Fermentation of Corn Starch to Ethanol with Genetically Engineered Yeast

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Expression of the glucoamylase gene from *Aspergillus awamori* by laboratory and distiller's strains of *Saccharomyces cerevisiae* allowed them to ferment soluble starch. Approximately 95% of the carbohydrates in the starch were utilized. Glycerol production was significantly decreased when soluble starch was used instead of glucose. Ethanol yield on soluble starch was higher than that on glucose. The rate of starch fermentation was directly related to the level of glucoamylase activity. Strains with higher levels of glucoamylase expression fermented starch faster. The decline in starch fermentation rates toward the end of the fermentation was associated with accumulation of disaccharides and limit dextrins, poor substrates for glucoamylase. The buildup of these products in continuous fermentations inhibited glucoamylase activity and complete utilization of the starch. Under these conditions maltose-fermenting strains had a significant advantage over nonfermenting strains. The synthesis and secretion of glucoamylase showed no deleterious effects on cell growth rates, fermentation rates, and fermentation products.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is commonly used in the brewing industry and in the commercial fermentation of carbohydrates to ethanol. *Saccharomyces cerevisiae* strains lack significant amylase and glucoamylase activities and are unable to ferment starch. Consequently, before commercial fermentation the starch must be liquefied and then saccharified to glucose. The liquefaction stage is a thermal process with α-amylase, commonly derived from *Bacillus* strains. The saccharification stage is either an enzymatic process, commonly done with glucoamylase from *Aspergillus* strains, or an acid hydrolysis process.[1] Yeast strains with glucoamylase activity, e.g., *Saccharomyces diastaticus*, *Saccharomyces fibuligera*, and *Schwanniomyces alluvius* are inefficient ethanol producers, sensitive to high ethanol concentration, or can not degrade starch completely.[2–4]

A combined saccharification and fermentation process would eliminate the saccharification stage and could reduce fermentation costs. In addition, keeping the glucose at low concentration would avoid enzymatic reversion and transglucosylation reactions that repolymerize the glucose and decrease glucose yield.[1,5] The *Aspergillus awamori* glucoamylase (EC 3.2.1.3; 1,4-α-D-glucose gluconohydrolase) gene has been cloned into a laboratory yeast strain[6] and a distiller's yeast strain[7] to allow combined saccharification and fermentation of soluble starch. The distiller's strain was selected because of its ethanol tolerance, osmotolerance, and temperature tolerance. Direct fermentation of soluble corn starch to ethanol by addition of glucoamylase to the broth is a common practice in the industry.[8] In addition, it has been demonstrated in a mixed culture of *Zymomonas mobilis* and *Aspergillus awamori*.[9] However, glucoamylase in these processes is produced by aerobic fermentation of carbohydrates, so substrate is wasted and ethanol yield is low. The cost of the enzyme in ethanol fermentation is \$0.02–\$0.03/gal ethanol produced.[10] This study evaluates the performance of glucoamylase-producing recombinant yeast strains for direct fermentation of soluble starch to ethanol and analyzes the rate-limiting step in this combined process.

MATERIAL AND METHODS

Strains and Plasmids

C468 is a *S. cerevisiae* haploid laboratory strain (α *leu*2-3 *his*3-11 *his*3-15 *mal*). C162 is a distiller's yeast strain provided by National Distillers (MAL). Plasmid pAC1 is an autonomously replicating yeast expression vector with the *leu*2 structural gene as a selectable marker. The vector also contains the promoter and the terminator of the yeast enolase gene, *eno*1 and a *hind*III site at the junction of the yeast *eno*1 promoter and terminator DNA. Plasmid pGAC9 is pAC1 with the *A. awamori* glucoamylase gene inserted at the site *hind*III. Construction of the plasmid pGAC9 is detailed elsewhere.[6] Plasmid pPM18 is a modification of pGAC9 that results in a high level of glucoamylase expression. Plasmid pPM20 is similar to pPM18, except it is designed for integration into the yeast chromosomal DNA and contains the kanamycin resistance gene that infers re-

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sistance to the antibiotic geneticin sulfate (G418).[7] Strain C468 was transformed with the plasmids pGAC9 and pPM18. Strain C162 was transformed with the plasmid pPM20.

Cultivation Techniques

In these studies a defined minimal medium was used. It contains the following ingredients (per L): ammonium succinate, 13 g; H_3PO_4, 0.58 g; H_2SO_4, 0.31 g; KCl, 0.37 g; $MgCl_2 \cdot 6H_2O$, 0.20 g; $FeSO_4$, 6.1 mg; $MnSO_4 \cdot H_2O$, 1.7 mg; $CoCl_2 \cdot 6H_2O$, 1.2 mg; $Na_2MoO_4 \cdot 2H_2O$, 1.2 mg; H_3BO_3, 3.1 mg; $CaCl_2 \cdot 2H_2O$, 14.7 mg; pyridoxine HCl, 0.5 mg; thiamine HCL, 1 mg; biotin, 10 μg; calcium pantothenate, 1 mg; and myo-inositol, 40 mg. Final pH was 4.5. Vitamins, trace elements, and carbon source were sterilized and added aseptically to the medium. Carbon sources in these studies were glucose, maltose, soluble starch, and dextran. We used Maltrin 150 (Grain Processing Corporation, Mucatin, IA) as soluble starch. Maltrin 150 is produced by enzymatic hydrolysis with α-amylase and was chosen for its similarity to the soluble starch obtained before the saccharification stage of commercial processes. The soluble starch was precipitated and washed before use with 80% ethanol to remove traces of glucose. For continuous-culture studies, we substituted ammonium chloride (4.0 g/L) for the ammonium succinate and the pH was 2.1 (to avoid back growth). The media for C468 cultivation also contained 100 mg/L L-histidine.

Growth-rate studies were conducted in 125-mL Erlenmeyer flasks with side arm for Klett readings. The flasks contained 50 mL minimal defined medium and a carbon source. The specific growth rates were calculated from turbidity measurements between 20 and 50 Klett units. Fermentation studies were conducted in 125-mL flasks equipped with air restrictors. Fermentation rates were calculated from the measurements of weight loss due to CO_2 evolution. These studies showed a stoichiometric relationship between weight loss due to CO_2 evolution and the accumulation of ethanol in the medium. One mole of CO_2 produced was equal to one mole of ethanol produced (as confirmed by ethanol measurement). All flask studies were done in a gyrotory shaker (New Brunswick Co., New Brunswick, NJ) at 200 rpm and at 30°C for C468 strains and 32°C for C162 strains.

Continuous-culture studies were conducted in a 1-L LH chemostat (LH Fermentation, Stoke Poges, England) with a 700-mL working volume. Carbon source was either 6% (w/v) soluble starch or 5% (w/v) glucose. The fermentation conditions were pH 4.0; the temperature was 30°C for C468 strains and 32°C for C162 strains; aeration adjusted to 10 mmol O_2/g cells (adequate to maintain a predominantly anaerobic metabolism); agitation was 800 rpm; and the dilution rate (D) was controlled from 0.025 to 0.20 h^{-1}, as specified. Steady-state conditions were defined for the purpose of analysis as the time when no changes were observed after more than six medium volume changes in the growth vessel.

Assays

Cell-free extract was prepared from 50-mL samples of culture. The cells were centrifuged, washed twice with 0.05*M* sodium phosphate buffer (pH 7.5), and resuspended in 10 mL of the same buffer. Cells were broken by cycling three times with French Press at 2×10^4 lb/in^2. The cell suspension was centrifuged at 1.5×10^4 g for 20 min, and the supernatant was kept on ice until the enzyme assays were performed.

Glucoamylase assay was done by diluting the supernatant fivefold in 0.1*M* citrate buffer (pH 4.0) and 1.25% (w/v) soluble starch, followed by incubation for 30 min at 32°C. The reaction was stopped in boiling water for 5 min, and the glucose concentration was estimated with a YSI glucose analyzer. One enzyme unit activity (U) was defined as 1 μmol glucose produced/min. Enzyme concentration (mg/L) was estimated assuming the specific activity of pure glucoamylase as 30 U/mg enzyme (based on experimental results with purified enzyme). Percent enzyme expression was calculated by dividing enzyme concentration by the concentration of the cell protein (mg/L) and multiplying the result by 100.

Maltase (α-D-glucoside gluconohydralase; EC 3.2.1.20) activity in the cell-free extract was determined by the method of Halvorson, by using *p*-nitrophenyl α-D-glucopyranoside (Sigma, St. Louis, MO) as a substrate in 0.05*M* sodium phosphate buffer (pH 7.5) at 30°C.[11] Glucoamylase activity at pH 7.5 was negligible.

Cell concentration was determined from 25-mL samples of culture. The cells were centrifuged, washed twice in deionized water, then dried in a vacuum oven at 80°C until a constant weight was obtained.

Total carbohydrate in the medium was determined by the phenol sulfuric acid assay with glucose as a standard.[12] Glucose was analyzed by means of an automatic analyzer (YSI, Yellow Springs, CO) and with glucose oxidase (procedure No. 510, Sigma). Glycerol was analyzed with an enzymatic kit, (Catalog No. 148270) from Boehringer Mannheim (Indianapolis, IN). Ethanol was assayed by gas chromatography. Protein was analyzed by the Biuret method, with BSA as a standard.[13]

Oligosaccharides were analyzed via gradient HPLC elution followed by on-line post column derivatization and fluorometric detection.[14] This method allows semiqualitative analysis of reducing sugars from DP-1 to DP-15. Postcolumn reaction with boric acid, ethanolamine, and fluorescence detection allows the use of acetonitrile gradients, and greatly enhances the specificity of detection.[15] Consequently, fermentation broths may be analyzed directly, as only reducing sugars are detected. Glucoamylase (*Rhizopus*) and pullulanase (*Enterobacter aerogenes*) were

purchased from Sigma. All other materials were reagent grade.

RESULTS AND DISCUSSION

The Role of Glucoamylase in the Fermentation of Starch

Cloning of the glucoamylase gene into C468 allowed this strain (C468pGAC9) to ferment soluble starch (Fig. 1). In this example, ca. 95% of the carbohydrates were utilized in eight days. Batch fermentation conditions were not optimized. The relatively long fermentation times were due to the low inocula used [1% (v/v)] and the use of starch that had been washed free of glucose and low oligosaccharides. Addition of commercial glucoamylase to the medium allowed the glucoamylase-negative strain (C468pAC1) to ferment soluble starch and enhanced soluble-starch fermentation by C468pGAC9 to a rate that is similar to that on glucose. *In vitro* analysis of glucoamylase activity showed that ca. 80–90% of the activity was secreted into the medium and 10–20% was cell wall associated, independent of cell growth rate. These data indicate that expression of the cloned glucoamylase gene is both necessary and sufficient to allow C468pGAC9 to grow on soluble starch. In addition it shows that insufficient expression can account for the slower growth on soluble starch compared to the growth on glucose.

In a similar system (Fig. 1) strain C468pPM18 fermented 95% of the carbohydrates in less than five days. The performance of C162pPM20 was similar to that of C468pGAC9.

The fermentation rate of soluble starch by different recombinant strains varied and is directly related to the level of glucoamylase expression by these strains (Table I). Higher expression of glucoamylase allowed faster fermentation of soluble starch. Expression of glucoamylase also allowed C468pGAC9 (mal) to ferment maltose (α-1,4 disaccharide) and dextran (α-1,6 polysaccharide). The fermentation rates of these substrates are related to the *in vitro* activity of recombinant glucoamylase against these substrates (Table II). The control strain C468pAC1 did not ferment these substrates. The specificity of glucoamylase activity against different carbohydrates is similar to that found with glucoamylase from an *Aspergillus niger* strain.[16]

Table I. Glucoamylase level and fermentation rates of different yeast strains on soluble starch.

Strain	Glucoamylase level (% cell protein)	Fermentation rate (h^{-1})
C468	ND	NG
C468pGAC9	0.28	0.05
C468pPM18	1.1	0.14
C162	ND	NG
C162pPM20	0.26	0.05

Fermentation studies were carried out on minimal defined medium with 10% soluble starch. Glucoamylase level was estimated from glucoamylase activity in the supernatant divided by cell protein.
ND means not detected.
NG means no growth.

Continuous-Culture Studies

We initiated chemostat studies to examine combined saccharification and fermentation of soluble starch in a continuous process. The recombinant C468 strains were grown under selective conditions, without addition of leucine. Strain C162, with the integrated plasmid, was grown nonselectively without addition of the antibiotic G418 to the medium. The fraction of plasmid-containing cells among C468 strains under selective conditions was about

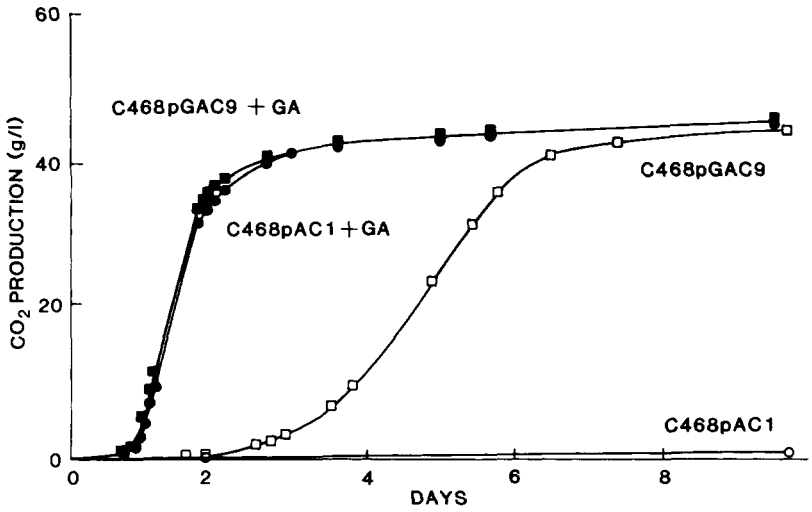

Figure 1. Fermentation of soluble starch by recombinant yeast strains. Fermentation was on minimal defined medium with 10% soluble starch and 1% (v/v) inoculum. Commercial glucoamylase was added as indicated at ca. 1 U/mL. Recombinant yeast strain C468pGAC9 produces glucoamylase. Control yeast strain C468pAC1 does not produce glucoamylase. Used with permission from Science, copyright 1985 by the AAAS (ref. 6).

Table II. Fermentation rates of C468pGAC9 and recombinant glucoamylase activity versus different saccharides.

Saccharide	Fermentation rate (h^{-1})	Relative fermentation rate (%)	Relative glucoamylase activity[a] (%)
Soluble starch	0.05	100	100
Maltose	0.03	60	24
Dextran	0.004	8	0.5
Pullulan	NT	NT	2.5
Glucose	0.25	500	ND

Fermentation studies were in minimal media with 10% soluble starch, 10% maltose, 10% dextran, or 10% glucose. Glucoamylase activity against these substrates was tested at 1% final concentration except dextran, which was tested at 10% (w/v).

[a] This is a combination of two different assays.

NT means not tested.

ND means not detected.

70% and 60% of the population for strains C468pGAC9 and C468pPM18, respectively. Strain C162pPM20 showed no signs of plasmid loss under nonselective conditions (without G418) for over 800 generations. Detailed stability studies with these strains will be published elsewhere.[7]

The steady-state data for the chemostats of the three strains are summarized in Table III. The residual glucose concentrations in these fermentations are low (0.26–0.45 g/L) suggesting that ethanol production and cell growth were limited by glucoamylase activity. The glucoamylase levels in C468pGAC9 and C162pPM20 are similar (0.26–0.28% of total protein), while that of C468pPM18 is about fourfold higher (1.08% of total protein). Interestingly, however, the carbohydrate utilization by C468pPM18 is only 2- and1.2-fold higher than that of C468pGAC9 and C162pPM20, respectively. The same difference exists between the *in vitro* and the estimated *in vivo* activities: different levels of glucoamylase expression and the amount of carbohydrate utilized. The reasons for this discrepancy are most likely because of the mechanism of action and specificity of glucoamylase (Table II).[1,16] Glucoamylase removes glucose units from the nonreducing ends of oligosaccharides and has a strong preference both for oligosaccharides longer than tetramaltose and for α-1,4- over α-1,6-glucosidic bonds. Consequently, the broth is enriched with small oligosaccharides and oligosaccharides with α-1,6 glucosidic bonds at the nonreducing end that are poor substrates for further degradation by glucoamylase. About 3% of the glucosidic bonds in corn starch are of the α-1-6 type.[17] The large difference between the *in vivo* and the *in vitro* activity in strain C468pPM18 is because the *in vitro* assay is done with the addition of undegraded soluble starch. The better carbohydrate utilization by C162pPM20 compared to C468pGAC9 is most likely due to maltose utilization by the host strain, C162. The characterization of starch degradation by these recombinant strains, evidence for maltose utilization, and support for this hypothesis are described below.

Ethanol-yield values in these fermentations are indicative of predominately fermentative metabolism (Table III). Yeast metabolism at low-glucose concentration tends to be aerobic unless the dissolved oxygen concentration is restricted.[18] In these fermentations the aeration was not truly restrictive. In later continuous fermentations in which the aeration was restricted to below 10 mmol oxygen/g cells, the ethanol yield values were 0.42–0.46 g/g (Table IV), similar to what we obtained in batch fermentations which restricted aeration (Table VIII).

The Effect of Growth Rate on Glucoamylase Expression

The effect of growth rate on glucoamylase production by C162pPM20 was tested in a chemostat by varying the dilution rates. The substrate was 5% glucose. There was a

Table III. Continuous fermentation of soluble starch by recombinant yeast strains.

	C468pGAC9	C468pPM18	C162pPM20
Carbohydrates in feed (g glucose equivalents/L)	59.8	58.5	60.9
Residual carbohydrates (g glucose equivalents/L)	35.4	7.8	18.1
Carbohydrates used (%)	41	87	70
Residual glucose (g/L)	0.45	0.40	0.25
Cell concentration (g/L)	3.9	5.0	5.0
Cell yield (g cells/g glucose utilized)	0.16	0.10	0.12
Ethanol (g/L)	6.5	19.6	15.3
Ethanol yield (g ethanol/g glucose utilized)	0.27	0.39	0.37
In vivo[a] glucoamylase activity (mmol glucose/L h)	6.67	13.89	11.67
In vitro[b] glucoamylase activity (mmol glucose/L h)	10.0	48.8	11.11
Glucoamylase level[c] (% cell protein)	0.28	1.08	0.26

Continuous fermentation studies were carried out in minimal medium with 6% soluble starch and at $D = 0.05\ h^{-1}$.

[a] *In vivo* activity is assumed to be equal to the rate of glucose utilization.

[b] *In vitro* activity is GA activity in the supernatant after addition of undegraded soluble starch.

[c] Glucoamylase level is the *in vitro* activity of the supernatant divided by glucoamylase specific activity and cell protein concentration.

Table IV. The effect of dilution rate on glucoamylase expression.

	Dilution rate (h^{-1})			
	0.025	0.05	0.1	0.2
Cell concentration (g/L)	5.14	5.58	5.95	5.18
Cell yield	0.10	NT	0.12	0.10
Ethanol (g/L)	21.1	NT	22.9	22.5
Glucoamylase level (% cell protein)	0.52	0.18	0.12	0.10
Glucoamylase volumetric productivity (U/L h)	10.2	7.9	10.8	11.3
Glucoamylase specific productivity (U/g cell protein h)	4.2	3.0	3.6	5.7

Strain C162pPM20 was grown in chemostat in minimal medium plus 5% (w/v) glucose.
NT means not tested.

Table V. The effect of growth rate on glucoamylase level.

Carbon source	Growth rate (h^{-1})	Glucoamylase expression (% cell protein)	Glucoamylase specific productivity (U/g cell protein h)
Soluble starch	0.09	0.14	4.2
Glucose	0.45	0.096	6.6

Strain C162pPM20 was grown on minimal medium plus 10% soluble starch or glucose.

Table VI. The effect of pullulanase addition on apparent glucoamylase activity.

	Glucoamylase activity (U/mL)
Supernatant	126
Boiled supernatant	ND
Supernatant + 1 U/mL pullulanase	202
Boiled supernatant + 1 U/mL pullulanase	2
Supernatant + 1% soluble starch	281
Supernatant + 1% soluble starch + 1 U/mL pullulanase	296

The assay was performed with continuous culture supernatant of C468pPM18 (Table III). Conditions for glucoamylase assay are described in the Methods section.
ND means not detected.

Table VII. Maltase activity of C162pPM20 on various substrates.

Growth substrates	Maltase (U/mg protein)	Maltase relative activity (%)
Maltose (batch culture)	4.6	100
Glucose (batch culture)	0.03	6
Soluble starch (continuous culture)	2.1	47

Batch growth was on minimal medium plus 10% glucose or maltose. The conditions for continuous-culture fermentation were described in Table III. The preparation of cell-free extract and maltase assay are described in the Methods section.

fivefold decrease in glucoamylase expression level when the dilution rate was increased from 0.025 to 0.2 h^{-1} (Table IV). Enzyme specific productivity was largely constant and independent of cell growth rate. Batch-culture studies confirm the continuous-culture results (Table V). C162pPM20 was grown on soluble starch to provide slow growth and on glucose for fast growth. The results obtained at the exponential phase of growth showed similar specific glucoamylase production rates. Therefore, lower glucoamylase levels were obtained on glucose.

Dynamics of Starch Degradation

Because of glucoamylase preference toward long oligosaccharides and α-1,4 glycosidic bonds, we expect that the composition of the soluble starch will vary during the combined saccharification fermentation process (Table 2).[1,16] The dynamic changes in the residual starch composition during batch fermentation with C468pPM18 are presented in Figure 2. At the initial phases of fermentation there is a preferential degradation of the long oligosaccharides and accumulation of mono-, di-, and trisaccharides. Later the mono-, di-, and trisaccharides are degraded and utilized at lower rates. The pattern of these changes fits well with glucoamylase substrate specificity and the inability of C468 strain (*mal*) to utilize disaccharides directly. Strains C468pGAC9 and C162pPM20 with lower glucoamylase expression did not accumulate glucose in similar batch fermentations (less than 0.2 g/L).

Table VIII. Fermentation products of C162 and C162pPM20 on glucose and soluble starch.

	Carbohydrates utilized (g/L)	Cells (g/L)	Ethanol (g/L)	CO_2 (g/L)	Glycerol (g/L)	Ethanol yield (g/g)	Carbon recovery (%)
Glucose							
C162	99.0	5.61	44.7	42.0	6.13	0.45	99
C162pPM20	99.0	5.45	44.5	41.8	6.47	0.45	99
Soluble starch							
C162	5.0	2.44	NT	3.5	0.84	NT	101
C162pPM20	93.0	7.07	44.8	43.0	1.62	0.48	104

Studies were conducted on minimal medium plus 10% (w/v) glucose or soluble starch.
Analyses were made at the end of the fermentation.
NT means not tested.

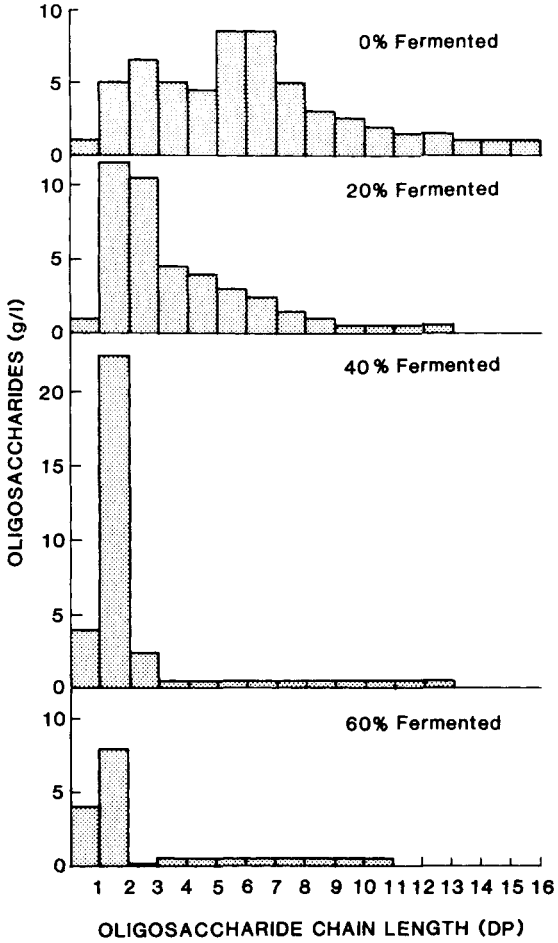

Figure 2. Degradation of soluble starch by C468pPM18 in batch fermentation. Growth was in minimal medium with 10% soluble starch. Samples for analysis were taken after 0, 20, 40, and 60% of the total carbohydrates fermented (based on weight loss).

Figure 3 compares the steady-state composition of the soluble starch that is fed to the chemostat to that in the growth vessel for strains C468pGAC9 and C468pPM18. The composition of the starch that is fed to the vessel is different from that in the vessel. The trend seen is a significant degradation of the long-chain oligosaccharides and an accumulation of disaccharides, which are poor substrates for glucoamylase (Table II).[1,16] C468pPM18, which has the highest glucoamylase expression, degraded most of the soluble starch. These results support the previous reasoning as to why higher glucoamylase expression by C468pPM18 does not result in a proportional increase in the amount of carbohydrate utilized in the chemostat. C162pPM20, which is capable of directly utilizing maltose and isomaltose, did not accumulate disaccharides (not shown). This explained why C162pPM20 performs better than C468pGAC9.

The *in vitro* glucoamylase activity of C468pPM18 supernatant (Table III) is enhanced by about twofold with the addition of soluble starch or pullulanase (Table VI). Pullulanase alone does not have any glucoamylase activity in the culture supernatant (i.e., release of free glucose). Pullulanase from *Aerobacter aerogenes* degrades the α-1,6 glucosidic bonds in pullulan (maltotriose α-1,6 maltotriose), and the minimal oligosaccharide length for this enzyme is maltose α-1,6 maltose.[1] This suggests that the residual starch in this fermentation is enriched in limit dextrins (oligosaccharides with α-1,6-glucosidic bonds near or at the nonreducing end), and addition of pullulanase converts these oligosaccharides to better substrates for glucoamylase. Dextrins are produced during hydrolysis of starch by α-amylase and can be accumulated because they are poor substrates for glucoamylase.[1,19]

Utilization of Maltose

Further indications for simultaneous utilization of maltose with glucose during continuous fermentation of starch by C162pPM20 are 1) the *in vitro* measurements of maltase activity and 2) fermentation experiments with maltose as a substrate. Table VII shows that maltase activity is induced in maltose medium, repressed on glucose medium, and ca. 47% of that on maltose during continuous-culture growth on soluble starch ($D = 0.05\ h^{-1}$). The low glucose levels in starch fermentation (0.25 g/L; Table III) apparently allowed partial derepression of maltase synthesis. Cells from a continuous culture (Table III) grown on soluble starch and transferred to a similar medium containing maltose were able to ferment this substrate without a significant lag (less than 30 min). On the other hand, cells grown on glucose and transferred to a similar medium with maltose as a substrate could ferment this substrate only after a significant lag (greater than 3 h).

Fermentation Balance

Table VIII lists the fermentation products of C162 and C162pPM20 grown on 10% glucose and 10% soluble starch in batch culture. No difference in the fermentation products, cell concentration, or ethanol yield were observed between the two strains on glucose. Synthesis and secretion of glucoamylase up to 0.26% of cell protein do not seem to affect C162 fermentation. Similar conclusions were obtained when the specific growth rate of C162 was compared to that of C162pPM20 on glucose (not shown). Starch fermentation by C162pPM20 is characterized by lower glycerol and higher ethanol yields than occur with comparable fermentations on glucose as a substrate. The lower glycerol production on starch is most likely due to the lower osmolarity of the starch medium.[20–22] The higher ethanol yield is probably due to lower glycerol production. Carbohydrate utilization varied between 93 to 95%, and ethanol yield varied between 0.44 and 0.48 (g/g) of the carbohydrates utilized. The residual carbohydrates (by Phenol sulfuric acid assay) could be retrograded starch or other polysaccharides.[1,23] Preliminary evidence suggests that ca. 30% of the remaining carbohydrates can be converted to glucose by crude cellulase from *Trichoderma reesei*.

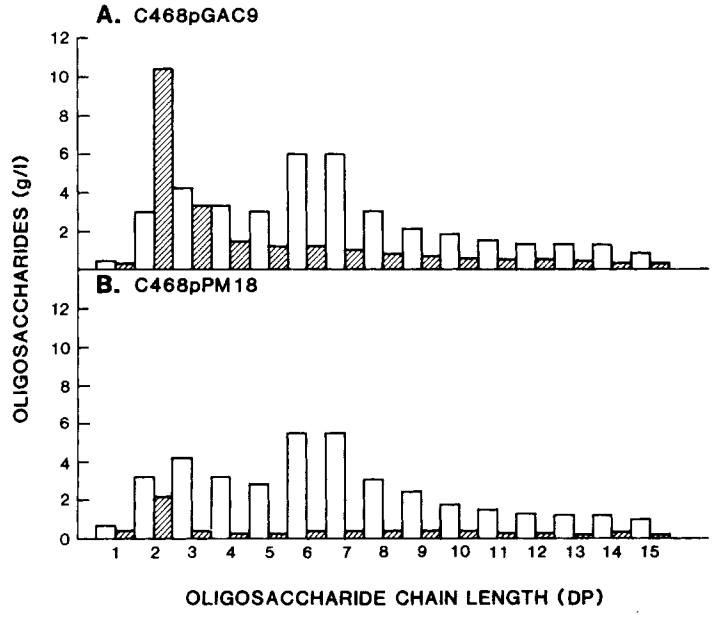

Figure 3. Degradation of soluble starch by (A) C468pGAC9 and (B) C468pPM18 in continuous culture. Oligosaccharide concentrations in the feed and in the growth vessel are marked by nonshadowed and shadowed columns, respectively. Conditions are described in Table III.

Process Considerations

Extrapolating our results, we believe that a recombinant yeast strain (Mal) with an expression level of 1–2% of cell protein would be sufficient for direct fermentation of soluble starch to ethanol within the 48-h standard batch fermentation time [the apparent μ or D would be 0.062 h^{-1}, assuming 5% (v/v) inoculum]. An alternative to high glucoamylase expression is a glucoamylase with higher activity against α-1,6-glucosidic bonds. Our results suggest that expression of glucoamylase to 1–2% (of total protein) levels does not impair the strain performance. A process based on direct fermentation of soluble corn starch to ethanol would eliminate the saccharification stage, increase ethanol yield, and reduce production costs.

CONCLUSIONS

A combined process for saccharification and fermentation of soluble starch with genetically engineered yeast that produce and secrete glucoamylase was demonstrated; 93–95% of the carbohydrates were utilized, glycerol production was particularly low, and ethanol yield of 0.44–0.48 g/g was obtained. No deleterious effects were observed on yeast growth and metabolism due to glucoamylase synthesis and secretion (up to 1.1% cell protein for the laboratory strain and 0.26% for the distiller's strain). Starch fermentation by the genetically engineered strains was limited by glucoamylase activity. The rate of starch fermentation can be increased by increasing the level of glucoamylase expression, and especially by increasing the ability to degrade maltose, isomaltose, and limit dextrins. Industrial yeast strains that ferment maltose and isomaltose have an important advantage. A commercial process for direct conversion of soluble corn starch to ethanol can be technically feasible at glucoamylase expression of 1–2% of cell protein.

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