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New Amylolytic Yeast Strains for Starch and Dextrin Fermentation

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Yeast strains capable of fermenting starch and dextrin to ethanol were isolated from samples collected from Brazilian factories in which cassava flour is produced. Considerable alcohol production was observed for all the strains selected. One strain (DI-10) fermented starch rapidly and secreted 5 times as much amylolytic enzyme than that observed for *Schwanniomyces alluvius* UCD 54-83. This strain and three other similar isolates were classified as *Saccharomyces cerevisiae* var. *diastaticus* by morphological and physiological characteristics and molecular taxonomy.

The capacity to degrade starch is not widespread among yeasts, but yeasts that are capable of degrading starch have been investigated as promising microorganisms for the conversion of starchy materials to "single-cell" protein or ethanol (i.e., the ability to produce proteins or ethanol not derived from animal or vegetable tissues). Starch is found in high concentrations in cassava roots. Cassava can be grown on very dry tropical lands and may be considered as a primary alternative to maize and other grains that are used as distillery feed stocks. Panchal et al. (9) have shown that *Saccharomyces diastaticus* is more appropriate than a non-amylolytic strain of *Saccharomyces cerevisiae* for the fermentation of starch hydrolyzed by conventional methods. By using *Saccharomyces diastaticus*, Whitney et al. (14) achieved a 50% savings in the glucoamylase concentration which is normally used in the commercial processes for the production of ethanol from starch.

In this report we describe the isolation procedure and the properties of yeast strains that are able to ferment starch isolated from samples collected in Brazilian factories in which cassava flour is produced.

MATERIALS AND METHODS

Yeast sources. *Schwanniomyces alluvius* UCD 54-83 was a gift from W. M. Ingledew of the Praire Regional Laboratory, Medical Research Council, Saskatoon, Saskatchewan, Canada. The flocculent strain 62 of *Saccharomyces diastaticus* derived from the Labatt culture collection was obtained by courtesy of C. J. Panchal. *Saccharomyces diastaticus* NCYC-361 and Y2044 were obtained from the Food Research Institute, United Kingdom, and the U.S. Department of Agriculture, Northern Regional Laboratory, Peoria, Ill. Strain CL-9 was a hybrid diploid strain that was prepared in a previous study (6). The amylolytic strains DI-10 to DI-22 were from this study. Strains CBS 604 and CBS 1171 were *Saccharomyces cerevisiae* and were obtained from the Central Burreau voor Schimmelcultures (CBS), Delft, The Netherlands.

Media. The complete medium contained 1% yeast extract (Y), 2% peptone (P) (Difco Laboratories, Detroit, Mich.), and an appropriate carbon source. Stock cultures were grown on 2% glucose medium (YPD). The buffered starch medium (BYPS) contained Lintner starch (Sigma Chemical

Co., St. Louis, Mo.) and 1% succinic acid (pH 4.2). The dextrin medium (YPDex) contained commercial dextrin 1920 (Refinações de Milho Brasil, Ltda., Sao Paulo, Brazil) as a carbon source. Dextrin 1920 was obtained by acid hydrolysis of corn starch and contains 3% monosaccharides, 6% disaccharides, 8% trisaccharides, 7% tetrasaccharides, 7% pentasaccharides, and 69% hexasaccharides and higher polysaccharides. The solid starch medium (YPSI) used for the isolation of new strains contained 0.003% (wt/vol) rose bengal and 0.19% (vol/vol) propionic acid (pH 4.7). YPSI medium was heated to dissolve the soluble starch and agar, and the propionic acid was added before medium was poured into the plates, when the temperature fell to 40°C. Rose bengal and propionic acid were used to avoid or reduce the growth of sample contaminants. The medium described by McClary et al. (8) was used for sporulation.

Isolation procedures. Samples were collected from ponds containing the wastewater from manioc flour factories, and soil samples were also collected from those areas around the factories that are frequently exposured to waste spillages. Samples suspended in sterile water were streaked onto YPSI medium, and the small colonies that formed after 5 days were transferred to YPS medium. After growth on the second medium containing starch, the plates were refrigerated at 4°C (1 to 2 days) to convert dissolved starch into an insoluble and highly turbid suspension within the agar. The strains producing glucoamylase exhibited clear zones around the colonies. Yeast colonies showing a clear zone diameter that was 1.5 to 4.3 times greater than their own growth diameter (ranging from 2 to 19 mm) were tested by the inverted Durham tube method for their abilities to ferment starch (BYPS medium).

Determination of morphological and physiological properties. Routine methods described by Kreger-van Rij (5) were used for studies of fermentative and assimilative abilities.

DNA purification, determination of G+C content, and reannealing reactions. DNA purification, determination of the G+C content, and reannealing reactions were carried out by the procedures used by Vaughan Martini and Kurtzman (12).

Growth and fermentation procedure. All growth and fermentation experiments were carried out at 30°C. Stock cultures were grown in YPD slants. One loopful from a fresh slant was inoculated into 6 ml of YPD medium in a test tube and was incubated for 24 h. The resulting preinoculum was mixed with 150 ml of YPDex (5% dextrin) or BYPS (2%

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TABLE 1. Fermentation of 5% starch in YPS medium and 15% dextrin in YPDex medium at 30°C by selected yeast strains[a]

| Strain | Ethanol (%; vol/vol) from: | | | | Viability (%) of: | | | | Enzyme activity (U/ml) of: | |
| | Starch | | Dextrin | | Starch | | Dextrin | | Starch (4 days) | Dextrin (4 days) |
	2 days	4 days	2 days	4 days	2 days	4 days	2 days	4 days		
DI-2[b]	0.4	1.3	7.0	8.2	85	80	86	70	1.2	0.33
DI-3[b]	0.4	1.2	7.0	7.8	92	85	86	70	0.9	0.28
DI-7[b]	0.6	1.6	6.1	7.5	81	75	75	65	1.8	0.23
DI-10	1.8	2.0	8.0	8.7	85	50	60	40	5.0	0.72
DI-11	0.4	1.7	6.2	8.0	86	80	81	70	2.3	0.32
DI-12	0.7	1.6	6.7	8.2	90	83	80	75	1.7	0.31
DI-13	0.7	1.7	6.7	8.5	93	82	68	60	2.0	0.34
DI-20	0.5	1.0	6.7	7.5	96	89	90	80	1.0	0.30
DI-21	0.7	1.7	7.0	8.3	89	80	75	70	2.1	0.40
CL-9[c]	0.6	1.8	6.8	8.4	90	88	81	75	1.9	0.36

[a] Initial cell density, 10 mg/ml; fermentation was done without shaking. The initial cell viability was about 95%.
[b] Strains from our culture collection (strain DI-2 is NCYC-361, DI-3 is Y2044, and DI-7 is 62).
[c] Hybrid strain (*Saccharomyces cerevisiae* × *Saccharomyces cerevisiae* var. *diastaticus*).

starch) medium in a 500-ml Erlenmeyer flask. BYPS medium was incubated on a rotary shaker (model G-25; Superohm, Piracicaba, Brazil) that was operated at 300 rpm, while YPDex medium was incubated without shaking for 48 h. The cells were allowed to settle for 12 h at 4°C, and the supernatant was aspirated. The cells were washed with 200 ml of sterile water and then suspended in 2 ml of sterile water. This small volume of concentrated cell suspension was the inoculum that was grown aerobically in BYPS and semianaerobically in YPDex medium.

For fermentation, a sterile 125-ml Erlenmeyer flask fitted with a one-hole rubber stopper was used. A short glass tube, as described by Laluce and Mattoon (6), was placed into the hole. Each flask was inoculated to give an initial cell concentration of 10 mg (dry weight)/ml, and the fermentation flasks containing 100 ml of BYPS medium (5% starch) or YPDex medium (15% dextrin) were incubated without shaking for several days at 30°C.

Measurement of ethanol and total sugar. Ethanol was assayed in a gas chromatograph (model CG-37; CG Instrumentos Científicos Ltda., Sao Paulo, Brazil) that was equipped with an integrator-processor (model CG-300; CG Instrumentos Científicos) and a column (model PAD-2499; CG Instrumentos Científicos) and was operated with a flame ionization detector. The vaporizer temperature was 130°C, and the detector temperature was 140°C. The column was operated isothermally at 94°C. Total sugar was measured by the colorimetric anthrone-sulfuric acid procedure, as described by Weiner (13).

Determination of amylolytic activity. The extracellular amylolytic activity that was produced during the fermentation of dextrin and starch by the amylolytic yeasts was determined by measuring the reducing sugar groups released from starch by a colorimetric method, based on the reduction of 3,5-dinitrosalicylic acid. Each reaction mixture contained, in a final volume of 2.2 ml, 0.1 mM sodium acetate-acetic acid buffer (pH 5.0), 4.6 mM sodium chloride, and 4.5 mg of Lintner starch per ml. Supernatant fluids obtained by centrifugation of yeast cultures were used as the samples (0.2 ml in the case of starch fermentation or 0.05 ml in the case of dextrin fermentation). Incubation was for 60 min at 45°C for reaction mixtures containing supernatants derived from starch fermentation and 90 min for reaction mixtures containing supernatants derived from dextrin fermentation. The reaction was terminated by adding 2 ml of 3,5-dinitrosalicylic reagent, and the reducing sugar was determined

colorimetrically at 546 nm, as described by Bernfeld (1). One unit of enzyme activity was defined as the amount that liberated 1 μmol of reducing group per min per ml of enzyme sample. A standard curve for the colorimetric assay was constructed by using maltose as the reducing sugar.

Viable cell counts. Cell number was determined with a hemacytometer. Cell viability was examined by using the staining technique of Lee et al. (7).

RESULTS

Many yeast colonies (1,200) that were isolated from samples collected in cassava flour factories located in the state of São Paulo, Brazil, were able to grow on starch, but a reduced number were able to ferment it. Only four fermenting strains (DI-10, DI-11, DI-12, and DI-13) were isolated from samples collected in five new factories (10 years old or less). On the other hand, a high number of fermenting strains (ca. 90%) was isolated from a 50-year-old factory located in Nazaré, state of Bahia, Brazil, but only two of these strains (DI-20 and DI-21) were described in this study because of their higher ethanol production and survival after 2 days of starch fermentation at 30°C.

The ethanol produced by the fermentation of 5% starch and 15% dextrin by the fermenting strains that were previously selected by the Durham assay and the survival and ethanol levels are shown in Table 1. Strain DI-10 excreted more amylolytic enzyme(s) during the fermentation of starch or dextrin and was able to accumulate more ethanol in 2 days than the other strains. Otherwise, the lowest viability was observed for strain DI-10. All the strains excreted more enzyme during starch fermentation than during dextrin fermentation. Ethanol production from dextrin showed that the isolated strains (except strain DI-10) fermented dextrin almost as fast as strain CL-9, which was prepared for a previous study (6) by sexual hybridization.

Alcohol production, residual sugar, and enzyme excretion during the fermentation of 5% starch by the isolated strains *Saccharomyces cerevisiae* DI-10 and DI-21 and *Schwanniomyces alluvius* UCD 54-83 are shown in Fig. 1. The *Schwanniomyces alluvius* strain produced more ethanol than did strain DI-10 (Fig. 1A); however, strain DI-10 showed faster fermentation and produced much more enzyme (about 6 times more after 12 days) than the *Schwanniomyces alluvius* strain did (Fig. 1C). Finally, alcohol production, enzyme excretion, and residual sugar during the fermenta-
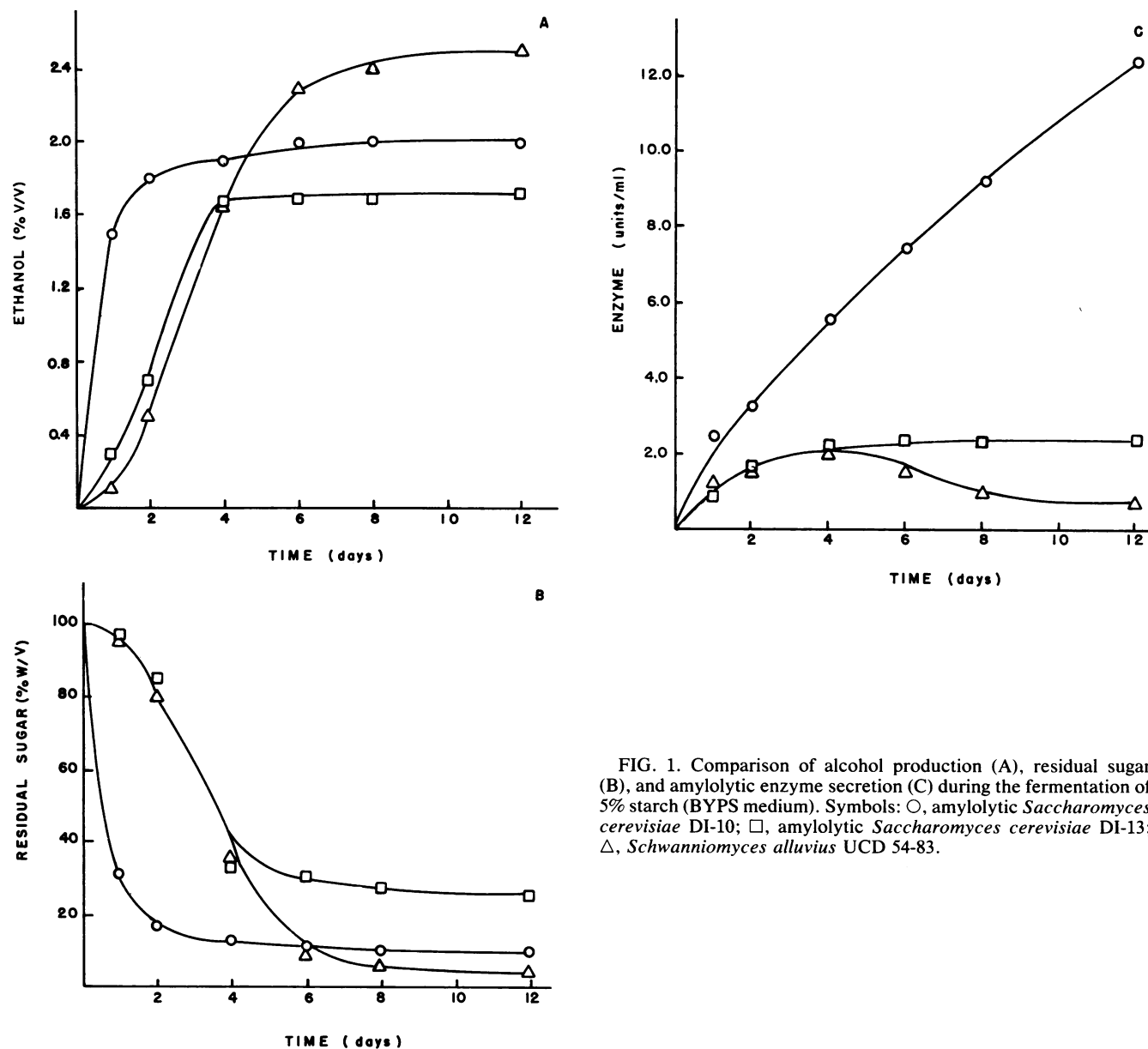
FIG. 1. Comparison of alcohol production (A), residual sugar (B), and amylolytic enzyme secretion (C) during the fermentation of 5% starch (BYPS medium). Symbols: O, amylolytic *Saccharomyces cerevisiae* DI-10; □, amylolytic *Saccharomyces cerevisiae* DI-13; △, *Schwanniomyces alluvius* UCD 54-83.

tion of 15% dextrin by strains DI-10 (Fig. 2A) and DI-21 (Fig. 2B) are shown in Fig. 2.

Some physiological properties were determined for strains DI-10, DI-11, DI-12, and DI-13; and the same behavior was observed for all of them. Fermentations of maltose, D-mannose, D-galactose, D-glucose, D-trehalose, and raffinose were positive. Negative assimilations and fermentations were observed for lactose, *meso*-inositol, L-arabinose, *meso*-erythritol, adonitol, D-xylose, D-cellobiose, dulcitol, D-melibiose, succinic acid, citric acid, and DL-lactic acid. Weak assimilations were detected with L-rhamnose, L-inulin, starch (Lintner), and D-melezitose. Positive assimilations were observed for ethanol and glycerol. Negative assimilations were observed for nitrate, L-lysine, and urea, which were used as nitrogen sources. Weak growth was observed in vitamin-free medium, and negative growth was observed in the presence of 100 ppm (μg/ml) of cycloheximide. Vigorous growth was observed at 37°C.

Usual cell morphologies in malt extract were observed for strains DI-11, DI-12, and DI-13, while cells of strain DI-10 occurred mainly in small clusters or occasionally singly. For strain DI-10, cell clusters showing short cell chains were observed, instead of the typical pseudomycelia formed in corn meal agar by strains DI-11, DI-12, and DI-13.

All strains were assayed for ascospore formation. The vegetative cells were transformed directly into asci after 3 days of incubation at 30°C in sporulation medium. The asci contained one to four spheroid ascospores. No ascospores were observed for strain DI-10 after 7 days of incubation.

The guanine and cytosine content of strains DI-10, DI-11, DI-12, and DI-13, as well as the results of DNA reassociation experiments, were those expected for *Saccharomyces cerevisiae* var. *diastaticus*. The guanine and cytosine content of the type strain CBS 604 (38.4%) and the new strains DI-10 (40.3%), DI-11 (39.2%), DI-12 (39.3%), and DI-13 (39.4%), as well as their genetic relatedness to another type strain of
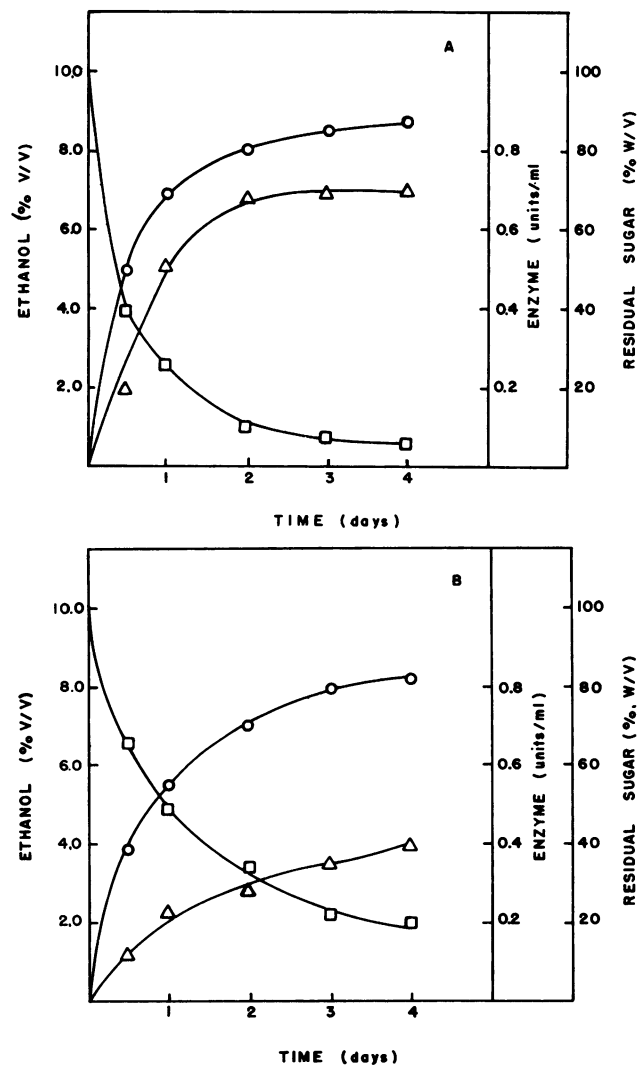
FIG. 2. Fermentation of 15% dextrin (YPDex medium) by amylolytic *Saccharomyces cerevisiae* DI-10 (A) and DI-21 (B). Symbols: ○, ethanol production; □, residual sugar; △, amylolytic enzyme excretion.

Saccharomyces cerevisiae (CBS 1171), was determined through DNA-DNA reassociation experiments. The nuclear DNA of the strains listed above was highly related (homology, >90%) to the type strain of *Saccharomyces cerevisiae*, CBS 1171.

DISCUSSION

By using the simple procedure described in this report, we isolated amylolytic strains from samples collected in cassava flour factories. Morphological and physiological characteristics described for a few strains (DI-10, DI-11, DI-12, and DI-13) and DNA-DNA reassociation homologies indicated that such strains must be considered strains of *Saccharomyces cerevisiae*.

The degree of ethanol production, enzyme excretion, and viability during the starch and dextrin fermentations by the newly isolated strains were compared quantitatively (Table 1). Clearer differences were observed among the strains when starch instead of dextrin was used as the carbon

source. Strain DI-10 showed a better capability to ferment both substrates, and it was even more efficient at fermentation than the hybrid strain obtained by Laluce and Mattoon (6). Strain DI-10 fermented 5% starch more rapidly than did *Schwanniomyces alluvius* UCD 54-83, probably because of its higher level of enzyme excretion. De Mot et al. (3) obtained 9.6% (wt/vol) ethanol by fermentation of 22.5% dextrin (Maldex 15) by using *Saccharomyces diastaticus* NCYC 914. Our strain was able to produce 8.7% (vol/vol) ethanol, which is equivalent to 6.96% (wt/vol) ethanol by fermentation of 15% dextrin (Mor-Rex 1920). This yield appears to be better, even when one considers the differences in medium and dextrin composition. Ingledew (4) reported production of about 3.2% (wt/vol) ethanol after 10 days of fermentation of 9.25% starch when mutant R69 of *Schwanniomyces castellii* was used. Strain DI-10 produced 2% (vol/vol) or 1.6% (wt/vol) ethanol after only 24 h of fermentation of 5% starch at 30°C. The *Schwanniomyces* species excrete α-amylase and amyloglucosidase (15). Therefore, they should produce ethanol more efficiently than the other species; however, their velocity of starch fermentation seems to be lower compared with that of the *Saccharomyces* species, which only produce glucoamylases (10). This is probably because the *Schwanniomyces* species produce less glucoamylase. Calleja et al. (2) have described a direct and quantitative conversion of starch to ethanol by *Schwanniomyces alluvius* ATCC 26074 after 72 h of fermentation of a low concentration (2.5%) of starch.

Our results and those obtained by De Mot et al. (3) suggest that amylolytic yeasts cannot be used for the direct alcoholic fermentation of starch in alcohol factories at this time. One of the problems related to the direct fermentation of dextrin or starch by our amylolytic strains was the low velocity of sugar consumption when the residual sugar concentration reached 40% of the initial sugar concentration. However, Whitney et al. (14) have shown the possibility of saving as much as 50% (vol/wt) of glucoamylase by using *Saccharomyces diastaticus* instead of *Saccharomyces cerevisiae* in the conventional process of ethanol production from starch. Several advantages of using amylolytic yeasts in industrial processes have been well described recently by Tubb (11). A superior, newly isolated strain could be useful for genetic studies carried out to obtain an ideal strain.

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