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High-Efficiency Carbohydrate Fermentation to Ethanol at Temperatures above 40°C by *Kluyveromyces marxianus* var. *marxianus* Isolated from Sugar Mills

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A number of yeast strains, isolated from sugar cane mills and identified as strains of *Kluyveromyces marxianus* var. *marxianus*, were examined for their ability to ferment glucose and cane syrup to ethanol at high temperatures. Several strains were capable of rapid fermentation at temperatures up to 47°C. At 43°C, >6% (wt/vol) ethanol was produced after 12 to 14 h of fermentation, concurrent with retention of high cell viability (>80%). Although the type strain (CBS 712) of *K. marxianus* var. *marxianus* produced up to 6% (wt/vol) ethanol at 43°C, cell viability was low, 30 to 50%, and the fermentation time was 24 to 30 h. On the basis of currently available strains, we suggest that it may be possible by genetic engineering to construct yeasts capable of fermenting carbohydrates at temperatures close to 50°C to produce 10 to 15% (wt/vol) ethanol in 12 to 18 h with retention of cell viability.

Sugar cane (as chips, juice, or molasses) and cassava are important raw materials for the production of ethanol from renewable energy resources (21). The tropical or subtropical nature of these two crops and the necessity of situating process plants close to the source of fermentable substrate to offset prohibitive transport costs make the development and application of tropical fermentation technology a prerequisite for the commerical exploitation of these crops in ethanol production. Considerable difficulties are associated with fermentation in tropical areas owing to the lack of heat tolerance in conventional industrial yeast strains. These difficulties include high ambient temperatures, especially in the summer, coupled with an exothermic fermentation reaction, the compound effect of which leads to inhibition of yeast fermentation ability. Furthermore, competition from heat-tolerant contaminating bacteria and low-ethanol-producing wild yeast strains may be allowed at such temperatures. Alternatively, lower fermentation temperatures, 35°C or below, may be achieved by refrigerated temperature control, albeit at considerable capital and running costs.

It is clear, therefore, that tropical fermentation technology, as applied to ethanol fermentation by yeasts, requires strains capable of substrate conversion at high efficiency at temperatures above 40°C.

There are surprisingly few publications on high-temperature fermentation by yeasts. Hacking et al. (6) recently evaluated several strains of *Kluyveromyces*, *Candida*, and *Saccharomyces* spp. for glucose fermentation to ethanol at 40°C and concluded that yeast strains with promising commercial performance at 40°C could be isolated. A major drawback was that high concentrations of ethanol (6 to 7% [wt/vol]) were only achieved after fermentation for 48 to 72 h. Much faster rates of fermentation were reported by Hughes et al. (9) for a strain of *Kluyveromyces marxianus* utilizing glucose as substrate at temperatures above 40°C. A novel approach, involving protoplast fusion, was used by Seki et al. (23) to genetically engineer a *Saccharomyces* hybrid capable of rapid ethanol production (6% [wt/vol]) after fermentation at 24 h at 42°C.

There are distinct advantages in high-temperature (40 to 50°C) fermentation by yeasts. These include faster rates of substrate consumption and product (ethanol) formation, facilitation of ethanol recovery, and considerable savings on capital and running costs of refrigerated temperature control. On the other hand, it is generally accepted that the inhibitory effect of ethanol increases with increasing temperature (17, 18, 22).

In the work described in the present report, a detailed study was undertaken on high-temperature ethanol fermentation by naturally occurring yeast strains isolated from Australian sugar cane mills. We report the isolation and characterization of naturally occurring strains of *K. marxianus* var. *marxianus* which ferment carbohydrate to ethanol at efficiencies between 85 and 90% at temperatures up to 47°C.

MATERIALS AND METHODS

Yeast strains. Wild yeast strains were isolated from Mackay district sugar mill process streams which had a temperature range of 40 to 70°C. A loopful of the mill sample was streaked onto plates containing wort agar (50 g per liter; Oxoid Ltd., London, England) medium containing 50 mg of aureomycin and 2 mg of actinomycin per liter (4), and the plates were incubated at 45°C for 2 to 3 days. Purified cultures were freeze-dried and stored at 4°C.

Type strains were obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, and are listed in Table 1.

Growth of organisms. Stock cultures were maintained on slopes containing (per liter) 10 g of yeast extract, 5 g of peptone, 3 g of $(NH_4)_2SO_4$, 3 g of KH_2PO_4, 25 mg of $CaCl_2 \cdot 2H_2O$, 25 mg of $MgSO_4 \cdot 7H_2O$, 25 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.), and either 100 g of glucose or sugar cane syrup (14% total sugars; Pleystowe Mill, Mackay) as carbon source. Liquid medium was as above but without the agar and contained glucose at concen-

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TABLE 1. Fermentation screen[a] of various strains in order of decreasing ethanol production after 24 h

Strain no.	Glucose[b] (% [wt/vol])	Ethanol production	
		% (wt/vol)	% Theoretical yield[c]
K. marxianus var. *marxianus* 974	0.39	6.26	85.6
K. marxianus var. *marxianus* 972	1.11	6.07	87.3
K. marxianus var. *marxianus* 469	1.17	5.88	85.0
K. marxianus var. *marxianus* 970	1.56	5.86	87.2
K. marxianus var. *marxianus* 467	1.51	5.80	85.9
K. marxianus var. *marxianus* 977	1.21	5.77	83.6
K. marxianus var. *marxianus* 951	1.97	5.74	88.0
K. marxianus var. *marxianus* 982	2.15	5.58	88.3
K. marxianus var. *marxianus* 976	1.45	5.68	83.8
K. marxianus var. *marxianus* 957	2.17	5.63	87.7
K. marxianus var. *marxianus* 973	1.87	5.61	85.4
K. marxianus var. *marxianus* 954	2.34	5.54	87.5
K. marxianus var. *marxianus* 985	2.10	5.53	85.7
K. marxianus var. *marxianus* 953	2.21	5.50	85.4
K. marxianus var. *marxianus* CBS 712	4.50	4.16	79.2
K. marxianus var. *marxianus* CBS 397	4.67	4.06	78.5
K. marxianus var. *bulgaricus* CBS 2762	10.43	1.08	47.6
K. marxianus var. *lactis* CBS 683	11.40	0.53	29.4
K. wickerhamii CBS 2745	11.95	0.26	16.7

[a] Fermentation was determined at 45°C with glucose (15% [wt/vol]) as substrate.

[b] Glucose remaining after 24 h.

[c] A molar reaction stoichiometry of glucose to ethanol to CO_2 of 1:2:2 has been assumed for a theoretical yield.

trations indicated above. Simulated cane juice (15.5 to 22° Bx Bx syrup) contained (per liter) 10 g of yeast extract, 5 g of peptone, 3 g of $(NH_4)_2SO_4$, 3 g of KH_2PO_4, and concentrated sugar cane syrup. The pH of the liquid medium was 5.4 to 5.5. Cells were grown aerobically in a temperature-controlled orbital or, less frequently, in a reciprocal shaker operating at 100 to 120 rpm. Starter cultures were grown in 125-ml flasks (30 ml of media) to late-exponential phase and used as a 10% inoculum for duplicate 100-ml (final volume) portions of medium in 250-ml flasks. Growth temperatures were as indicated above.

Identification of organisms. The standard identification procedures used in yeast taxonomy were followed (1, 2, 11, 14). The CLUSTAN numerical taxonomy program of Wishart (33) showed that all wild-type strains listed in Table 1 clustered closely with *K. marxianus* var. *marxianus* CBS 712 and CBS 397 (P. J. Anderson, M.Sc. thesis, James Cook University of North Queensland, Townsville, Australia, 1986).

Determination of viable cells. Cell viability was determined by a modification of the methylene blue procedure of Lee et al. (13). A 2- to 5-ml portion of test culture was added to 5 ml of methylene blue (0.03%, wt/vol) in Ringer solution containing (per liter) 9 g of NaCl, 0.42 g of KCl, 0.48 g of $CaCl_2 \cdot 6H_2O$, 0.2 g of $NaHCO_3$, and 10 g of glucose. The mixture was vortexed for 5 s and allowed to stand for 5 to 10 min. A Neubauer improved, bright-line-counting chamber (Weber International, U.K.) was used to count percent viable cells by taking clear cells as alive and stained (blue) cells as dead. A total of 100 to 300 cells were counted for each determination.

Measurement of ethanol and substrate consumption. Ethanol and glucose were routinely determined by enzymatic analysis (Sigma Chemical Co., St. Louis, Mo.) or by high-pressure liquid chromatography (Waters Associates, Inc., Milford, Mass.). Degassed, 0.2-μm filtered glass-distilled H_2O was used as the eluant (0.8 ml/min) for Dextro-Pak and C_{18} Rad-Pak (Waters) columns operating at 25°C. A Waters model 450 variable wavelength detector in the refractive index mode was used. Glucose, fructose, and sucrose were determined on a Shodex S-801 ion exchange column (Showa Denko, Tokyo, Japan) operating at 50°C with H_2O as the eluant (0.8 ml/min). All samples were filtered (0.2 μm) before dilution and analyses. The data reported represent the mean of duplicate determinations. Standards consisted of Analar-grade sugars and redistilled ethanol.

RESULTS

High-temperature (45°C) fermentation screen. Thirty-five yeast strains isolated from sugar mills were screened for the ability to ferment glucose (15% [wt/vol]) to ethanol at 45°C in 24 h. Five type strains were also screened for comparison. Table 1 summarizes the results obtained for the high-temperature screen.

Of the 35 strains, 14 produced >5.5% (wt/vol) ethanol after 24 h at 45°C and the remaining 21 strains produced <5.5% (wt/vol) ethanol (results not shown). *K. marxianus* var. *marxianus* CBS 712 and CBS 397 were the only type strains to produce >4% (wt/vol) ethanol. Strain CBS 397 was previously described as the type strain for *K. fragilis*, now designated *K. marxianus* var. *marxianus* (29).

The efficiency of glucose conversion to ethanol for the strains isolated from sugar -mills was 84 to 88% and <80% for *K. marxianus* var. *marxianus* CBS 712 and CBS 397, respectively. The other type strains were considerably less efficient (17 to 48% efficiency).

Following the initial high-temperature screen, several strains, including strains 972, 974, and 977, were selected for more detailed studies on high-temperature fermentation. These strains have all been classified as strains of *K. marxianus* var. *marxianus* (see Materials and Methods). Two strains, *K. marxianus* var. *marxianus* CBS 712 and CBS 397, were included as controls.

Effect of temperature on fermentation of glucose. The fermentation of glucose (18% [wt/vol]) to ethanol by the selected strains was tested at 25, 39, and 47°C. Cells were grown aerobically in shake culture, as outlined in Materials and Methods. The parameters measured were ethanol production, glucose utilization, and cell viability. Strains 972 (Fig. 1), 974 (Fig. 2), and 977 (results not shown) were capable of rapid fermentation at temperatures at high as 47°C to produce about 6% (wt/vol) ethanol after 24 h (Fig. 1A and 2A). At 39°C, approximately 7% (wt/vol) ethanol was produced after 20 h. Ethanol production at 25°C reached a maximum at about 80 h and remained relatively steady up to 5 days. Cell death after 20 h was extremely rapid at 47 and 39°C (Fig. 1C and 2C), with death being more rapid at the higher temperature. At 25°C, loss of viability was first noted at around 40 h, after which viability gradually decreased.

The two control strains, *K. marxianus* var. *marxianus* CBS 712 and CBS 397, were unable to grow at 47°C, and, for comparison, fermentation was determined at 25 and 39°C. Fermentation was rapid at 39°C, with 7% (wt/vol) ethanol produced after 19 to 23 h (Fig. 3A). Cell death, however, was significantly more rapid (Fig. 3C) than in strains 972, 974, and 977. For example, at 16 to 19 h, cell viability was approximately 90% in strains 972 (Fig. 1C) and 974 (Fig. 2C),

less than 70% in strain CBS 397 (results not shown), and 30% in strain CBS 712 (Fig. 3C).

Differences in cell viability were also apparent at the lower temperature. At 25°C, after fermentation for 48 h, corresponding to almost complete utilization of substrate, the proportion of viable cells was around 20% for the two control strains (Fig. 3C), but was more than 70% for strain 974 (Fig. 2C) and was close to 100% for strain 972 (Fig. 1C). Prolonged exposure (>72 h) of cells to 7 to 8% (wt/vol) ethanol at 25°C led to essentially 100% cell death in the type strains, whereas strains 972 and 974 were still 70 to 80% viable.

Fermentation of 16 to 22° Bx cane syrup solutions. All strains tested were capable of rapid fermentation of 16 to 22° Bx cane syrup solutions at temperatures up to 43°C (type strains) and 47°C (strains 972, 974, and 977). To gain more detailed insight into high-temperature fermentation, temperatures from 39 to 47°C were selected with 16, 19, and 22° Bx cane syrup as substrate.

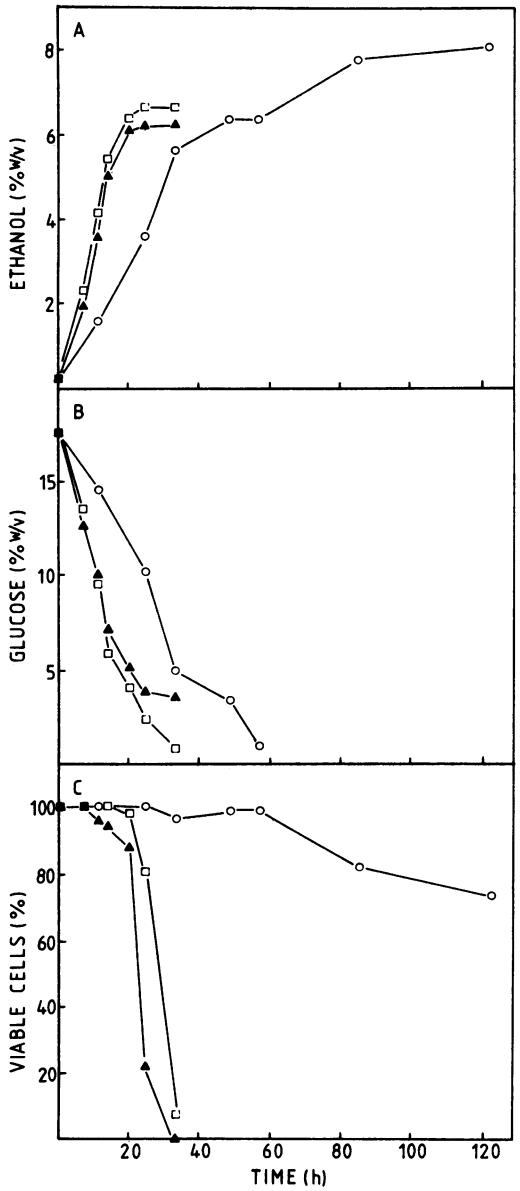

FIG. 1. Effect of temperature on fermentation of glucose (18%, wt/vol) by strain 972. (A) Ethanol production; (B) glucose utilization; (C) cell viability. Symbols: ▲, 47°C; □, 39°C; ○, 25°C.

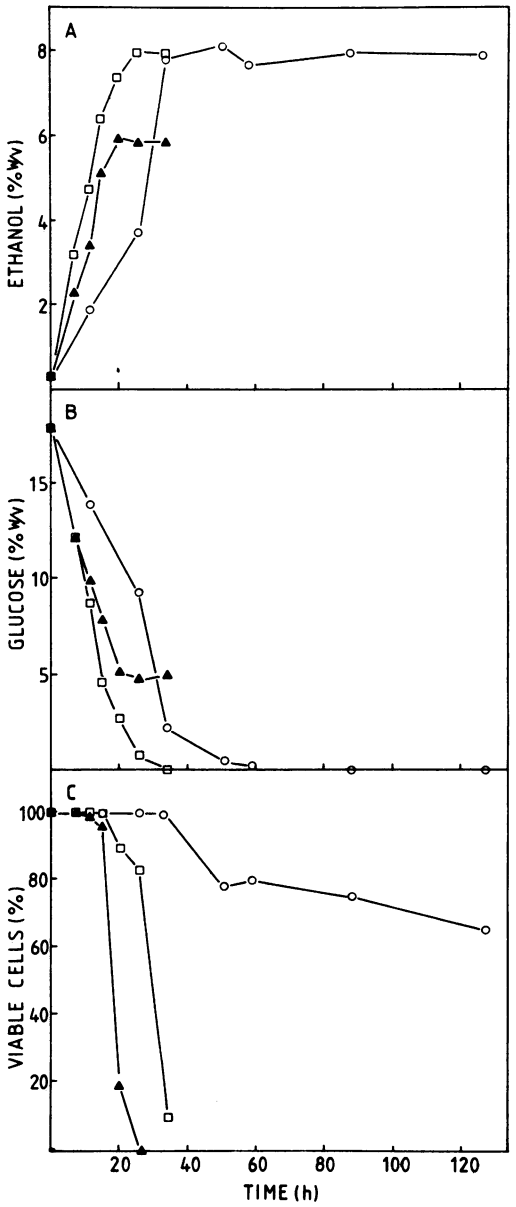

FIG. 2. Effect of temperature on fermentation of glucose (18%, wt/vol) by strain 974. Symbols: (A) Ethanol production; (B) glucose utilization; (C) cell viability. ▲, 47°C; □, 39°C; ○, 25°C.

Representative results for *K. marxianus* var. *marxianus* 974 with 19° Bx cane syrup are shown in Fig. 4. Fermentation at 39, 41, and 43°C resulted in rapid ethanol production, with a maximum approaching 8% (wt/vol) after 16 h (Fig. 4A). Total sugars, measured as the concentrations of sucrose, glucose, and fructose, decreased correspondingly (Fig. 4B). Fermentations at 45 and 47°C were significantly slower (Fig. 4A). Cells remained essentially 100% viable at all temperatures for up to 12 h (Fig. 4C). At 16 h, there was a distinct drop in cell viability to 80%, followed by a marked decrease. Cells fermenting at 45°C were the least viable (10%

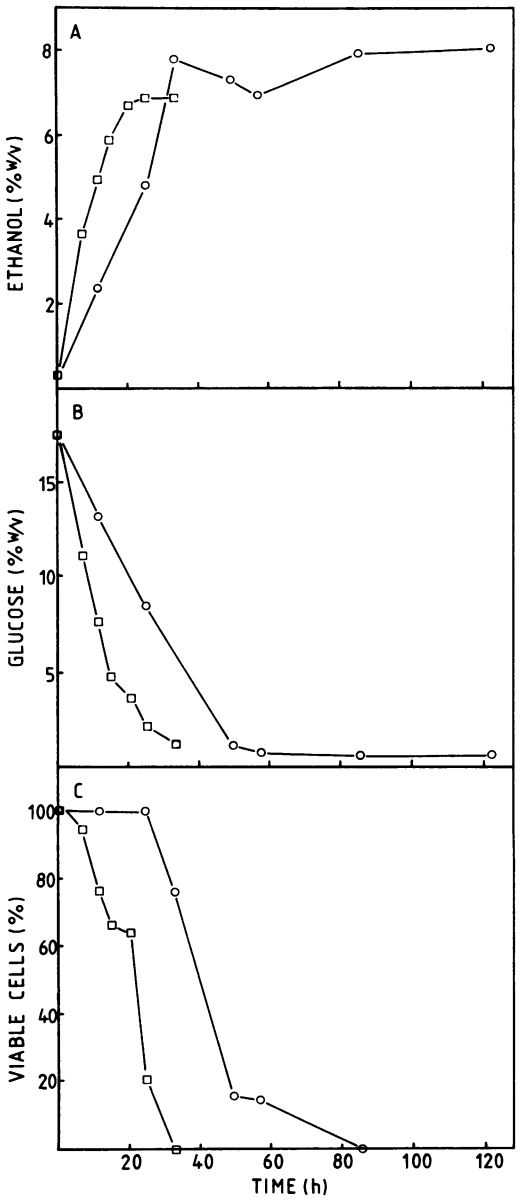

FIG. 3. Effect of temperature on fermentation of glucose (18%, wt/vol) by strain CBS 712. (A) Ethanol production; (B) glucose utilization; (C) cell viability. Symbols: □, 39°C, ○, 25°C.

after 18 h), while cells at 39°C retained moderate viability (70%) for up to 20 h.

Essentially analogous results were obtained when 19° Bx cane syrup was used with strains 972 and 977 (results not shown). These results led to a series of experiments to determine the optimum conditions for rapid ethanol production concurrent with retention of cell viability at temperatures above 40°C.

Figure 5 shows typical results obtained at 43°C with *K. marxianus* var. *marxianus* CBS 712 and CBS 397 with 15.5° Bx cane syrup (14% [wt/vol] total sugars as 11.5% sucrose and 2.5% glucose plus fructose). The two control strains produced maximum ethanol levels, approaching 6% (wt/vol), between 24 to 30 h (Fig. 5A). At 24 h, cell viability was 70 and 50% for strains CBS 712 and CBS 397, respectively (Fig. 5B). However, after 36 h at 43°C, cell viability was essentially 0% for both strains.

K. marxianus var. *marxianus* 974 and 977 produced ethanol more rapidly, and maximum ethanol levels (6 to 6.5% [wt/vol]) were reached after only 15 to 18 h at 43°C (Fig. 5A). It was noteworthy that strain 974 retained viability significantly longer than did strain 977, although populations of both strains died exponentially after 18 h (Fig. 5B).

Fermentation at 43°C was also determined with glucose (14% [wt/vol]) as substrate. As for dilute cane syrup, strains 974 and 977 produced ethanol more rapidly than did CBS 712 and CBS 397 (results not shown). In all experiments, cell viability was lower with glucose than with dilute cane syrup as substrate.

Growth curves. Growth curves for the various strains were determined at 39, 43, and 47°C with 18% (wt/vol) glucose or

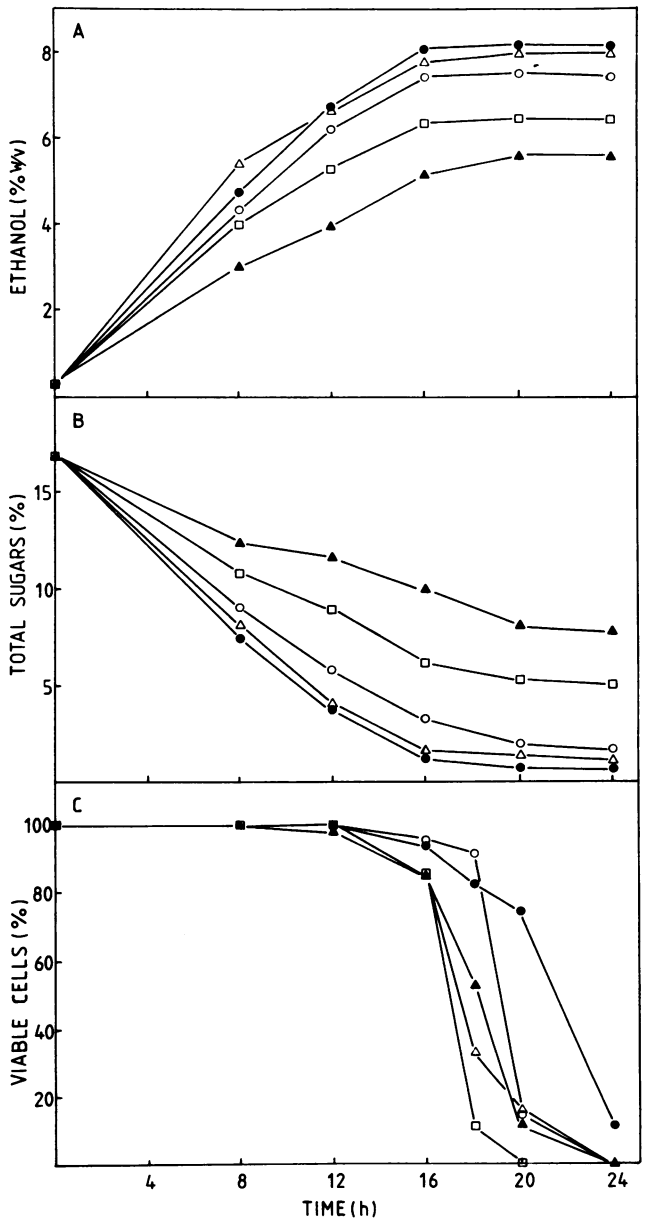

FIG. 4. Effect of temperature on fermentation of 19° Bx cane syrup by strain 974. (A) Ethanol production; (B) glucose utilization; (C) cell viability. Symbols: ▲, 47°C; □, 45°C; ○, 43°C; △, 41°C; ●, 39°C.

22° Bx cane syrup as carbon source. A representative graph (for strain 974) is shown in Fig. 6. Similar growth curves were exhibited by strains 972, 977, and CBS 712, except that CBS 712 did not grow as rapidly. For a given temperature, growth was more prolific and rapid with cane syrup than with glucose as carbon source. Furthermore, the rate of growth and cell numbers decreased as the growth temperature was increased from 39 to 43 to 47°C (Fig. 6).

Effect of temperature and ethanol on cell viability. In these experiments, cells were grown at 37°C to around 5×10^7 cells per ml, corresponding to mid- to late-log phase and then suspended (10% inoculum) in fresh medium containing 18% (wt/vol) glucose and 7% (wt/vol) ethanol. Incubations were at 37, 43, and 47°C, and cell viability was monitored over time. At 37°C, strain 974 remained viable (>75%) for up to 12 h, after which the viability decreased to about 50% at 24 h. At high temperatures, cell viability decreased from 60 to 70% at 47°C after 2 h to 20 to 25% after 4 h. Cell viability was marginally improved at 43°C, with viabilities of 80 and 30% after 2 and 4 h, respectively. Cells were essentially all dead after 12 h at 43 or 47°C in the presence of 7% (wt/vol) ethanol. Similar trends were noted for type strain CBS 712, except that cell viability was significantly lower at the higher temperatures, with 5 to 10% and 0% viability after 4 h at 43 and 47°C, respectively.

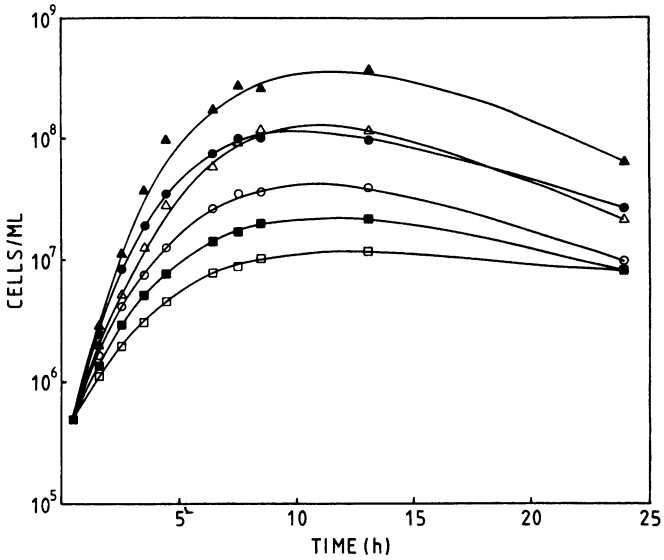

FIG. 6. Growth curves for strain 974 with 18% (wt/vol) glucose as carbon source (symbols: △, 39°C; ○, 43°C; □, 47°C and 22° Bx cane syrup as carbon source (symbols: ▲, 39°C; ●, 43°C; ■, 47°C).

DISCUSSION

In the present study, several basic criteria considered crucial for the commercial development of tropical fermentation technology were applied. These were the ability of strains to ferment carbohydrate (as sucrose, fructose, and glucose) to ethanol at temperatures above 40°C and the rapid production of ethanol (>6% [wt/vol] ethanol after 12 h) concurrent with retention of cell viability (>80% viable cells). These criteria were closely met by several strains of *K. marxianus* var. *marxianus* with glucose (Fig. 1 and 2) and dilute sugar cane syrup (Fig. 4 and 5) as substrates.

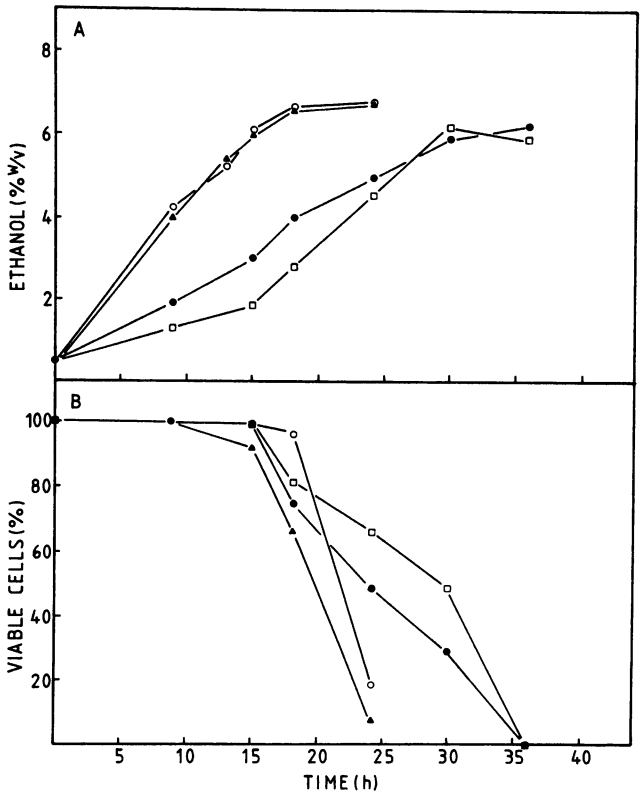

FIG. 5. Fermentation of 15.5° Bx cane syrup at 43°C by *K. marxianus* var. *marxianus*. Symbols: ○, strain 974; ▲, strain 977; □, strain CBS 712; ●, strain CBS 397. (A) Ethanol levels; (B) cell viability.

Our results are superior to those of previous studies on high-temperature ethanol fermentation by yeasts. Moreover, previous reports have placed little or no emphasis on the viability of cells subjected to prolonged high-temperature exposure to high concentrations of ethanol. Viability was considered to be a key parameter in the present study and served to differentiate among our isolated strains as well as the type strains of *K. marxianus*. The strains isolated and tested in the present studies performed significantly better than the type strains with respect to ethanol production, high-temperature fermentation, and cell viability. Nevertheless, the type strains performed surprisingly well under the experimental conditions, and *K. marxianus* var. *marxianus* CBS 712 and CBS 397 were capable of fermentation at 43°C, producing up to 6% (wt/vol) ethanol in 24 to 30 h (Fig. 5).

There are a few previous reports on high-temperature (>40°C) fermentation of cane molasses. Hughes et al. (9), using a naturally occurring strain of *K. marxianus*, reported a marked drop in ethanol yield at temperatures above 40°C. Haraldson and Bjorling (7) screened commercially available strains of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* for ethanol productivity at high osmotic pressure (30 to 50° Bx cane molasses) at temperatures up to 45°C. However, values for final ethanol concentration, cell viability, and time required for peak ethanol concentration were not reported. It was difficult, therefore, to make a direct comparison between these results and the present studies.

Improvements in high-temperature fermentations by yeasts may come from lipid nutritional studies. As long ago as 1959, Sherman (24) showed that at 40°C the addition of

yeast extract and oleic acid eliminated the initial death phase. In thermophilic enteric yeasts, sterol synthesis decreases as the growth temperature is raised above 40°C (28), and unsaturated fatty acid stimulates growth (32).

Lipid supplements have been shown to markedly influence ethanol production and ethanol tolerance in yeasts (8, 10, 12, 19, 30). Work by Rose and co-workers (3, 26, 27) has further emphasised the importance of the plasma membrane lipid composition in influencing ethanol tolerance in *S. cerevisiae*. Casey et al. (5) recently reported that supplementation of high-gravity brewing worts with oleic acid and ergosterol markedly improved the rate of ethanol production and viability of brewing yeast. It was further noted that oleic acid alone was much more effective than ergosterol alone, thus confirming a previous report by Watson (30) on unsaturated fatty acid stimulation of high ethanol production in *Saccharomyces* strains.

In the present study there was a general trend for a decrease in unsaturated fatty acid with increasing temperature (Watson and Anderson, unpublished results), a phenomenon well characterized in yeasts (31). Supplementation of media with lipids, in particular unstaurated fatty acids, may well improve ethanol tolerance and cell viability at high temperatures, and studies along these lines are currently in progress in this laboratory.

Genetic manipulation of yeasts for rapid ethanol production at high temperatures offers an alternative means for strain improvement. Panchal et al. (20) and Seki et al. (23) reported improved ethanol tolerance in genetically constructed hybrid strains of *Saccharomyces*. From the results of Seki et al. (23), it was estimated that 6% (wt/vol) ethanol was produced in 24 h at 42°C and >10% (wt/vol) ethanol was produced in 40 h at 40°C by their hybrid strains.

The naturally occurring *Kluyveromyces* spp. used in the present study were capable of rapid fermentation of carbohydrate to ethanol at temperatures up to 47°C, which is close to the upper temperature limit for growth of yeasts (25). There is little room for improvement, therefore, with respect to natural selection or genetic manipulation in the isolation of a strain capable of a higher fermentation temperature. On the other hand, ethanol tolerance and, to a lesser extent, the rate of ethanol production may be subject to improvement in our strains. Brewing and sake yeasts, for example, can produce up to 15% (wt/vol) ethanol, although relatively moderate temperatures (<30°C) and long incubation times are required (8, 30).

On the basis of currently available strains, it may be possible by genetic engineering to construct a yeast capable of fermenting carbohydrates at temperatures close to 50°C to produce 10 to 15% (wt/vol) ethanol in 12 to 18 h with retention of cell viability. These specifications are unlikely to be surpassed in the immediate future without a quantum step in our knowledge of an area of fermentation technology other than microbial genetics and biochemistry.

The use of alternative organisms such as *Zymomonas* and thermoanaerobic bacteria for high-temperature ethanol fermentation has severe limitations. Fermentation of carbohydrate (sucrose, fructose, and glucose) by *Z. mobilis* at temperatures above 40°C is slow and inefficient (16). On the other hand, although high-temperature (50 to 60°C) fermentation poses no problem with thermoanaerobics, the low ethanol concentrations (<4%, wt/vol) and efficiency (<50%) of substrate conversion (15) are fundamental drawbacks to their use in an industrial process.

At present, yeasts are the organisms of choice for efficient high-temperature fermentation of simple carbohydrates, such as fructose, glucose, and sucrose, to ethanol. In the longer term, and given the rapid advances in molecular biology, hybrid yeasts capable of high-temperature fermentation of cellulosic substrates and starches may be anticipated.

ACKNOWLEDGMENTS

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