APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1981, p. 284-289
0099-2240/81/080284-06$02.00/0

Vol. 42, No. 2

D-Xylulose Fermentation to Ethanol by *Saccharomyces cerevisiae*

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Received 22 December 1980/Accepted 5 May 1981

We used commercial bakers' yeast (*Saccharomyces cerevisiae*) to study the conversion of D-xylulose to ethanol in the presence of D-xylose. The rate of ethanol production increased with an increase in yeast cell density. The optimal temperature for D-xylulose fermentation was 35°C, and the optimal pH range was 4 to 6. The fermentation of D-xylulose by yeast resulted in the production of ethanol as the major product; small amounts of xylitol and glycerol were also produced. The production of xylitol was influenced by pH as well as temperature. High pH values and low temperatures enhanced xylitol production. The rate of D-xylulose fermentation decreased when the production of ethanol yielded concentrations of 4% or more. The slow conversion rate of D-xylulose to ethanol was increased by increasing the yeast cell density. The overall production of ethanol from D-xylulose by yeast cells under optimal conditions was 90% of the theoretical yield.

Previously, we described the production of ethanol from isomerized D-xylose (D-xylulose) by *Saccharomyces cerevisiae*. We also demonstrated that D-xylose isomerase is required for the production of ethanol from D-xylose by yeasts (5). In this report, we describe the characteristics of D-xylulose fermentation to ethanol by industrial bakers' yeast.

D-Xylose is the major product of the hydrolysis of hemicellulose from many plant materials. It often comprises more than 60% of the recoverable sugars derived from hemicelluloses (9). To maximize the conversion of biomass-derived sugars to ethanol, it is important to ferment not only six-carbon sugars, but also five-carbon sugars.

Many efforts have been made to convert cellulose to ethanol via saccharification and fermentation steps. Attempts to convert D-xylose to ethanol have not yielded satisfactory results. Primarily, this is because yeast cannot ferment D-xylose to ethanol (2). Several bacteria and mycelial fungi are known to ferment D-xylose to a mixture of fermentation products, including ethanol. However, no yeasts have been reported to ferment pentoses, although some are known to metabolize D-xylose aerobically (2). Recently, we have observed that many yeasts are able to produce ethanol from D-xylose in the presence of D-xylose-isomerizing enzyme. Similar observations have also been reported by Wang et al. (19, 20). The conversion of D-xylose to ethanol is thought to proceed by the enzyme-catalyzed isomerization of D-xylose to D-xylulose and the subsequent fermentation of D-xylulose to ethanol (5).

These observations indicated that production of ethanol from D-xylose can be accomplished by using yeast and commercially available xylose isomerase (glucose isomerase) in a simultaneous isomerization and fermentation process. Since D-xylose isomerization and sugar fermentation have different optimal conditions (5, 15), it is necessary to determine the compatible environmental conditions for the isomerization and fermentation of D-xylose by yeast and isomerase. In addition to pH and temperature effects, factors such as yeast cell density and the effects of various sugars present in the fermentation broth must be investigated.

The objectives of this study were to determine the optimal conditions for D-xylulose fermentation by bakers' yeast and to evaluate the ability of yeast to ferment D-xylulose to ethanol in the presence of other sugars.

We used commercial bakers' yeast rather than pure cultures of *S. cerevisiae* because bakers' yeast provides a large supply of yeast cells in an active form that can be added easily to fermentation substrates. We used a high density of yeast for fermentations to overcome the slow rate of ethanol production from D-xylulose (5).

MATERIALS AND METHODS

Organism. An industrial strain of bakers' yeast (*S. cerevisiae*) was purchased in dried pellet form from

Universal Food Co., Milwaukee, Wis. This yeast was kept in a refrigerator and was added to a fermentor directly as an inoculum without any pretreatment.

Substrate. A stock solution of pentose syrup (50% D-xylulose and 50% D-xylose) was prepared by using immobilized glucose isomerase (Sweetzyme; type Q; lot no. BA-117-0298; 220 IU/g; Novo Industries, Inc.). The isomerase was swollen in 0.05 M phosphate buffer (pH 7.5) containing 0.5 g of sodium bisulfite per liter and 3×10^{-3} mM Mg^{2+} and was packed in a 220-ml glass jacket column (3.5 by 30 cm). A D-xylose solution (500 g/liter) was pumped at a flow rate of 50 ml/h through the column at constant temperature of 70°C and a pH of 7.5. At this flow rate the effluent had an equilibrium conversion of total sugar to D-xylulose of 30%. A concentrated solution containing 50% D-xylulose was then prepared by differential ethanol precipitation of D-xylose from the concentrated isomerized D-xylose solution.

Fermentation. Fermentations were performed in a 5-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) equipped with pH and temperature controls. Agitation was fixed at 350 rpm, and fermentation conditions (such as pH, temperature, substrate composition, and inoculum size) were varied. The substrate used for studying the effects of pH, temperature, yeast cell density, and ethanol concentration was pentose syrup at a concentration of 150 g/liter. The substrate solution used for comparing sugar utilization values contained 120 g of pentose syrup per liter and 30 g of D-glucose or D-fructose per liter. The effect of D-xylose on D-xylulose fermentation was investigated by using substrates containing 30 g of D-xylulose per liter and different concentrations of D-xylose. Yeast growth nutrients, such as yeast extract and peptone, were not added to any of the substrates, nor were the substrates autoclaved before fermentation.

Bacterial contamination. Slight bacterial contamination (average, 35 cells per g of dry yeast) was present in commercial bakers' yeast. During fermentation experiments, samples were removed periodically from the fermentation broth and observed microscopically for the presence of bacterial cells. No significant bacterial growth was observed during fermentations unless the pH was 7 or more and the incubation time was 48 h or longer.

Analysis. During fermentation experiments, samples were withdrawn and centrifuged, and the supernatants were collected, frozen, and later analyzed. Ethanol concentration was measured by gas chromatography, and sugar consumption was analyzed by low-pressure liquid chromatography (10).

RESULTS AND DISCUSSION

The early steps of yeast metabolism of D-xylose proceed through the oxidoreduction reaction. In this reaction, D-xylose is reduced to xylitol by reduced nicotinamide adenine dinucleotide phosphate-dependent aldoreductase, and xylitol is then oxidized to D-xylulose by nicotinamide adenine dinucleotide phosphate-dependent D-xylulose reductase. D-Xylose is then phosphorylated to D-xylulose 5-phosphate, which is then converted to pyruvate through both the pentose phosphate pathway and the Embden-Myerhof pathway (1, 3, 4, 14).

In most bacteria, the direct isomerization of D-xylose to D-xylulose by D-xylose isomerase is the first step in D-xylose metabolism (11, 16, 21). In certain yeasts, such as *Candida utilis* and *Rhodotorula gracilis*, the presence of an inducible enzyme, D-xylose isomerase, has been demonstrated (6, 18). However, the oxidoreduction pathway is believed to be the predominant pathway for D-xylose metabolism in yeasts (1).

Previous observations of D-xylose metabolism by yeasts in our laboratory indicated that many yeasts convert D-xylose to xylitol readily, but none of the yeasts tested was able to ferment either D-xylose or xylitol to ethanol (5). Several yeasts, such as *S. cerevisiae*, *Candida utilis*, and *Candida diddensii*, readily ferment both D-glucose and D-xylulose to ethanol; *S. cerevisiae* is the most effective in producing ethanol from D-xylulose.

In this study we used industrial bakers' yeast to examine the effects of many environmental factors on the production of ethanol from D-xylulose.

Effect of cell density on ethanol production. The rate of ethanol production from D-xylulose was linear over a concentration range from 50 to 150 mg of cells per ml. The ethanol production rate reached its maximum at 150 mg of cells per ml (Fig. 1). A high concentration of yeast cells decreased the fermentation time, resulting in a higher ethanol yield. At a lower concentration of yeast cells (<100 g/liter), the fermentation time increased significantly. This

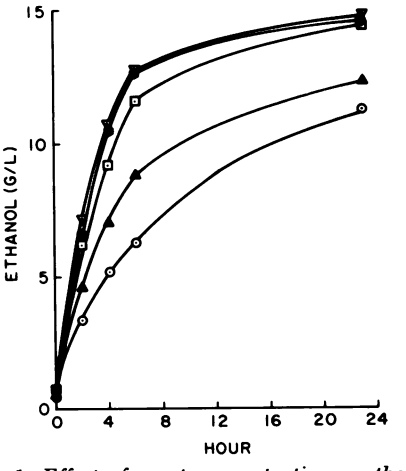

FIG. 1. *Effect of yeast concentration on the production of ethanol from D-xylulose by bakers' yeast. The temperature was 37°C, and the pH was 6. Symbols: ⊙, 50 g/liter; ▲, 100 g/liter; ⊡, 150 g/liter; ⬢ 200 g/liter; ▽, 250 g/liter.*

resulted in a difference of 30% in ethanol yield between yeast cells at concentrations of 250 and 50 g/liter after 24 h of incubation.

The initial rate of D-xylulose fermentation was linear as a function of yeast cell density at low cell concentrations. Figure 2 shows the effect of yeast concentration on the specific ethanol production rate (grams of ethanol produced per hour per gram of yeast cells). The specific ethanol production rate was approximately 0.03 g/liter per h when 75 g of yeast cells per liter was used.

A decrease in specific ethanol production rate with increasing cell density reportedly occurs in *S. cerevisiae* during "rapid fermentation" of glucose under high-cell density conditions (12). This is due to the effect of accumulated ethanol within yeast cells, which decreases alcohol dehydrogenase activity and cell viability.

To evaluate the effects of various fermentation conditions on D-xylulose fermentation, we used a concentration of 75 g of active dried yeast per liter as a standard inoculum. At this yeast concentration, the fermentation of D-xylulose was complete within 24 h, whereas the yeast growth during this period of fermentation was limited.

Effect of temperature. The initial fermentation rate of D-xylulose was relatively constant between 35 and 40°C, with an optimal temperature around 35°C (Fig. 3). At temperatures of more than 40°C, the rate of ethanol production decreased faster than at lower temperatures. Final ethanol concentrations varied slightly between 35 and 40°C. The ethanol yields from D-xylulose at these temperatures remained high (<90%).

The optimal temperature for fermentation is usually higher than the optimal growth temperature (17). However, it is more difficult to maintain the ability of a yeast to ferment at higher temperatures (13). During glucose fermentation, yeasts lose the ability to ferment within 16 h at 39.6°C, whereas at lower temperatures they retain this ability much longer. This is because continuous yeast growth is required for fermentation (8). We attempted to prolong yeast activity at high temperatures by supplementing media with yeast growth factors, but no significant yeast growth was observed after 16 h of fermentation, even when we used a relatively small inoculum (40 g/liter).

Effect of pH. Figure 4 shows the effect of pH on D-xylulose fermentation. We observed few differences in the rates of ethanol production and sugar consumption in the pH range from 4 to 6. At pH 7, the initial fermentation rate and

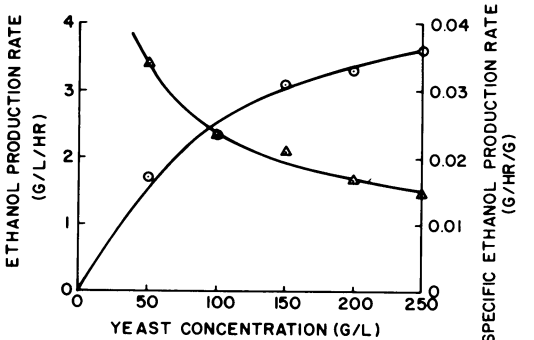

FIG. 2. *Effect of yeast concentration on ethanol production rate and yeast activity. Symbols: ⊙, ethanol production rate; △, specific ethanol production rate.*

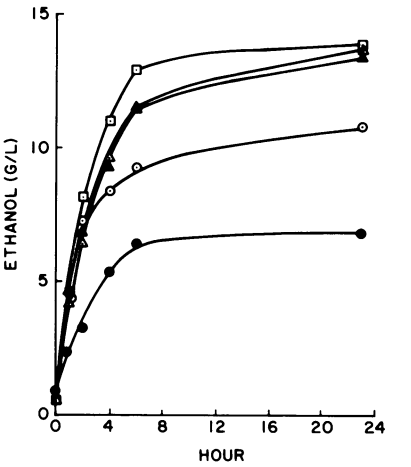

FIG. 3. *Effect of temperature on the production of ethanol from D-xylulose by bakers' yeast. The pH was 6, and the cell density was 75 g/liter. Symbols: ⊙, 30°C; ⊡, 35°C; ▲, 37°C; △, 40°C; ●, 45°C.*

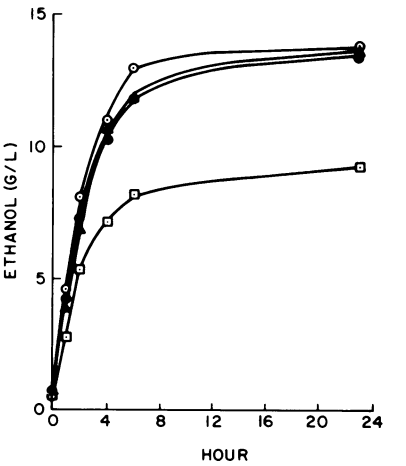

FIG. 4. *Effect of pH on the production of ethanol from D-xylulose by bakers' yeast. The temperature was 37°C, and the cell density was 75 g/liter. Symbols: ⊙, pH 4; ●, pH 5; ▲, pH 6; ⊡, pH 7.*

the final ethanol yield were 75 and 67% of the values of pH 4, respectively. The effect of pH on D-xylulose fermentation differed from the effect on the D-xylose isomerization reaction. The optimal pH range was narrower and the optimal pH was higher for the isomerization of D-xylose to D-xylulose than for the D-xylulose fermentation (15; H. Y. Hsiao, L.-F. Chen, C.-S. Gong, and G. T. Tsao, manuscript in preparation).

The reduced ethanol yield at pH 7 could have been caused by bacterial contamination which decreased the amount of D-xylulose available for the yeast. By-product formation could also have been an important factor contributing to the reduced ethanol yield at the high pH. We measured the concentrations of some fermentation by-products, such as xylitol and glycerol. The formation of xylitol and the formation of glycerol were greater at a higher pH and a lower temperature.

Normally, during the fermentation of D-glucose and D-xylulose the pH decreases to approximately 4, and this decrease in pH coincides with maximal ethanol production (5). Since a decrease in pH is detrimental to the rate of D-xylose isomerization, the maintenance of a pH of approximately 6 is important in a simultaneous isomerization and fermentation process.

Effect of D-xylose. In the simultaneous isomerization and fermentation of D-xylose to ethanol, the presence of some D-xylose is unavoidable due to the equilibrium of the isomerization reaction catalyzed by the isomerase. At high pentose concentrations, the effect of high osmotic pressure, the hinderance of D-xylulose transport, and the substrate inhibition exerted by D-xylose on D-xylulose fermentation must be considered. The total sugar concentration used in this study was 150 g/liter or less. It is doubtful that the osmotic pressure significantly affected the fermentation.

Information concerning D-xylulose transport is not available. In the red yeast *Rhodotorula*, D-xylose is taken up and accumulates rapidly, but the metabolism of D-xylose does not occur until after a lag period (7). The effect of D-xylose on D-xylulose uptake has not been reported in yeasts. As Fig. 5 shows, the presence of different concentrations of D-xylose had no significant effect on D-xylulose fermentation. This indicted that the uptake mechanism of D-xylulose is probably different from that of D-xylose. Since we observed no lag period between the uptake and utilization of D-xylulose, these results indicated that D-xylulose was taken up and metabolized by yeast cells readily and that the presence of D-xylose had no inhibitory effect.

Effect of ethanol. The effect of ethanol on D-xylulose fermentation was studied by adding different amounts of ethanol to the fermentation substrate before inoculating with yeast. Figure 6 shows that there was strong end product inhibition by ethanol. Increasing the ethanol concentration in the fermentation broth decreased the rate of ethanol production over the 24-h sampling period. The decrease in fermentation rate due to the increasing concentrations of ethanol produced could be avoided by using ethanol-tolerant yeast strains.

Substrate specificity. We examined the substrate specificity for yeast fermentation of D-xylulose, D-glucose, and D-fructose in the presence of equal amounts of D-xylose. As Fig. 7 shows, there was little difference between the rates of ethanol production when glucose and

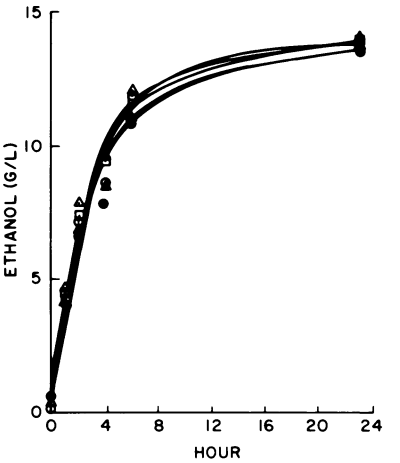

FIG. 5. *Effect of D-xylose on the production of ethanol from D-xylulose by bakers' yeast. The temperature was 37°C, the pH was 6, and the cell density was 75 g/liter. Symbols: ⊙, 30 g/liter; △, 45 g/liter, ▲, 60 g/liter; ●, 75 g/liter; ⊡, 90 g/liter.*

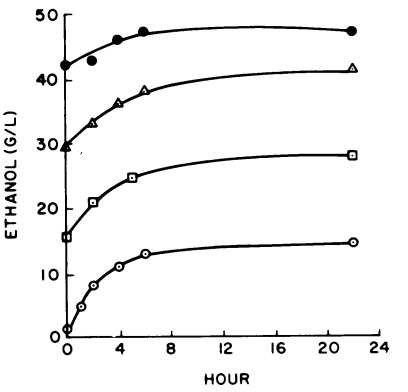

FIG. 6. *Effect of initial ethanol concentration on the production of ethanol from D-xylulose by bakers' yeast. The temperature was 37°C, the pH was 6, and the cell density was 75 g/liter. Symbols: ⊙, 0.5 g/liter; ⊡, 15.6 g/liter; △, 29.6 g/liter; ●, 42.3 g/liter.*

fructose were used as substrates; a linear relationship between fermentation time and ethanol yield was obtained. The ethanol production rate was 20 g/liter per h for both glucose and fructose. When D-xylulose was used as a substrate, a much slower fermentation rate was observed. Since more enzymes are involved in the fermentation of pentose than in glycolysis, the steps of the pentose phosphate pathway could be the possible rate-limiting steps for D-xylulose fermentation.

Effect of D-xylulose concentration. The effect of D-xylulose concentration on the ethanol production rate was studied by adding different amounts of pentose syrup as the fermentation substrate before adding yeast cells. Figure 8 shows that the initial ethanol production rate increased with increasing concentrations of D-xylulose. Although the initial rate of ethanol production varied with initial substrate concentration, the rate of ethanol production after approximately 8 h of fermentation was reduced. This resulted in the accumulation of ethanol at a concentration of approximately 13 g/liter after 24 h of fermentation regardless of the initial substrate concentration used.

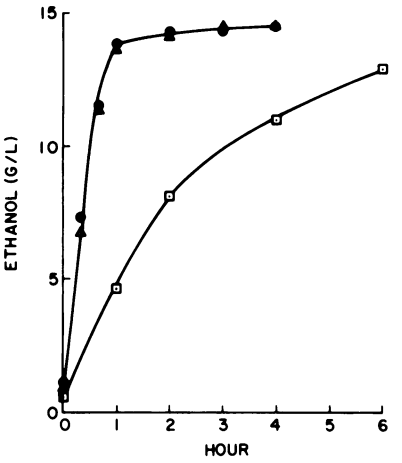

FIG. 7. *Fermentation of D-glucose (●), D-fructose (△), and D-xylulose (□) to ethanol by bakers' yeast. The temperature was 37°C, the pH was 6, and the cell density was 75 g/liter; the D-glucose and D-fructose concentrations were 30 g/liter, and the D-xylulose concentration was 60 g/liter.*

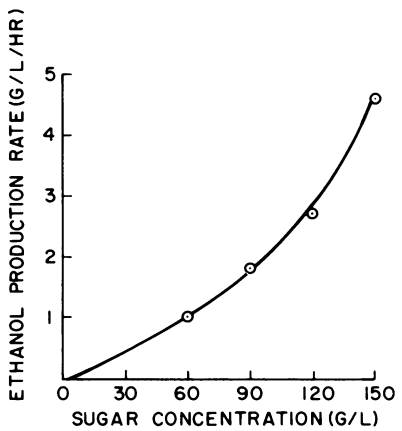

FIG. 8. *Effect of D-xylulose concentration on ethanol production rate.*

By-product formation. The major by-product formed when D-xylulose is fermented is xylitol, but small amounts of glycerol were also produced. The final yields of xylitol and glycerol at 35°C and pH 6 were 11 and 0.5 g/liter, respectively. Low temperatures, high pH values, and high D-xylose concentrations favored xylitol production (Table 1). Xylitol could be derived from D-xylose or D-xylulose or both (5).

Conclusion. Temperature, pH, isomerizing enzyme activity, end product inhibition, cell density, and substrate concentration are the important factors in determining the rate of ethanol production from D-xylulose in simultaneous isomerization and fermentation of D-xylose. For the D-xylulose fermentation by yeast, a temperature of 35 to 40°C and a pH of 4 to 6 are optimal. Higher pH values and lower temperatures reduce fermentation activity and increase by-product formation. At lower pH values and temperatures, isomerization activity is decreased. However, since the rate of fermentation is slower than the rate of isomerization, the slower rate of isomerization has no effect on the rate of fermentation.

On the basis of the experimental results described here, acceptable pH and temperature conditions can be chosen for the simultaneous isomerization and fermentation of D-xylose by

TABLE 1. *Xylitol production by bakers' yeast under various fermentation conditions*

Fermentation conditions			Concn of xylitol produced (g/liter)
pH	Temp (°C)	Concn of xylose (g/liter)	
4	30	120	11.0
4	35	120	8.0
4	37	120	7.7
4	40	120	7.6
4	45	120	4.2
5	35	120	9.6
6	35	120	11.4
7	35	120	12.0
4	35	30	2.1
4	35	45	2.5
4	35	60	2.8
4	35	75	4.0
4	35	90	5.5

D-xylose isomerase and yeast. The optimal pH is 6, and the optimal temperature is 37°C. Under these optimal fermentation conditions, a typical fermentation resulted in the production of 0.45 g of ethanol, 0.1 g of xylitol, and 0.02 g of glycerol per g of D-xylulose consumed.

ACKNOWLEDGMENTS

We express our appreciation to Savannah Foods and Industries, Inc., and United Sugar Corp. for sponsoring this research.

LITERATURE CITED

1. **Barnett, J. A.** 1968. Biochemical differentiation of taxa, with special reference to the yeasts, p. 557-595. *In* G. C. Ainsworth and A. S. Sussman (ed.), The fungi, vol. 3. Academic Press, Inc., New York.
2. **Barnett, J. A.** 1976. The utilization of sugars by yeasts. Adv. Carbohydr. Chem. Biochem. **32**:125-234.
3. **Chakravorty, M., L. A. Veiga, M. Bacila, and B. L. Horecker.** 1962. Pentose metabolism in *Candida*. II. The diphosphopyridine nucleotide-specific polyol dehydrogenase of *Candida utilis*. J. Biol. Chem. **237**: 1014-1020.
4. **Chiang, C., and S. G. Knight.** 1960. Metabolism of D-xylose by moulds. Nature (London) **188**:79-81.
5. **Gong, C. S., L. F. Chen, M. C. Flickinger, L. C. Chiang, and G. T. Tsao.** 1981. Production of ethanol from D-xylose using D-xylose isomerase and yeasts. Appl. Environ. Microbiol. **41**:430-436.
6. **Höfer, M. A., A. Betz, and A. Kotyk.** 1971. Metabolism of the obligatory aerobic yeast *Rhodotorula gracilis*. IV. Induction of an enzyme necessary for D-xylose catabolism. Biochim. Biophys. Acta **252**:1-12.
7. **Kotyk, A., and M. Höfer.** 1965. Uphill transport of sugars in the yeast *Rhodotorula gracilis*. Biochim. Biophys. Acta **102**:410-422.
8. **Krouwel, P. G., and L. Barber.** 1979. Ethanol production by yeast at supraoptimal temperatures. Biotechnol. Lett. **1**:403-408.
9. **Krull, L. H., and G. E. Inglett.** 1980. Analysis of neutral carbohydrates in agricultural residues by gas-liquid chromatography. J. Agric. Food Chem. **2**:917-919.
10. **Ladisch, M. R., and G. T. Tsao.** 1978. Theory and practice of rapid liquid chromatography at moderate pressure using water as eluent. J. Chromatogr. **166**:85-100.
11. **Mortlock, R. P., and W. A. Wood.** 1964. Metabolism of pentoses and pentitols by *Aerobacter aerogenes*. I. Demonstration of pentose isomerase, pentulokinase, and pentitol dehydrogenase enzyme families. J. Bacteriol. **88**:838-841.
12. **Nagodawithana, T. W., and K. H. Steinkraus.** 1976. Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in "rapid fermentation." Appl. Environ. Microbiol. **31**: 158-162.
13. **Navarro, J. M., and G. Durand.** 1978. Alcohol fermentation: effect of temperature on ethanol accumulation within yeast cells. Ann. Microbiol. (Inst. Pasteur) **129B**: 215-224.
14. **Osmond, C. B., and T. A. Rees.** 1969. Control of the pentose phosphate pathway in yeast. Biochim. Biophys. Acta **184**:35-42.
15. **Sanchez, S., and K. L. Smiley.** 1975. Properties of D-xylose isomerase from *Streptomyces albus*. Appl. Microbiol. **29**:745-750.
16. **Shamanna, D. K., and K. E. Sanderson.** 1979. Uptake and metabolism of D-xylose in *Salmonella typhimurium* LT 2. J. Bacteriol. **139**:64-70.
17. **Stokes, J. L.** 1971. Influence of temperature on the growth and metabolism of yeasts, p. 114-134. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 2. Academic Press, Inc., New York.
18. **Tomoyeda, M., and H. Horitsu.** 1964. Pentose metabolism by *Candida utilis*. Agric. Biol. Chem. **28**:139-143.
19. **Wang, P. Y., B. F. Johnson, and H. Schneider.** 1980. Fermentation of D-xylose by yeasts using glucose isomerase in the medium to convert D-xylose to D-xylulose. Biotechnol. Lett. **2**:273-278.
20. **Wang, P. Y., C. Shopsis, and H. Schneider.** 1980. Fermentation of a pentose by yeasts. Biochem. Biophys. Res. Commun. **94**:248-254.
21. **Wood, W. A.** 1966. Carbohydrate metabolism. Annu. Rev. Biochem. **35**:521-553.