High-Efficiency, One-Step Starch Utilization by Transformed Saccharomyces Cells Which Secrete Both Yeast Glucoamylase and Mouse α-Amylase

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Transformed, hybrid Saccharomyces strains capable of simultaneous secretion of glucoamylase and α-amylase have been produced. These strains could carry out direct, one-step assimilation of starch, with conversion efficiency greater than 93% during a 5-day growth period. One of the transformants converted 92.8% of available starch into reducing sugars in only 2 days. Glucoamylase secretion by these strains resulted from expression of one or more chromosomal STA genes derived from Saccharomyces diastaticus. The strains were transformed by a plasmid (pMS12) containing mouse salivary α-amylase cDNA in an expression vector containing yeast alcohol dehydrogenase promoter and a segment of yeast 2μm plasmid. The major starch hydrolysis product produced by crude amylases found in culture broths was glucose, indicating that α-amylase and glucoamylase acted cooperatively.

The conversion of starch biomass to industrial and fuel ethanol by Saccharomyces cerevisiae or Saccharomyces carlsbergensis employs a three-step process: (i) liquefaction of starch with bacterial α-amylase, (ii) enzymatic saccharification of the liquefied starch to produce fermentable sugars, and (iii) fermentation of the sugars. The commercial enzymes used for starch degradation represent a significant cost in the production of fermentation alcohol. Several yeasts of genera other than Saccharomyces assimilate starch, and a number of these organisms secrete enzymes with debranching or α-amylase activity or both (15). Recent interest in such yeasts has focused on their potential for one-step starch fermentation (1, 4). However, these amylolytic yeasts are generally not suitable for alcohol production because they have a low tolerance for ethanol and exhibit slow fermentation rates (4).

On the other hand, one amylolytic yeast, Saccharomyces diastaticus (2), does exhibit high tolerance for alcohol and high fermentation rates (4, 12). Laluce and Mattoon (12) examined a strain that produced 12% (vol/vol) ethanol in a 4-day fermentation. S. diastaticus is very closely related to S. cerevisiae genetically, and the two species are readily hybridized (12, 25). The primary difference between them is that S. diastaticus secretes a glucoamylase, whereas S. cerevisiae lacks this ability. Glucoamylase secretion is determined by the presence of one or more linked glucoamylase structural genes in S. diastaticus (7, 14, 19, 25). Three such genes, STA1, STA2, and STA3, have been identified (7, 25) and cloned (8, 14, 18, 20, 30). The nucleotide sequence of the STA1 gene has been determined (31).

Laluce and Mattoon (12) evaluated a variety of S. diastaticus strains for direct conversion of starch and dextrans to ethanol. Through a combination of strain selection, hybridization, and systematic optimization of fermentation conditions, up to 80% conversion of Lintner starch was attained by using S. diastaticus. The residual 20% carbohydrate represents some type of limit dextrin which is refractory to hydrolysis by S. diastaticus glucoamylases. However, this refractory starch residue can be almost entirely eliminated by prior treatment of starch with α-amylase. When starch was first digested by commercial α-amylase (Taka-therm; Miles Laboratories, Inc., Elkhart, Ind.), 97% conversion of starch was achieved with S. diastaticus (12). Several laboratories have introduced heterologous α-amylase genes derived from various organisms into S. cerevisiae to produce transformants which secrete active α-amylase into the culture medium (9, 22, 26, 27, 29). For example, Thomsen (26, 27) constructed chimeric plasmids containing mouse salivary α-amylase cDNA under control of the promoter of the S. cerevisiae alcohol dehydrogenase I (ADH1) gene ADC1. A transformant of S. cerevisiae bearing plasmid pMS12 is capable of direct fermentation of starch from various sources, but conversion of carbohydrate to alcohol is not efficient, varying from 10 to 50% (27).

The present report describes the preparation of a yeast strain which secretes both α-amylase and glucoamylase. This strain was obtained by transforming a S. diastaticus derivative with the mouse salivary α-amylase plasmid pMS12. With this yeast, 97% degradation and 93% utilization of Lintner starch was obtained.

MATERIALS AND METHODS

Yeast strains. Strains containing both STA (glucoamylase) genes and the transformation selection marker trp1 were constructed by crossing S. cerevisiae SHU32a with S. diastaticus 5301-17B and S. cerevisiae SHU32α with hybrid strain CL1-17B. The resulting diploids were sporulated, and segregants KK1-R1 and KK2-R1, exhibiting both tryptophan auxotrophy and glucoamylase secretion, were selected. The genotypes of these various strains are listed in Table 1. All strains listed in this table exhibited a Mal+ (maltose fermentation) phenotype.

Plasmid. Plasmid pMS12 (27), containing a cDNA coding for mouse salivary α-amylase in yeast expression vector pMA56 (26), was generously provided by Karl K. Thomsen, Carlsberg Laboratory, Valby, Denmark. The expression vector contains the Escherichia coli origin of replication and the β-lactamase gene of pBR322, a segment of yeast 2μm
TABLE 1. Genotypes and sources of yeast strains used

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHU32a</td>
<td>a leu2 trp1 ura3</td>
<td>M. Breitenbach, Austria</td>
</tr>
<tr>
<td>SHU32a</td>
<td>a leu2 trp1 ura3</td>
<td>M. Breitenbach, Austria</td>
</tr>
<tr>
<td>5301-17B</td>
<td>a lys7 STA3</td>
<td>H. Tamaki, Japan</td>
</tr>
<tr>
<td>CL1-17B*</td>
<td>a ade6 his2 STA*</td>
<td>J. Mattoo, this laboratory</td>
</tr>
<tr>
<td>KK1-R1*</td>
<td>trp1 trp1 ade6</td>
<td>This work</td>
</tr>
<tr>
<td>KK2-R1*</td>
<td>trp1 ade6 his2 STA*</td>
<td>This work</td>
</tr>
</tbody>
</table>

* A hybrid strain containing STA gene(s) derived from S. diastaticus. The genome is derived from both S. cerevisiae and S. diastaticus.
* Contains one or more STA genes of undefined genetic locus.
* Descendent from the cross SHU32a × 5301-17B (KK1).
* The mating type could not be determined, and the strain is probably homothallic.
* Descendent from the cross SHU32a × CL1-17B (KK2).

DNA containing an origin of replication, the yeast TRP1 gene, and the promoter of the alcohol dehydrogenase I gene ADO1. In chimeric plasmid pMS12, the α-amylase cDNA has been inserted by means of an EcoRI linker downstream of position −14 of the ADO1 gene. The cDNA includes the mature-amylase signal peptide, which is 15 amino acid residues long. Plasmid pMS12 was maintained in E. coli C600 SF8 grown in LB medium containing an ampicillin concentration of 50 μg/mL. Amplification and extraction of the plasmid were performed as described by Maniatis et al. (13).

Media. Complete yeast growth media contained 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% Difco peptone, and an appropriate carbon source. YPD medium contained 2% glucose. YPD153 medium contained 1% glucose and 3% Lintner soluble potato starch (Sigma Chemical Co., St. Louis, Mo.). Buffered starch medium contained 0.1 M succinic acid and either 2 or 4% Linter starch (BYPS2 and BYPS4). The pH of buffered media was adjusted to 4.2. Minimal medium contained 0.6% Difco yeast nitrogen base (without amino acids), 2% glucose, and nutritional supplements as required (24). Sporulation medium contained 1.0% potassium acetate, 0.1% Difco yeast extract, and 0.05% glucose. LB medium (pH 7.5) was used for E. coli cultures and contained 1% Difco tryptone, 0.5% Difco yeast extract, and 1% NaCl.

Genetic methods. Construction of recipient yeast strains KK1-R1 and KK2-R1, as described above, was by standard genetic procedures as described by Mortimer and Hawthorne (16). After the treatment of asci with Glusulase (Du Pont Co., Wilmington, Del.), spores were isolated by micro-manipulation or by plating on YPD medium, incubating 2 days, and then selecting putative haploid colonies as those which were significantly smaller than diploid colonies. Haploids containing the trp1 marker were then tested for the presence of the STA gene by the ability of colonies to produce halos on YPD153 plates after incubation for 5 days followed by refrigeration at 4°C for 2 days. Glucoamylase-producing strains produced clear halos in a turbid background, as described by Laluce and Mattoo (12). Mating-type tests and auxotrophic marker analysis were performed as described by Sherman et al. (24).

Transformation. Yeast cells were transformed by the method of Ito et al. (10), and transformants were selected on minimal medium lacking tryptophan.

Growth. All growth experiments were conducted at 30°C. Stock cultures were grown on YPD medium, except for transformants carrying pMS12, which were grown on minimal medium lacking tryptophan. For the assay of amylase secreted by yeasts, a half loopful of cells previously grown on minimal medium for 2 days was used to inoculate a 125-ml flask containing 30 ml of BYPS2 medium; the inoculated medium was incubated aerobically on a rotary shaker operated at 300 rpm for 3 days. For the measurement of residual carbohydrate and yeast growth, 2 loopfuls of cells previously grown on minimal medium for 2 days were used to inoculate a 500-ml flask containing 150 ml of BYPS4 medium; the inoculated medium was incubated at 30°C on a rotary shaker for 5 days at 300 rpm.

Carbohydrate assay. Glucose was assayed with a glucose oxidase-peroxidase method by using PGO-enzymes supplied by Sigma. Reducing sugar was determined by the colorimetric method by using 3,5-dinitrosalicylic acid as described by Bernfeld (3), using glucose to make a standard curve. For the analysis of residual starch in the culture medium, 1 ml of culture supernatant was mixed with 1 ml of 2 N HCl, and hydrolysis was accomplished by heating the mixture in a boiling water bath for 35 min, the time required for complete hydrolysis of starch (11). After neutralization of the hydrolysate with 1 ml of 2 N NaOH, the reducing sugar released from starch was determined, and the starch content was calculated by using the following equation: percent reducing sugar after hydrolysis = percent reducing sugar before hydrolysis) × (1.134 + 0.0594). The conversion factor 1.134 was established by following the hydrolysis of Lintner potato starch (1, 2, and 4%) by using exactly the same procedure and by dividing starch content before hydrolysis by reducing sugar content after hydrolysis.

The percentage of sugar taken up by yeast during growth was determined by using the following equation: percent sugar uptake = 100 – percent residual starch – percent residual sugar. The presence of residual starch was also assayed qualitatively by a starch-iodine reaction: 1 volume of centrifuged culture fluid was mixed with 0.1 volume of 0.4% iodine in 2% KI and examined for the purple color of starch-iodine complex.

All carbohydrase assays were performed with two different transformants of each strain, and the averages were recorded in the tables and figure.

Amylase assay. The reaction mixture for the enzyme assay contained 0.2 ml of 1.6% Lintner potato starch, 0.1 ml of sodium acetate buffer (1 M, pH 5.0), and 0.7 ml of centrifuged culture fluid as crude enzyme solution. After a 30-min incubation at 55°C, the reaction was stopped by immersing the tube containing the reaction mixture in a boiling water bath for 10 min. The tube was then cooled, and the contents of glucose and reducing sugars were measured. A reaction mixture containing inactive culture fluid which had been previously heat treated for 10 min in a boiling water bath was used as a blank. One unit of the enzyme is defined as the amount that liberated 1 μmol of glucose or reducing sugar per min per ml of enzyme sample.

Growth measurement. Growth of yeast strains in BYPS4 was estimated from measurement of A420 and multiplication by a conversion factor to obtain dry weight (12). The growth measurement was performed with two different transformants of each strain, and the average was recorded.

Mitotic stability. A diluted sample of cell suspension grown aerobically in 150 ml of BYPS4 for 3 or 5 days was plated onto plates of minimal medium with or without added tryptophan. After 5 days of incubation, the mitotic stability was determined by using the following equation: mitotic stability (percent) = 100 × (number of colonies on medium without tryptophan/number of colonies on medium with
tryptophan). The mitotic stability was measured for two different transformants of each strain, and the average was recorded.

RESULTS AND DISCUSSION

Effects of α-amylase gene on growth and starch utilization. Strains SHU32a, KK1-R1, and KK2-R1 were transformed to tryptophan prototrophy with plasmid pMS12 with an efficiency ranging from 3,500 to 6,300 transformants per μg of DNA. All transformant colonies produced clear halos on plates of starch medium.

The time courses of starch hydrolysis for each transformant and for the corresponding recipient strain (untransformed) during a 5-day growth period are shown in Fig. 1. S. cerevisiae SHU32a (untransformed) exhibited only a trace of growth and no detectable hydrolysis of starch (Fig. 1A). In contrast, the transformant gave moderate growth and decreased the starch content to 12.9% of the initial concentration of 4 g/100 ml. As shown below, a substantial fraction of the starch was only partially hydrolyzed to oligosaccharides which could not be assimilated.

The S7A-containing hybrids and their corresponding transformants behaved quite differently (Fig. 1B and C). The untransformed strains, because they secreted glucoamylase, gave substantial growth on starch medium, and by the end of 5 days decreased the starch content of the medium significantly, by 71.0 and 72.1% for KK1-R1 and KK2-R1, respectively. Strain KK2-R1 grew and hydrolyzed starch more rapidly than did strain KK1-R1.

The transformants of these hybrid strains exhibited more rapid and more extensive growth than did the S. cerevisiae (SHU32a) transformant. Moreover, starch hydrolysis was almost complete, exceeding 97% by the end of 5 days in the transformants producing both α-amylase and glucoamylase. The hydrolysis rate of KK2-R1 was excellent, and 92.8% of initial starch content was already degraded after 2 days.

These observations were confirmed by using the starch-iodine reaction. After only 1 day, the culture fluid produced by the KK2-R1 transformant no longer exhibited the typical

FIG. 1. Time course of starch hydrolysis and growth of untransformed and transformed strains of yeast.
purple color of the starch-iodine complex but only a faint red color, indicating the presence of residual dextrins. In contrast, the 1-day culture fluids of all untransformed strains as well as transformants of KK1-R1 and SHU32a exhibited strong purple colors. After 2 days of growth, culture fluids from all three transformants gave negative starch-iodine reactions, whereas the untransformed strains continued to exhibit positive results. Even at the end of the 5-day growth period, cultures of all of the untransformed strains tested positive for starch, indicating that a significant fraction of starch was not hydrolyzed by glucoamylase alone. In contrast, all of the transformants gave negative results for the starch-iodine test.

**Amylolytic activities secreted by transformed and untransformed strains.** Cell-free culture fluids from transformed and untransformed strains were examined for amylolytic activity (Table 2). Important differences were obtained when activity measured enzymatically as glucose produced was compared with activity determined in terms of reducing sugar formed. As expected, untransformed *S. cerevisiae* SHU32a culture fluid exhibited no detectable amylolytic activity measured by either technique. Individual pMS12 transformants of this strain produced reducing sugar equivalents measured chemically with the 3,5-dinitrosalicylic acid reagent, but none of this material was free glucose. This result reflects the fact that α-amylase produces primarily maltose, maltotriose, and oligosaccharides from starch (5). Thus, the sole amylolytic activity secreted by these *S. cerevisiae* transformants was apparently α-amylase.

Unlike the *S. cerevisiae* transformants, the transformed *S. diastaticus*/*S. cerevisiae* hybrids secreted amylolytic activities which could be measured both by the chemical method and by the glucose oxidase procedure. Reducing sugar produced by untransformed strains KK1-R1 and KK2-R2 consisted almost entirely of glucose, reflecting the fact that glucoamylase is an exo-amylase, acting on the nonreducing ends of starch chains to release free glucose (15). The amylolytic activity produced by transformants of the hybrid strains also produced reducing sugar that was largely glucose, although the slightly higher values obtained by chemical assay may reflect the presence of a small percentage of oligomeric hydrolysis products.

The effect of the α-amylase gene on the production of amylolytic activity by the hybrid strains appeared to be more or less additive when reducing sugar was assayed. For example, the sum of the α-amylase activity produced by transformed strain SHU32a (0.26 U/ml) plus the activity of the glucoamylase produced by untransformed strain KK2-R1 (0.37 U/ml) was 0.63 U/ml, very similar to the values obtained with KK2-R1 transformants (0.60 and 0.66). However, when activity was assayed enzymatically with glucose oxidase, it was evident that secreted α-amylase was in fact acting cooperatively with the glucoamylase produced as a result of STA gene expression. Whereas enzyme obtained from SHU32a transformants produced no detectable glucose, enzymes from transformants of the hybrid strains produced much more glucose than did the enzyme secreted by corresponding untransformed strains. Because the additional hydrolysis product was largely glucose, it may be concluded that the oligomeric products of α-amylase action were serving as substrates for glucoamylase.

McCann and Barnett (15) have noted that α-amylase is an endoglucanase which hydrolyzes the linkages of starch in a random fashion, producing mostly oligosaccharides containing two to seven glucose residues. Therefore, the cooperative interaction just described most probably resulted from the increased concentration of molecules with nonreducing ends produced from starch by α-amylase, which in turn could serve as substrate molecules for glucoamylase, thereby increasing the rate of formation of free glucose.

As noted above, starch hydrolysis by α-amylase produces primarily maltose, maltotriose, and oligosaccharides from starch, with maltose becoming the predominant product as hydrolysis proceeds (5, 27). Since maltose and maltotriose can reduce the 3,5-dinitrosalicylic acid reagent, it is very likely that these substances represent a major fraction of the reducing sugar produced by amylolytic activity in culture fluid produced by transformed SHU32a.

**Sugar uptake and residual carbohydrate resulting from growth of transformed and untransformed strains.** Because secreted amylases are present continuously during growth of yeast cells, the carbohydrate content of the culture broth at a given time will reflect both the types and activities of the amylolytic enzymes present as well as the ability of the cells to assimilate various starch hydrolys products. Table 3 presents an analysis of sugar uptake and residual carbohydrates present in 5-day culture medium after growth of the various transformants and their untransformed counterparts.

As expected, untransformed *S. cerevisiae* SHU32a consumed little or no sugar, and almost all the starch remained at the end of the incubation period. In contrast, less than 30% of the starch remained in the broth obtained from cultures of the two untransformed hybrids KK1-R1 and KK2-R1, reflecting the activity of the glucoamylase secreted by these strains. The very low residual sugar content of the

**Table 2. Amylolytic activities of transformed and untransformed yeast strains**

<table>
<thead>
<tr>
<th>Yeast strain and condition</th>
<th>Amylolytic activity (U/ml) with product:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>SHU32a</td>
<td></td>
</tr>
<tr>
<td>Untransformed</td>
<td>0</td>
</tr>
<tr>
<td>Transformant 1</td>
<td>0</td>
</tr>
<tr>
<td>Transformant 2</td>
<td>0</td>
</tr>
<tr>
<td>KK1-R1</td>
<td></td>
</tr>
<tr>
<td>Untransformed</td>
<td>0.21</td>
</tr>
<tr>
<td>Transformant 1</td>
<td>0.44</td>
</tr>
<tr>
<td>Transformant 2</td>
<td>0.34</td>
</tr>
<tr>
<td>KK2-R1</td>
<td></td>
</tr>
<tr>
<td>Untransformed</td>
<td>0.33</td>
</tr>
<tr>
<td>Transformant 1</td>
<td>0.51</td>
</tr>
<tr>
<td>Transformant 2</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Table 3. Sugar uptake and residual carbohydrates in culture medium after growth for 5 days**

<table>
<thead>
<tr>
<th>Yeast strain and condition</th>
<th>Sugar taken up</th>
<th>Residual sugar*</th>
<th>Residual starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHU32a</td>
<td>2.6 ± 0.18</td>
<td>2.7 ± 0.24</td>
<td>2.8 ± 0.18</td>
</tr>
<tr>
<td>Untransformed</td>
<td>77.0 ± 0.26</td>
<td>10.2 ± 0.26</td>
<td>12.9 ± 0.53</td>
</tr>
<tr>
<td>Transformated</td>
<td>1.0 ± 2.24</td>
<td>1.6 ± 0.08</td>
<td>29.0 ± 2.83</td>
</tr>
<tr>
<td>KK1-R1</td>
<td>93.8 ± 0.41</td>
<td>3.0 ± 0.30</td>
<td>3.0 ± 0.73</td>
</tr>
<tr>
<td>Untransformed</td>
<td>69.8 ± 0.24</td>
<td>2.3 ± 0.74</td>
<td>27.9 ± 0.18</td>
</tr>
<tr>
<td>Transformated</td>
<td>93.6 ± 0.09</td>
<td>3.8 ± 0.26</td>
<td>2.6 ± 0.18</td>
</tr>
</tbody>
</table>

*Residual sugar measured as reducing sugar.
broths indicates that the primary starch hydrolysis product was glucose, which was rapidly assimilated and fermented by the yeast cells. Comparison of the results obtained with the three types of transformants reflects the differences in the type or types of amylases secreted. The transformant of SHU32a, which secretes only α-amylase, utilized less sugar (77.0%) than did the transformants of KK1-R1 or KK2-R1 (94%). Moreover, the SHU32a transformant left a substantial amount of residual starch (12.9%) and unutilized reducing sugar (10.2%) in the culture medium. In contrast, hybrid transformants left only about 3% unused starch and 3% unused reducing sugar. Clearly, transformants of KK1-R1 or KK2-R1 which secreted both types of amylases could utilize starch much more completely than could a strain which secreted only α-amylase or glucoamylase.

The relatively high concentration (10.2%) of residual sugar in culture medium of the SHU32a transformant, which secretes only α-amylase, is consistent with the observations of Panchal et al. (17) that this enzyme produces significant quantities of maltotetraose and larger oligosaccharides which cannot be transported into the yeast cell.

Although, as judged by the absence of the purple color of starch-iodine complex, starch appeared to be completely degraded by the SHU32a transformant, the data in Table 3 show that considerable starch remained after 5 days of growth. This apparent paradox may be explained by postulating a disruption of the tertiary structure of the starch macromolecule resulting from a limited action of α-amylase. This hypothesis is consistent with the work of De Mot and Verachtert (6), who observed a rapid loss of iodine staining accompanied by a relatively low production of reducing sugar during action of α-amylase on starch.

Filho et al. (9) reported that a transformant of S. cerevisiae secreting mouse pancreatic α-amylase degrades all the starch in the medium. However, in their investigation, starch degradation was judged by the disappearance of iodine staining, and no additional data concerning the efficiency of carbohydrate utilization were presented. The results presented in Table 3 are consistent with those of Thomsen (27), who reported that a different strain of S. cerevisiae transformed with the same α-amylase plasmid (pMS12) ferments only 10 to 30% of potato starch in a 7-day incubation at 30°C.

In a previous report, Mattoon et al. (14) reported that direct conversion of 4% Lintner starch to ethanol is no greater than 80% even after 12 days of fermentation by S. diastaticus. However, pretreatment of starch with α-amylase permits more than 95% conversion by the same organism. The present study demonstrates that by constructing a yeast transformant capable of secreting both α-amylase and glucoamylase, direct conversion of more than 93% of starch in 5 days can be attained. Thus, it is now possible to attain almost the same efficiency of conversion in one step as was previously attained in two steps (12, 14). Moreover, by using such a yeast strain, the cost of commercial α-amylase in the conversion of starch to ethanol could be eliminated.

**Mitotic stability of yeast strains transformed with plasmid pMS12.** It is generally observed that yeast transformants bearing plasmids carrying the 2μm origin of replication exhibit various degrees of mitotic instability. That is, during many generations of growth under nonselective conditions, cells with plasmids are gradually diluted out of the population. The stabilities of the various transformants obtained with plasmid pMS12 were examined (Table 4). Cells were grown on BYPS4 medium, which contains tryptophan as a component of yeast extract and of peptone. Therefore, there was no selective pressure to retain the TRP1 gene residing on the plasmid. However, since starch was the primary carbon source, there was some selective pressure to retain the ability to produce amylase. Plasmid loss, as measured by retention of tryptophan prototrophy, occurred progressively during growth of the three different transformants (Table 4). The more limited loss of the plasmid by the S. cerevisiae (SHU32a) transformants may reflect the fact that the only source of amylolytic activity in this strain was the plasmid α-amylase gene, whereas cells of KK1-R1 and KK1-R2 could produce glucoamylase even after they had lost all plasmid. An alternative explanation for the difference between the plasmid stabilities of the transformed hybrid and the SHU32a transformant could be that fewer generations occurred in the culture of the latter strain, which grew more slowly than the transformed hybrid.

Further improvement in strains possessing both glucoamylase genes and α-amylase genes could be made by incorporating the α-amylase gene into a centromere vector (23) or by integrating the gene, together with the attached ADCl promoter, into a chromosome of the host cell. Although Filho et al. (9) reported that a plasmid vector containing 2μm DNA, a LEU2 gene, and mouse pancreatic α-amylase cDNA was integrated into a yeast chromosome with low frequency, the presence of 2μm sequences may destabilize such integrated fragments. Integration by using gene replacement with linear fragments carrying α-amylase DNA, as described by Rothstein (21), would seem to be a preferable method.

In conclusion, efficient one-step starch utilization has been achieved by constructing *Saccharomyces* strains capable of secreting glucoamylase and α-amylase simultaneously.

**ACKNOWLEDGMENTS**

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