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Development of Rapidly Fermenting Strains of *Saccharomyces diastaticus* for Direct Conversion of Starch and Dextrins to Ethanol

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Alcoholic fermentation, growth, and glucoamylase production by 12 strains of *Saccharomyces diastaticus* were compared by using starch and dextrins as substrates. Haploid progeny produced from a rapidly fermenting strain, SD2, were used for hybridization with other *S. diastaticus* and *Saccharomyces cerevisiae* haploids. Alcoholic fermentation and enzyme production by hybrid diploids and their haploid parents were evaluated. Although the dosage of the *STA* or *DEX* (starch or dextrin fermentation) genes may enhance ethanol production, epistatic effects in certain strain combinations caused decreases in starch-fermenting activity. Both the nature of the starch or dextrin used and the fermentation medium pH had substantial effects on alcohol production. Commercial dextrin was not as good a substrate as dextrins prepared by digesting starch with α-amylase. Crude manioc starch digested by α-amylase was fermented directly by selected hybrids with almost 100% conversion efficiency. The manioc preparation contained adequate minerals and growth factors. This procedure should be suitable for direct commercial application in manioc-producing regions in Brazil and elsewhere. A rapidly fermenting haploid strain, SD2-A8, descended from strain SD2, contains two unlinked genes controlling formation of extracellular amylase. A convenient method for detecting these genes (*STA* genes) in replica plates containing large numbers of meiotic progeny was developed.

The production of industrial and fuel ethanol from starchy biomass commonly involves a three-step process: (i) liquefaction of starch by an endoamylase such as α-amylase, (ii) enzymatic saccharification of the low-molecular-weight liquefaction products (dextrins) to produce glucose, and (iii) fermentation of glucose. Commercial enzymes are used for liquefaction and saccharification and represent a significant expense in the production process. The object of the present investigation was to assess the possibility of developing a direct, one-step process for starch fermentation employing the starch-digesting yeast *Saccharomyces diastaticus*.

Since the first description of *S. diastaticus* by Andrews and Gilliland (1), a number of investigators have isolated strains of *Saccharomyces* capable of direct fermentation of starch (4, 6, 8, 9, 14, 16–19). Takahashi (16) isolated a strain from nonpasteurized beer which he compared with that isolated by Andrews and Gilliland. Through hybridization studies it was shown that each strain contained at least one starch fermentation (*STA*) gene which was unlinked to a *STA* gene in the other strain. Subsequently Tamaki (17) demonstrated the existence of three unlinked *STA* alleles (*STA1*, *STA2*, and *STA3*) in five independently isolated *S. diastaticus* cultures. Similarly, Erratt and Stewart (4) described three unlinked genes which permitted fermentation of commercial dextrins. Two of these genes were designated *DEX1* and *DEX2*, and the third was shown to be functionally allelic to the *STA3* gene of Tamaki. Since both *DEX1* and *DEX2* strains produce extracellular glucoamylases, these *DEX* genes may also be considered to be *STA* genes. It may be concluded that starch fermentation by *S. diastaticus* is controlled by a polygenic system of *STA* genes, any one of which suffices for fermentation.

In this study, a variety of *S. diastaticus* strains of different origins were collected and used to prepare haploid, starch-fermenting strains. A series of hybrids were then constructed and compared for efficiency of direct starch fermentation.

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MATERIALS AND METHODS

Yeast strains. The *S. diastaticus* strains surveyed in this study are listed in Table 1 together with *S. cerevisiae* strains used in genetic studies. Additional strains were obtained by hybridizing various haploids and by sporulating diploids.

Genetic methods. Haploids were isolated from sporulated diploid cultures by selecting large colonies with morphological traits differing significantly from the parental diploid. Both colony size and texture differences could be used. Morphological changes were also employed in selecting new hybrid diploids. Adenine auxotrophs (*ade1* or *ade2*) of several haploids were isolated as pink colonies after mutagenesis with ethyl methanesulfonate (15). Other auxotrophic markers were introduced by hybridizing *STA* haploids with haploid *S. cerevisiae* strains bearing genes for different nutritional defects and for canavanine resistance (*can*^{r}). Random spores produced from diploids heterozygous for *can*^{r} were isolated by plating on minimal medium containing 60 mg of canavanine per liter. Because *can*^{r} is recessive to the normal canavanine-sensitive allele, only resistant haploid segregants produce colonies. Tetrad dissection and auxotrophic marker analysis were performed as described by Mortimer and Hawthorne (10). *STA* genes were detected by replication of segregants on BYPS medium (see below) and incubation for 3 to 5 days to allow glucoamylase action to occur. Plates were then refrigerated for at least 3 days to allow dissolved starch to produce an insoluble, highly turbid suspension in the agar. Those strains producing glucoamylase (*STA* or *DEX* strains) exhibited clear zones around the colony. Usually a narrow, turbid halo of retrograde starch was produced within the clear zone (Fig. 1). In an alternative detection technique, the plates were exposed to iodine vapor for a few seconds. Starch in the plates stained deep blue, and glucoamylase-producing colonies were surrounded by a clear, unstained zone.

To produce hybrid diploids, it was first necessary to obtain a variety of haploid strains. This was accomplished in

TABLE 1. List of yeast strains used

Strain	Genotype	Source	Reference
SD1[a]	a/α *STA*	Northern Regional Laboratory	
SD2[a]	a/α *STA*	U.S. Department of Agriculture, Peoria, Ill.	
SD7-01	a/α *STA*	T. Takahashi (Japan)	16
Y83	a/α *STA*	T. W. Young (U.K.)	18
NCYC361	a/α *STA*	Food Research Institute (U.K.)	17
708-7B	α *arg4 STA1*	H. Tamaki (Japan)	17
5692-10A	α *lys7 STA2*	H. Tamaki (Japan)	17
5301-7b	α *lys7 STA3*	H. Tamaki (Japan)	4
1354	a *DEX1*	G. G. Stewart (Canada)	4
1355	α *DEX1*	G. G. Stewart (Canada)	4
1359	α *DEX2*	G. G. Stewart (Canada)	4
1360	a *DEX2*	G. G. Stewart (Canada)	
IT-6A[b]	a/α *DEX*	C. Laluce	
1268-2B	α *ade6 his2 sta*	J. Mattoon	
JP293-25A/C1	a *ade6 can*^{r} *his sta*	J. Mattoon	
D261	a/α *sta/sta*	J. Mattoon	

[a] Strains SD1 and SD2 were single-colony isolates prepared from cultures Y2044 and Y2416, respectively. The number of *STA* genes in these cultures is not known.

[b] *Saccharomyces* strain isolated from a production vat at Zanin Ethanol Plant, near Araraquara, São Paulo, Brazil.

several ways. (i) A group of seven haploid *S. diastaticus* strains were obtained from other laboratories (Table 1). Each strain contained a single starch-dextrin fermentation gene. (ii) Strain SD2 was allowed to sporulate, and haploid progeny (SD2-A1, SD2-A2, etc.) were selected according to morphological traits (colony types). Some of these haploid strains were further mutagenized to produce adenine auxotrophs. Spontaneous canavanine-resistant strains were also selected. (iii) Canavanine-sensitive haploid segregants obtained from sporulated SD2 were crossed with *Saccharomyces cerevisiae* haploids containing auxotrophic markers and a *can*^{r} (canavanine resistance) gene. Resulting diploids were selected by morphological differences and sporulated. Spores were plated, and *can*^{r} *STA* segregants, which also inherited auxotrophic markers, were selected.

Media. Complete media contained 1% Difco yeast extract, 2% Difco peptone, and an appropriate carbon source. Stock cultures were grown on 2% glucose medium (YPD). Buffered starch medium (BYPS) contained 4% Lintner starch (Sigma Chemical Co.) and 0.1 M succinic acid (pH 4.2). Unbuffered starch medium (YPS) was the same as YPD except 2% Lintner starch was used instead of 2% glucose. The concentrations and types of starch were varied as indicated below. Buffered dextrin medium (BYPdex) contained either commercial dextrin (Sigma type 1) or α-limit dextrin prepared by hydrolysis of starch with commercial α-amylase (Taka-therm; Miles Laboratories) as described below. Minimal medium contained 0.6% Difco yeast nitrogen base (without amino acids, 2% glucose, or other carbohydrate), and nutritional supplements as required (15). The pH was 5.0. L-Canavanine · HCl (60 mg/liter) was added as required. In the initial stages of this work a medium based on that described by Ogur and St. John (11) was employed. It contained 0.3% yeast extract, 0.35% peptone, 0.2% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, and 2% starch. It was designated Ogur medium. For solid media, 3% agar was added. Media for manioc starch fermentation consisted of a solution of crude manioc meal (about 75% carbohydrate) liquified by α-amylase treatment. The pH of the preparation was adjusted to 4.2. This preparation was used with and without other nutrient additions as indicated below.

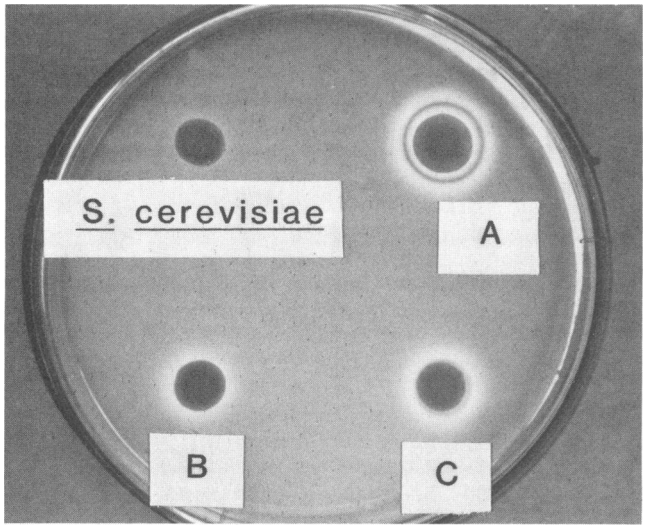

FIG. 1. Detection of *STA* genes on solid starch-containing medium. *S. cerevisiae* strain JP293-25A/C1 was used. (A) Strain CL1-17B. (B) Strain CL1-21A. (C) Strain CL1-21B.

Enzymatic preparation of dextrin solution. A starch paste was prepared by mixing starch, water, and α-amylase stock solution (Taka-therm) in the proportions 1 g:3 ml:0.01 ml. The mixture was then heated and rapidly stirred continuously until the paste was completely liquified (about 4 min). Gentle heating was continued for about 15 min, and the progress of digestion was tested at intervals for residual starch by mixing 1 drop of reaction mixture and 1 drop of 0.4% iodine in 2% KI with about 5 ml of water. When the starch-iodine reaction no longer occurred, heating was discontinued. The resulting concentrated dextrin solution was used in preparing medium.

Dextrins from manioc were prepared as follows. Whole manioc root was ground and dried and then treated directly by α-amylase by the same procedure used for treating pure

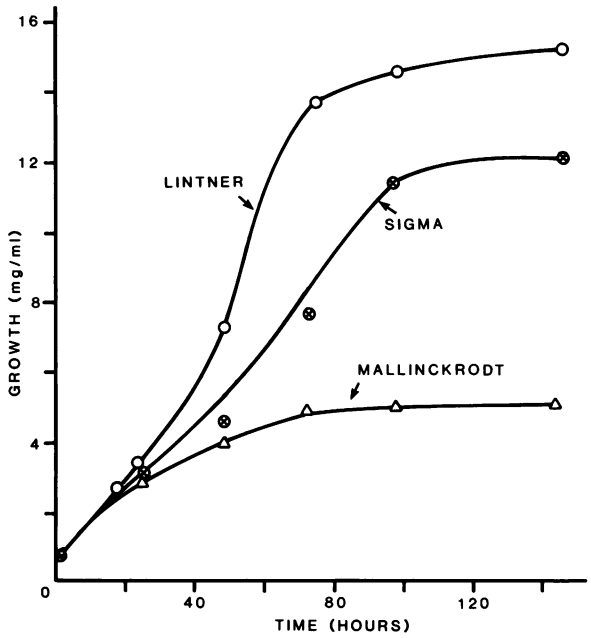

FIG. 2. Effect of different types of starch on growth of *S. diastaticus* in liquid media. Cultures were grown in Ogur medium containing 37 mM citrate buffer (pH 5.6) and different types of starch (2%): Lintner starch (Sigma), Sigma potato starch, and Mallinckrodt potato starch. Inoculum was grown on Ogur medium containing 2% Mallinckrodt starch.

starch. The solubilized material was diluted without additions of yeast extract, peptone, or other nutrients.

Growth and fermentation procedures. All growth and fermentation experiments were conducted at 30°C. Stock cultures were grown on YPD slants. For semianaerobic fermentation, a preinoculum was prepared by inoculating 150 ml of YPD medium in a 500-ml Erlenmeyer flask with 2 loopfuls of culture from a slant and incubating aerobically on a rotary shaker operated at 300 rpm for 24 h. A suspension containing 70 mg (dry weight) of preinoculum cells in 20 ml was prepared, with sterile water added as required. In some instances it was necessary to concentrate the preinoculum by allowing cells to sediment and then removing a portion of the clear supernatant fluid by aspiration with a sterile pipet. The 20 ml of cell suspension was then mixed with 150 ml of 2% starch (BYPS) medium in a 500-ml Erlenmeyer flask and incubated aerobically for 72 h on a rotary shaker operated at 300 rpm. To remove secreted glucoamylase, which would interfere in subsequent comparisons of enzyme production by various strains, cells were allowed to settle. The clear supernatant fluid was then aspirated from each flask as completely as possible and replaced with 200 ml of sterile water. After the cells had settled again, all but 20 to 40 ml of the clear supernatant fluid was removed by aspiration. The residual liquid was then used to suspend the cells, and the cell concentration of the resulting inoculum suspension was adjusted to 0.5 or 1.0 g dry weight per 25 ml with sterile water.

For fermentation, a sterile 125-ml Erlenmeyer flask fitted with a one-hole rubber stopper was used. A short glass tube was fitted into the hole so that the lower end just protruded into the flask. The other end of the tube was fitted with a 2-in. (5.08-cm) length of rubber tubing containing a loose cotton plug at the upper end. Initial evolution of CO_2 from the fermentation mixture scrubbed out most of the O_2 from the tubing, creating an almost anaerobic environment. Each flask contained 100 ml of sterile medium to which 2 or 3 drops of polypropylene glycol had been added as an antifoaming agent. Twenty-five-milliliter portions of inoculum suspension were then added, and the fermentation flasks were incubated at 30°C without agitation for various periods. The inoculum was 0.5 g (dry weight) of cells per flask if starch or dextrin concentrations were 4% or less. For higher carbohydrate concentrations 1 g of cells was used. All fermentations were run in duplicate.

Ethanol determination. Ethanol production was determined enzymatically by a modification of the method of Bernet and Gutmann (3). Each reaction mixture (5 ml) contained 75 mM sodium PP_i, 21 mM glycine, 75 mM semicarbazide · HCl, 1.35 mM β-NAD, ethanol (0.005 to 0.03% [vol/vol], and 0.12 mg of lyophilized alcohol dehydrogenase (Sigma, no. A7011). The pH of the reaction mixture was 8.7, and the reaction temperature was 30°C. The reaction was initiated by adding 0.1 ml of an appropriately diluted ethanol-containing sample to the reaction tube. After a 12-min incubation, the reduced NADH produced was determined by measuring absorbance at 340 nm. Prior deproteinization of fermentation fluid by perchloric acid was not required for measuring ethanol. A standard curve was made for each set of assays performed.

Determination of amylolytic activity excreted by yeast. The extracellular amylolytic activity produced during growth of *S. diastaticus* strains and various derived hybrids containing *STA* or *DEX* genes was determined by measuring reducing sugar released from starch by a colorimetric method in which reduction of 3,5-dinitrosalicylic acid to nitroaminosalicylic acid is determined. Each reaction mixture contained, in a final reaction volume of 2.2 ml, 18.2 mM sodium acetate, 9.1 mM sodium chloride, and 9.1 mg of Lintner starch per ml. The pH was 5.0. The enzyme samples (0.2 ml) consisted of supernatant fluid obtained by centrifuging yeast cultures grown on BYP medium with 4% starch. Incubation of reaction mixtures was for 10 min in a 35°C water bath. The reaction was terminated by adding 2 ml of 3,5-dinitrosalicylic acid reagent, and reducing sugar was determined colorimetrically at 546 nm as described by Bernfeld (2). One unit of enzyme was defined as the amount that liberated 1 μmol of reducing group per min per ml of enzyme sample. A standard curve for colorimetric assay was constructed, using maltose as reducing sugar. A linear response was obtained up to 2.8 μmol in a 2.2-ml reaction mixture.

Growth measurements. Growth of strains in liquid medium was estimated from measurement of absorbance at 570 nm and multiplication by a conversion factor to obtain dry weight. Cultures were diluted as required to bring absorbance values at 570 nm into the range in which cell concentration is a linear function of absorbance (0.05 to 0.30).

RESULTS

Effect of medium composition and pH on growth of *S. diastaticus*. In the initial stages of this study, several different media were tested with *S. diastaticus* SD1. The type of starch employed had a striking effect on both the rate and extent of growth (Fig. 2). Potato starch treated by the Lintner process (dilute acid washing) (Sigma) proved to be a far better substrate than untreated potato starch from Mallinckrodt Inc. or from Sigma. In other experiments (data not shown) the Lintner preparation (Merck & Co. or Sigma) was a better substrate than potato starch from Fisher Scientific Co. or starch from Matheson Coleman Bell (Curtin Matheson). Growth on starch medium gives a characteristic growth

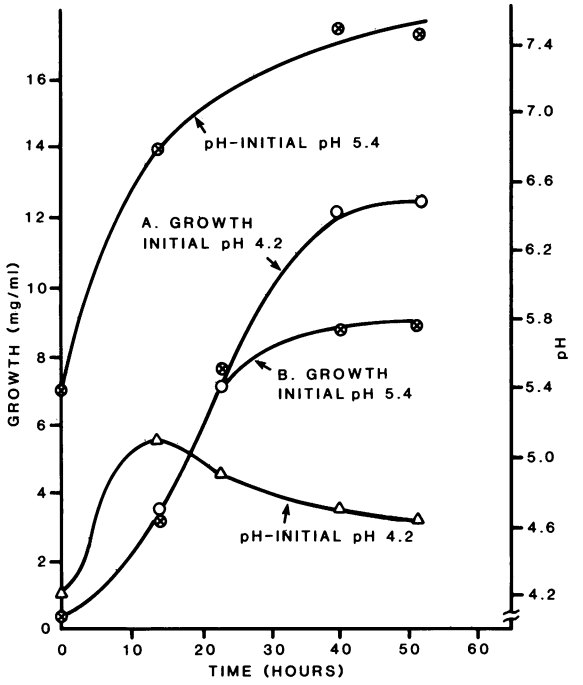

FIG. 3. Effect of initial pH on growth and pH change in starch medium. Strain SD1 was grown on YPS medium with 2% starch. Lintner starch was used.

curve with an initial phase which gradually accelerates until a rapid exponential phase is attained.

It was also observed (data not shown) that the size of the inoculum used had a marked effect on the shape of the growth curve. Apparently, the concentration of amylolytic enzymes introduced with the inoculum is an important determinant of this effect. Consequently, in comparing fermentation by different strains (see below), the bulk of the enzyme-containing medium was removed from inoculum cells to ensure reproducible growth behavior.

There is a pronounced effect of pH on the growth of *S. diastaticus* on starch medium. Strain SD1 gave substantially more growth on unbuffered YPS medium with 2% starch when the initial pH was 4.2 (Fig. 3, curve A) rather than 5.4 (curve B). Moreover, there was a marked difference in the pattern of pH change during growth. Whereas pH rose continuously to 7.5 from an initial value of 5.4, it rose to 5.0 and then gradually declined to 4.6 when the initial value was 4.2. It seems likely that the marked decline in growth rate after 20 h (curve B), was largely a consequence of the high pH (7.0) attained. At least two factors may be involved. First, yeast does not grow rapidly at this pH, and second, the pH optimum for three different glucoamylases produced by *S. diastaticus* strains was found to be 5.4 at 25°C (J. Erratt, Ph.D. thesis, University of Western Ontario, London, Ont., Canada, 1980), far below the medium pH of 7.0 (at 20 h). Further improvement in both growth and fermentation was obtained by adding 0.1 M succinate buffer to the YPS medium (data not shown).

Comparison of ethanol production and enzyme production by different strains. Figure 4 illustrates the progress of ethanol production by three different yeasts, strain D261, a diploid laboratory strain of *S. cerevisiae* (Fig. 4A), strain IT-6A, a diploid (or polyploid) brewing strain from a Brazilian ethanol plant (Fig. 4B), and strain SD2, a diploid *S. diastaticus* strain (Fig. 4C). Each strain was tested individually with three carbohydrates: glucose, dextrin (prepared from starch with commercial α-amylase), and Lintner starch. Both *S. cerevisiae* D261 and brewing strain IT-6A exhibited very weak starch fermentations, whereas strain SD2 fermented starch actively. With this strain, the yield of ethanol from 4% starch after 12 days was 80% of that obtained from 4% glucose. Prior digestion of starch to dextrin by α-amylase increased the ethanol yield to 97% of that obtained from glucose during a 4-day period. A and B in Fig. 4 show that strain IT-6A, although unable to ferment starch, is capable of fermenting dextrin slowly. In contrast, strain D261 (Fig. 4A) produces very little ethanol from the same preparation.

For most strains tested, a rough correlation between the production of extracellular amylolytic activity and ethanol production was found. Figure 5 compares the course of enzyme excretion and ethanol production in three strains, SD1, SD2, and a new hybrid strain, CL9 (described below). Both enzyme formation and ethanol production by strain SD1 were relatively slow, with ethanol concentration attaining only 0.16% (vol/vol) in 2 days. Although both enzyme and alcohol were produced faster by strain SD2, the new hybrid, strain CL9, was clearly superior to either SD1 or SD2, attaining an ethanol concentration of 0.9% in 2 days, six times that obtained with strain SD1 during the same period. There was only a twofold difference in the enzyme titers of SD1 and CL9. The slower-fermenting strains usually attained about the same final yield of ethanol if fermentation time was extended up to 12 days. However, because rapid fermentation is economically desirable, ethanol production from starch within 2 days was used subsequently as a criterion for selecting new starch-fermenting yeast varieties. Amylolytic enzyme production and aerobic growth rate were also used in comparing strains.

Hybridization of yeast and selection of rapidly fermenting strains. Table 2 shows that diploid *S. diastaticus* strains obtained from different laboratories varied considerably with respect to growth on starch and production of amylolytic enzymes and ethanol. All strains tested gave substantial aerobic growth on BYPS medium, but strain Y83 gave a low yield. Since strain SD2 produced the greatest amount of amylolytic activity and produced ethanol rapidly, it was selected for further study.

Also shown in Table 2 are data for strain SD2-A8, a haploid segregant obtained from a random population of haploid strains obtained by plating a sporulated culture of strain SD2 on YPS plates. Both enzyme production and ethanol formation by this haploid were greater than that by the parental strain. Consequently, this strain was chosen for subsequent genetic studies and was used in producing new hybrid strains.

When appropriate haploids had been collected, hybrid diploids containing at least two *STA* (*DEX*) genes were constructed by mating various haploid pairs (Table 3). Diploids were selected either by morphological markers (CL4 through CL12; CL21 and CL22) or by prototrophic selection (CL13 through CL20).

Table 3 also summarizes the 2-day ethanol production obtained when the hybrids and their respective haploid parents were tested on BYPS medium. Also shown are data for diploids resulting from three crosses between SD2 segregants (*STA*) and *S. cerevisiae* haploids (CL1, CL2, and CL3). As others have observed (4, 15, 16), the *STA* genes derived from SD2 are essentially dominant.

The relative ethanol productions for the haploids obtained

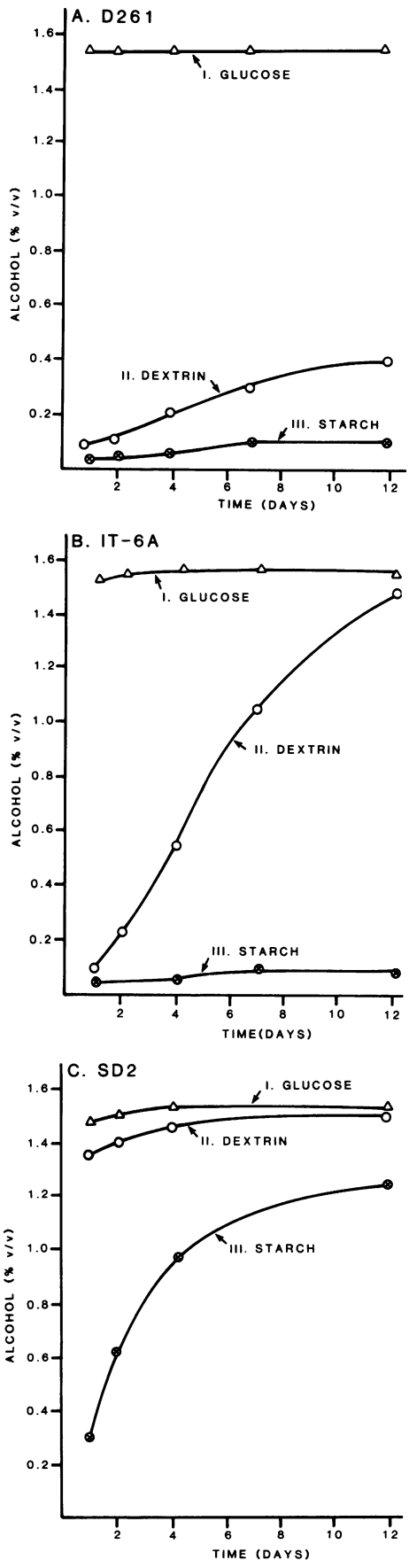

FIG. 4. Alcohol production from starch, dextrin, and glucose by different yeast strains. Fermentation media contained BYP plus 4% carbohydrate: I, glucose; II, dextrin prepared by α-amylase digestion of starch; III, Lintner starch. (A) Strain D261. (B) Strain IT-6A. (C) Strain SD2.

from Canada and Japan are compared in Table 3. Two-day ethanol production ranged from 0.24% (vol/vol) for *DEX1* strain 1355, to 0.87% for *STA2* strain 5962-10A. These values may be compared with those obtained with SD2-A8 (1.18%) and SD2-A6/C1 (0.80).

Tamaki (17) identified three unlinked loci, *STA1*, *STA2*, and *STA3*, in *S. diastaticus* strains. In an independent investigation, Errat and Stewart (4) also identified three loci, using dextrin as fermentable carbohydrate. They showed that one of the genes controlling dextrin fermentation was allelic to the *STA3* gene found by Tamaki. The other two loci were designated *DEX1* and *DEX2*, and their allelism with *STA1* and *STA2* was not determined. As shown below, strain SD2-A8 also contains two *STA* genes, which may or may not be alleles of the genes described by Tamaki and Errat. Although a full knowledge of allelic relationships would have been ideal, the fact that all *STA* and *DEX* genes appear to be dominant to their respective nonfermenting alleles (note diploids CL1, CL2, and CL3) still permits one to test the effect of gene dosage on ethanol fermentation.

The hybrids CL4 through CL22 listed in Table 3 all received at least one *STA* gene inherited from strain SD2 together with a single *STA* or *DEX* gene from the Japanese and Canadian stocks. When two-day ethanol production was compared, only six of the hybrids exhibited additive or partially additive (apparent gene dosage) effects (CL6, CL8, CL9, CL17, CL19, and CL21).

Except for hybrids CL6, CL8, and CL9, the diploid production values were less than the sum of the corresponding haploid values. In CL6, however, there was an apparent synergism between *STA* and *DEX* genes.

In contrast, several of the hybrid strains produced the same amount or even less ethanol than one or both parental haploids. This effect was particularly apparent in strain CL16, which was the least productive of all the hybrids tested.

A group of seven hybrid strains was selected as showing promise. The strains chosen were CL4, CL5, CL6, CL9, CL13, CL18, and CL19. Strain CL16 was retained for comparison purposes. Two-day alcohol production values for these strains ranged from 0.82% (CL18) to 1.09% (CL9), all substantially higher than the value for strain SD2 (0.48%). The time course of ethanol production and amylolytic enzyme excretion for strain CL9 is shown in Fig. 5C. The behavior of other selected hybrids was quite similar.

Determination of the number of *STA* genes in strains SD2-A8. Since haploid strain SD2-A8 had ethanol fermentation characteristics superior to those of all other haploids tested, it was of interest to determine the number of *STA* genes in this strain. Therefore, diploid CL1 (SD2-A8 × JP293-25A/C1) was subjected to tetrad analysis, and segregants were scored for auxotrophic markers and *STA* genes by the technique illustrated in Fig. 1. Although the sizes of the zones of starch digestion varied, no clear pattern indicating the probable number of *STA* genes in the segregants was evident. Of 22 tetrads exhibiting normal segregation of auxotrophic markers, all contained three *STA* segregants and one *sta* (nonfermenting) segregant.

Fermentation of high concentrations of starch and dextrin. In commercial application of *S. diastaticus* it would be

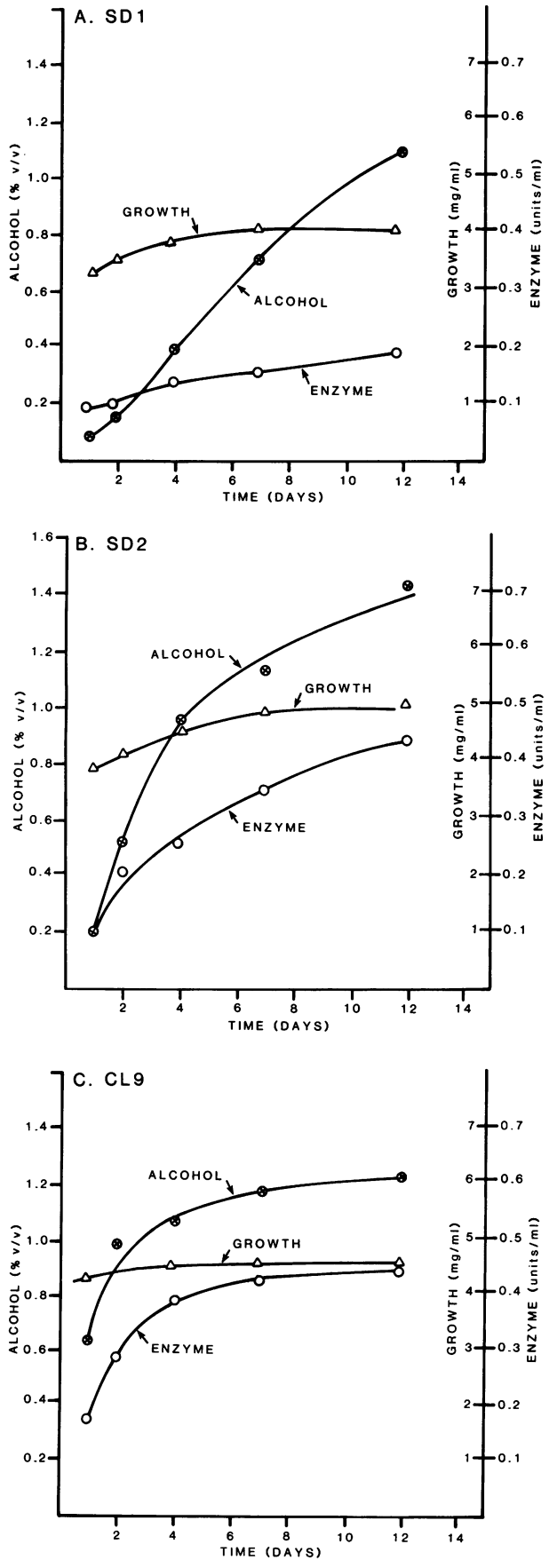

FIG. 5. Comparison of amylolytic enzyme secretion and ethanol production by different yeast strains. BYPS medium (4% starch) was used for all experiments.

TABLE 2. Comparison of growth, amylolytic activity, and ethanol formation by *S. diastaticus* strains from different laboratories[a]

Strain	Growth (mg/ml)[b]	Amylolytic activity (U/ml)[b]	Ethanol production (% [vol/vol] ± SD)[c]
SD1	15.1	0.19	0.18 ± 0.04
SD2	12.6	0.62	0.48 ± 0.05
SD7-01	15.4	0.23	0.10 ± 0.00
Y83	5.6	0.11	0.12 ± 0.07
NCYC 361	15.4	0.32	0.57 ± 0.00
SD2-A8[d]	9.1	0.72	1.18 ± 0.04

[a] Each flask was inoculated with 0.5 g (dry weight) of washed yeast cells.
[b] Growth and amylolytic activity were measured after 3 days of aerobic growth.
[c] Ethanol production was determined after 2 days of anaerobic fermentation. BYPS medium was used for both types of experiment.
[d] Strain SD2-A8 is a haploid segregant obtianed by sporulating strain SD2.

desirable to utilize high starch concentrations. The effects of increasing starch concentrations on alcohol production were therefore studied (Fig. 6). There was a linear increase in alcohol production with starch concentration up to 6%, and then efficiency dropped off sharply.

An alternative method for increasing carbohydrate and ethanol concentrations involves the prior conversion of starch to dextrin by α-amylase (Taka-therm). Dextrin concentrations up to 25% were converted to ethanol as efficiently as a similar concentration of glucose, provided that additional fermentation time was allowed (Fig. 7). With 25% carbohydrate, ethanol concentration reached 9.29% (vol/vol).

Ethanol production from crude manioc starch. As a practical test of the applicability of *S. diastaticus* fermentation to production of ethanol from an abundant source of starch biomass, crude manioc meal was employed. Nutrients present in the "milk" of the whole manioc root, which are preserved in the crude meal, are adequate to support fermentation. As shown in Fig. 8, 12% ethanol was producd in a 4-day fermentation of a 40% suspension of α-amylase-treated manioc meal. Comparable results were obtained with added nutrients.

DISCUSSION

The results shown in Fig. 4 indicate that *S. cerevisiae* D261 produces very little ethanol from α-amylase-digested starch. This limited fermentation may represent slow utilization of very small dextrin molecules, because no free glucose could be detected in the digested preparation by using glucose oxidase. The very limited starch-digesting capacity of strain D261 is sufficient to permit slow aerobic growth on minimal medium containing dialyzed starch as the sole carbon source.

The results with the hybrid yeasts indicate that genes in the background genomes, other than *DEX* and *STA* genes, can play a significant part in determining rates of ethanol production from starch. These epistatic effects may be either positive (strain CL6) or negative (strain CL16). No specific *DEX* or *STA* gene can be considered specifically susceptible to either type of effect.

In the analysis of tetrads from diploid CL1, the percentage of nonfermenting segregants (25%) is what would be expect-

TABLE 3. Two-day alcohol production by haploid and hybrid diploid yeast strains

Strains		Ethanol production (% [vol/vol] ± SD)	Genotype
Haploid parents	Hybrid diploid		
SD2-A8		1.18 ± 0.04	α *STA*
JP293-25A/C1		0.00 ± 0.00	**a** *ade6 his2 can*^{r}
	CL1	0.81 ± 0.07	
1268-2B		0.00 ± 0.00	α *ade6 his2*
SD2-A6		0.58 ± 0.06	**a** *STA*
	CL2	0.68 ± 0.06	
SD2-A1		0.42 ± 0.13	α *STA*
JP293-25A/C1		0.00 ± 0.00	**a** *ade6 his2 can*^{r}
	CL3	0.42 ± 0.000	
SD2-A8		1.18 ± 0.04	α *STA*
1354		0.39 ± 0.10	**a** *DEX1*
	CL4	0.97 ± 0.09	
SD2-A8/C5		1.03 ± 0.15	α *STA ade*
1354		0.39 ± 0.10	**a** *DEX1*
	CL5	1.03 ± 0.03	
CL1-A2		0.21 ± 0.09	α *ade6 can*^{r} *STA*
1354		0.39 ± 0.10	**a** *DEX1*
	CL6	0.94 ± 0.04	
SD2-A1/C1		0.88 ± 0.04	α *ade STA*
1354		0.39 ± 0.10	**a** *DEX1*
	CL7	0.79 ± 0.00	
1355		0.24 ± 0.02	α *DEX1*
CL1-A1		0.42 ± 0.13	**a** *ade6 can*^{r} *STA*
	CL8	0.66 ± 0.09	
1355		0.24 ± 0.02	α *DEX1*
SD2-A6/C1		0.80 ± 0.05	**a** *ade STA*
	CL9	1.09 ± 0.09	
1355		0.42 ± 0.13	α *DEX1*
CL1-A3		0.57 ± 0.07	**a** *his2 can*^{r} *STA*
	CL10	0.55 ± 0.05	
1359		0.57 ± 0.01	α *DEX2*
CL1-A1		0.91 ± 0.08	**a** *ade6 can*^{r} *STA*
	CL11	0.51 ± 0.18	
1359		0.57 ± 0.01	α *DEX2*
CL1-A3		0.57 ± 0.07	**a** *his2 can*^{r} *STA*
	CL12	0.47 ± 0.18	
5962-10A		0.87 ± 0.06	α *lys7 STA2*
SD2-A6/C1		0.80 ± 0.02	**a** *ade STA*
	CL13	0.88 ± 0.07	
5962-10A		0.87 ± 0.06	α *lys7 STA2*
CL1-A1		0.18 ± 0.08	**a** *ade6 can*^{r} *STA*
	CL14	0.52 ± 0.14	
5962-10A		0.87 ± 0.06	α *STA2 lys7*
CL1-A3		0.42 ± 0.13	**a** *his2 can*^{r} *STA*
	CL15	0.39 ± 0.08	
708-7B		0.65 ± 0.01	α *arg4 STA1*
CL1-A3		0.57 ± 0.07	**a** *can*^{r} *his2 STA*
	CL16	0.26 ± 0.05	
708-7B		0.65 ± 0.01	α *arg6 STA1*
CL1-A1		0.57 ± 0.07	**a** *ade6 can*^{r} *STA*
	CL17	0.79 ± 0.03	
5301-17B		0.78 ± 0.11	α *lys7 STA3*
SD2-A6/C1		0.80 ± 0.05	**a** *ade STA*
	CL18	0.82 ± 0.01	
5301-17B		0.78 ± 0.11	α *lys7 STA3*
CL1-A3		0.57 ± 0.07	**a** *can*^{r} *his STA*
	CL19	0.92 ± 0.02	
5301-17B		0.78 ± 0.11	α *lys7 STA3*
CL1-A1		0.18 ± 0.08	**a** *ade can*^{r} *STA*
	CL20	0.49 ± 0.16	
SD2-A1		0.42 ± 0.13	α *STA*
1354		0.39 ± 0.10	**a** *DEX1*
	CL21	0.68 ± 0.04	
SD2-A8		1.18 ± 0.04	α *STA*
1360		0.64 ± 0.04	**a** *DEX2*
	CL22	0.59 ± 0.04	

ed for two unlinked *STA* genes. However, for normal two-gene segregation, about one in six should have had four *STA* segregants (nonparental ditype). Since 19 other tetrads were dissected which showed some abnormality in marker segregation, we tentatively conclude that strain SD2-A8 may have been aneuploid for some chromosomes. Consequently, strain SD2 could be polyploid.

This investigation demonstrates that direct fermentation of starch by *S. diastaticus* can be accomplished with consid-

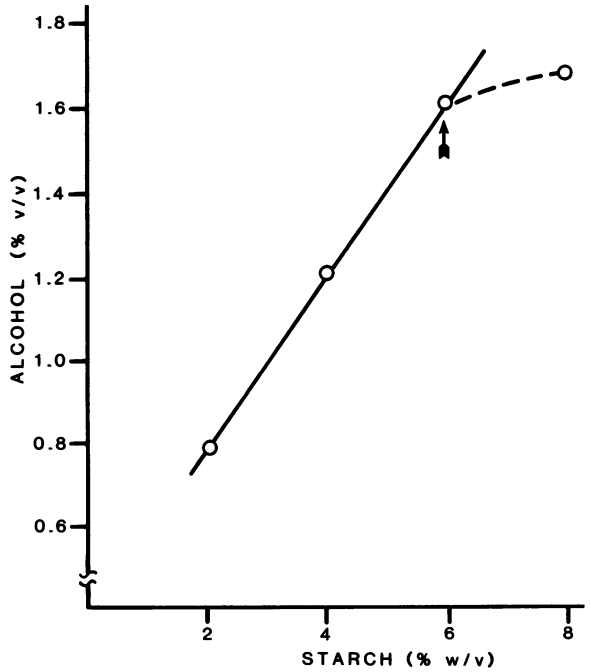

FIG. 6. Effect of increasing starch concentration on ethanol yield. Strain SD2 was used with BYP medium containing the indicated concentrations of Lintner starch. Fermentation time was 12 days. Above 6% starch concentration, strong retrogradation (precipitation) of starch occurred (arrow).

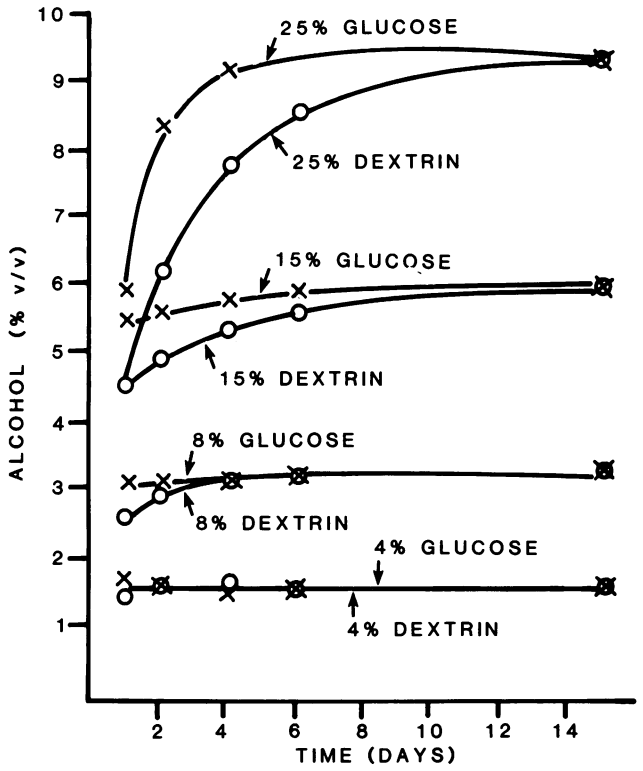

FIG. 7. Effects of dextrin and glucose concentrations on long-term ethanolic fermentation by strain SD2. Media consisted of BYP plus the indicated concentrations of glucose or dextrin prepared by hydrolysis of Lintner starch with α-amylase.

erable efficiency through a combination of strain selection, hybridization, and systematic optimization of fermentation conditions. Up to 80% conversion of Lintner starch to ethanol was attained by *S. diastaticus* alone, and 97% conversion of carbohydrate occurred when starch was first digested to dextrins by commercial α-amylase. Under the latter conditions, 40% (wt/vol) carbohydrate could be fermented by *S. diastaticus* to produce 12% (vol/vol) ethanol. With α-amylase treatment, crude manioc (cassava) starch was converted to ethanol with almost 100% efficiency. Since the manioc preparation contained sufficient growth factors and minerals, no additional medium supplements were required. Consequently, the procedure should be suitable for direct application in manioc-producing regions in Brazil and elsewhere.

From an industrial perspective, the direct fermentation of starch by *S. diastaticus* has two limitations. Firstly, the low solubility of starch limits the carbohydrate concentration which can be used in the fermentation mash. A relatively inexpensive acid treatment (Lintner process) produces a product which is soluble up to 6% (12). Attempts to exceed this concentration led to loss of solubility and interfered with conversion of the starch to ethanol (Fig. 6). In agreement with other investigators (7, 14), we found that there is a component of starch which resists attack by *S. diastaticus* enzymes acting alone. This component, which represents about 20% of Lintner starch, can be rendered fermentable by prior action of the α-amylase preparation (Taka-therm) employed. It is not known whether this preparation contains α-1→6-glucosidase activity as well as α-amylase.

There is a wide range of rates of ethanol production from Lintner starch by strains of *S. diastaticus* from various laboratories. Evidently, the most important determinant of these differences is the rate of glucoamylase production (Fig. 5). The hybridization experiments reported here do not indicate a simple relationship between gene-dosage and rate of ethanol production. Among 19 hybrids investigated, only 6 showed additive effects, and 1 of these behaved synergistically. In 11 other hybrids the rate of ethanol production by the hybrid either was no greater than that of the best parent or had an intermediate value. Two hybrids were inferior to either parent. Clearly, the rate of ethanol production is not simply a function of *STA* gene dosage, but involves other (probably several) interacting genetic loci. Although Erratt and Stewart found gene-dosage effects on dextrin fermentation with strains containing *DEX1* or *DEX2* genes or both, similar effects were not obtained with *STA3* (4).

The possible existence of a separate α-1→6-glucosidase which is excreted by *S. diastaticus* might be invoked to explain the synergism obtained in diploid CL6 (Table 3). However, because synergism is a relatively rare event, it seems doubtful that a gene for an extracellular debranching enzyme is present in any of the genomes investigated.

The presence of negative epistatic effects (hybrids CL14 and CL16) are reminiscent of the studies of Gilliland (5) and Hopkins (7). From a hybrid obtained from *S. diastaticus* and *Saccharomyces chevalieri*, Gilliland obtained a haploid which exhibited very slow dextrin fermentation and low levels of glucoamylase secretion (5). Although a relationship between a maltose-fermentation gene (*MAL*) and rapid fermentation was inferred, the actual basis for the epistatic effects merits further investigation.

The ability of Brazilian strain IT-6A to ferment dextrins may be related to the work of Gilliland (6) in which he reports the existence of a gene which permits fermentation of maltotetraose in a *Saccharomyces* strain. Conceivably,

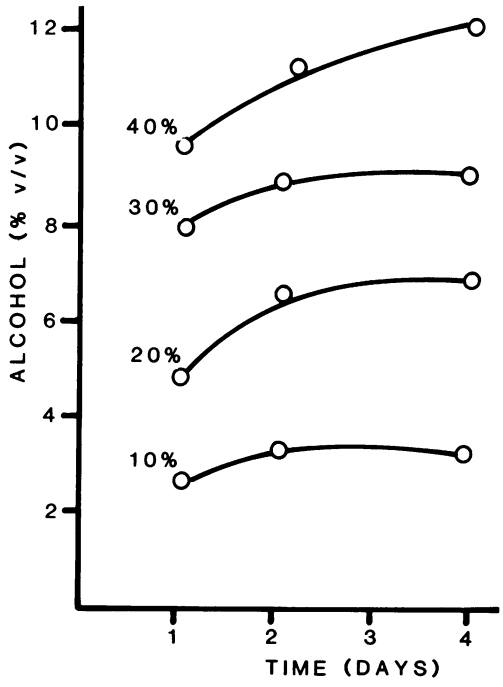

FIG. 8. Production of ethanol from crude manioc meal solubilized by α-amylase treatment. Strain SD2 was used.

the behavior of strain IT-6A reflects the presence of a similar gene.

An important feature of the present investigation is the control of pH. Since glucoamylase is extracellular, it must be protected from fermentation-related pH changes to behave in a controlled, predictable manner.

Since this study was completed, a report has appeared (13) which shows that some strains of *S. cerevisiae* contain a dominant gene, *STA10*, which is epistatic to starch fermentation genes (*STA1*, *DEX1*, etc.). *STA10* suppresses expression of the fermentation genes.

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