

Isolation and Characterization of Acetic Acid-Tolerant Galactose-Fermenting Strains of *Saccharomyces cerevisiae* from a Spent Sulfite Liquor Fermentation Plant

TORBJÖRN LINDÉN,* JOHAN PEETRE, AND BÄRBEL HAHN-HÄGERDAL

Applied Microbiology, Chemistry Center, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

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From a continuous spent sulfite liquor fermentation plant, two species of yeast were isolated, *Saccharomyces cerevisiae* and *Pichia membranaefaciens*. One of the isolates of *S. cerevisiae*, no. 3, was heavily flocculating and produced a higher ethanol yield from spent sulfite liquor than did commercial baker's yeast. The greatest difference between isolate 3 and baker's yeast was that of galactose fermentation, even when galactose utilization was induced, i.e., when they were grown in the presence of galactose, prior to fermentation. Without acetic acid present, both baker's yeast and isolate 3 fermented glucose and galactose sequentially. Galactose fermentation with baker's yeast was strongly inhibited by acetic acid at pH values below 6. Isolate 3 fermented galactose, glucose, and mannose without catabolite repression in the presence of acetic acid, even at pH 4.5. The xylose reductase (EC 1.1.1.21) and xylitol dehydrogenase (EC 1.1.1.9) activities were determined in some of the isolates as well as in two strains of *S. cerevisiae* (ATCC 24860 and baker's yeast) and *Pichia stipitis* CBS 6054. The *S. cerevisiae* strains manifested xylose reductase activity that was 2 orders of magnitude less than the corresponding *P. stipitis* value of 890 nmol/min/mg of protein. The xylose dehydrogenase activity was 1 order of magnitude less than the corresponding activity of *P. stipitis* (330 nmol/min/mg of protein).

Our earlier work has shown that *Saccharomyces cerevisiae* in combination with xylose isomerase ferments lignocellulose hydrolysates more efficiently than do the pentose-fermenting yeasts *Pichia stipitis*, *Candida tropicalis*, and *Pachysolen tannophilus* (23). The main purpose of the present study was to find adapted yeasts that were better suited for lignocellulose hydrolysates than the yeasts used in the work mentioned above.

No known yeast fulfills all requirements for industrial-scale fermentation of lignocellulose hydrolysates. The requirements are high productivity and yield, pentose and hexose fermentation, and resistance to inhibitors produced or released during the pretreatment and hydrolysis of lignocellulose (17, 31). Yeasts partly fulfilling these demands are *P. stipitis* (28, 36, 40), *Candida shehatae* (18, 28, 41), and *P. tannophilus* (13). Another approach is to use a highly productive and inhibitor-resistant hexose-fermenting yeast, such as *S. cerevisiae* or *Schizosaccharomyces pombe*. These yeasts cannot ferment xylose, the most common pentose in lignocellulose, but they can ferment the isomer xylulose. Xylose can be isomerized to xylulose and fermented to ethanol, either with a yeast transformed with the gene for xylose isomerase (EC 5.3.1.5) (10) or by adding xylose isomerase to the medium (16, 23, 38).

Inhibition of yeast activity in lignocellulose hydrolysates can be partially overcome by either detoxifying the substrate (11, 28, 37) or adapting the yeast (4, 25) or by using a combination of both (26, 43). One way of adapting yeasts is to recirculate them in an appropriate substrate. Few yeasts can have been more recirculated than those obtained for the present study from a spent sulfite liquor (SSL) fermentation plant at a pulp mill which has been in continuous operation since the 1940s. Commercial baker's yeast, *S. cerevisiae*, is used in such a fermentation, and only the hexose sugars are

fermented, xylose remaining unfermented. Thus, the chances of finding a xylose-fermenting yeast were considered to be much poorer than those of finding a strain of *S. cerevisiae* with high resistance to lignocellulose-derived inhibitors.

Another interesting possibility was that of discovering in this environment (SSL) an *S. cerevisiae* strain with xylose reductase (XR) and/or xylitol dehydrogenase (XDH) activity, XR and XDH being the first two enzymes involved in the xylose metabolism in yeasts and fungi. Although the inability of *S. cerevisiae* to ferment xylose has generally been ascribed to the absence of these enzyme activities, their presence in this yeast has in fact been reported (5). Access to a non-ethanol-producing *S. cerevisiae* strain with one or both of these enzymatic activities would enable the regulatory mechanisms preventing ethanol formation to be studied, and knowledge of these mechanisms might facilitate work on the transformation of *S. cerevisiae* with genes for XR and XDH from other yeasts (15, 19, 35).

MATERIALS AND METHODS

Strains. The yeasts *S. cerevisiae* and *Pichia membranaefaciens* were isolated from SSL. Commercial baker's yeast was purchased from a local distributor. In addition, *S. cerevisiae* ATCC 24860 and *P. stipitis* CBS 6054 were also used.

The strains were maintained on YM agar (see "Isolation media" below)—slants with 10 g (each) of glucose, galactose, and xylose per liter for *S. cerevisiae* and 10 g of xylose per liter for *P. membranaefaciens* and *P. stipitis*.

Sampling. Liquid and solid samples were taken from the fermentation line of a sulfite pulpmill (MoDo, Örnsköldsvik, Sweden), a continuous overflow system with six tanks, the first two being stirred with compressed air and the other four being stirred with pumps. Two liquid samples, one from each of the first two tanks, were pooled as sample 1+2 (L) (Table

* Corresponding author.

TABLE 1. Sample sources and isolation media (isolates 1 to 22)

Fermentation tank	Sample liquid/solid	Isolate no. on:				
		Galactose		Xylose		SSL, pH 6.0
		pH 3.8	pH 4.8	pH 3.8	pH 4.8	
1+2	L	1	6	11	16	
3+4	L	2	7	12	17	21
3+4	S		9	14	19	22
5+6	L	5	8	13	18	
5+6	S	3, 4	10	15	20	

1). From the third and fourth tanks, both liquid (L) and solid (S) samples were taken and separately pooled as sample 3+4 (L) and sample 3+4 (S); the same procedure was followed with the fifth and sixth tanks, yielding sample 5+6 (L) and sample 5+6 (S).

Isolation media. The medium YM contained the following components: yeast extract (Difco, Detroit, Mich.), 3 g/liter; malt extract broth (Difco), 3 g/liter; Bacto Peptone (Difco), 5 g/liter; and xylose (Sigma Chemical Co., St. Louis, Mo.) or galactose (Sigma), 10 g/liter. YM agar consisted of the foregoing YM medium supplemented with 20 g of Bacto Agar (Difco) per liter. SSL supplemented with 3 g of yeast extract (Difco) per liter, 3 g of malt extract broth (Difco) per liter, and 5 g of Bacto Peptone (Difco) per liter was also used as an isolation medium; SSL agar consisted of the foregoing SSL isolation medium supplemented with 20 g of Bacto Agar (Difco) per liter.

Isolation. The samples were used as inocula for enrichment cultures on three different carbon sources: xylose, galactose, and sterile-filtered unfermented SSL. Enrichment cultures with xylose and galactose were used to select for yeasts different from the originally added baker's yeast with respect to fermentation of xylose and galactose. SSL was used to select for yeasts that grew well on the substrate it should ferment. Xylose and galactose were used at pHs 3.8 and 4.8, respectively, and SSL was used at pH 6.0. In all enrichment and isolation studies, YM broth or YM agar without glucose was used. The enrichment cultures were incubated at 30°C on a rotary shaking water bath. From the 25 enrichment cultures, agar plates with the same media as for the enrichment cultures were plated every 24 h for a week or until growth was detected. From each sample, the largest well-isolated colony was replated twice on enrichment medium and microscopically examined for bacterial contamination. Isolates were then transferred to agar slants of enrichment medium and stored at 4°C.

Fermentation medium. For fermentation purposes, SSL was supplemented with the following components: yeast extract (Difco), 2.5 g/liter; $(\text{NH}_4)_2\text{HPO}_4$, 0.25 g/liter; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 g/liter.

Identification. After transfer to YM agar slants with glucose, the yeast isolates were sent to the National Collection of Yeast Cultures (Norwich, United Kingdom) for identification.

Screening of fermentation in SSL. One loopful of each isolate was added to 1.5 ml of sterile-filtered SSL supplemented with medium components. The fermentations were performed in filled and sealed 1.5-ml glass flasks provided with an outlet tube to lead evolved carbon dioxide into water. The temperature was maintained at 30°C by a thermostat. A pure culture of commercial baker's yeast (replated twice and maintained on YM agar with glucose, galactose, and xylose) was used for comparison.

Cell mass production. Cell mass was produced in a medium containing the following: yeast extract (Difco), 3 g/liter; malt extract broth (Difco), 3 g/liter; Bacto Peptone (Difco), 5 g/liter; KH_2PO_4 , 19 g/liter; $(\text{NH}_4)_2\text{HPO}_4$, 3 g/liter; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1 g/liter; and 10 g (each) of xylose, galactose, and glucose per liter. The cells were harvested in late log phase by centrifugation ($14,000 \times g$, 10 min, 4°C; Beckman model J2-21) and washed with sterile 0.9% NaCl solution.

XR and XDH activities. Inocula (100 ml) were prepared by growing the cells in aerobic conditions as overnight cultures in growth medium for cell mass production. For *P. stipitis*, 30 g of xylose per liter only was used. For preparation of cell extracts, two to four 1-liter shake flasks containing 500 ml of growth medium were inoculated with 10 to 20 ml from the inoculum culture. All cultures were incubated on a rotary shaker at 30°C. The cells were harvested in late log phase, and 10 g (wet weight) of cells was suspended in a total volume of 25 ml of disintegration buffer (0.1 M phosphate buffer, 5 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.0) and freeze-pressed twice with an X-press (Biox). Cell debris was removed by centrifugation ($15,000 \times g$, 15 min), and the clear supernatant was stored frozen (-80°C) until used for enzymatic assays (within days).

NADPH-dependent XR activity and NAD^+ -dependent XDH activity were measured spectrophotometrically following the oxidation-reduction of the cofactors at 340 nm. The XR assay mixture contained 0.7 ml of 0.1 M phosphate buffer, pH 7.0, 0.1 ml of 1.5 mM NADPH, 0.1 ml of 0.1 M D-xylose, and 0.1 ml of sample (34). For the measurement of nonspecific activity, xylose was replaced by phosphate buffer. The XDH assay mixture contained 0.7 ml of 0.1 M phosphate buffer, pH 7.0, 0.1 ml of 50 mM NAD^+ , 0.1 ml of 0.5 M xylitol, and 0.1 ml of the sample (34). For the measurement of nonspecific activity xylitol was replaced by phosphate buffer. Enzyme units were defined as nanomoles of cofactor oxidized or reduced per minute at 24°C. Specific activities were defined as units per milligram of protein. Protein concentrations were determined by the method of Bradford (8) with bovine serum albumin fraction V (Sigma) as the standard.

Modified method of XR and XDH activities. Because with the method outlined above it was not possible to obtain linearity with respect to enzyme concentration in the assay, the method was modified by increasing the final substrate concentration to 0.33 M for xylose and to 1 M for xylitol (32).

Fermentation of SSL. The SSL sugar content was not the same in all fermentations, as different amounts—80 or 300 g/liter (wet weight)—of yeast cells were used, which resulted in dilution of the substrate. The initial sugar content for each fermentation is given in the footnotes of Tables 3 and 4.

The cell masses of isolate 3 and baker's yeast were produced on growth medium for cell mass production. Fermentations were made in 25-ml flasks filled to the neck and sealed with a rubber stopper, equipped with a cannula for carbon dioxide removal. The flasks were incubated at 30°C in a water bath equipped with a multimagnetic stirring plate. Centrifuged and washed wet cells (2 g [80 g/liter]), fermentation medium components, 0.25 g of an immobilized xylose isomerase (XI) preparation of *Lactobacillus brevis* (DSM 20054) (24), 50 μl of 0.25 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 100 μl of 0.9 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added to SSL, the pH of which had been adjusted to 6.0 with 10 M KOH. The addition of yeast and medium components caused a minor decrease of the pH, which was readjusted to 6.0 with 1 M KOH. The total volume was 25 ml. Fermentations were made with and without pH control.

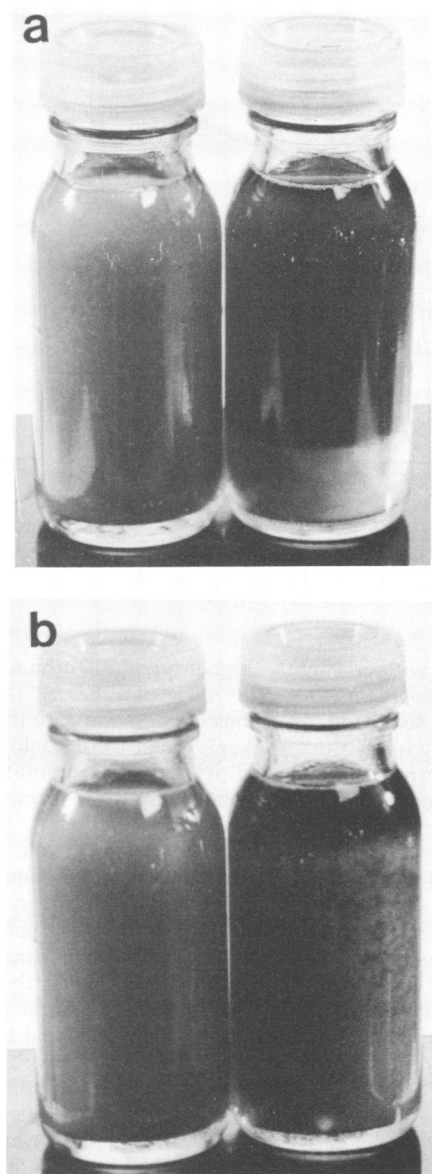


FIG. 1. Flocculation in SSL. Baker's yeast (left) and isolate 3 (right) after 30 min without stirring (a) and with magnetic stirring (b).

The pH optimum for the combination of XI and isolate 3 was determined within the pH range of 4.5 to 8.0. The pH was adjusted with 10 M KOH and readjusted with 1 M KOH each time a sample was taken.

Recirculation of yeasts in SSL. Cells of isolate 3 and baker's yeast were grown, harvested, and used as described above except that 9.1 g of wet yeast (300 g/liter), 1.14 g of XI, 200 μ l of 0.25 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 400 μ l of 0.9 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were used. The total volume here was 30 ml, and the pH was adjusted to 5.5, being readjusted with 1 M KOH each time a sample was taken. Five repeated 48-h fermentations were made in centrifuge tubes equipped with magnetic stirring rods and rubber stoppers with cannulae. The cells and the XI were recovered by centrifugation after each 48-h fermentation and weighed, and new SSL with medium components was added.

Acetic acid tolerance. The medium used to assay acetic

TABLE 2. XR and XDH activities

Species	Isolate no.	Activity (nmol/min/mg of protein) of:	
		XR	XDH
<i>S. cerevisiae</i>	3	1.1 (12 ^a)	0.4 (22 ^a)
<i>S. cerevisiae</i>	10	0.8 (9 ^a)	0.4 (26 ^a)
<i>P. membranaefaciens</i>	12	5.1	73
<i>P. membranaefaciens</i>	16	5.6	86
<i>P. membranaefaciens</i>	21	6	275
<i>P. membranaefaciens</i>	22	12	56
<i>S. cerevisiae</i> ATCC 24860		0.9	0
Commercial baker's yeast		1.3 (10 ^a)	0.4 (22 ^a)
<i>P. stipitis</i> CBS 6054		332 (890 ^b)	615 (330 ^b)

^a Values obtained with the modified method (see Materials and Methods).

^b Values from Skoog and Hahn-Hägerdal (32) (modified method).

acid tolerance consisted of the following: xylose, 10 g/liter; galactose, 7 g/liter; mannose, 16 g/liter; arabinose, 4 g/liter; yeast extract (Difco), 2.5 g/liter; $(\text{NH}_4)_2\text{HPO}_4$, 0.25 g/liter; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/liter. The nutrients and sugars were suspended in 0.1 M citrate buffer, pH 4.5 or 6, acetic acid being added at 4 or 8 g/liter. The yeasts were grown as described above for the fermentation of SSL, 10 g (dry weight) of yeasts per liter being used; fermentation conditions were also the same as in SSL fermentation.

Analyses. All sugars and fermentation products were analyzed on a Shimadzu high-pressure liquid chromatograph equipped with a refractive index detector. Ethanol, xylitol, glycerol, and acetic acid were analyzed with a Bio-Rad HPX 87-H column at 65°C, with 0.005 M H_2SO_4 as the eluent at a flow rate of 0.6 ml/min. Sugars were measured with a Bio-Rad HPX 87-P column at 85°C, with water as the eluent at a flow rate of 0.6 ml/min. Xylulose and arabitol were measured as described previously (22).

RESULTS

Enrichment and isolation. From 25 enrichment cultures 22 isolates of yeast were obtained (Table 1). Isolates were obtained on galactose and xylose at pH 3.8 and pH 4.8. On SSL, pH 6, only two of the five samples yielded isolates.

Fermentative screening of isolates. The 22 isolates and baker's yeast were then used to ferment SSL at low cell density. Isolates 3 and 10 from galactose, at pH 3.8 and 4.8, respectively, produced more ethanol than did baker's yeast; these isolates originated from a sample taken from the walls of fermentation tanks 5 and 6, which are at the end of the fermentation line. In addition, isolate 3, which was sampled from the wall of a fermentation tank, showed extreme flocculation at all stages of growth (Fig. 1). In general, isolates from the xylose and SSL media were poorly fermenting.

Identification. The best ethanol-producing isolates (no. 3, 10, 12, and 16) from each enrichment substrate and both isolates (no. 21 and 22) from the SSL enrichment were identified (Tables 1 and 2). Isolates 3 and 10, originating from the walls of the fermentation tanks 5 and 6 and isolated on galactose, were identified as *S. cerevisiae*; both gave a better ethanol yield than did baker's yeast. The others were identified as *P. membranaefaciens* and produced much less ethanol than did baker's yeast.

XR and XDH activities. XR and XDH activities were measured both in the identified isolates and in the strains of *S. cerevisiae* and *P. stipitis* (Table 2). XR activity was

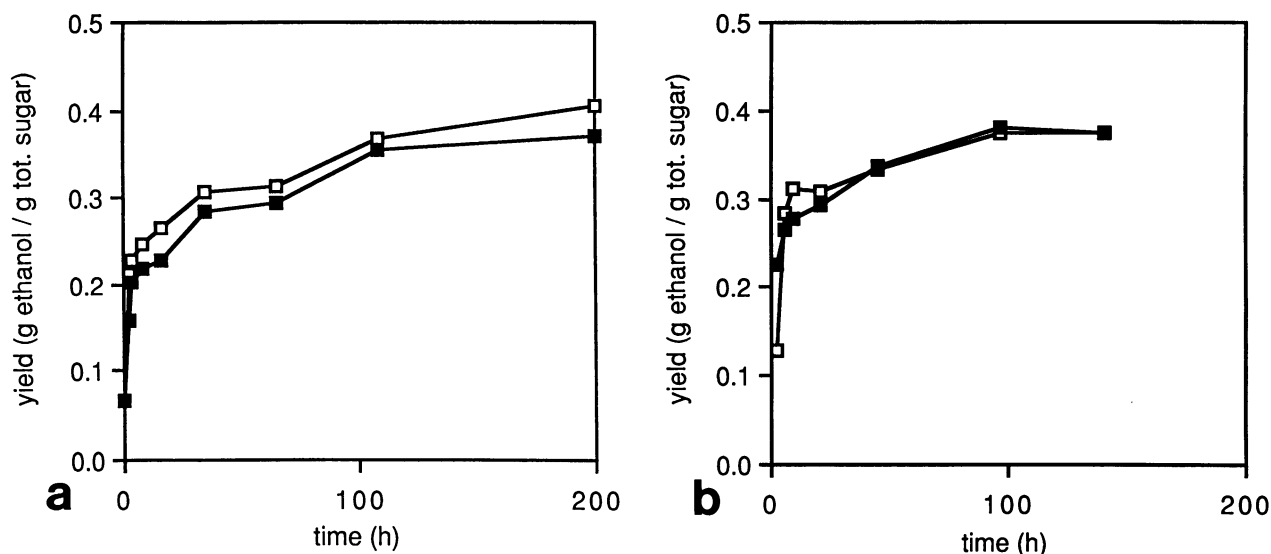


FIG. 2. Fermentation of SSL without pH control (a) and at pH 6 (b). Ethanol yield with baker's yeast (■) and isolate 3 (□).

considerably (2 orders of magnitude) lower both in the isolates and in the *S. cerevisiae* strains than the corresponding *P. stipitis* value (332 nmol/min/mg of protein). XDH activity was found in all isolates, the value for isolate 21, *P. membranaefaciens*, being half that of *P. stipitis*. By contrast, XDH activity was hardly detectable in either of the *S. cerevisiae* strains (being 3 orders of magnitude less than the *P. stipitis* value of 615 nmol/min/mg of protein). With a modified assay procedure (32), increased values both of XR and XDH activities were obtained for *S. cerevisiae* (Table 2). These activities agreed well with earlier reported activities (5), except for a factor of 1,000. The unit used in the present investigation is nanomoles per minute per milligram of protein. In the earlier investigation the unit was micromoles per minute per milligram of protein.

Fermentation of SSL. Isolates 3 and 10 and baker's yeast were compared with respect to ethanol fermentation in SSL, pH 6, with and without pH control. A gelatin-immobilized whole-cell preparation of *L. brevis* containing the enzyme XI (24) was added to the fermentations of SSL to supply the yeast with fermentable xylulose. In Fig. 2, only the ethanol yields of isolate 3 and baker's yeast are shown. All yields are calculated on total available sugar. Isolate 10 produced more ethanol than did baker's yeast but less than isolate 3.

Without pH control (final pH of 5.3), isolate 3 fermented

the SSL with a slightly higher yield of ethanol than did baker's yeast (Fig. 2a). With pH kept at 6.0, no difference in yield was seen (Fig. 2b). These results indicate that isolate 3 was less sensitive than baker's yeast to pH below 6.

To determine the optimal pH for the fermentation of SSL with the combination of isolate 3 and XI, fermentations of SSL were made at pHs 4.5, 5, 5.5, 6, 7, and 8. The optimal pH for ethanol production was around 5.5 (Table 3), whereas both total sugar consumption and xylose consumption were optimal at pH 6. However, the production of xylitol, arabitol, glycerol, and acetic acid increased with increasing pH, which lowered the ethanol yield. At pH values below 6, total sugar and xylose consumption decreased but the formation of by-products also decreased. The decrease in yield at low pH can be attributed to the pH dependence of the XI (24). The differences in total consumed sugar between pH 6 and pHs 5.5, 5, and 4.5 were 1.8, 7.8, and 9.6 g/liter, respectively, the corresponding residual xylose values being 2.2, 7.8, and 9.0, respectively. Therefore, the lower ethanol yield with baker's yeast, compared with that of isolate 3, in the fermentation without pH control must be due to some other factor.

Acetic acid tolerance. The pH tolerance in the presence of acetic acid was investigated in greater detail. To ascertain the effect of acetic acid alone on fermentation, isolate 3 and

TABLE 3. Optimal pH for isolate 3 and XI in SSL^a

pH	Sugar consumption (g/liter)		Accumulated xylulose (g/liter)	Product formation (g/liter) of:				Maximum ethanol concn [g/liter (h)]	Maximum ethanol yield (g/g of total sugar)
	Total	Xylose		Xylitol	Arabitol	Glycerol	Acetic acid		
4.5	27.7	2.2	0	0.6	0	2.0	0.5	13.0 (88)	0.32
5.0	29.5	3.4	0	1.7	0	2.0	0.9	15.0 (145)	0.37
5.5	35.5	9.0	0	2.0	0	2.9	1.0	16.4 (145)	0.40
6.0	37.3	10.1	0	1.8	1.2	3.6	1.3	15.0 (145)	0.37
7.0	36.6	9.8	0	2.0	2.6	4.2	1.9	12.0 (265)	0.30
8.0	36	8.4	0.14	2.2	3.2	5.0	2.4	9.6 (265)	0.24

^a Sugar content in SSL (per liter): Glu, 4.6 g; Man, 17.7 g; Gal, 5.6 g; Xyl, 11.2 g; Ara, 1.9 g; and total, 40.6 g.

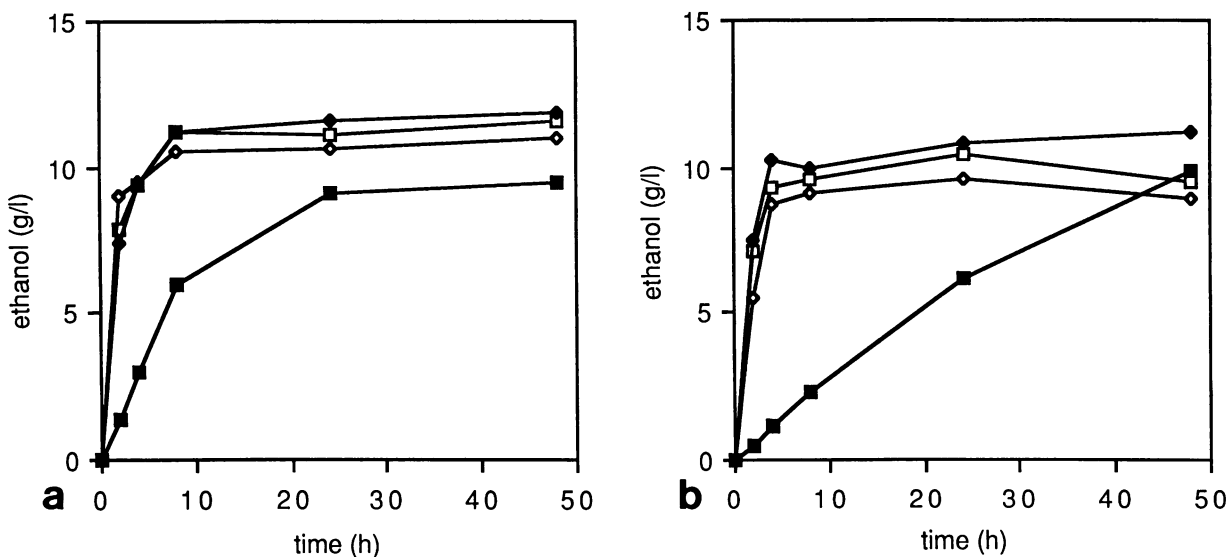


FIG. 3. Acetic acid tolerance of baker's yeast (a) and isolate 3 (b) in a sugar medium. Ethanol concentration at pH 4.5 with 0 g (□) or 8 g (■) of acetic acid per liter and at pH 6 with 0 g (◇) or 8 g (◆) of acetic acid per liter.

baker's yeast were studied at pHs 4.5 and 6 in a sugar (glucose, mannose, galactose, and xylose) medium with different amounts of acetic acid (4 and 8 g/liter) added. The amounts of acetic acid and sugars were chosen to approximate those occurring in a lignocellulose hydrolysate such as SSL.

Without added acetic acid, ordinary baker's yeast produced more ethanol than isolate 3, both at pH 4.5 and at pH 6.0 (Fig. 3).

At pH 4.5 the addition of acetic acid resulted in slower ethanol production (Fig. 3). For clarity, only the values for 0 and 8 g of added acetic acid per liter are shown in the figures, but those for 4 and 8 g/liter were similar. Ethanol formation was slower with isolate 3 than with baker's yeast but reached the same concentration as when no acetic acid was added. This was not the case with the initially better baker's yeast, for which the final concentration of ethanol was decreased with increasing amounts of acetic acid (Fig. 3a); the decrease was so large that the ethanol production of isolate 3 became

as good as or even a shade better than that of baker's yeast (Fig. 3b).

The sugar utilization curves (Fig. 4) for baker's yeast and isolate 3 at pH 4.5 with 0 and 8 g of added acetic acid per liter show that the galactose consumption rate of isolate 3 was unaffected by acetic acid both at pH 4.5 and at pH 6. Again, only the values for pH 4.5 with 0 and 8 g of acetic acid per liter are presented. The sugar consumption rates at pH 4.5 without acetic acid were similar for baker's yeast and isolate 3. Glucose and mannose were consumed first, and then galactose was consumed. However, adding acetic acid made isolate 3 ferment glucose, mannose, and galactose simultaneously with no great difference in rates. Adding acetic acid to baker's yeast at pH 4.5 resulted in almost total inhibition of galactose fermentation, but the glucose and mannose consumption rates were less affected than were those of isolate 3. The 2-g/liter difference in final ethanol concentration between 0 and 8 g of acetic acid, pH 4.5, per liter for

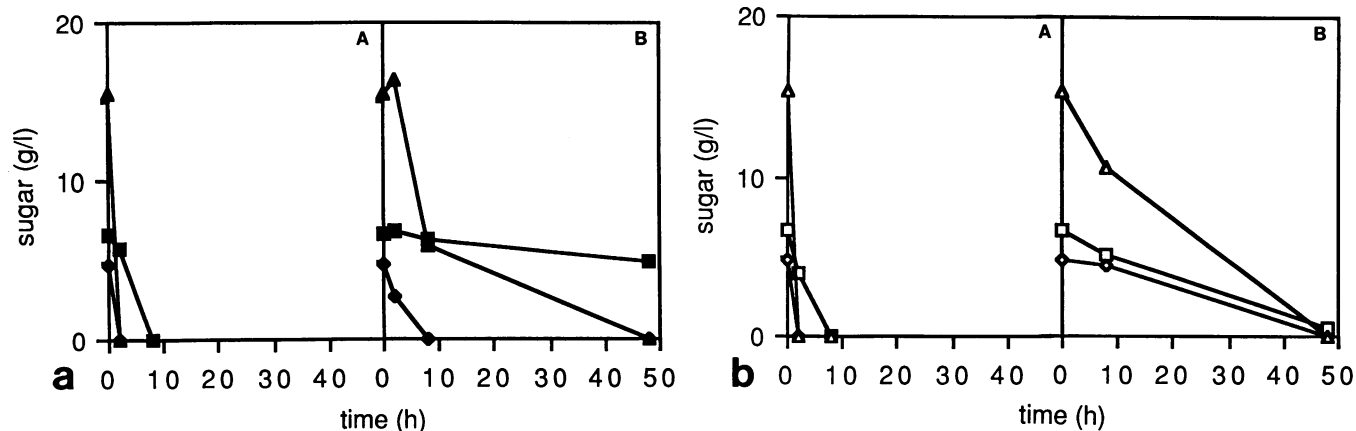


FIG. 4. Acetic acid tolerance of baker's yeast (a) and isolate 3 (b) in a sugar medium with 0 (A) and 8 (B) g of acetic acid per liter (pH 4.5). Sugar consumption: glucose (◆ and ◇), mannose (▲ and △), and galactose (■ and □).

TABLE 4. Recirculation of isolate 3 and baker's yeast in SSL (pH 5.5)^a

Yeast and recirculation no.	Xylose (g/liter)		Accumulated xylulose (g/liter)	Product formation (g/liter) of:			
	Consumed	Residual		Xylitol	Glycerol	Acetic acid	Ethanol
Baker's yeast							
1	7.2	2.4	0.15	7.5	1.4	0.6	10.6
2	7.7	2.4	0.24	1.7	1.2	1.0	10.8
3	7.3	3.0	0.38	2.3	1.5	0.7	10.6
4	4.7	4.7	0.65	1.5	1.3	0.2	11.4
5	5.7	5.2	0.99	1.2	2.2	0.4	11.7
Isolate 3							
1	7.1	2.5	0.02	1.6	2.0	1.0	12.2
2	8.7	2	0.15	1.2	2.9	1.0	14.0
3	7.2	3.4	0.31	1.1	2.6	0.8	11.7
4	5.5	5.3	0.60	1.0	2.7	0.7	12.5
5	5.5	5.7	0.59	1.1	3.1	0.4	13.0

^a Sugar content in SSL (per liter): Glu, 3.6 g; Man, 14 g; Gal, 4.5 g; Xyl, 9.1 g; Ara, 1.4 g; and total, 32.6 g.

baker's yeast approximately matched the 5-g/liter value of unfermented galactose.

Recirculation of yeasts in SSL. Recirculation of baker's yeast and isolate 3 in the presence of XI was investigated to compare the long-term performance of the two yeast strains. To minimize contamination of the fermentations, a large cell mass, 300 g (wet weight) of yeast per liter, was used. In the first fermentation, SSL was diluted with a pure yeast cell mass and immobilized XI. In subsequent recirculations, the centrifuged pellet was not washed but contained both unconsumed sugars and fermentation products. Table 4 shows the substances actually consumed and produced in each recirculation, with correction for the composition of each pellet. After each recirculation, the amounts of sugars and products in the pellet were estimated by weighing, subtracting the dry weight of yeast and enzyme, and estimating the volume that contained sugars and products.

In all recirculations, isolate 3 produced more ethanol than did baker's yeast. There were also differences in the product formation (Table 4). Baker's yeast produced slightly more xylitol but less glycerol than isolate 3. The fermentation rate of isolate 3 was not significantly affected by recirculation (Fig. 5), whereas the ethanol production of baker's yeast was considerably retarded after five recirculations.

Xylulose accumulation was higher for baker's yeast in all five recirculations, especially the last one (Table 4), indicating that xylose utilization was not limited by the XI and that isolate 3 fermented xylulose better than did baker's yeast.

The fourth recirculation was also studied with respect to sugar utilization (Fig. 6). The greatest difference between baker's yeast and isolate 3 was the galactose consumption. All the hexose sugars were consumed simultaneously and at quite similar rates by isolate 3, whereas baker's yeast consumed glucose and mannose only, leaving galactose unfermented. A similarity between acetic acid tolerance and recirculation data is that a major part of the difference between isolate 3 and baker's yeast in produced ethanol (1.1 g/liter), glycerol (1.4 g/liter), and acetic acid (0.5 g/liter) in the fourth recirculation (Table 4) could be attributed to the corresponding difference in galactose utilization, 3 g/liter.

DISCUSSION

The isolation procedure resulted in two kinds of isolates, either adapted strains of the *S. cerevisiae* originally added

(isolated on galactose) or contaminating yeasts (isolated on xylose). No xylose-fermenting yeasts were isolated. The hexose-fermenting yeasts isolated on xylose were all identified as *P. membranaefaciens*, which assimilates xylose without producing ethanol. Isolates 3 and 10 were isolated from solid samples taken from the walls of fermentation tanks 5 and 6. It is to be expected that galactose isolates show up late in the fermentation line, as the fermentation rate on galactose is generally lower than the fermentation rates on glucose and mannose (1, 43). Isolate 3 was heavily flocculating, a property which facilitates recirculation in large-scale fermentation processes (27).

Batt et al. (5) have reported strains of *S. cerevisiae* that manifest XR and XDH activity. It might be expected to find an *S. cerevisiae* strain with either or both of these activities in an SSL fermentation plant where the yeasts had been in contact with xylose for almost 50 years. However, our results on XR and XDH activity were a factor of 1,000 lower than the activities reported by Batt et al. (5).

At a pH below 6 and in the presence of acetic acid, isolate

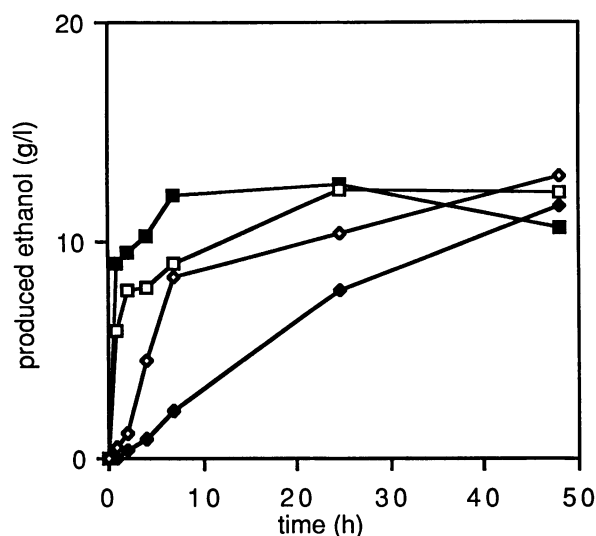


FIG. 5. Recirculation of yeasts in SSL. Ethanol production in baker's yeast fermentations 1 (■) and 5 (◆) and isolate 3 fermentations 1 (□) and 5 (◇).

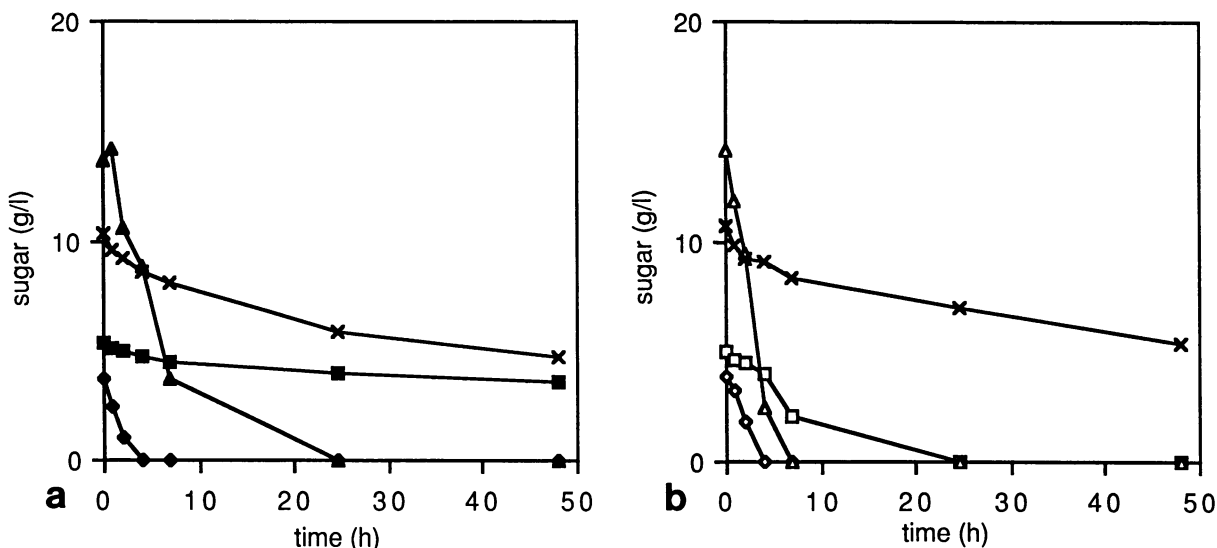


FIG. 6. Recirculation of baker's yeast (a) and isolate 3 (b) in SSL. Sugar consumption: xylose (x), glucose (◆ and ◇), mannose (▲ and △), and galactose (■ and □).

3 produced more ethanol than did baker's yeast. The difference could be ascribed to galactose fermentation which was inhibited in baker's yeast. The pH affects the yield of ethanol. At pHs above 6 the yield is reduced by production of polyols and acetic acid (30). On the other hand, when the pH approaches or passes the pK_a (4.76) for acetic acid, the yield of ethanol decreases when acetic acid is present. Acetic acid, which is present in lignocellulose hydrolysates (6, 37, 40), is a well-known antimicrobial compound which in its protonated form can penetrate the cell membrane (7) and dissociate, which decreases the intracellular pH. This pH decrease is counteracted by an ATP-consuming transport of hydrogen ions out of the cell (33, 39).

There are similarities in sugar utilization between isolate 3 and baker's yeast on one hand and mutants of *S. cerevisiae* described by Bailey et al. (3) on the other hand (Table 5). These mutants were derived from a parent strain which did not ferment galactose. The mutants fell into two general phenotypic classes. The first class showed catabolite repression and fermented glucose and galactose sequentially. The second class was resistant to catabolite repression and fermented glucose and galactose simultaneously. In the absence of acetic acid, both baker's yeast and isolate 3 utilized glucose and galactose sequentially, i.e., showed catabolite repression like the mutants belonging to the first class (Table 5). The presence of acetic acid in baker's yeast fermentations resulted in an inhibition of galactose fermentation resembling that of the parent strain of the mutants. For isolate 3, the presence of acetic acid changed the sugar

utilization so that it lost catabolite repression and fermented glucose and galactose simultaneously, which resembled the second class of mutants. The inhibition of galactose fermentation in baker's yeast at low pH and in the presence of acetic acid might be because the intracellular pH decreases and might affect (i) the sugar transport systems (14, 29) or galactose permease (20, 21), (ii) the three initial enzyme steps in galactose catabolism, or (iii) regulation of gene expression (9, 42).

There is probably also a decreased intracellular pH in isolate 3. However, the decrease could be less pronounced than in baker's yeast. There are at least three different mechanisms that could counteract a decrease in intracellular pH: (i) simultaneous sugar utilization, (ii) flocculation, and (iii) thermotolerance.

The ability to change from catabolite repression to simultaneous glucose and galactose utilization when the intracellular pH decreases could be an adapted trait selected for in the SSL fermentation. A simultaneous sugar utilization would give a higher rate of ATP production and hence a higher rate of proton efflux, which would counteract a further pH decrease.

Isolate 3 was heavily flocculating; the aggregation of cells in flocs or the changed properties of the cell wall might decrease the diffusion of acetic acid into the cells, similar to the decreased diffusion rates obtained with immobilized cells (2). This could result in a higher intracellular pH for isolate 3 compared with that for baker's yeast.

There could have been a selection for thermotolerant

TABLE 5. Galactose fermentation by mutants of *S. cerevisiae*, baker's yeast, and isolate 3

Phenotypic fermentation characteristic(s)	Strains exhibiting phenotype		
	Mutants of <i>S. cerevisiae</i> (3)	Baker's yeast (present study)	Isolate 3 (present study)
Gal ⁻	Parent strain	With HAc ^a	
Gal ⁺ , sequentially	First class of mutants (catabolite repression)	Without HAc	Without HAc
Gal ⁺ , simultaneously	Second class of mutants (resistant to catabolite repression)		With HAc

^a HAc, acetic acid.

strains in the last uncooled tanks of the SSL fermentation plant, which hold at around 35°C. Temperatures above optimum presumably affect the cell membrane, making it more permeable to protons, which led Coote et al. (12) to postulate that increased thermotolerance was due to an increased ability to maintain normal intracellular pH values. The somewhat elevated temperatures at the end of the fermentation line could therefore result in a selection for strains with an improved ability to maintain a normal intracellular pH.

Therefore, the decrease in intracellular pH might be less in isolate 3 than in baker's yeast. It may not be large enough to induce inhibition of galactose fermentation observed with baker's yeast but may be enough to affect, directly or indirectly, the gene regulation only, since the change seen was a shift from catabolite repression to constitutive galactose fermentation.

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