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Novel strategy for yeast construction using δ-integration and cell fusion to efficiently produce ethanol from raw starch

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Abstract We developed a novel strategy for constructing yeast to improve levels of amylase gene expression and the practical potential of yeast by combining δ-integration and polyploidization through cell fusion. *Streptococcus bovis* α-amylase and *Rhizopus oryzae* glucoamylase/α-agglutinin fusion protein genes were integrated into haploid yeast strains. Diploid strains were constructed from these haploid strains by mating, and then a tetraploid strain was constructed by cell fusion. The α-amylase and glucoamylase activities of the tetraploid strain were increased up to 1.5- and tenfold, respectively, compared with the parental strain. The diploid and tetraploid strains proliferated faster, yielded more cells, and fermented glucose more effectively than the haploid strain. Ethanol productivity from raw starch was improved with increased ploidy; the tetraploid strain consumed 150 g/l of raw starch and produced 70 g/l of ethanol after 72 h of fermentation. Our strategy for constructing yeasts resulted in the simultaneous overexpression of genes integrated into the genome and improvements in the practical potential of yeasts.

Keywords Cell fusion · δ-integration · Ethanol fermentation · Raw starch

Introduction

Along with the exhaustion of fossil fuels and environmental issues such as global warming and acid rain, utilization of the biomass as a fuel source particularly of ethanol has recently become an attractive option. Utilization of the biomass to produce alternative fuels such as ethanol is globally desirable because it is abundant, renewable, and has favorable environmental properties. Therefore, an efficient method of producing ethanol from the biomass is required for worldwide consumption.

Starchy materials are abundant in the biomass, and *Saccharomyces cerevisiae* is a yeast that can efficiently produce ethanol. However, this yeast lacks the amylolytic activity required to utilize starch as a carbon source, and thus ethanol production from the starchy biomass by classical yeast fermentation is expensive, and the process is complex and time-consuming. This process typically consists of the gelatination of raw starch by cooking, liquefaction using α-amylase, and enzymatic saccharification to glucose by glucoamylase. The costs associated with the extensive energy and enzyme consumption involved in this process are extremely high. Several investigators have described ethanol production from starch using recombinant *S. cerevisiae* that expresses amylolytic enzymes (Innis et al. 1985; Cole et al. 1988; Inlow et al. 1988; Ibragimova et al. 1995; Nakamura et al. 1997; Birol et al. 1998). A recent study suggests that recombinant *S. cerevisiae* that co-expresses *Streptococcus bovis* α-amylase and *Rhizopus oryzae* glucoamylase/α-agglutinin fusion protein can directly and efficiently ferment raw starch to ethanol (Shigechi et al. 2002; Yamada et al. 2009).

The yeast episomal plasmid (YEp) has been used to genetically manipulate yeasts to overexpress foreign genes (Broach 1983). However, YEp vectors are mitotically unstable under non-selective conditions such as long-term industrial operation in poorly defined media (Murray and Szostak 1983; Romanos et al. 1992). Although, yeast integrative plasmids (YIp) allow stable foreign gene expression, YIp is unsuitable as an overexpression vector because only one copy of a gene can be integrated into the yeast genome (Romanos et al. 1992). The rate-limiting step

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is starch degradation during direct ethanol fermentation from starch materials, thus both high amylolytic activity and stable expression are critical to efficient ethanol production (Khaw et al. 2005).

Here, we developed an efficient method of ethanol fermentation from raw starch using α-amylase that was overexpressed by combining δ-integration with polyploidization. Multi-copy integration methods such as δ-integration and rDNA-integration are evidently the most suitable methods for overexpressing foreign genes (Sakai et al. 1990; Lopes et al. 1991; Lee and Da Silva 1997; Nieto et al. 1999). We amplified multi-copy integrated genes by polyploidization, which allowed significant overexpression. Moreover, two studies have shown that breeding polyploid strains can improve ethanol productivity or the quality of fermentation products of industrial yeast strains without recombinant techniques (Higgins et al. 2001; Hashimoto et al. 2006). We investigated ethanol fermentation from raw starch using a tetraploid recombinant yeast strain constructed by δ-integration and cell fusion.

Materials and methods

Strains, plasmids, and media

Table 1 summarizes the genetic properties of all strains and plasmids used in this study. In brief, the host for recombinant DNA manipulation was the *Escherichia coli* strain, NovaBlue (Novagen, Madison, WI, USA), and α-amylase and glucoamylase were expressed in the haploid yeast strains *S. cerevisiae* MT8-1 and NBRC1440ΔHUWL that can be polyploidized (Yamada et al. 2009). The diploid strain *S. cerevisiae* MNII/δGS and the tetraploid strain MNIV/δGS were constructed by mating and protoplast fusion from MT8-1/δGS and NBRC1440/δGS as described below.

E. coli transformants were grown in Luria-Bertani medium (10 g/l of tryptone, 5 g/l of yeast extract, and 5 g/l of sodium chloride) supplemented with 100 μg/ml of ampicillin. Transformants and fusants were screened in SD medium (6.7 g/l of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA), 20 g/l of glucose)

Table 1 Characteristics of strains and plasmids used in this study

Strains or plasmids	Relevant features	Reference
Bacterial strain		
E. coli NovaBlue	*endA1 hsdR17*($r_{K12}^{-}m_{K12}^{+}$) *supE44 thi-I gyrA96 relA1 lac recA1/F'*[*proAB* $^{+}$*lacI*^{q} ZΔM15::Tn*10*(Tet^{r})]	Novagen
S. cerevisiae yeast strains		
MT8-1	*MAT*a *ade his3 leu2 trp1 ura3*	Tajima et al. (1985)
MT8-1/IGS	*MAT*a *ade his3 leu2* integration of glucoamylase gene and α-amylase gene	This study
MT8-1/δGS	*MAT*a *ade his3 leu2* δ-integration of glucoamylase gene and α-amylase gene	This study
MT8-1/δGS/403	*MAT*a *ade leu2* δ-integration of glucoamylase gene and α-amylase gene	This study
MT8-1/δGS/405	*MAT*a *ade his3* δ-integration of glucoamylase gene and α-amylase gene	This study
NBRC1440	Wild type strain	NBRC[a]
NBRC1440ΔHUWL[b]	*MAT*α *his3 leu2 trp1 ura3*	Our laboratory[b]
1440/δGS	*MAT*α *his3 leu2* δ-integration of glucoamylase gene and α-amylase gene	This study
MNII/δGS	*MAT*a/α *leu2* δ-integration of glucoamylase gene and α-amylase gene	This study
MNII/δGS/405	*MAT*a/α *his3* δ-integration of glucoamylase gene and α-amylase gene	This study
MNIV/δGS	*MAT*a/a/α/α δ-integration of glucoamylase gene and α-amylase gene	This study
Plasmids		
pBluescript II KS+	No expression	Stratagene
pRS403	*HIS3* no expression	Stratagene
pRS404	*TRP1* no expression	Stratagene
pRS405	*LEU2* no expression	Stratagene
pRS406	*URA3* no expression	Stratagene
pIU-PGGluRAG	*URA3* surface expression of glucoamylase fused with 3′-half of α-agglutinin gene by integration	This study
pIW-GPSBA	*TRP1* secretory expression of *S. bovis* α-amylase gene by integration	This study
pδU-PGGluRAG	*URA3* surface expression of glucoamylase fused with 3′-half of α-agglutinin gene by δ-integration	This study
pδW-GPSBA	*TRP1* secrete expression of *S. bovis* α-amylase gene by δ-integration	This study

[a] NITE Biological Research Center, Chiba, Japan

[b] NBRC1440ΔHUWL was bred by gene deletion as described by Akada et al. (2006)

supplemented with appropriate amino acids and nucleic acids. Yeast cells were aerobically cultured in SDC medium (6.7 g/l of yeast nitrogen base without amino acids, 20 g/l of glucose, 20 g/l of casamino acids (Difco Laboratories, Detroit, MI, USA)) supplemented with appropriate amino acids and nucleic acids, and in YPD medium (10 g/l of yeast extract, 20 g/l of Bacto-peptone (Difco Laboratories, Detroit, MI, USA), 20 g/l of glucose). Ethanol fermentation proceeded in YPRS medium (10 g/l of yeast extract, 20 g/l of Bacto-peptone, and 200 g/l of raw corn starch (Wako Pure Chemical Industries Ltd., Osaka, Japan)) containing 0.5 g/l of potassium disulfide to prevent contamination by anaerobic bacteria such as lactic acid bacteria.

Plasmid construction and yeast transformation

Table 2 summarizes the polymerase chain reaction (PCR) primers used in this study. The δ-integrative fundamental-vector plasmids pδW (*TRP1* as selective marker) and pδU (*URA3* as selective marker) were constructed as follows: The *Sac* I-*Sac* I DNA fragment encoding the 5′ half of the δ-sequence was amplified from *S. cerevisiae* genomic DNA by PCR using the primer pair, 5′DSF (*Sac* I) and 5′DSR (*Sac* I). The *Kpn* I-*Kpn* I DNA fragment encoding the 3′ half of the δ-sequence was amplified from *S. cerevisiae* genomic DNA by PCR using the primer pair, 3′DSF (*Kpn* I) and 3′DSR (*Kpn* I). These fragments were subcloned into the *Sac* I and *Kpn* I sites of the plasmid pBluescript II KS+ (Stratagene, La Jolla, CA, USA). The resulting plasmids were named pδseq. The *Xho* I-*Xho* I DNA fragment encoding large portion of promoter-deficient *TRP1* (*TRP1d*) was amplified from pRS404 by PCR using the primer pair, *TRP1d*F (*Xho* I) and *TRP1d*R (*Xho* I) (Lopes et al. 1991). The *Xho* I-*Xho* I DNA fragment encoding large portion of promoter-deficient *URA3* (*URA3d*) marker gene was amplified from pRS406 (Stratagene) by PCR using the primer pair *URA3d*F (*Xho* I) and *URA3d*F (*Xho* I; Lopes et al. 1991). These fragments were subcloned into the *Xho* I site of plasmid pδseq, and the resulting plasmids were named pδW and pδU, respectively.

The secretory expression of α-amylase proceeded in plasmid pδW-GPSBA constructed from the δ-integrative vector plasmid pδW. The *Not* I-*Not* I DNA fragment encoding the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter from *S. cerevisiae*, the secretion signal of the *R. oryzae* glucoamylase gene, the *S. bovis* α-amylase mature gene, and the GAPDH terminator from *S. cerevisiae* was obtained by digesting pIU-GluRAG-SBA (Yamada et al. 2009) with *Not* I. The fragment was inserted into the *Not* I site of the plasmid pδW to generate the plasmid pδW-GPSBA (Fig. 1A). The plasmid pδU-PGGluRAG was constructed from the δ-integrative vector plasmid pδU, which expressed glucoamylase on the cell surface expression via the 3′-half of α-agglutinin. The *Xba* I-*Xba* I DNA

Table 2 Polymerase chain reaction primers used in this study

Amplification fragment	Primers (restriction endonuclease)	Sequence	Source of DNA sequence
5′ half of δ-sequence	5′DSF (*Sac* I)	5′-ATGCGAGCTCTGTTGGAATAGAAATCAACT-3′	*S. cerevisiae* genome DNA
	5′DSR (*Sac* I)	5′-GCATGAGCTCGGCGCGCCATGTTTATATTC ATTGATCCTA-3′	
3′ half of δ-sequence	3′DSF (*Kpn* I)	5′-ATGCGGTACCGGCGCGCCATAAAATGA TGATAATAATATT-3′	*S. cerevisiae* genome DNA
	3′DSR (*Kpn* I)	5′-GCATGGTACCTGAGAAATGGGTGAATGTTG-3′	
*TRP1*d	*TRP1*dF (*Xho* I)	5′-ATGCCTCGAGTGGAGTATGTCTGTTATTAA-3′	pRS404
	*TRP1*dR (*Xho* I)	5′-GCATCTCGAGTGCAGGCAAGTGCACAAACA-3′	
*URA3*d	*URA3*dF (*Xho* I)	5′-ATGCCTCGAGGAAACGAAGATAAATCATGT-3′	pRS406
	*URA3*dR (*Xho* I)	5′-CGATCTCGAGGTAATAACTGATATAATTAA-3′	
pPGK-signal sequence-glucoamylase gene-3′ half of alpha agglutinin gene-tAGα1	pPGKF (*Xba* I)	5′-ATGCTCTAGACGATTTGGGCGCGAATCCTT-3′	*S. cerevisiae* genome DNA and pRS406_Psed1-glaR-CAS1
	pPGKRO.L.	5′-TGCATTTTTTTTGTTTTATATTTGTTGTAAAAA GTAGATAA-3′	
	GluRFO.L.	5′-ATATAAAACAAAAAAATGCAACTGTTCAAT TTGCCATTGA-3′	
	tAGα1R (*Xba* I)	5′-GCATTCTAGATTTGATTATGTTCTTTCTAT-3′	

fragment encoding the *S. cerevisiae PGK1* promoter, the *R. oryzae* glucoamylase gene with a secretion signal sequence, and the 3′-half of the α-agglutinin gene with a terminator was amplified from *S. cerevisiae* genomic DNA and pIU-GluRAG-SBA by overlap extension PCR with the primers pPGKF(*Xba* I), pPGKRO.L., GluRFO.L., and tAGα1R(*Xba* I). The fragment was digested with *Xba* I and inserted into the *Xba* I site of the plasmid pδU to generate pδU-PGGluRAG (Fig. 1B).

The control plasmids for α-amylase and glucoamylase expression via classical integration were pIW-GPSBA and pIU-PGGluRAG, respectively. The plasmid pIW-GPSBA was constructed as follows: The *Not* I-*Not* I DNA fragment encoding the GAPDH promoter from *S. cerevisiae*, the secretion signal of the *R. oryzae* glucoamylase gene, the *S. bovis* α-amylase mature gene, and the GAPDH terminator from *S. cerevisiae* was obtained by *Not* I digestion of pδW-GPSBA and inserted into the *Not* I site of the classical integration vector plasmid pRS404 (Stratagene) to generate pIW-GPSBA. The plasmid pIU-PGGluRAG was constructed as follows: The *Xba* I-*Xba* I DNA fragment encoding the *PGK1* promoter from *S. cerevisiae*, *R. oryzae* glucoamylase gene with a secretion signal sequence, and the 3′-half of the α-agglutinin gene with a terminator was obtained by *Xba* I digestion of pδU-PGGluRAG. The fragment was inserted into the *Xba* I site of the classical integration vector plasmid pRS406 to yield pIU-PGGluRAG.

Plasmids were transformed into *S. cerevisiae* using lithium acetate as described (Chen et al. 1992). The transformants with the highest amylase activity was selected from several colonies and used in the subsequent experiments.

Mating and protoplast fusion

The diploid MNII/δGS strain was constructed by mating the haploid strains MT8-1/δGS and NBRC1440/δGS. Both strains were grown on SDC liquid medium for 24 h, harvested, spread together on SDC plates, and incubated for 72 h at 30°C. The strains were then replica-plated onto SD plates and incubated for 3 days at 30°C. The resulting diploid strain formed single colonies on SD plates.

The tetraploid MNIV/δGS strain was constructed by protoplast formation and fusion between the diploid MNII/δGS and MNII/δGS/405 strains as described (Harashima et al. 1984).

Determination of DNA content of yeast cells

Yeast genomic DNA was extracted from mid-exponential phase cells as described (Hereford et al. 1979) and then the DNA concentrations were adjusted based on the assumption that the coefficient of one absorbance unit is equal to 50 μg of DNA/ml at 260 nm. The cell number was counted microscopically in a Bürker Türk hemocytometer with appropriate dilution of cultures.

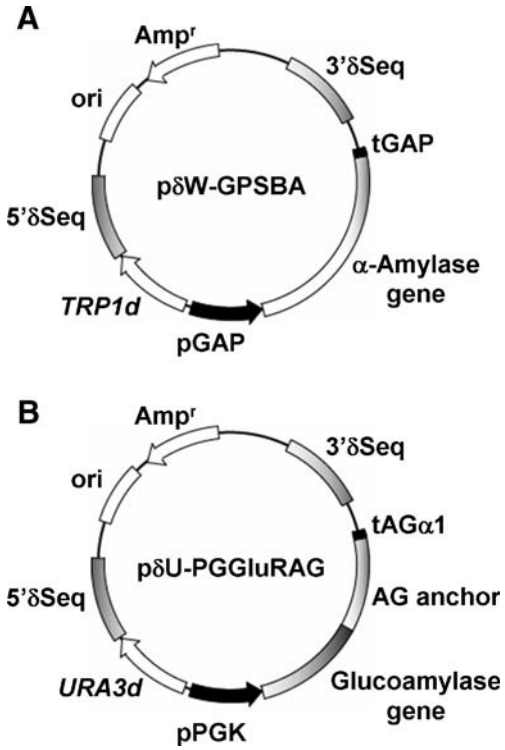

Fig. 1 Expression plasmids for secretion of *Streptococcus bovis* α-amylase (**a**) and display of *Rhizopus oryzae* glucoamylase (**b**) by δ-integration

Amylase activities

Yeast cells were grown aerobically in YPD medium at 30°C for 72 h, and then α-amylase and glucoamylase activities in the culture broth were measured as described (Yamada et al. 2009).

Cell growth and ethanol fermentation from glucose

Yeast cells were cultivated aerobically in 50 ml of YPD liquid medium at 30°C for 96 h, and then the cell density was determined at OD_{600} by spectrophotometry (Model U-2000A, Hitachi, Tokyo, Japan).

Yeast cells were grown aerobically in YPD medium at 30°C for 72 h, washed twice with water, and then 50 g of wet yeast cells/l were inoculated into YP medium containing glucose (100 g/l). Ethanol fermentation then proceeded under anaerobic conditions.

Ethanol fermentation from raw starch

Yeast cells were grown aerobically in YPD medium at 30°C for 72 h, harvested by centrifugation at 3,000×g for 5 min,

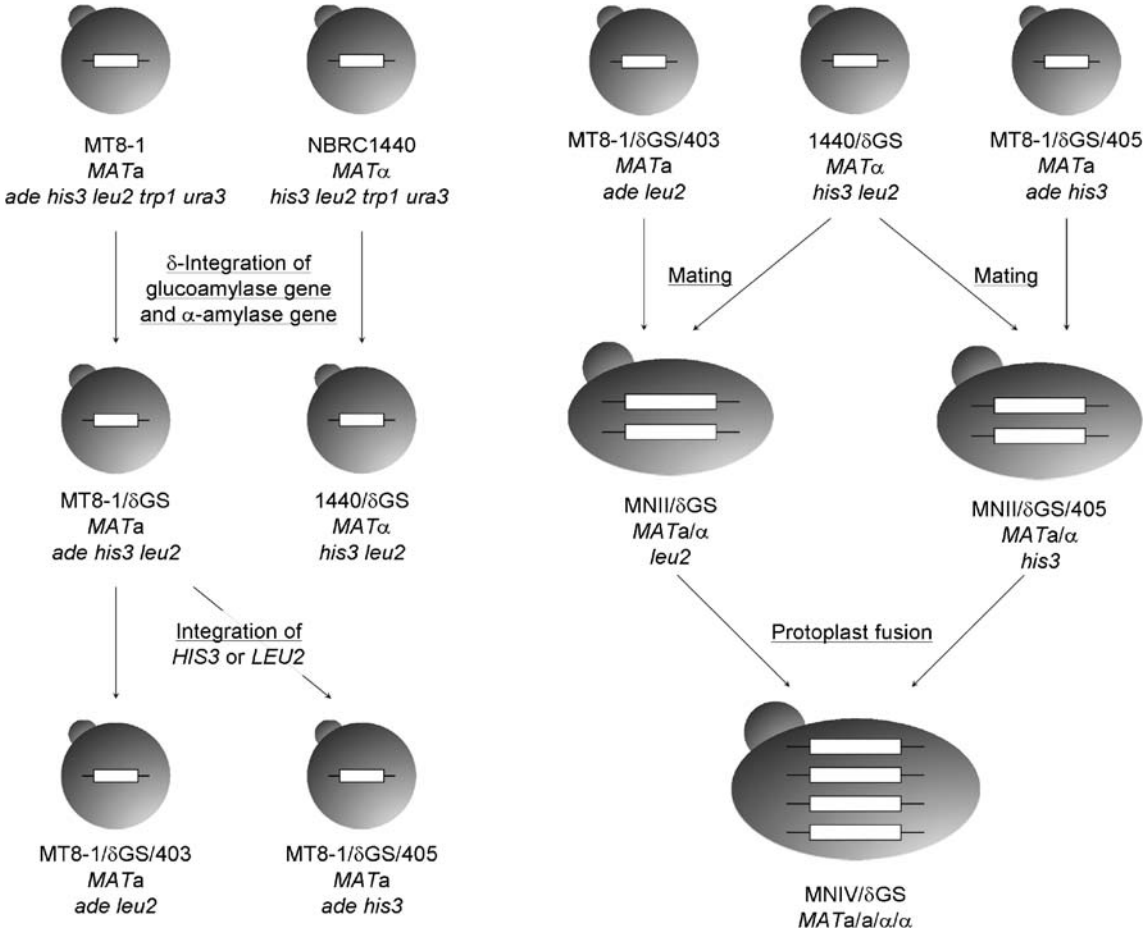

Fig. 2 Strategy for constructing haploid, diploid, and tetraploid yeast strains. Some auxotrophy of parental strain was complemented by vacant vectors to screen cell fusants

washed twice with water, and re-suspended in 50 ml of YPRS medium. Ethanol fermentation proceeded at 30°C with mild agitation in 100 ml bottles equipped with a bubbling CO_2 outlet. The initial cell concentration was adjusted to 50 g of wet cells/l. Wet cell weight was determined by weighing a cell pellet that was harvested by centrifugation at 3,000×g for 5 min. The estimated dry cell weight for all strains was approximately 0.15-fold the wet cell weight.

Other analytical methods

Ethanol and glucose concentrations were simultaneously determined by high performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) using a Shim-pack SPR-Pb column (Shimadzu, Kyoto, Japan). The operating conditions were 80°C, water mobile phase, flow rate of 0.6 ml/min, and then ethanol and glucose concentrations were determined using a refractive index detector (Shimadzu RID-10A). Culture supernatant was separated from the culture broth by centrifugation at 14,000×g for 10 min and then analyzed by HPLC. Total sugars were determined using the phenol sulfuric acid method as described (Dubois et al. 1956). The α-amylase and glucoamylase activities of the recombinant yeast strain were assayed using kits for α-amylase and saccharification-ability (Kikkoman Corp., Chiba, Japan), respectively.

Results

Construction of yeast strains

The five recombinant yeast strains constructed in this study are shown in Fig. 2. The haploid strains MT8-1/δGS and 1440/δGS were constructed by δ-integration of the glucoamylase

Table 3 DNA contents of haploid, diploid, and tetraploid strains

Strain	DNA contents (μg/10^7 cells)	Predicted ploidy
MT8-1/δGS	0.93±0.30	Haploid
MNII/δGS	2.50±0.44	Diploid
MNIV/δGS	5.99±0.57	Tetraploid

Table 4 Amylase activities by strains cultured under aerobic conditions

Strain	α-Amylase activity (U/l)	Glucoamylase activity (U/l)
MT8-1/IGS	37±5.5	14.3±6.8
MT8-1/δGS	722.6±7.6	33.1±2.0
MNII/δGS	725.0±30.2	85.5±18.3
MNIV/δGS	1,115.8±77.4	330.0±8.5

and α-amylase genes into their chromosomal DNA, respectively. Integrated amylase gene expression was enhanced in the diploid strains MNII/δGS, MNII/δGS/405, and mating or protoplast fusion of these δ-integrant strains, respectively, yielded the tetraploid strain MNIV/δGS. Because the amylase activities and fermentation abilities of the haploid strains MT8-1/δGS and 1440/δGS and of the diploid strains MNII/δGS and MNII/δGS/405 were similar (data not shown), the amylase δ-integrant strains MT8-1/δGS, MNII/δGS, and MNIV/δGS were included in subsequent experiments.

Determination of DNA contents of yeast cells

To confirm the formation of the polyploid yeast fusants, we determined the cellular DNA contents of individual yeast cells. Table 3 shows that the DNA contents per cell increased with increasing ploidy, thus confirming the formation of polyploid fusants.

Amylase activities of δ-integrant polyploid fusants

To investigate the effect of δ-integration, mating, and protoplast fusion on amylase activities, amylase activities of each recombinant yeast cells were measured after cultivation in YPD medium. Table 4 shows that the activities of glucoamylase (33.1 U/l) and of α-amylase (722.6 U/l) in the δ-integrant haploid strain MT8-1/δGS were about twofold and 20-fold higher, respectively, than those of the general integrant strain MT8-1/IGS. The activities of glucoamylase and α-amylase improved with increasing ploidy, reaching 330.0 and 1,115.8 U/l, respectively, in the tetraploid strain MNIV/δGS. These values were about 1.5- and tenfold higher than the respective activities of the δ-integrant haploid strain, MT8-1/δGS.

Effect of increasing of ploidy on growth and fermentation in δ-integrant polyploid fusants

The relatively low growth rate and cell yield of the haploid strain MT8-1/δGS under aerobic conditions is shown in Fig. 3a. The growth rate and cell yield of the diploid and tetraploid strains MNII/δGS and MNIV/δGS, respectively, were similarly increased. Their abilities to ferment ethanol from glucose under anaerobic conditions were also similar and higher than that of the haploid strain (Fig. 3b).

Ethanol fermentation from raw starch

We examined the abilities of the recombinant yeast strains to directly ferment ethanol from raw starch under anaerobic conditions. The ethanol productivity from raw starch was improved with increasing ploidy, as shown in Fig. 4; the tetraploid strain consumed 150 g/l of raw starch and produced 70 g/l of ethanol after 72 h of fermentation. The amounts of ethanol produced by the haploid, diploid, and tetraploid strains after 72 h of fermentation were 0.55, 0.72, and 0.93 g/l/h, respectively. Table 5 shows that directions of maximal glucoamylase and α-amylase activities were identical during ethanol fermentation and that amylase activities were highest in the tetraploid strain.

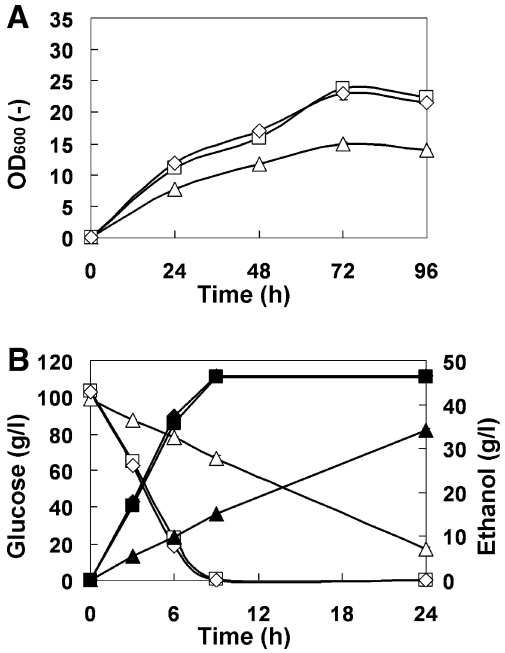

Fig. 3 **a** Aerobic cultivation of haploid MT8-1/δGS (*triangles*), diploid MNII/δGS (*squares*), and tetraploid MNIV/δGS (*diamonds*). **b** Time course of ethanol fermentation from glucose by haploid MT8-1/δGS (*triangles*), diploid MNII/δGS (*squares*), and tetraploid MNIV/δGS (*diamonds*). *Filled and unfilled symbols* represent ethanol and glucose concentrations, respectively. Data are means from three independent experiments

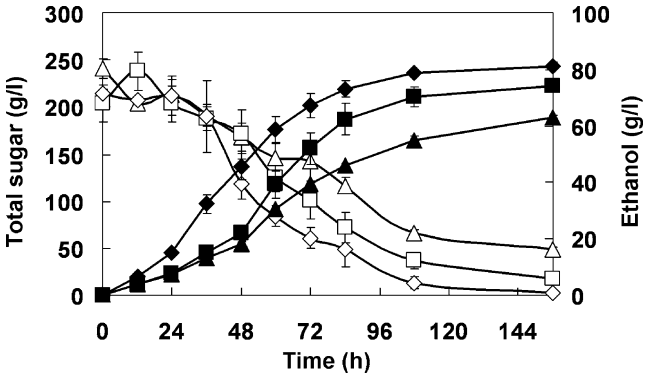

Fig. 4 Time course of ethanol fermentation from raw starch by haploid MT8-1/δGS (*triangles*), diploid MNII/δGS (*squares*), and tetraploid MNIV/δGS (*diamonds*). *Filled and unfilled symbols* represent ethanol and total sugar concentrations, respectively. Data are averages from three independent experiments

Discussion

The combination of δ-integration and cell fusion of yeasts generated an efficient direct fermentation process from raw starch. Two mitotically stable recombinant haploid yeast strains were constructed by the δ-integration of multiple α-amylase and glucoamylase genes into their chromosomal DNA. Two diploid strains were constructed by mating from these δ-integrant haploid strains, and one tetraploid strain was constructed by protoplast fusion. The cell growth, amylase activities, and ethanol productivity of these strains were investigated.

Increasing the amylase activity is critical to efficiently produce ethanol from starchy materials. Here, we repeated two δ-integrations through two types of auxotrophic markers to introduce as many copies of glucoamylase and α-amylase into the yeast genome. We finally increased glucoamylase and α-amylase activities two- and 20-fold improved, respectively, in the haploid strain MT8-1/δGS, compared with the haploid strain MT8-1/IGS, in which genes were introduced by general integration (Table 4). The expression of a single δ-integrant gene is usually two- to tenfold higher than that obtained with general integration (Parekh et al. 1996; Lee and Da Silva 1997; Choi et al. 2002). This is the first study to demonstrate that repeated δ-integration through two types of auxotrophic markers significantly improves the activities of two amylases. Neither gene was overexpressed in the glucoamylase and α-amylase double cassette δ-integrated vector (data not shown) because this vector is very large. These results agreed with other findings (Ghang et al. 2007) and suggested that repeated δ-integration using a single cassette vector would be a more useful strategy for overexpression.

In our previous study, amylase expressing diploid strain constructed by mating of general integrant haploid strains showed high ethanol productivity (0.46 g/l/h) with 290 U/l of glucoamylase and 950 U/l of α-amylase maximum activity (Yamada et al. 2009). In contrast, in this study, δ-integrant tetraploid strain MNIV/δGS showed appropriately twofold higher ethanol productivity (0.93 g/l/h) than our previous results with 843 U/l of glucoamylase and 1,580 U/l of α-amylase maximum activity (Fig. 4 and Table 5). This result suggests that ethanol productivity from raw starch deeply depend on the amylase activities, and our novel strategy using δ-integration and cell fusion is effective for overexpression of genes and improvement of ethanol productivity from raw starch.

The rates of ethanol fermentation by diploid and tetraploid strains from glucose were similar (Fig. 3), whereas those from raw starch were significantly higher in the latter than in the former strain (Fig. 4). This is because α-amylase and glucoamylase activities were significantly improved in the tetraploid strain (Table 5). These findings indicated that the rate-limiting step in ethanol production from raw starch is the degradation of raw starch to glucose, even though it is very difficult to determine the exact amount of insoluble raw corn starch in the early fermentation phase using phenol sulfuric methods. Hence, combining δ-integration with polyploidization is efficient to construct yeasts that can directly ferment ethanol from raw starch.

We constructed a polyploid strain based on MT8-1/δGS and 1440/δGS, which significantly improved amylase activity after δ-integration (Table 4), suggesting that polyploidization is useful for increasing and maintaining the stable expression of integrated genes. In addition, tetraploid fusants MNIV/δGS also maintained high ethanol productivity from raw starch for several batch fermentations (data not shown), showing tetraploid fusants MNIV/δGS is mitotically stable sufficiently for practical fermentation process (Brigidi et al. 1988; Hashimoto et al. 2006). Our novel strategy of combining δ-integration and polyploidization thus significantly improved the growth rate, cell yield, amylase activities, and the abilities of yeast strains to efficiently ferment ethanol.

Table 5 Maximum amylase activities by strains cultured under anaerobic fermentation conditions

Strain	Maximum α-amylase activity (U/l)	Maximum glucoamylase activity (U/l)
MT8-1/δGS	451±19.8	307±41.9
MNII/δGS	837±122	647±42.7
MNIV/δGS	1,580±397	843±71.8

In conclusion, we established a novel strategy for constructing yeasts that can efficiently produce ethanol from raw starch. The fermentation process might be further improved by investigating the reusability of recombinant yeast cells or the application of more practical medium such as corn steep liquor. Our strategy simultaneously achieved the overexpression of genes integrated into the yeast genome and improved the practical potential of yeasts. Recombinant yeasts with an engineered cell surface can also be used as a whole cell biocatalyst in other bioconversions or in fine chemical production processes.

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