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Phosphorus-31 and Carbon-13 Nuclear Magnetic Resonance Studies of Glucose and Xylose Metabolism in Cell Suspensions and Agarose-Immobilized Cultures of *Pichia stipitis* and *Saccharomyces cerevisiae*

ELKE M. LOHMEIER-VOGEL,* DEANE D. MCINTYRE, AND HANS J. VOGEL

Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

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The metabolism of glucose and xylose as a function of oxygenation in *Pichia stipitis* and *Saccharomyces cerevisiae* cell suspensions was studied by ^{31}P and ^{13}C nuclear magnetic resonance spectroscopy. The rate of both glucose and xylose metabolism was slightly higher and the production of ethanol was slightly lower in aerobic than in anoxic cell suspensions of *P. stipitis*. As well, the cytoplasmic pH of oxygenated cells was more alkaline than that of nonoxygenated cells. In contrast, in *S. cerevisiae*, the intracellular pH and the rate of glucose metabolism and ethanol production were the same under aerobic and anoxic conditions. Agarose-immobilized *Pichia stipitis* was able to metabolize xylose or glucose for 24 to 60 h at rates and with theoretical yields of ethanol similar to those obtained with anoxic cell suspensions. Cell growth within the beads, however, was severely compromised. The intracellular pH [$pH_{(int)}$] of the entrapped cells fell to more acidic pH values in the course of the perfusions relative to corresponding cell suspensions. Of importance was the observation that no enhancement in the rate of carbohydrate metabolism occurred in response to changes in the $pH_{(int)}$ value. In contrast to *P. stipitis*, agarose-immobilized *Saccharomyces cerevisiae* showed a dramatic twofold increase in its ability to metabolize glucose in the immobilized state relative to cell suspensions. This strain was also able to grow within the beads, although the doubling time for the entrapped cells was longer, by a factor of 2, than the value obtained for log-phase batch cultures. Initially, the $pH_{(int)}$ of the immobilized cells was more alkaline than was observed with the corresponding *S. cerevisiae* cell suspensions; however, over time, the intracellular pH became increasingly acidic. As with immobilized *P. stipitis*, however, the $pH_{(int)}$ did not play a key role in controlling the rate of glucose metabolism.

Whole-cell immobilization is a valuable technique for the continuous conversion of substrates to products in bioreactors; in addition, it also allows for recycling of the biocatalyst (15). It may have even further beneficial effects; for example, the fermentation of D-glucose to ethanol is more rapid when immobilized *Saccharomyces* strains rather than cell suspensions of similar concentration are used (9, 11, 13, 22, 27, 30, 33). However, our recent attempts to improve the rate of D-glucose and D-xylose fermentation by using agarose-entrapped *Candida tropicalis* cells were not successful (19); in fact, the metabolism of both carbon sources was considerably slower for the immobilized cells than could be predicted on the basis of studies with cell suspensions (20). Potential reasons for the different behavior of these two yeast strains upon cell entrapment are threefold. (i) Our immobilization study was conducted with *C. tropicalis* over the course of a day, rather than the 2- to 6-h time frame in previous comparative in vivo nuclear magnetic resonance spectroscopy (NMR) studies with *Saccharomyces cerevisiae* (9, 11, 30). This time frame afforded the cells the opportunity to grow, thus possibly imposing mass transfer limitations on the fermentation process. (ii) Aside from *Saccharomyces* species, yeast strains have oxygen requirements for vital processes such as growth and sugar uptake (12, 32), thus compromising their performance in the anoxic to anaerobic environments which generally exist in the interior of polymer beads used for cell immobilization. (iii) In our study, we have used agarose as the encapsulation matrix rather than the calcium alginate (9, 11) or κ-carrageenan (30) used in previous studies. It is possible that the counterions required to maintain bead integrity with the last two matrices affect the cell metabolism; in contrast, agarose does not require Ca^{2+} or K^+ to stabilize the beads.

In the present study, we have used in vivo NMR to compare glucose metabolism in long-term studies of growing, agarose-immobilized *S. cerevisiae* to investigate which of the above factors is important in contributing to faster glucose fermentation kinetics with this strain. As well, we hope to determine if it is possible to enhance the rate of glucose and xylose fermentation in a similar fashion with *Pichia stipitis*. *P. stipitis* is one of the best xylose-fermenting yeast strains currently available (14) and is capable of producing close to theoretical yields of ethanol from D-xylose under anaerobic conditions (25). As such, this organism should function well in the immobilized state. Enhancement of the rate of xylose and other pentose sugar fermentations through whole-cell immobilization is potentially important for efficient ethanol production from lignocellulosic biomass (2).

MATERIALS AND METHODS

Organism and cultivation conditions. *P. stipitis* CBS 6054 and *S. cerevisiae* ATCC 28460 were maintained at 4°C on xylose-complex plates as previously described (20). Colonies of *S. cerevisiae* were much smaller when grown with xylose as the sole carbon source and took longer to establish than those of *P. stipitis*, suggesting that growth occurred mainly on the peptone or yeast extract components of the media. Both yeast strains were cultivated in yeast nitrogen base (YNB) minimal medium (Difco) supplemented with either 50 g of glucose or xylose per liter of medium, depending on which carbon source was used in subsequent experiments. The cells were cultivated in unbaffled Erlenmeyer flasks filled to 50% capacity and were incubated at 30°C with shaking at 140 rpm. Measurements of doubling times in "perfusion medium," i.e., YNB glucose or

* Corresponding author. Fax: (403) 289-9311.

xylose diluted 1:1 with MES salts buffer (50 mM morpholineethanesulfonic acid [MES; pH 6.0], 2.5 mM $MgCl_2$, 10 mM KCl, 2.4 mM $CaCl_2$) (23) were performed in a similar manner at 25°C by monitoring the increase in optical density at 620 nm.

Preparation of cell suspensions for NMR. Cells were harvested at mid-log phase by cooling on ice to 4°C with gentle shaking, followed by centrifugation and washing in cold MES-salts buffer. The final NMR sample (15-mm tube) contained 1 g (wet weight) of cells in 5.5 ml of perfusion medium (see above)–0.5 ml of D_2O–0.25 ml of SAG 471 silicon antifoam fluid (Harrison and Crossfield Ltd.). At time zero, either glucose or xylose was added to a final concentration of 64 mM. The samples were bubbled with either oxygen or nitrogen at 45 ml/min. Under these conditions, cells are close to being fully oxygenated (20); more vigorous bubbling leads to deterioration of the NMR spectra. A capillary (inner diameter, 1.0 mm) containing 0.2 M methylene diphosphonic acid was used as an external chemical shift and intensity reference. It was set to −18.59 ppm. For calibration, peak areas in the phosphorus spectra were compared with this standard, which was arbitrarily set to 100 integration units (IU). Experiments were run an average of three times each. When possible, data are plotted with standard error analysis.

Preparation of immobilized cells for NMR studies. Whole-cell agarose immobilization of both yeast strains was performed exactly as previously described (19). Beads larger than 0.5 mm but smaller than 1.0 mm in diameter were used in these experiments; this size minimizes substrate diffusion limitations (19). The final NMR sample contained 1 g (wet weight) of cells in 10 g of beads (drained weight) for *Pichia* perfusions and 0.5 g (wet weight) of cells in 5 g of beads for *Saccharomyces* perfusions. A capilliary of methylene diphosphonic acid was included as a phosphorus reference standard (see above). Agarose-immobilized cells were perfused at 1.5 ml/min with 100 ml of recycled oxygenated perfusion medium containing different amounts of either glucose or xylose as a carbon source (for details, see the figure legends).

NMR spectroscopy. NMR spectra were obtained at 25°C on a Bruker AM-400 wide-bore spectrometer operating in the Fourier transform mode, using a 15-mm ^{13}C-^{31}P switchable dual-tuned probe. ^{31}P-NMR spectra were recorded at 161.8 MHz with a recycle time of 1 s and a flip angle of 60°. Spectra of cell suspensions were acquired in 5-min blocks of 300 scans over a 1-h period with composite bilevel ^1H pulse decoupling (2-W power during acquisition); spectra of immobilized cells were acquired in 1-h blocks of 3,600 scans over a 24-h period.

Natural-abundance, proton-decoupled ^{13}C-NMR spectra were recorded at 100.6 MHz with a recycle time of 2 s and a flip angle of 70°. Spectra of cell suspensions were acquired in 5-min blocks of 150 scans (total time, 35 to 70 min), whereas spectra of immobilized cells were acquired in 1-h blocks of 1,800 scans over a 24-h period. Saturation factors were obtained for peaks of interest in the ^{13}C-NMR spectra by comparing the peak intensities obtained during standard pulsing with areas obtained under fully relaxed conditions.

RESULTS

^{13}C natural-abundance NMR studies of cell suspensions. Figure 1 shows representative ^{13}C-NMR spectra obtained at the beginning and end of an experiment in which xylose was metabolized by an oxygenated *P. stipitis* cell suspension. Although this sample had been bubbled at a rate of 45 ml/min with pure oxygen, the signal-to-noise ratio is acceptable for data analysis (20). The time course of the fermentation was monitored by observing the decrease in the α and β anomeric C-1 resonances of xylose and the increase in the ethanol peaks as a function of time. It is noteworthy here that *P. stipitis* produces considerably more ethanol than do suspensions of another xylose-fermenting yeast strain, *Candida tropicalis* (20). As well, xylitol by-product formation is essentially negligible with *P. stipitis*, and suspensions consuming glucose show mainly ethanol and only traces of glycerol in the final spectra (results not shown).

The integrated areas of the peaks of interest in a number of cell suspension experiments were corrected for saturation factors and plotted as a function of time in Fig. 2 and 3. Although the standard bars overlap somewhat, Fig. 2A and C shows that the rates of glucose assimilation with *P. stipitis* cell suspensions are slightly higher with oxygen bubbling (0.09 g/g [wet weight] of cells/h) than with nitrogen bubbling (0.077 g/g [wet weight] of cells/h). The rate of xylose consumption (in grams per gram [wet weight] of cells per hour) is 0.075 aerobically versus 0.056 under anoxic conditions. Figure 2B and D shows that ethanol production by *P. stipitis* from both carbon sources is higher under anoxic than aerobic conditions.

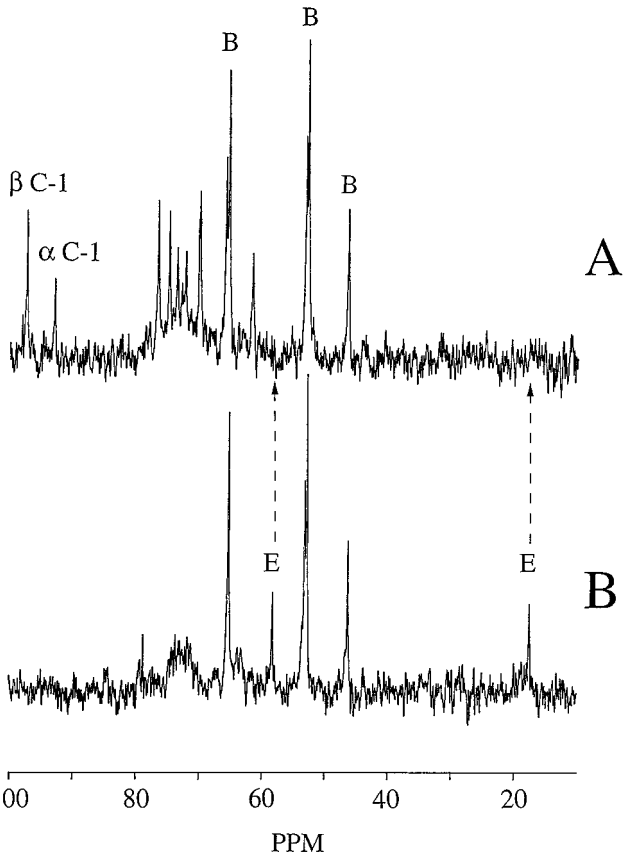

FIG. 1. (A) Natural-abundance ^{13}C-NMR spectrum of an aerobic *P. stipitis* cell suspension in buffered perfusion medium supplemented with 64 mM xylose. The α C-1 and β C-1 symbols refer to the anomeric carbons of xylose; MES buffer peaks in the spectrum are labelled B. (B) ^{13}C-NMR spectrum taken after 1 h, when the xylose had been fully consumed and only the fermentation product ethanol (E) was visible.

S. cerevisiae metabolizes xylose poorly, if at all; therefore, studies of xylose metabolism with cell suspensions of this yeast were not attempted here. Glucose metabolism by *S. cerevisiae*, however, is not dependent on aeration and is twice as fast (0.150 g/g [wet weight] of cells per h) as was observed with *P. stipitis* (compare Fig. 3A with Fig. 2A). As well, in contrast to *P. stipitis*, ethanol production by *S. cerevisiae* cell suspensions was similar under anoxic or aerobic conditions.

^{31}P-NMR studies of cell suspensions. ^{31}P-NMR spectra comparing *P. stipitis* and *S. cerevisiae* suspensions may be seen in Fig. 4. What is most obvious at first glance is that the phosphodiester (PDE) peak, representing cell wall phosphomannan (8), is significantly higher in Fig. 4A and B, representing *P. stipitis*, than in Fig. 4C, representing *S. cerevisiae*. By monitoring the PDE areas in subsequent 5-min spectra, it was ascertained that no significant cell growth occurred in the course of these experiments. *S. cerevisiae* suspensions metabolizing glucose (Fig. 4C) have close to twice the level of nucleoside tri- and diphosphates (mainly ATP and ADP) that *P. stipitis* suspensions metabolizing either glucose (Fig. 4B) or xylose do (Fig. 4A). This may be a reflection of the higher rate of sugar metabolism by *S. cerevisiae*. All other peak areas are not significantly different between the two strains.

The chemical shift (δ) of the pH-sensitive cytoplasmic P_i resonance [$P_{i(int)}$] can be used to estimate the intracellular pH of living cells (7). Figure 5 illustrates the fluctuations occurring

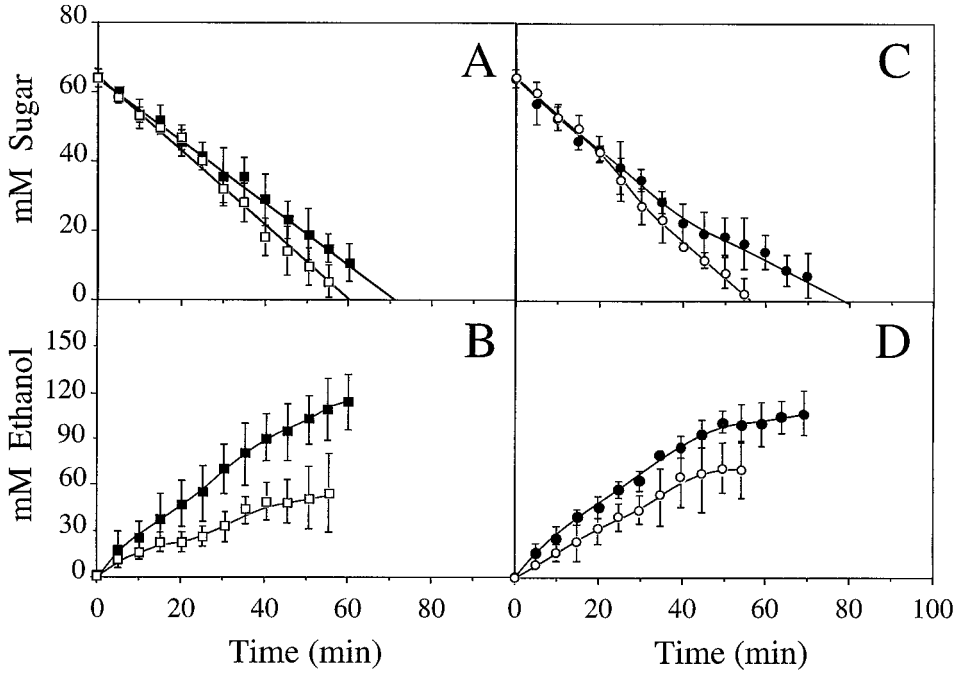

FIG. 2. The integrated peak areas in consecutive ^{13}C-NMR spectra obtained with *P. stipitis* cell suspensions were plotted as a function of time. (A) Metabolism of 64 mM glucose (0.5 mmol total). Symbols: ■, results obtained with anoxic cell suspensions; □, results obtained under oxygenated conditions. (B) Corresponding increase in the amount of ethanol for glucose-metabolizing cell suspensions. Symbols are as in panel A. (C) Metabolism of xylose as a function of time with *P. stipitis* cell suspensions. Symbols: ●, results obtained with anoxic cell suspensions; ○, results obtained under oxygenated conditions. (D) Formation of ethanol in xylose fermentations as a function of time. Symbols are as in panel C.

in the chemical shift of the $P_{i(int)}$ resonance during representative ^{31}P-NMR experiments with yeast suspensions. Although the δ values are initially higher in all cases, steady-state values are obtained after 10 to 15 min. When these values are compared with a standard P_i titration curve obtained in medium with an ionic composition approximating that of the yeast cytoplasm (4), $pH_{(int)}$ values for the cell suspensions may be obtained. These values are summarized in Table 1. It is interesting that after 30 min, when glucose has been consumed by *S. cerevisiae*, the cells lose the ability to maintain alkaline $pH_{(int)}$ values and become increasingly more acidic (Fig. 5). Acidification of the cytoplasm of the *Pichia* cell suspensions is less evident on this timescale, because there is still some sugar available to be metabolized. However, in all cases, sugar-depleted cells suspensions have ^{31}P-NMR spectra which show an increase in the area of the $P_{i(int)}$ resonance and a decrease in the area of the nucleoside phosphate peaks (data not shown). These results are consistent with previous, more detailed, ^{31}P-NMR studies of glucose metabolism in *S. cerevisiae* (4, 5, 23, 24) and glucose and xylose metabolism in *C. tropicalis* (20).

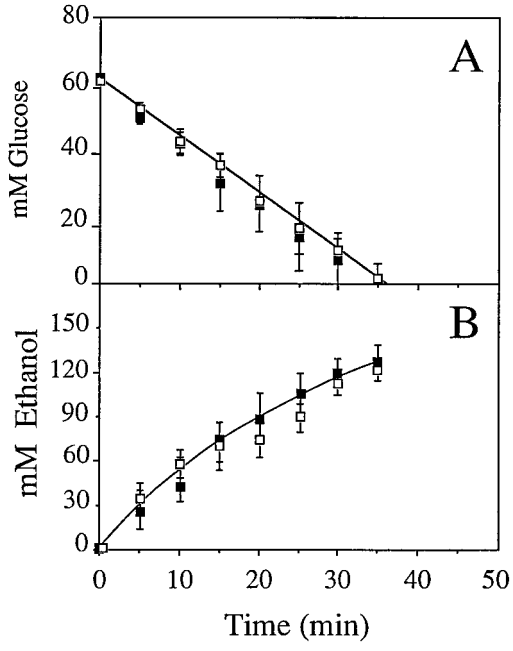

FIG. 3. Integrated peak areas in consecutive ^{13}C-NMR spectra obtained with *S. cerevisiae* cell suspensions metabolizing glucose, plotted as a function of time. Symbols: ■, results obtained with anoxic cell suspensions; □, results obtained under oxygenated conditions.

^{31}P- and ^{13}C-NMR studies of agarose-immobilized cells. ^{31}P-NMR studies were performed on agarose-immobilized yeast cells perfused with oxygenated glucose- or xylose-containing medium. Spectra similar to those in Fig. 4 were obtained. As we have previously shown for *C. tropicalis* (19), by monitoring the increase in the area of the PDE peak as a function of time and plotting the data on a semilog plot, as in Fig. 6, we were able to monitor cell growth noninvasively within the beads. Figure 6 shows that *P. stipitis*, even when perfused with oxygenated medium, is unable to grow significantly within the agarose beads. At most, an increase of 21% in the PDE area was observed after 24 h, translating to an estimated doubling time of approximately 120 h for growth on both carbon sources. Batch cultures of *P. stipitis* in perfusion medium at 25°C have doubling times of 2.7 h for glucose and 3.3 h for xylose (results not shown). In contrast to *P. stipitis*, agarose-immobilized *S. cerevisiae* cells double within 4.8 h.

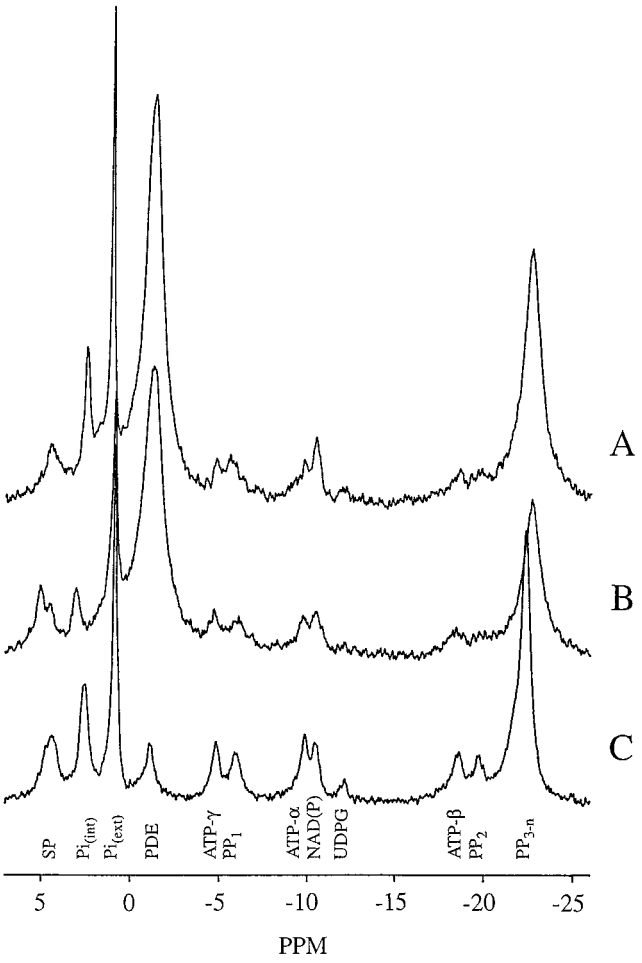

FIG. 4. ^{31}P-NMR spectra of an oxygenated cell suspension of *P. stipitis* metabolizing xylose (A), an oxygenated cell suspension of *P. stipitis* metabolizing glucose (B), and an anoxic cell suspension of *S. cerevisiae* metabolizing glucose (C). All spectra are the average of four spectra obtained between 20 and 40 min after the addition of the carbon source. SP, sugarphosphomonoesters; $P_{i(ext)}$, extracellular P_i; ATP-γ, contributions from both the ATP γ and the ADP β resonances; ATP-α, contributions from both the ATP α and the ADP α resonances; NDPG, nucleotide diphosphoglucose; PP_1, and PP_2, terminal and penultimate phosphates, respectively, in vacuolar polyphosphate chains (PP_{3-n}).

This compares well to the doubling time in cell suspensions of 2.0 h obtained in batch culture (result not shown).

In Fig. 7, the chemical shift (δ) of the $P_{i(int)}$ resonance in agarose-immobilized *P. stipitis* or *S. cerevisiae* cells is shown as a function of time. In three representative perfusions, we observed that the δ of the $P_{i(int)}$ peak moved upfield with time, implying that the intracellular pH of the immobilized yeast strains became progressively more acidic over the course of the experiments. A very dramatic drop in intracellular pH was observed in the *S. cerevisiae* perfusion after glucose had been exhausted from the medium, whereas *P. stipitis* perfusions, which still contained sugar after 24 h (see Fig. 8), had more stable $P_{i(int)}$ values.

Natural-abundance ^{13}C-NMR studies of agarose-immobilized *P. stipitis* show that the rates of both glucose and xylose metabolism over the course of 24 h are slightly lower than those calculated from the anoxic rates of sugar metabolism obtained with cell suspensions and adjustment to compensate for the small amount of cell growth within the beads. Of importance here is the observation that the fermentation kinetics

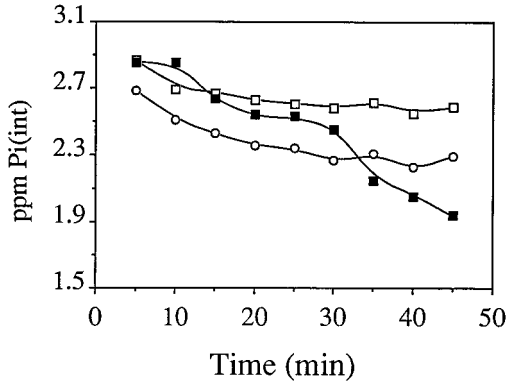

FIG. 5. The chemical shift (δ) of the cytoplasmic P_i resonance was monitored as a function of time in consecutive ^{31}P-NMR spectra of anaerobic yeast suspensions. Results are shown for *P. stipitis* metabolizing glucose (□), *P. stipitis* metabolizing xylose (○), and *S. cerevisiae* metabolizing glucose (■).

are not enhanced for either carbon source when using immobilized *P. stipitis*. About half of the initial 3 g of glucose or xylose is consumed in 24 h by an average of 1.1 g of cells (adjusting for cell growth). This translates into a rate of 0.057 g of sugar consumed per g of cells per h. Ethanol yields are 0.35 g of ethanol per g of glucose and 0.3 g of ethanol per g of xylose. These values are somewhat lower than those observed with anoxic cell suspensions but slightly higher than the yields obtained with aerobic cell suspensions.

Averaging the chemical shifts of the $P_{i(int)}$ resonances (Fig. 5) between 3 and 13 h and between 14 and 24 h for the *Pichia stipitis* perfusions allowed us to estimate the intracellular pH for the immobilized yeast strains during the early and later stages of these experiments. For xylose, the average $pH_{(int)}$ was 6.88 during the first half of the perfusion and 6.85 during the last half of the perfusion; the early value was slightly more alkaline than observed at steady state with aerobic cell suspensions (Table 1). In immobilized *P. stipitis* perfused with glucose, the average $pH_{(int)}$ was 7.15 during the first half of the perfusion and dropped to 6.9 during the latter stages. These values should be compared with a $pH_{(int)}$ value of 7.35 for steady state, obtained with aerobic cell suspensions (Table 1).

Close inspection of Fig. 8 shows a tendency for sugar consumption to slow after 20 h of perfusion. Concern arose about the possibility that long-term immobilization would lead to decreased viability of *P. stipitis*, since this strain requires oxygen for growth. For this reason, agarose-immobilized *P. stipitis* was perfused for 60 h with three fresh changes of medium containing 1.5 g of xylose each time. We observed that xylose consumption and ethanol production were not compromised over the course of 3 days (results not shown).

TABLE 1. Steady-state $pH_{(int)}$ of yeast suspensions as a function of oxygenation and carbon source

Cell type	$pH_{(int)}$[a] at degree of oxygenation:	
	Aerobic	Anoxic
P. stipitis		
Glucose-fed cells	7.35	6.85
Xylose-fed cells	6.85	6.50
S. cerevisiae glucose-fed cells	7.0	7.0

[a] The results are ±0.05 pH unit.

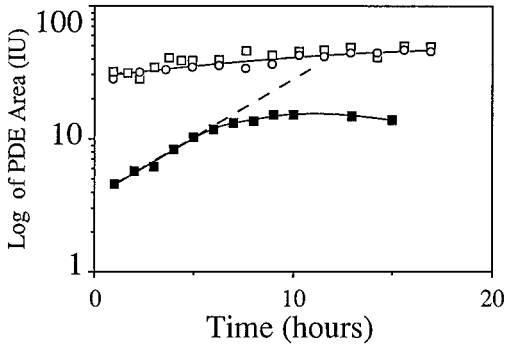

FIG. 6. The log value of the integrated PDE areas (in arbitrary integration units) from individual ^{31}P-NMR spectra of agarose-immobilized yeast perfusions was plotted as a function of time. Symbols: ■, glucose-perfused *S. cerevisiae*; □, glucose-perfused *P. stipitis*; ○, xylose-perfused *P. stipitis.*

The fermentation dynamics of agarose-immobilized *S. cerevisiae* perfused with glucose are shown in Fig. 9. In contrast to *P. stipitis*, there is an immediate enhancement of glucose consumption relative to what is theoretically predicted from cell suspensions, resulting in the depletion of 3 g of glucose from the medium after only 9 h. Theoretically, this amount of glucose should have been depleted in 20 h; hence, immobilized cells experienced a doubling in their rate of glucose consumption (0.33 g of glucose consumed per g of cells per h). Approximately 16% of the consumed glucose appeared as glycerol in these perfusions; the remaining carbon was converted to ethanol with a theoretical yield of 0.4 g of ethanol per g of glucose consumed.

Averaging the chemical shift of the $P_{i(int)}$ resonance over the first 4 h of the perfusion results in a $pH_{(int)}$ of 7.1 for the immobilized cells. This value is about 0.1 pH unit more alkaline than that observed with steady-state cell suspensions (Table 1). In the final 5 h of the perfusion experiment, however, the averaged chemical shift of the $P_{i(int)}$ resonance gives a more acidic value of 6.9 pH units. These values should be compared with the $pH_{(int)}$ value of 7.0 obtained with cell suspensions (Table 1).

DISCUSSION

Our ^{31}P- and ^{13}C-NMR studies with *P. stipitis* CBS 6054 and *S. cerevisiae* ATCC 28460 cell suspensions provided essential background data (i.e., rate of carbohydrate metabolism and intracellular pH) required for the analysis of results of long-term perfusion studies with these strains in the agarose-immobilized state. The results presented here show that cell suspensions of log-phase *P. stipitis* have fairly similar rates of glucose and xylose metabolism. Both carbon sources are consumed

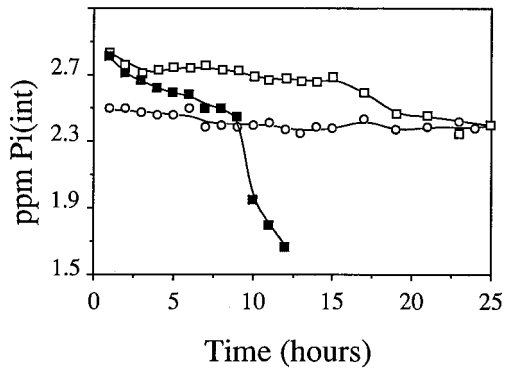

FIG. 7. The chemical shift (δ) of the cytoplasmic P_i resonance from individual ^{31}P-NMR spectra of agarose-immobilized yeast perfusions was monitored as a function of time. Symbols: ■, glucose-perfused *S. cerevisiae*; □, glucose-perfused *P. stipitis*; ○, xylose-perfused *P. stipitis.*

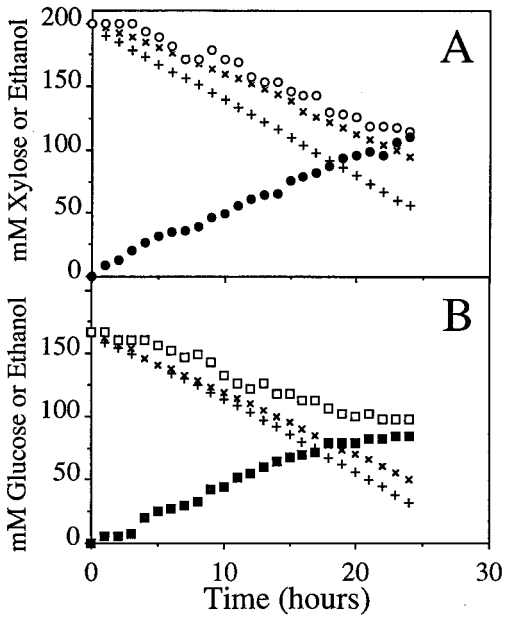

FIG. 8. Carbohydrate utilization and product formation by immobilized *P. stipitis* as determined by ^{13}C-NMR. The perfusion medium consisted of 100 ml of recycled, oxygenated medium containing 3 g of either glucose or xylose. (A) Rates of xylose metabolism (○) and ethanol production (●) as a function of time with xylose-perfused cells. The aerobic theoretical xylose uptake rate (+) was calculated from the value (grams of sugar consumed per gram [wet weight] of cells per hour) obtained with oxygenated *P. stipitis* cell suspensions metabolizing xylose (Table 1) and was adjusted hourly to correct for cell growth within the beads. The anoxic theoretical xylose uptake rate (×) was calculated from the value (grams of sugar consumed per gram [wet weight] of cells per hour) obtained with nitrogen-bubbled *P. stipitis* cell suspensions metabolizing xylose (Table 1). (B) Rates of glucose metabolism (□) and ethanol production (■) as a function of time with xylose-perfused *P. stipitis*. Theoretical uptake rates (+, ×) were calculated as described above from values obtained with glucose-consuming aerobic or anoxic *P. stipitis* cell suspensions.

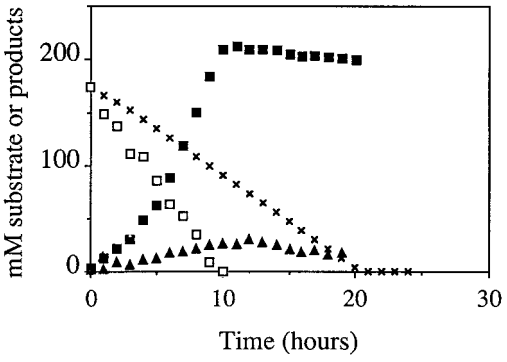

FIG. 9. Glucose metabolism (□), ethanol production (■), and glycerol production (▲) by immobilized *S. cerevisiae* as a function of time, as determined by ^{13}C-NMR. The perfusion medium consisted of 100 ml of recycled, oxygenated medium containing 3 g of glucose. The theoretical glucose uptake rate (+) was calculated as described in the legend to Fig. 8, using the value (grams of sugar consumed per gram [wet weight] of cells per hour) obtained with anoxic glucose-consuming *S. cerevisiae* suspensions (Table 1).

slightly faster in the presence of oxygen than under anoxic conditions. Previous studies on *P. stipitis* CBS 6054 in continuous culture have also shown that the rate of xylose and glucose consumption decreases as the rate of oxygen transfer to the cells is decreased (28, 29).

In contrast to *P. stipitis*, *S. cerevisiae* cell suspensions experienced similar rates of glucose consumption and ethanol production in both the presence and absence of oxygen. The "Pasteur effect," in which aerobic glucose metabolism is slower than anaerobic glucose metabolism, is evident only with derepressed *S. cerevisiae* cells, i.e., cells pregrown in the absence of glucose (see reference 5 for further discussion). Because the mitochondria of the glucose-repressed cells used in this study are not competent for respiration, ethanol production is the same aerobically and anaerobically. The observation that ethanol levels were significantly lower when glucose- or xylose-fed *Pichia* cell suspensions were bubbled with oxygen rather than nitrogen shows that glucose repression does not occur with *P. stipitis*, because mitochondrial function is not impaired. Thus, partial oxidation of ethanol is seen with this strain.

The observation that the steady-state $pH_{(int)}$ of glucose-metabolizing *S. cerevisiae* cell suspensions was the same under aerobic and anoxic conditions was not surprising, since the rates of glucose consumption in the two cases were similar. *P. stipitis*, on the other hand, had slightly higher $pH_{(int)}$ values in the presence of oxygen, probably because of the slightly increased rate of sugar metabolism in oxygenated cell suspensions relative to nitrogen-bubbled cells. These differences in $pH_{(int)}$ were, however, much less dramatic than the values previously observed with *C. tropicalis* metabolizing xylose as a function of aeration (20) or in the presence of azide (21). Because of a redox imbalance in the first two steps of xylose uptake (3), xylose metabolism in *C. tropicalis* is not possible under anaerobic conditions, even with cell suspensions (19). *P. stipitis*, on the other hand, circumvents the redox problem because it has a xylitol dehydrogenase with dual coenzyme specificity (31), thus allowing this organism to ferment xylose anaerobically. Since the interior of agarose beads can become fairly anoxic, even when oxygen is present in the perfusion medium (19), the ability of *P. stipitis* to ferment xylose anaerobically makes it a much better candidate for studying xylose fermentation in the encapsulated state than is *C. tropicalis* (20).

Our immobilization study cleared up a number of concerns. The first was that agarose-encapsulated *S. cerevisiae* was capable of growing within the polymer matrix. Even so, the twofold-faster glucose fermentation kinetics, which had previously been observed in studies of shorter duration involving nongrowing cells (9, 11, 30), were also observed in our long-term perfusion study. Thus, mass transfer effects imposed by the increased cell density within the beads did not prove to be a serious hindrance in the course of these experiments, as we had previously feared.

Recent articles have shown that facultatively fermentative yeasts like *P. stipitis* have oxygen requirements for growth and sugar transport (12, 32). Thus, a second concern arose, i.e., that enhanced glucose and xylose fermentation kinetics might not be observed with immobilized *P. stipitis* for reasons other than the redox problem discussed above. Indeed, although entrapped *P. stipitis* remains capable of fermenting both sugars over the course of 3 days, no increase in fermentation rates was observed relative to the rates in cell suspensions.

The third concern, about the potential effect of Ca^{2+} and K^+ counterions on fermentation kinetics, was also addressed in this study, since the agarose matrix does not require counterions to maintain bead integrity. Our observation that agarose-immobilized *S. cerevisiae* displayed the same enhancement of glucose fermentation as was previously observed for calcium alginate-entrapped (9, 11) or κ-carrageenan-entrapped (30) *Saccharomyces* strains suggest that the presence of counterions such as Ca^{2+} and K^+ does not stimulate the rate of ethanol production per se. Counterions may, however, have an effect on the intracellular pH of *Saccharomyces* strains. Earlier reports showed that calcium alginate-entrapped *S. cerevisiae* displayed an acidic $pH_{(int)}$ and faster glucose fermentation kinetics relative to cells suspensions (9, 11). A more acidic $pH_{(int)}$ should stimulate the flow of carbon through glycolysis, since protons have a positive allosteric effect on phosphofructokinase (1, 26). However, faster glucose fermentation kinetics but an alkaline $pH_{(int)}$ were observed in a recent study involving κ-carrageenan-entrapped *Saccharomyces bayanus* (30). It is possible that the variance in intracellular pH is due to the different counterions used in these two studies.

Our results with agarose-immobilized *S. cerevisiae* show that the $pH_{(int)}$ and the rate of sugar fermentation are not closely related. We observed a doubling in the rate of glucose fermentation, even though during the initial 4 h of the perfusions, the $pH_{(int)}$ of the entrapped cells was more alkaline (in agreement with the results in reference 30) than in steady-state cell suspensions, whereas in the last 5 h it was more acidic (in agreement with the results in references 9 and 11). Thus, although counterions may affect $pH_{(int)}$ values somewhat, changes in intracellular pH alone may play only a minor role in the whole fermentation scheme.

Indeed, a less prominent role for the effect of $pH_{(int)}$ on glucose fermentation kinetics was suggested in a study in which the flux control coefficients for glucose uptake and the major glycolytic enzymes were determined for entrapped versus free *S. cerevisiae* cells (10). In that study, it was shown that control at the level of phosphofructokinase, which plays a major role in determining the rate of sugar flux through the glycolytic pathway in cell suspensions, plays a secondary role in immobilized cells. Instead, these investigators suggested that in calcium alginate-entrapped, nongrowing *S. cerevisiae*, the rate of glucose transport is increased relative to that in free cells, leading to enhanced fermentation kinetics in the former case. Our results suggest that sugar transport in *P. stipitis* is not enhanced by immobilization. This may be due to the differing nature of sugar transport in these two organisms: facilitated diffusion for glucose in *S. cerevisiae* (17, 18) versus proton symport in *P. stipitis* (6, 16, 31). Evidence also exists to suggest that oxygen induces or activates *P. stipitis* symporters (28, 29), a process which is unlikely to occur within anoxic agarose beads.

In conclusion, our results in this and previous studies with *C. tropicalis* (19, 20) clearly show that although cell immobilization is beneficial for glucose fermentations in *S. cerevisiae*, there is no guarantee that faster fermentation kinetics with other yeast strains is possible. However, in contrast to *C. tropicalis* (19), xylose fermentation kinetics with *P. stipitis* were not significantly hindered by immobilization, making this organism the preferred choice for use in pentose fermentations in the entrapped state.

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