilus* was not greatly affected by the presence of inhibitors. This was in agreement with batch culture studies. The production of ethanol with immobilized *P. tannophilus* was inconsistent, however, and was observed in only two of 10 perfusions. It is possible for this strain to produce and to consume ethanol concurrently [16], thus ethanol in the recycled medium might be consumed to varying degrees in separate experiments. We have had a similar experience with another pentose-fermenting yeast strain, *Candida tropicalis* [14].

The rate of xylose metabolism with agarose-immobilized *P. stipitis* was more rapid than with *P. tannophilus*, and

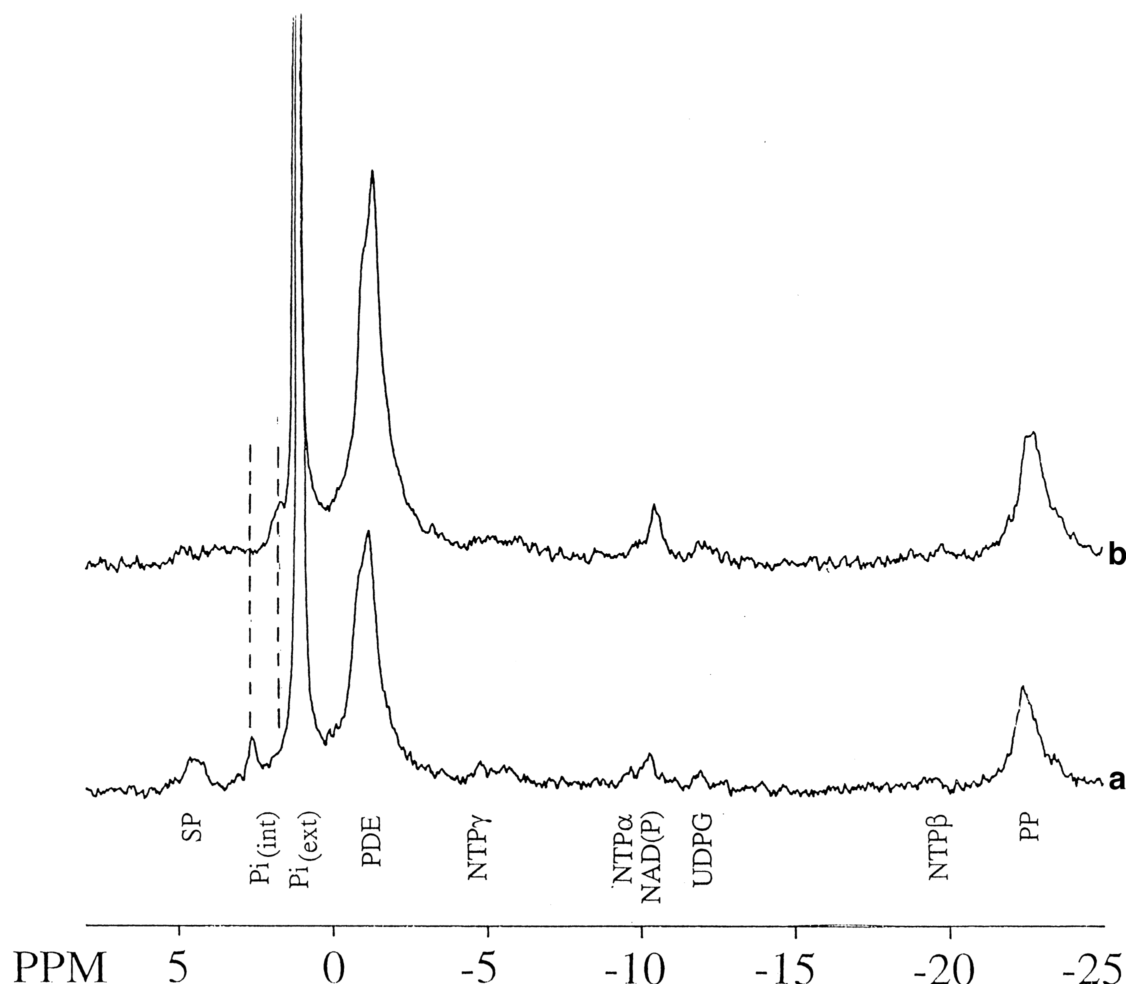

Figure 6 Fully relaxed, 1 h ^{31}P-NMR spectra of immobilized *P. stipitis* metabolizing xylose. Details and abbreviations as in Figure 5.

enough ethanol was produced to be detectable in the natural abundance ^{13}C-NMR spectra. The addition of inhibitors to immobilized *P. stipitis* resulted in a 50% reduction in the rate of xylose fermentation. This was an improvement over the 90% inhibition observed with batch cultures. It would appear that the agarose matrix protects *P. stipitis* from the effects of the inhibitors.

In *P. stipitis* and *P. tannophilus* the first enzyme in xylose assimilation, xylose reductase (XR), can function with NADH as the coenzyme [12,23] and will thus form a closed redox loop with the following enzyme, xylitol dehydrogenase. NADH-XRs theoretically enable these yeasts to ferment xylose anaerobically [6]. We therefore expected both organisms to be fairly energized within the anoxic environment of the beads. Our ^{31}P-NMR spectra, however, showed *P. tannophilus* to have low levels of nucleoside triphosphates and an intracellular pH of only 6.5. *P. stipitis* exhibited slightly higher levels of nucleoside triphosphates and had a more alkaline $pH_{(int)}$ value of 6.8. The discrepancy between the two pentose-fermenting yeast strains might be explained by the fact that XR has dual coenzyme specificity in *P. stipitis* (NADH or NADPH; [23]) whereas *P. tannophilus* has two XRs. The major XR is specific for NADPH while the minor XR can use either NADH or NADPH [19]. Only the NADH-XR would be expected to function well when the cells are in an anoxic immobilized environment.

C. tropicalis, a yeast which has only NADPH-XR (and would thus be expected to assimilate xylose poorly when immobilized), displays a $pH_{(int)}$ of 6.3 when entrapped in agarose beads [14]. From a comparison of the three strains, it could be suggested that *P. tannophilus* may have a 60/40 split between its NADPH-XR and NADH-XR activities under the test conditions since its $pH_{(int)}$ lies closer to *C. tropicalis* than *P. stipitis*. It is noted that none of the pentose-fermenting strains grew significantly under the anoxic environment found within the agarose beads, as all three strains require oxygen for growth [8,21].

The presence of inhibitors had little effect on the $pH_{(int)}$ of *P. tannopilus*. This is not surprising in light of the fact that fermentation kinetics, both in batch cultures and with immobilized cells were not greatly affected when inhibitors were added to the medium. *P. stipitis*, on the other hand, showed a marked decrease of 0.68 pH units in the presence of inhibitors. Intracellular acidification could explain the slower rate of xylose metabolism in the presence of inhibitors, which was observed with this yeast strain.

During acid hydrolysis, pentoses and hexoses in hemicellulose can be broken down to form furfurals and hydroxymethylfurfurals, respectively [17,22]. Their precise mode

of action on yeasts remains poorly understood. The present study shows that cellular de-energization may be one of the mechanisms involved in their inhibitory effect. Whether this de-energization is a direct effect of these compounds on cellular membranes or an indirect effect mediated by other means remains to be investigated.

Acetate is a by-product of some alcoholic yeast fermentations [1]. In lignocellulosic hydrolysates, it is mainly derived from the release of acetyl groups from acetylated xylan. The mode of action of acetic acid on yeast cells may be via reduction of intracellular pH below the physiological range, resulting in decrease in growth and metabolism, as seen with other weak acids [9]. As with furfural and hydroxymethylfurfural, the mechanism by which acetate affects intracellular pH homeostasis is not known. One possibility may be through its actions on the proton-pumping plasma membrane ATPase which is responsible for maintaining intracellular pH in yeast cells [2,9].

Acknowledgements

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