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Development of innovative recombinant yeast strains for efficient bioethanol production from various biomasses by consolidated bioprocess

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博士論文

Development of innovative recombinant yeast strains for efficient bioethanol production from various biomasses by consolidated bioprocess

多様なバイオマスからの統合型高効率エタノール生産プロセスを実現する革新的な酵母菌体触媒の開発

2011 年 1 月

神戸大学大学院工学研究科

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PREFACE

This is a thesis submitted by the author to Kobe University as partial fulfillment of the requirements for the degree of Doctor of Engineering. The studies reported here were carried out between 2005 and 2011 under the direction of Professor Akihiko Kondo in the Laboratory of Biochemical Engineering, Department of Chemical Science and Engineering, Graduate school of Engineering, Kobe University.

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CONTENTS

Introduction 1

Synopsis 9

Part I. Direct ethanol production from starch-rich biomass 14

Chapter 1. Efficient production of ethanol from raw starch by a mated diploid *Saccharomyces cerevisiae* with integrated alpha-amylase and glucoamylase genes 15

Chapter 2. Novel strategy for yeast construction using delta-integration and cell fusion to efficiently produce ethanol from raw starch 32

Chapter 3. Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that express amylases 52

Part II. Direct ethanol production from lignocellulosic biomass 65

Chapter 1. Cocktail delta-integration: a novel method to construct cellulolytic enzyme expression ratio-optimized yeast strains 66

Chapter 2. Direct ethanol production from cellulosic materials using a cellulolytic enzymes expression optimized diploid *Saccharomyces cerevisiae* strain 83

General conclusion 104

Publication lists 107

INTRODUCTION

Bioethanol - Alternative fuels for the future

In recent years, because of the exhaustion of fossil fuels and environmental problems such as global warming and acid rain, the utilization of biomass as a source of renewable, environmentally friendly energy has attracted much attention. In particular, bioethanol produced from biomass has been suggested as a promising alternative fuel or gasoline enhancer, and it has been used all over the world (Table 1). Bioethanol has been produced from sugar cane and starch-rich biomass, and the level of global ethanol production was reached 51,000 million ton in 2006 and increases annually (Table 2). Therefore, the development of an efficient and cost-effective process for bioethanol production from biomass is required.

Table 1 Usage of bioethanol as alternative fuel in some countries.

Country	Main feedstock for bioethanol	Commo persentage of ethanol in gasoline blends or gasolin enhancer
United States	Corn	10
Brazil	Sugar cane	24
China	Corn, wheat	10
India	Sugar cane	5
France	Sugar beet, wheat, corn	ETBE[a]
Canada	Corn, wheat, barley	7.5-10
Spain	Wheat, barley	ETBE[a]

[a] ETBE; ethyl tert-butyl ether, gasoline enhancer

Adapted from Berg (2004), Murray (2005), and Sánchez and Cardona (2008)

Table 2 World production of ethanol (million liters).

Country	2005	2006
United States	16,139	18,376
Brazil	15,999	16,998
China	3,800	3,849
India	1,699	1,900
France	908	950
Germany	431	765
Russia	749	647
Canada	231	579
Spain	352	462
South Africa	390	386
World amounts	45,988	51,056

Adapted from Londoño (2007), Renewable Fuels Association (2007), and Sánchez and Cardona (2008)

Bioethanol production from starch-rich biomass

At present, most used feedstock for bioethanol production is starch-rich biomass represented by corn in United States which is one of the biggest ethanol produced country (Table 1 and 2). In general, bioethanol is produced by fermentation of sugar using efficient ethanol producing yeast *Saccharomyces cerevisiae* (Sánchez and Cardona 2008). However, because *S. cerevisiae* lacks the ability to utilize starch as a carbon source, ethanol production from starch-rich biomass by the classical yeast fermentation method requires a complex and multi-step process. The process typically consists of gelatination of raw starch by heating with high energy cost, liquefaction by α-amylase, and saccharification to glucose by glucoamylase with high enzyme cost. In recent years, there are some reports about reduction of energy cost by conducting hydrolyzing starch at low temperature (Robertson et al. 2006) and reduction of enzyme cost by developing amylolytic enzyme expressing yeast (Shigechi et al. 2004, Chi et al. 2009). However bioethanol production cost from starch-rich biomass remains high to use as low cost alternative fuel, and that

the retail price of bioethanol is suppressed to the level of gasoline by subsidy is current situation all over the world (Solomon et al. 2007). Therefore the development of an efficient and cost-effective process for ethanol production from starch-rich biomass is required.

Bioethanol production from lignocellulosic biomass

Lignocellulosic biomass has attracted much attention as feedstock for bioethanol production alternative to starch-rich biomass in recent days. Starch-rich biomass is relatively well decomposed by amylolytic enzymes such as α-amylase and glucoamylase, and thus easily used for feedstock for bioethanol. However it is also consumed as food, so if too much bioethanol is made from them, food prices would rise and shortages should be occurred in some countries. In contrast, lignocellulosic biomass is not consumed as food and is most abundant biopolymer in the Earth. It is considered that lignocellulosic biomass accounts about 50% of world biomass and its annual production was estimated in 10-50 billion ton (Claassen et al. 1999). However it requires high cost and complex hydrolyzing steps such as pretreatment by high temperature, high pressure, acid, and alkaline and/or numerous amount of cellulase treatment to use lignocellulosic biomass as feedstock for bioethanol. Although, many lignocellulosic biomasses have been tested for feedstock of bioethanol (Sánchez and Cardona 2008), the structure of lignocellulosic biomass is very rigid and complex, and the structure and composition is vary depending on the origin of lignocellulosic biomass. Thus, bioethanol production process from lignocellulosic biomass remains very low efficiency and very high cost. Therefore, it would be drastically reduction of producing cost for industrial production and prevalence of lignocellulosic ethanol.

Consolidated bioprocessing

A promising strategy to reduce bioethanol production cost drastically from starch-rich and lignocellulosic biomass should be consolidated bioprocessing (CBP) (Lynd et al. 2002 and 2005). The process consolidates multi and complex steps such as production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to ethanol into just single step (Fig. 1). To realize this process efficiently, it is absolutely imperative to develop high performance microorganisms which can convert starch-rich and/or lignocellulosic biomass into ethanol directly and efficiently. Some reports suggested the prospect of achieving efficient CBP (Fujita et al. 2004, Shigechi et al. 2004, Lynd et al. 2005). However, in previous reports, there are numerous limitations associated with achieving CBP efficiently, such as the low hydrolysis enzymes activity, low robustness and stability of microorganisms.

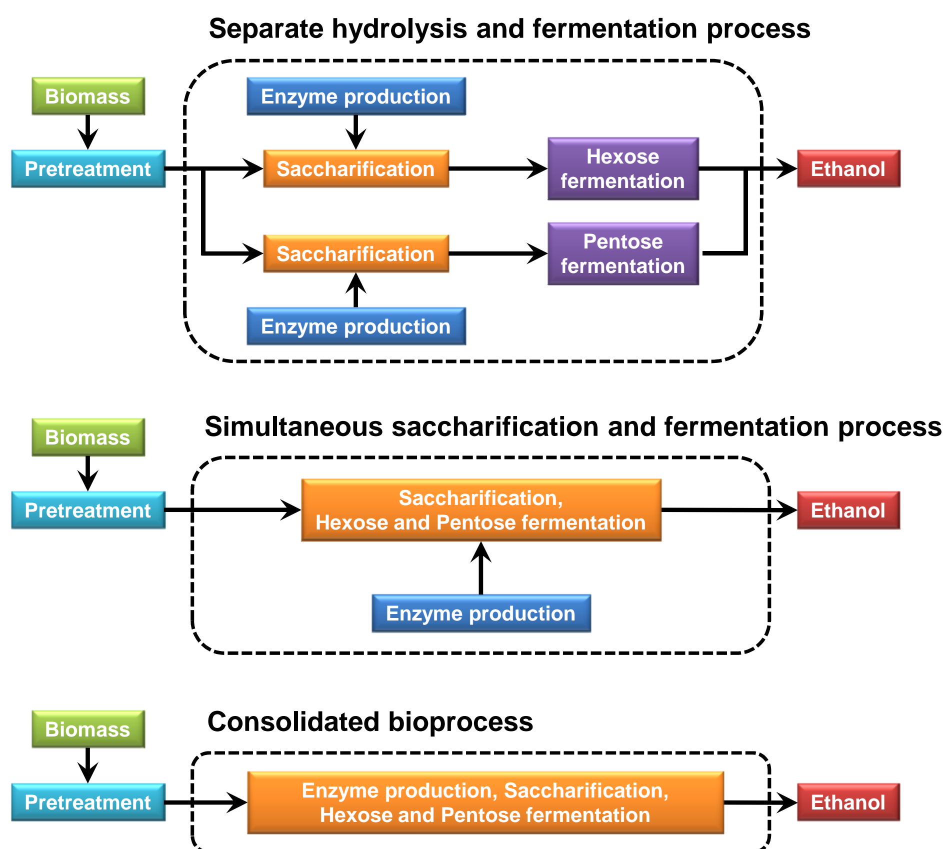

Figure 1 Ethanol production by consolidated bioprocess.

Constituent of this thesis

In this study, high performance yeast strains to achieve efficient and cost-effective bioethanol production from starch-rich or lignocellulosic real biomass by CBP were developed. In Part I, development of high performance yeast to produce bioethanol from starch-rich biomass, and in Part II, from lignocellulosic biomass was described.

The objectives in each chapter were described as below.

Part I. Direct ethanol production from starch-rich biomass

Chapter 1.

To construct a stable and efficient process for the direct production of ethanol from raw starch using a recombinant *S. cerevisiae*, haploid and diploid recombinant strain with expressing two types of amylase were constructed. Then their ethanol productivity and reusability was evaluated.

Chapter 2.

To improve levels of amylase gene expression and the practical potential of yeast, we developed novel recombinant strategy combining δ-integration and polyploidization through cell fusion. Then the ethanol productivity and practical potential of the polyploid yeast strain was evaluated.

Chapter 3.

To achieve cost-effective and efficient ethanol production by CBP from starch-rich biomass, the ethanol productivity from real biomass high-yielding rice was evaluated using the polyploid yeast strain. In addition, to reduce production cost much further, ethanol fermentation without any nutrient supplementation was also carried out.

Part II. Direct ethanol production from lignocellulosic biomass

Chapter 1.

To construct engineered yeast with efficient cellulose degradation, we developed a simple method to optimize cellulase expression levels named cocktail δ-integration. In cocktail δ-integration, several kinds of cellulase expression cassettes are integrated into yeast chromosomes simultaneously in one step, and strains with high cellulolytic activity (i.e., expressing an optimum ratio of cellulases) are easily obtained.

Chapter 2.

To produce ethanol by CBP from rice straw, one of the most abundant lignocellulosic biomass, cellulolytic enzymes expression optimized diploid strain was constructed. Then their growth profile in industrial cheap medium and ethanol productivity from rice straw was evaluated.

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SYNOPSIS

Part I.
Direct ethanol production from starch-rich biomass

Chapter 1. Efficient production of ethanol from raw starch by a mated diploid *Saccharomyces cerevisiae* with integrated alpha-amylase and glucoamylase genes

The goal of this research was to construct a stable and efficient process for the production of ethanol from raw starch, using a recombinant *Saccharomyces cerevisiae*, which is productive even under conditions such as non-selection or long-term operation. Three recombinant yeast strains were used, two haploid strains (MT8-1SS and NBRC1440SS) and one diploid strain (MN8140SS). The recombinant strains were constructed by integrating the glucoamylase gene from *Rhizopus oryzae* fused with the 3'-half of the α-agglutinin gene as the anchor protein, and the α-amylase gene from *Streptococcus bovis,* respectively, into their chromosomal DNA by homologous recombination. The diploid strain MN8140SS was constructed by mating these opposite types of integrant haploid strains in order to enhance the expression of integrated amylase genes. The diploid strain had the highest ethanol productivity and reusability during fermentation from raw starch. Moreover, the ethanol production rate of the integrant diploid strain was maintained when batch fermentation was repeated three times (0.67, 0.60, and 0.67 g/l/h in each batch). These results clearly show that a diploid strain developed by mating two integrant haploid strains is useful for the establishment of an efficient ethanol production process.

Chapter 2. Novel strategy for yeast construction using delta-integration and cell fusion to efficiently produce ethanol from raw starch

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 10-fold, 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.

Chapter 3. Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that express amylases

Efficient ethanol producing yeast *Saccharomyces cerevisiae* cannot produce ethanol from raw starch directly. Thus the conventional ethanol production required expensive and complex process. In this study, we developed a direct and efficient ethanol production process from high-yielding rice harvested in Japan by using amylase expressing yeast without any pretreatment or addition of enzymes or nutrients. Ethanol productivity from high-yielding brown rice (1.1 g/L/h) was about 5 fold higher than that obtained from purified raw corn starch (0.2 g/L/h) when nutrients were added. Using an inoculum volume equivalent to 10% of the fermentation volume without any nutrient supplementation resulted in ethanol productivity and yield reaching 1.2 g/L/h and 101%, respectively, in a 24-hour period. High-yielding rice was demonstrated to be a suitable feedstock for bioethanol production. In addition, our polyploid amylase-expressing yeast was sufficiently robust to produce ethanol efficiently from real biomass. This is first report of direct ethanol production on real biomass using an amylase-expressing yeast strain without any pretreatment or commercial enzyme addition.

Part II.

Direct ethanol production from lignocellulosic biomass

Chapter 1. Cocktail delta-integration: a novel method to construct cellulolytic enzyme expression ratio-optimized yeast strains

The filamentous fungus *T. reesei* effectively degrades cellulose and is known to produce various cellulolytic enzymes such as β-glucosidase, endoglucanase, and cellobiohydrolase. The expression levels of each cellulase are controlled simultaneously, and their ratios and synergetic effects are important for effective cellulose degradation. However, in recombinant *Saccharomyces cerevisiae*, it is difficult to simultaneously control many different enzymes. To construct engineered yeast with efficient cellulose degradation, we developed a simple method to optimize cellulase expression levels, named cocktail δ-integration. In cocktail δ-integration, several kinds of cellulase expression cassettes are integrated into yeast chromosomes simultaneously in one step, and strains with high cellulolytic activity (i.e., expressing an optimum ratio of cellulases) are easily obtained. Although the total integrated gene copy numbers of cocktail δ-integrant strain was about half that of a conventional δ-integrant strain, the phosphoric acid swollen cellulose (PASC) degradation activity (64.9 mU/g-wet cell) was higher than that of a conventional strain (57.6 mU/g-wet cell). This suggests that optimization of the cellulase expression ratio improves PASC degradation activity more so than overexpression. To our knowledge, this is the first report on the expression of cellulase genes by δ-integration and optimization of various foreign genes by δ-integration in yeast. This method should be very effective and easily applied for other multi-enzymatic systems using recombinant yeast.

Chapter 2. Direct ethanol production from cellulosic materials using a cellulolytic enzymes expression optimized diploid *Saccharomyces cerevisiae* strain

The breakdown of cellulose requires the synergistic action of the cellulolytic enzymes endoglucanase, cellobiohydrolase, and β-glucosidase, whose expression ratios and synergetic effects significantly influence the efficiency of cellulose degradation. In this study, using our previously developed method to optimize cellulase expression levels in yeast, we constructed a cellulolytic enzymes expression optimized diploid *Saccharomyces cerevisiae* strain in an attempt to improve cellulose degradation activity and enable direct ethanol production from rice straw, one of the most abundant sources of lignocellulosic biomass. The engineered diploid strain, which contained multiple copies of three cellulase genes integrated into its genome, precultured in molasses medium (381.4 mU/g wet cell) displayed approximately six-fold higher phosphoric acid swollen cellulose (PASC) degradation activity than the parent haploid strain (63.5 mU/g wet cell). In ethanol fermentation from PASC, the diploid strain produced 7.6 g/L ethanol in 72 h, with an ethanol yield that reached 75% of the theoretical value. Moreover, the diploid strain produced 7.5 g/L ethanol from pretreated rice straw in 72 h. We developed a cellulolytic enzymes expression optimized diploid yeast strain that has high ethanol productivity from cellulosic materials. To our knowledge, this is the first report of ethanol production from agricultural waste biomass using cellulolytic enzyme-expressing yeast without the addition of exogenous enzymes. Our results suggest that combining multi-gene expression optimization and diploidization in yeast is a promising approach for enhancing ethanol production from various types of lignocellulosic biomass.

Part I.
Direct ethanol production from starch-rich biomass

Chapter 1. Efficient production of ethanol from raw starch by a mated diploid *Saccharomyces cerevisiae* with integrated alpha-amylase and glucoamylase genes

Introduction

Because of the exhaustion of fossil fuels and environmental problems such as global warming and acid rain, the utilization of biomass as a source of renewable, environmentally friendly energy has attracted much attention in recent years. In particular, bioethanol produced from biomass, including fast-growing plant species and waste paper, has been suggested as a promising alternative fuel. Bioethanol has been produced from sugar cane and starch-rich grains for use as automobile fuel in Brazil and the United States of America, and the level of global ethanol production increases annually. Therefore, the development of an efficient process for ethanol production from biomass is required.

Starch is mainly composed of two fractions of high molecular weight: amylose and amylopectin. The minor fraction, amylose (20-30%), is mainly a linear glucose polymer formed by α-1,4-glucosidic linkage and some α-1,6-branching points. Amylopectin represents the major fraction of starch (70-80%) and is highly branched. Because the yeast *Saccharomyces cerevisiae* lacks the ability to utilize starch as a carbon source, ethanol production from starchy biomass by the classical yeast fermentation method is expensive and requires a complex, multi-step process. The process typically consists of gelatination of raw starch by cooking, liquefaction by α-amylase, and saccharification to glucose by glucoamylase. There are many reports on the production of ethanol from starch utilizing recombinant *S. cerevisiae* that express 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 suggested that recombinant *S. cerevisiae* that co-express *Streptococcus bovis* α-amylase and *Rhizopus oryzae* glucoamylase/α-agglutinin fusion protein can ferment raw starch to ethanol directly and efficiently (Khaw et al. 2005). In the Khaw *et al.* report, the amylase genes were introduced by a yeast episomal plasmid (YEp) containing the origin of a 2 μm plasmid. YEp is suitable for overexpression of target genes because the copy numbers are very high (50 to 200

copies) under laboratory conditions (Broach 1983). However, YEp vectors are mitotically unstable under non-selective conditions such as long-term industrial operation in poorly defined media. In addition, the recombinant starch-fermenting *S. cerevisiae* constructed by YEp vectors was reported to have low reusability in repeated batch fermentation (Khaw et al. 2005).

It is well known that the breeding of polyploid strains can improve the ethanol productivity or the quality of fermentation products using industrial yeast strains without recombinant techniques (Higgins et al. 2001; Hashimoto et al. 2006). Since the laboratory haploid strains are difficult to use in practice, because they have low thermostability and low tolerance of acid, ethanol, and other fermentation inhibitors (Martín and Jönsson. 2003; Garay-Arroyo et al. 2004), it was expected that the breeding of diploid yeast from these haploid strains could solve these practical problems.

In this study, we bred a diploid recombinant yeast strain by mating haploid strains, in which multiple α-amylase and glucoamylase genes were integrated into the *URA3* and *HIS3* locus, in order to construct an efficient process for the production of ethanol from raw starch. Ethanol productivity was further improved by the use of a diploid recombinant yeast strain. In addition, three cycle repeated batch fermentation using the diploid recombinant yeast strain was then examined.

Material and methods

Strains, plasmids and media

The genetic properties of all strains and plasmids used in this study are summarized in Table 1. In brief, an *Escherichia coli* strain, NovaBlue (Novagen, Madison, Wisconsin, USA) was used as the host strain for recombinant DNA manipulation. The haploid yeast strains *S. cerevisiae* MT8-1 and NBRC1440ΔHUW were used for expression of α-amylase and glucoamylase, respectively. The diploid strain *S. cerevisiae* MN8140SS was constructed by the mating of MT8-1SS and NBRC1440SS, as described below.

E. coli transformant was grown in Luria-Bertani medium (10 g/l of tryptone, 5 g/l of yeast extract, 5 g/l of sodium chloride) supplemented with 100 μg/ml of ampicillin. SD medium (6.7 g/l

of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI, USA), 20 g/l of glucose) supplemented with appropriate amino acids was used for screening of yeast transformants. 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 YPD medium (10 g/l of yeast extract, 20 g/l of Bacto-peptone (Difco Laboratories, Detroit, MI, USA), 20 g/l of glucose) were used for the aerobic cultivation of yeast cells.

For ethanol fermentation, YPS medium (10 g/l of yeast extract, 20 g/l of Bacto-peptone, and 100 g/l of raw corn starch (Wako Pure Chemical Industries, Ltd., Osaka, Japan)) was used with 0.5 g/l of potassium disulfide to prevent contamination by anaerobic bacteria, such as lactic acid bacteria.

Table 1 Characteristics of strains and plasmids used in this study.

Strains or plasmids	Relevant features	Reference or source
Strains		
Bacterial strain		
E. coli NovaBlue	*endA1 hsdR17* ($r_{K12}^{-} m_{K12}^{+}$) *supE44 thi-I gyrA96 relA1 lac recA1/F'* [*proAB* $^{+}$ *lacI* ^{q} *Z*ΔM15::Tn*10*(Tet^{r})]	Novagen
S. cerevisiae yeast strains		
MT8-1	*MAT*a *ade leu2 his3 ura3 trp1*	(Tajima et al. 1985)
MT8-1SS	*MAT*a integrating pRS404[a], pIH-GluRAG-SBA, and pIU-GluRAG-SBA	This study
NBRC1440	Wild type strain	NBRC[b]
NBRC1440ΔHUW	*MAT*α *his3 ura3 trp1*	Our laboratory[c]
NBRC1440SS	*MAT*α integrating pIH-GluRAG-SBA and pIU-GluRAG-SBA	This study
MN8140SS	*MAT*a/α integrating pIH-GluRAG-SBA and pIU-GluRAG-SBA	This study
Plasmids		
pRS403	*HIS3* No expression	Stratagene
pRS404	*TRP1* No expression	Stratagene
pRS406	*URA3* No expression	Stratagene
pIH-GluRAG-SBA	*HIS3* Secreting expression of *S. bovis* α-amylase gene and surface expression of glucoamylase fused with 3'-half of α-agglutinin gene	This study
pIU-GluRAG-SBA	*URA3* Secreting expression of *S. bovis* α-amylase gene and surface expression of glucoamylase fused with 3'-half of	This study

[a] Vacant vector pRS404 was integrated into MT8-1SS to screen diploid strain MN8140SS.

[b] NITE Biological Research Center, Chiba, Japan.

[c] NBRC1440ΔHUW was bred by the gene deletion method (Akada et al. 2006).

Construction of plasmids and yeast transformation

The plasmid pIU-GluRAG, used for cell-surface display of *R. oryzae* glucoamylase fused with 3'-half of α-agglutinin (Murai et al. 1997), was constructed as follows. The *Sma* I - *Eco* RI DNA fragment encoding *SED1* promoter was amplified from the *S. cerevisiae* genome DNA by PCR with the following single pair of primers: 5'-ATG CCC CGG GGA AAA ACG ACA ACA TTC CAC-3' and 5'-GCA TGA ATT CCT TAA TAG AGC GAA CGT ATT-3'. The *Eco* RI – *Xho* I and *Xho* I – *Kpn* I DNA fragments encoding the *R. oryzae* glucoamylase gene with a secretion signal sequence and 3'-half of α-agglutinin gene, respectively, were prepared by digestion of pGA11 (Murai et al. 1997). These fragments were subcloned into the *Sma* I – *Eco* RI, *Eco* RI – *Xho* I and *Xho* I – *Kpn* I sites of plasmid pRS406 (Stratagene, La Jolla, California, USA).

Plasmids pIU-GluRAG-SBA and pIH-GluRAG-SBA (Fig. 1 A) were used for the secretion of α-amylase and cell-surface display of glucoamylase fused with 3'-half of α-agglutinin in the presence of different promoters. The pIU-GluRAG-SBA plasmid was constructed from pIU-GluRAG, as described above. 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 amplified from pSBAA2 (Shigechi et al. 2002) by PCR using the following single pair of primers; 5'-ATA AGA ATG CGG CCG CAC CAG TTC TCA CAC GGA ACA CCA CTA ATG GAC AC-3' and 5'-ATA AGA ATG CGG CCG CTC AAT CAA TGA ATC GAA AAT GTC ATT AAA ATA GTA TAT AA-3'. The amplified fragment was inserted into the *Not* I site of the plasmid pIU-GluRAG. The resulting plasmid was named pIU-GluRAG-SBA (Fig. 1 A). For the construction of pIH-GluRAG-SBA, the *Aat* II – *Nae* I DNA fragment, encoding the *HIS3* gene from pRS403 (Stratagene), was inserted into the *Aat* II – *Nae* I site of plasmid pIU-GluRAG-SBA.

The transformation of plasmids into *S. cerevisiae* was carried out by the lithium acetate method, using the YEASTMAKER transformation system (Clontech Laboratories, Inc., Palo Alto, Calif.).

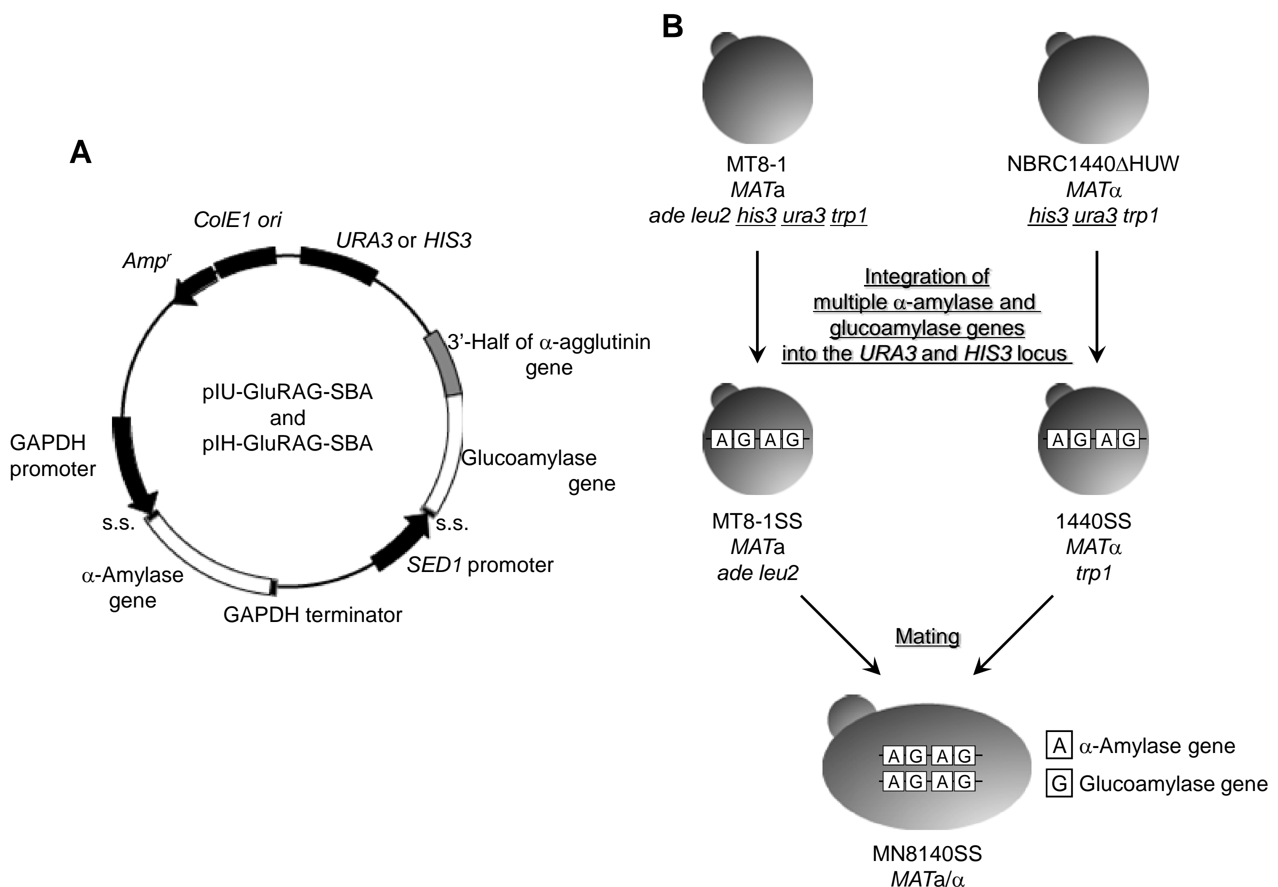

Figure 1 (A) Expression plasmids for secretion of *S. bovis* α-amylase and display of *R. oryzae* glucoamylase with *HIS3* (pIH-GluRAG-SBA) and *URA3* (pIU-GluRAG-SBA) as the homologous recombination sequence. s.s., Secretion signal sequence of glucoamylase gene.

(B) Construction scheme of haploid and diploid yeast strains. Tryptophan auxotrophy of haploid strain MT8-1was complemented by vacant vector pRS404 to screen diploid strain MN8140SS.

Mating method

Diploid strain MN8140SS was constructed by mating haploid strains MT8-1SS and NBRC1440SS. Each haploid strain was grown on YPD liquid medium for 24 h. Each was then harvested, and they were spread together on a YPD plate. After 72 h of incubation at 30°C, the plate was replica-plated to an SD plate, and subsequently incubated for 3 days at 30°C. The resulting diploid strain formed a single colony on the SD plate.

Cell growth rate and yield

The yeast cells were cultivated aerobically in 50 ml of SDC liquid medium at 30°C for 72 h. The specific growth rate was determined by measuring the cell concentration after the first 24 h of cultivation. The glucose concentration of culture supernatant and the cell concentration were measured after 72 h of cultivation. The cell yield was defined as the cell weight produced by 1 g of glucose consumption in aerobic cultivation conditions.

Amylase activities and fermentation ability

Yeast cells were aerobically grown in YPD medium at 30°C for 72 h. The α-amylase and glucoamylase specific activities were measured using culture broth with yeast cells. The fermentation ability was defined as the anaerobic ethanol specific production rate in YP medium with glucose (100 g/l) and yeast cells (50 g-wet-cell/l) after the first 3h.

Ethanol fermentation from raw starch

Yeast cells were aerobically grown in SDC medium at 30°C for 72 h, harvested by centrifugation for 5 min at 3,000 × g, and re-suspended into 50 ml of YPS medium. Ethanol fermentation was carried out in 100 ml bottles equipped with a bubbling CO_2 outlet line. All fermentation was performed at 30°C with mild agitation. The initial cell concentration was adjusted to 50 g wet cell weight per liter. The wet cell weight was determined by measuring the weight of a cell pellet that was harvested by centrifugation at 3,000 × g for 5 min. The dry cell weight was experimentally estimated to be approximately 0.15 times the wet cell weight in all the strains. For repeated batch fermentation, yeast cells were harvested by centrifugation at 3,000 × g for 5min after 60 h of fermentation. The yeast cells remaining in the residual starch were inoculated into fresh YPS medium, and the fermentation was repeated three times.

Other analytical methods

Ethanol and glucose concentrations were simultaneously determined using a high performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with a Shim-pack SPR-Pb column (Shimadzu, Kyoto, Japan). The operating conditions were carried out at 80°C, with

water as the mobile phase at a flow rate of 0.6 ml/min, and the detection was performed with a refractive index detector (Shimadzu RID-10A). HPLC analysis was applied to a sample of culture supernatant after separating culture broth by centrifugation at 14,000 × g for 10 min. Total sugar concentration was determined by the phenol sulfuric acid method, as described previously (Dubois et al. 1956). The α-amylase and glucoamylase activities of the recombinant yeast strain were determined using α-amylase and saccharifying-ability assay kits (Kikkoman Corp., Chiba, Japan), respectively.

Results

Construction of yeast strain

As shown in Fig. 1B, three recombinant yeast strains were constructed. Two haploid strains (MT8-1SS and NBRC1440SS) were constructed by integration of the glucoamylase gene and the α-amylase gene into their chromosomal DNA by homologous recombination, respectively. The diploid strain MN8140SS was constructed by mating these opposite types of integrant haploid strains in order to enhance the expression of integrated amylase genes. The amylase activities of twenty colonies of the diploid strain were investigated. Seven colonies showed improvement of amylase activities compared to the parental strains, and the colony with the highest amylase activity was used in the subsequent experiments.

Cell growth and yield

To investigate the effect of mating on cell growth, each of the recombinant cells was cultivated in SDC medium, and their cell growth and yields were determined in aerobic conditions (Table 2). The mated diploid strain MN8140SS showed the highest specific growth rate and cell yield. Since the specific growth rate of strain NBRC1440SS was significantly lower compared with the other strains, it may be dependent on the medium composition. In fact, when cultivated in YPD medium, the specific growth rate of strain NBRC1440SS was comparable to that of MT8-1SS, and MN8140SS showed the highest specific growth rate (data not shown). Additionally, the yield

attained by the diploid yeast cells was 0.74 g-wet-cell/g-glucose, which was approximately two times that of the haploid yeast strains MT8-1SS and NBRC1440SS (0.38 and 0.37 g-wet-cell/g-glucose, respectively).

Table 2 Summary of the properties of two haploid and one diploid yeast strains.

Strain	Aerobic condition				Anaerobic condition
	Specific growth rate (h^{-1})	Cell yield (g-wet-cell/ g-glucose -consumed)	α-Amylase activity (U/g-wet-cell)	Glucoamylase activity (U/g-wet-cell)	Ethanol production rate from glucose (g-ethanol/g-wet-cell/h)
MT8-1SS	0.44	0.38	15.8	0.93	0.069
NBRC1440SS	0.23	0.37	41.1	0.48	0.061
MN8140SS	0.47	0.74	55.4	1.86	0.107

Data are averages from three independent experiments.

Improvement of amylase activities and fermentation ability

To investigate the effectiveness of mating, each of the recombinant yeast cells was cultivated in YPD medium, and its amylase activities and ethanol productivity were measured. The haploid strain MT8-1SS showed relatively high specific activity of glucoamylase (0.93 U/g-wet-cell), and NBRC1440SS showed relatively high specific activity of α-amylase (41.1 U/g-wet-cell). On the other hand, the diploid strain MN8140SS showed the highest α-amylase and glucoamylase specific activities (55.4 U/g-wet-cell and 1.86 U/g-wet-cell) among all strains (Table 2). In anaerobic conditions, the fermentation ability of haploid strain MT8-1SS (0.069 g-ethanol/g-wet-cell/h) was quite similar to that of NBRC1440SS (0.061 g-ethanol/g-wet-cell/h). On the other hand, the fermentation ability of diploid strain MN8140SS (0.107 g-ethanol/g-wet-cell/h) was approximately 1.6-fold higher than that of the two haploid strains (Table 2).

Ethanol fermentation from raw starch

Each of the recombinant yeast strains was used for direct ethanol fermentation from raw starch in anaerobic conditions. The haploid strains, MT8-1SS and NBRC1440SS, hydrolyzed 58%

and 53% of the raw starch (which corresponded to total sugar), and produced 26 g/l and 28 g/l of ethanol after 84h, respectively. In contrast, the diploid strain MN8140SS hydrolyzed 89% of the starch and produced 39 g/l of ethanol after 84 h (Fig. 2A). The ethanol production rate of the diploid strain MN8140SS (0.46 g/l/h) was approximately 1.5-fold higher than that of the haploid strains (0.31 g/l/h and 0.33 g/l/h). As shown in Fig. 2B, the α-amylase and glucoamylase activities of the haploid strains, MT8-1SS and NBRC1440SS, reached maximum levels of 190 and 96 U/l, and 340 and 140 U/l, respectively. By comparison, the maximum α-amylase and glucoamylase activities of diploid strain MN8140SS were 950 and 290 U/l, respectively. Based on this result, it was hypothesized that the integrant diploid strain promoted the translation of both integrated amylase genes, and as a result, its ability to produce ethanol from raw starch was improved.

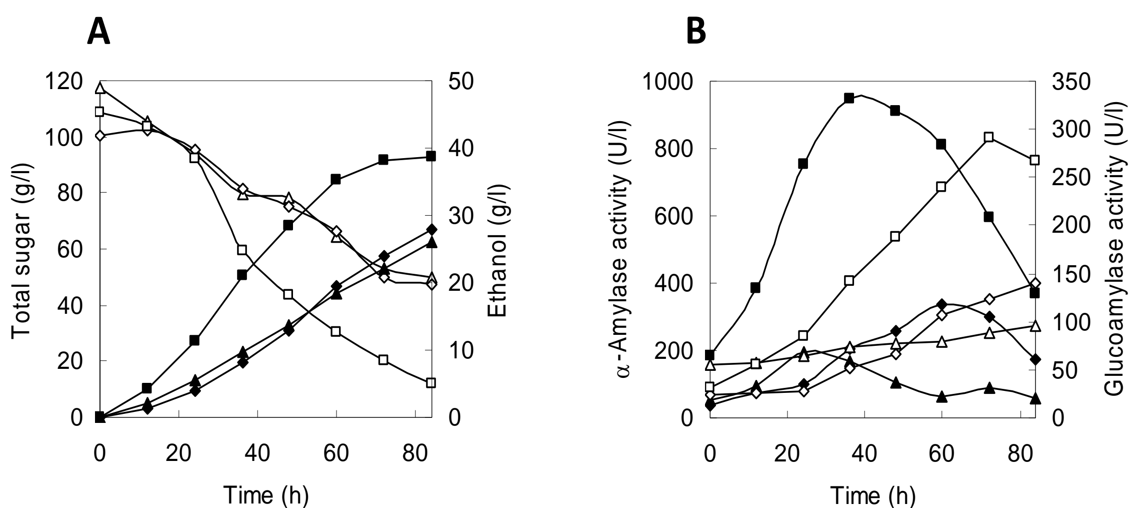

Figure 2 Time course of direct ethanol production from raw cornstarch by haploid yeast strains MT8-1SS (triangles), NBRC1440SS (diamonds), and diploid strain MN8140SS (squares). (A) Closed symbol, ethanol concentration; open symbol, total sugar. (B) Closed symbol, α-amylase activity; open symbol, glucoamylase activity. Data are averages from three independent experiments.

Repeated batch fermentation

To investigate the reusability of the yeast cells, repeated batch fermentation was performed three times with diploid strain MN8140SS (Fig. 3). Since the condition of initial cell weight 200 g-wet-cell/l was the most effective in the fermentation using diploid strain MN8140SS (data not shown), this cell concentration was used in this experiment. The ethanol production level was 40~50 g/l in each batch, and the ethanol production rate was maintained at almost the same level through three repeated batch fermentations (0.67, 0.60, and 0.67 g/l/h). The maximum α-amylase activity in the first, second and third batches was approximately 1,900, 1,600 and 1,000 U/l, respectively, and it decreased slightly with repetition. By contrast, the maximum glucoamylase activity in the first, second and third batch was approximately 180, 350 and 400 U/l, respectively, and tended to increase with repetition.

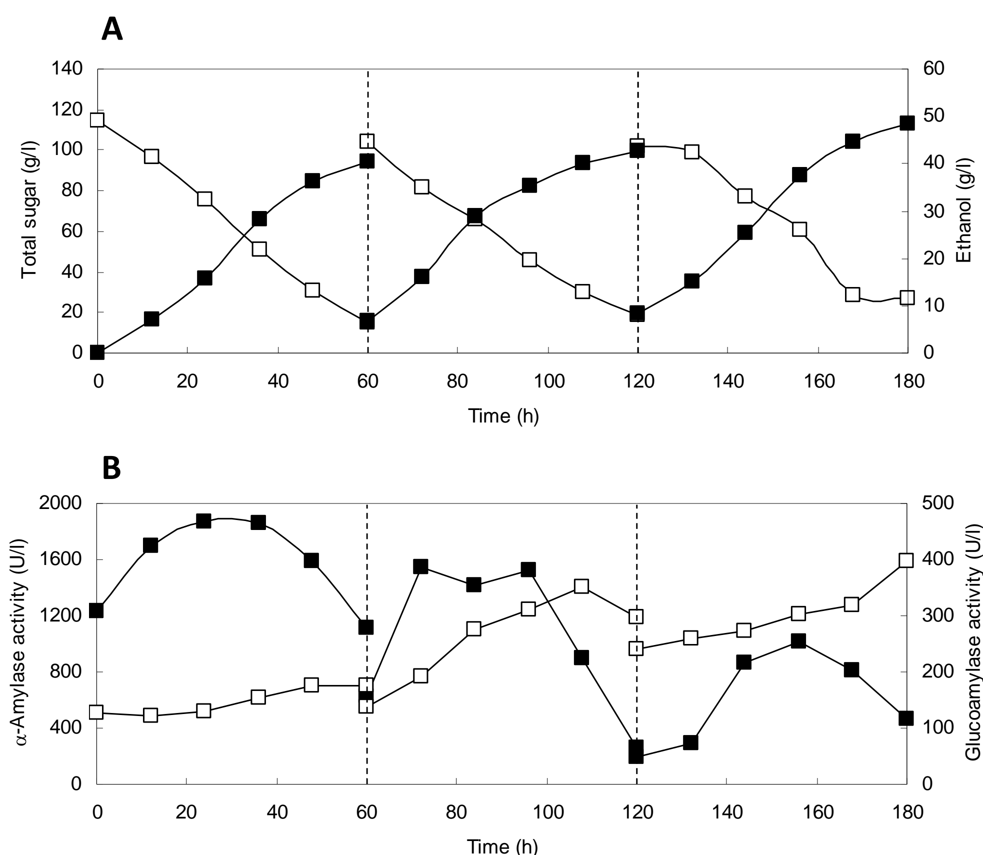

Figure 3 Time course of repeated batch fermentation by diploid yeast strain MN8140SS. (A) Closed symbol, ethanol concentration; open symbol, total sugar. (B) Closed symbol, α-amylase activity; open symbol, glucoamylase activity. Data are averages from three independent experiments.

Discussion

In this study, an efficient ethanol production process was constructed using recombinant diploid yeast cells that co-expressed α-amylase and glucoamylase. Two mitotically stable recombinant haploid yeast strains were constructed by integrating α-amylase and glucoamylase genes into their chromosomal DNA. A diploid yeast with high ethanol productivity and reusability was then bred by mating those two haploid strains, and its ethanol productivity from raw starch without any pretreatment was investigated.

At first, six combinations of amylase integrant diploid strains constructed by mating haploid strains, such as YF207 (*MAT*a), YF237 (*MAT*a) (Khaw et al. 2005), MT8-2 (*MAT*α) (Nishizawa et al. 1990), MT8-1 (*MAT*a), and NBRC1440ΔHUW (*MAT*α), were investigated (data not shown). Two combinations of diploid strains, YF207/MT8-2 and MT8-1/NBRC1440ΔHUW, showed improvement in amylase activities compared to the parental strain. These two diploid strains also showed improvement of cell growth, cell yield, and fermentation ability. Finally, since the combination of MT8-1 and NBRC1440ΔHUW showed the highest amylase activities of any investigated haploid pairs, this combination was selected in this study. Tanino *et al*. reported recombinant diploid strain is superior to parental haploid strains for protein production (Tanino et al. 2009). In this study, the diploidization of integrant haploid strains by mating was contributed to not only enzyme productivity but also fermentation potential (Table 2). And as shown in Fig. 2, the diploid strain showed the highest ethanol production rate in single batch fermentation from raw starch. This is because the diploid strain's productivity of two amylases was significantly improved. These findings clearly indicate the effectiveness of integrant diploid strain for direct ethanol fermentation from raw starch.

In this study, the ethanol production rate was maintained when batch fermentation was repeated three times (Fig. 3). Inlow *et al.* (1988) reported that the rate of starch fermentation by a recombinant yeast strain was directly related to the level of glucoamylase activity (Inlow et al. 1988). In this study, although the maximum α-amylase activity decreased to half its original level after the batch fermentation was repeated three times, the maximum glucoamylase activity increased two-fold at that point, and ethanol production was maintained at almost the same rate (Fig.

3). Based on these findings and previous papers, it was assumed that the reduction of α-amylase activity was covered by enhancement of glucoamylase activity in this study.

Western blot analysis of supernatant in each batch of repeated fermentation revealed that the α-amylase was not degraded proteolitically (data not shown). Hence, the reduction of α-amylase activities in the late phase of each batch would be due not to the degradation of the enzyme, but to the deactivation itself. In fact, half of the α-amylase activity in the supernatant without yeast was lost at 30°C for 24 h, and this phenomenon agreed well with previous papers (Okano et al. 2007; Tateno et al. 2007). In contrast, cell surface-displayed glucoamylase activity has been maintained for more than 300 h in repeated batch fermentation (Kondo et al. 2002). Therefore, for the construction of a more stable long-term fermentation process, improvement of α-amylase stability or the use of more stable α-amylase from another origin would be required.

In conclusion, efficient ethanol production was successfully achieved using a diploid strain of yeast, which was bred by mating two kinds of amylase gene integrated haploid strains that expressed α-amylase and glucoamylase, respectively. Although to develop more efficient and practical fermentation process, it would be required to investigate co-expression of other specific amylolitic enzymes such as pullulanase and isoamylase or using more practical medium such as corn steep liquor (Janse and Pretorius 1995; Kadam and Newman 1997; Ma et al. 2000). This yeast constructing strategy achieved the overexpression of genome integrated genes and the improvement of yeast capability simultaneously, and it can be applied to other bioconversion processes or to fine chemical production processes that use recombinant yeast as a whole cell biocatalyst.

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Chapter 2. Novel strategy for yeast construction using delta-integration and cell fusion to efficiently produce ethanol from 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 et al. 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 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 et al. 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.

Escherichia coli transformants were grown in Luria-Bertani medium (10 g/l of tryptone, 5 g/l of yeast extract, 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) 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.

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 glucomylase gene and α-amylase gene	This study
MT8-1/δGS	*MAT*a *ade his3 leu2* δ-integration of glucomylase gene and α-amylase gene	This study
MT8-1/δGS/403	*MAT*a *ade leu2* δ-integration of glucomylase gene and α-amylase gene	This study
MT8-1/δGS/405	*MAT*a *ade his3* δ-integration of glucomylase gene and α-amylase gene	This study
NBRC1440	Wild type strain	NBRC[a]
NBRC1440ΔHUWL[b]	*MAT*α *his3 leu2 trp1 ura3*	This study
1440/δGS	*MAT*α *his3 leu2* δ-integration of glucomylase gene and α-amylase gene	This study
MNII/δGS	*MAT*a/α *leu2* δ-integration of glucomylase gene and α-amylase gene	This study
MNII/δGS/405	*MAT*a/α *his3* δ-integration of glucomylase gene and α-amylase gene	This study
MNIV/δGS	*MAT*a/a/α/α δ-integration of glucomylase 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* Secrete 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).

Plasmid construction and yeast transformation

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 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. 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 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.

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). Amylase expressing transformants were selected based on the amylase activities assayed described below. At first, about 20 individual transformants were selected and assayed their amylase activities, and the transformants with the highest activity were selected and used in the subsequent experiments.

Table 2 PCR Primers used in this study.

Amplification fragment	Primers (restriction endonuclease)	Sequence	Source of DNA sequence
5'δ sequence	DS1- (F)-*Sac* I	5'-ATGCGAGCTCTGTTGGAATAGAAATCAACT-3'	*S. cerevisiae* genome DNA
	DS-167+*Asc* I (R)-*Sac* I	5'-GCATGAGCTCGGCGCGCCATGTTTATATTCATTGATCCTA-3'	
3'δ sequence	DS168-+*Asc* I (F)-*Kpn* I	5'-ATGCGGTACCGGCGCGCCATAAAATGATGATAATAATATT-3'	*S. cerevisiae* genome DNA
	DS-334 (R)-*Kpn* I	5'-GCATGGTACCTGAGAAATGGGTGAATGTTG-3'	
TRP1 d	*TRP1* d (F)-*Xho* I	5'-ATGCCTCGAGTGGAGTATGTCTGTTATTAA-3'	pRS404
	TRP1 d (R)-*Xho* I	5'-GCATCTCGAGTGCAGGCAAGTGCACAAACA-3'	
URA3 d	*URA3* d (F)-*Xho* I	5'-ATGCCTCGAGGAAACGAAGATAAATCATGT-3'	pRS406
	URA3 d (R)-*Xho* I	5'-CGATCTCGAGGTAATAACTGATATAATTAA-3'	
pPGK-signal sequence-glucoamylase gene-3' half of	pPGK (F)-*Xba* I	5'-ATGCTCTAGACGATTTGGGCGCGAATCCTT-3'	*S. cerevisiae* genome DNA and pRS406_Psed1-glaR-CAS1
	pPGK (R)-overlap	5'-TGCATTTTTTTGTTTTATATTTGTTGTAAAAAGTAGATAA-3'	
	GluR (F)-overlap	5'-ATATAAAACAAAAAAATGCAACTGTTCAATTTGCCATTGA-3'	
	CAS1 (R)-*Xba* I	5'-GCATTCTAGATTTGATTATGTTCTTTCTAT-3'	

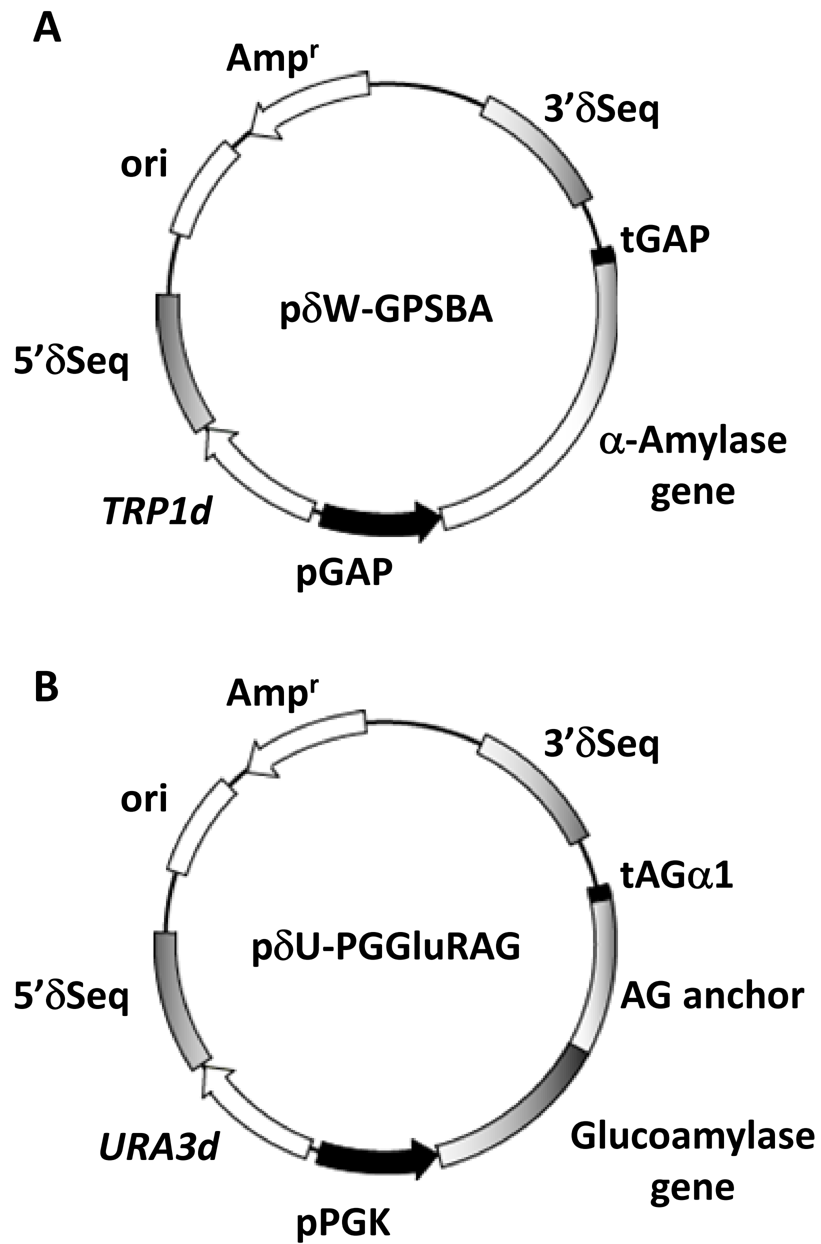

Figure 1 Expression plasmids for secretion of *S. bovis* α-amylase (A) and display of *R. oryzae* glucoamylase (B) by δ-integration.

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 (Herford 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.

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, 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 Figure 2. The haploid strains MT8-1/δGS and 1440/δGS were constructed by δ-integration of the glucoamylase 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.

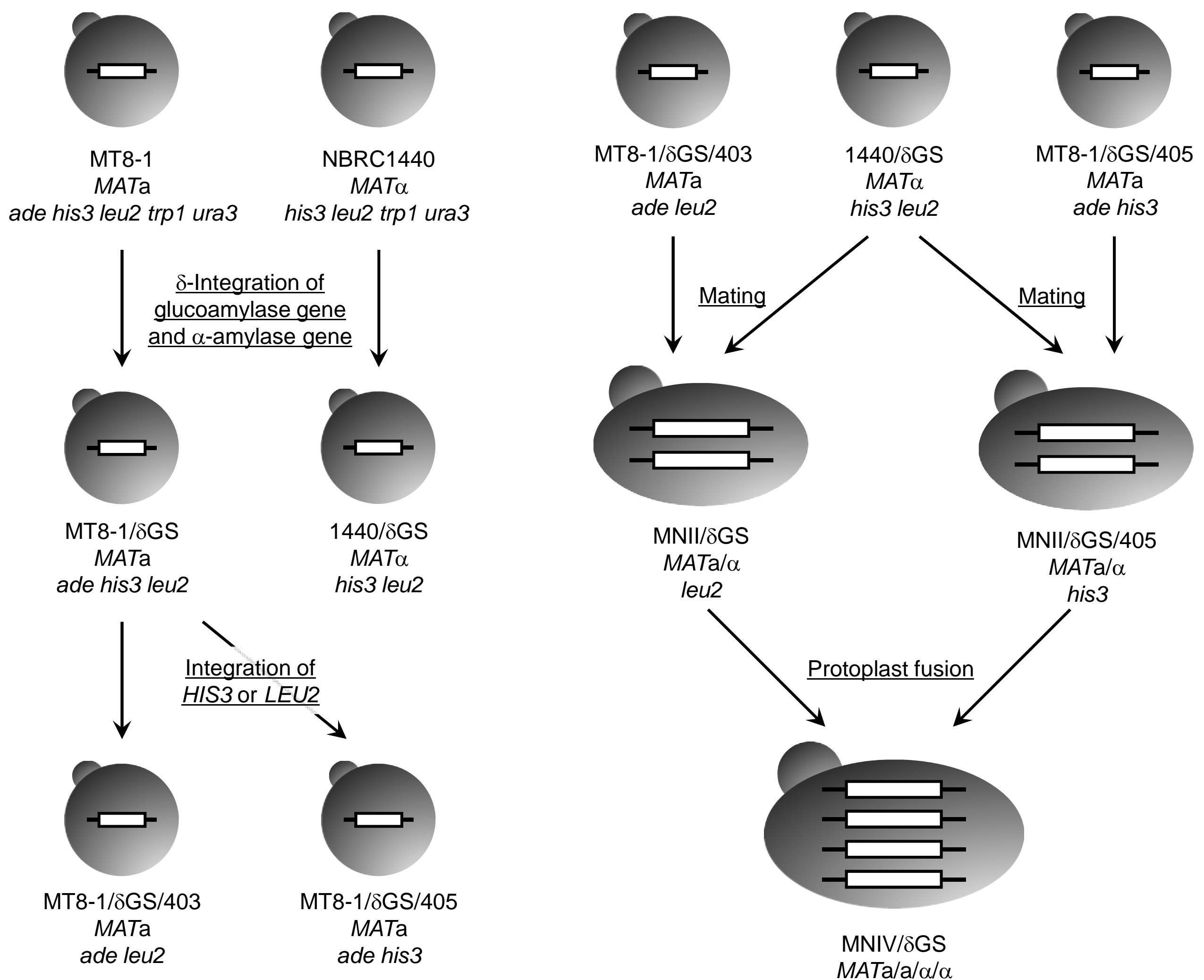

Figure 2 Strategy for constructing haploid, diploid, and tetraploid yeast strains.
Some auxotrophy of parental strain was complemented by vacant vectors to screen cell fusants.

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.

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

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 2-fold 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 1115.8 U/l, respectively in the tetraploid strain MNIV/δGS. These values were about 1.5- and 10-fold higher than the respective activities of the δ-integrant haploid strain, MT8-1/δGS.

Table 4 Amylase activities by strains cultured under aerobic conditions.

Strain	α-Amylase activity (U/l)	Glucoamylase activity (U/l)
MT8-1/IGS	37.0 ± 5.5	14.3 ± 6.8
MT8-1/δGS	722 ± 7.6	33.1 ± 2.0
MNII/δGS	725 ± 30.2	85.5 ± 18.3
MNIV/δGS	1120 ± 77.4	330 ± 8.5

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 Figure 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).

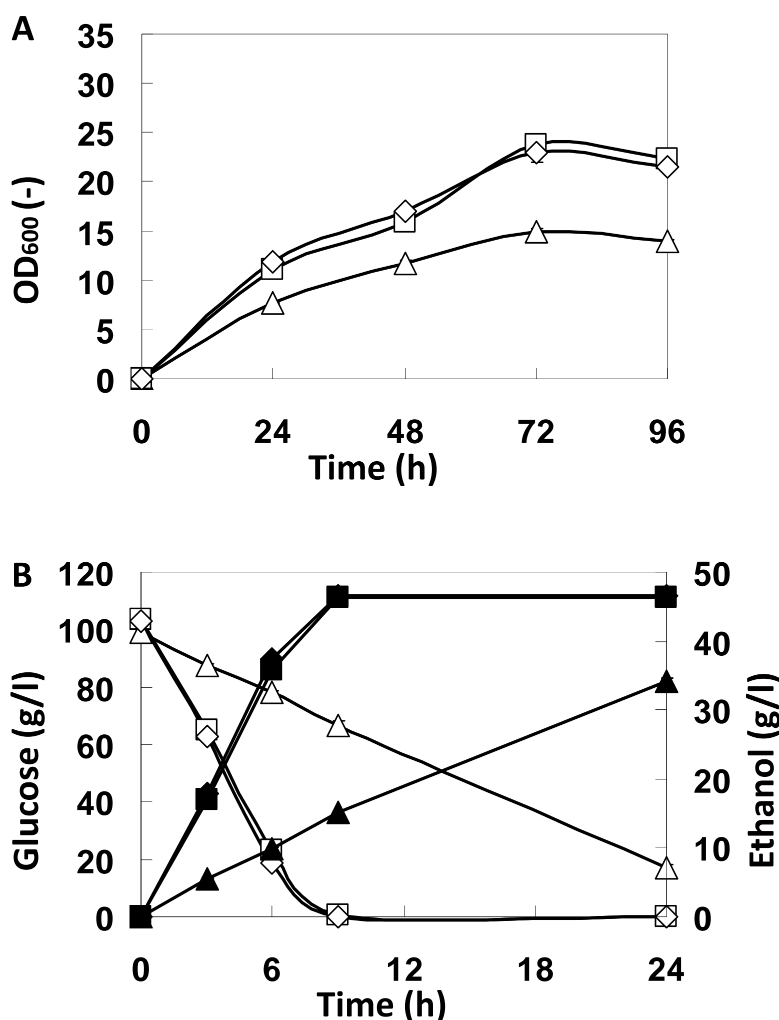

Figure 3 (A) Aerobic cultivation of haploid MT8-1/δGS (triangles), diploid MNII/δGS (squares) and tetraploid MNIV/δGS (diamonds).

(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.

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 Figure 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.

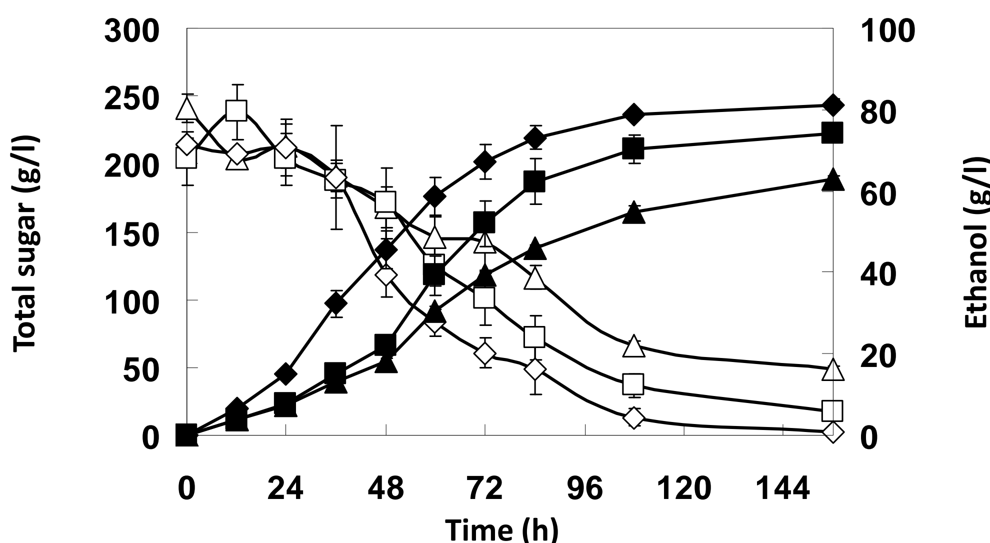

Figure 4 Time course of ethanol fermentation from raw starch by haploid MT8-1/δGS (triangles), diploid MNII/δGS (squares), and tetraploid MNIV/δGS (diamonds). Filled and unfilled symbols represent ethanol and total sugar concentrations, respectively. Data are averages from three independent experiments.

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	1580 ± 397	843 ± 71.8

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 was 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 2- 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 2 to 10-fold higher than that obtained with general integration (Parekh et al. 1996; Lee et al. 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 2-fold higher ethanol productivity (0.93 g/l/h) than our previous results with 843 U/l of glucoamylase and 1580 U/l of α-amylase maximum activity (Fig. 4 and Table 5). This result suggests that ethanol productivity from raw starch is 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.

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, the growth rate, cell yield, fermentation ability, and amylase activities detected using glucose as sole carbon source were improved by polyploidization, reflecting the fact that diploid or more polyploid industrial strains are highly tolerant to various stresses (Fig. 3A, Fig. 3B, and Table 4). 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.

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. Hence, combining δ-integration with polyploidization is efficient to construct yeasts that can directly ferment ethanol from raw starch.

The ethanol production rate of tetraploid strain MNIV/δGS is clearly higher than that of other two strains, however, the decrease of total sugar for first 36 hours are almost the same among three strains (Fig. 4). This is because the inaccuracy of phenol sulfuric acid method for determination of total sugar in fermentation medium. This method is difficult for determine the insoluble sugar concentration such as raw corn starch because of their scattering reaction character. Hence total sugar concentration of around first 36 hours may incorrect and not correlate to the ethanol production.

The α-amylase activities were almost same but glucoamylase activities were higher under anaerobic condition than that under aerobic condition in all the strains (Table 4 and Table 5). This is because the difference of production character of amylase. The glucoamylase activity was increasing slower than α-amylase both aerobic and anaerobic condition (data not shown). The glucoamylase activity in aerobic condition was assayed using 72h cultivated yeast cell, in contrast,

the maximum glucoamylase activity in anaerobic fermentation condition was indicated later phase of fermentation (data not shown).

Many reports suggested that polyploid fusants of *S. cerevisiae* was stable for many times of subculture on non-selective medium (Brigidi et al. 1988; Hashimoto 2006 et al.). In this study, to investigate the stability of tetraploid fusants, we conducted repeated batch fermentation with cell recycling using tetraploid fusants MNIV/δGS (data not shown). In this experiment, tetraploid fusants maintained high ethanol productivity from raw starch for several batch fermentations. From this result, tetraploid fusants MNIV/δGS is mitotically stable sufficiently for practical fermentation process.

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.

Acknowledgments

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Chapter 3. Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that express amylases

Introduction

The finite nature of fossil fuels combined with the environmental problems associated with their extraction and combustion, such as global warming and acid rain, has promoted an interest in biofuel production. Ethanol can be produced from sucrose and from starchy or lignocellulosic biomass (Sánchez and Cardona 2008) ethanol production from lignocellulosic biomass have recently been conducted. The attraction of these materials for fuel production is that they are abundant and cheaper than sucrose or starchy biomass (Sánchez and Cardona 2008; Margeot et al. 2009; Doran-Peterson et al. 2009). However, there are numerous limitations associated with using lignocellulosic biomass to produce ethanol, such as the slow rate of enzymatic degradation and high cost of enzymes (Sánchez and Cardona 2008). As a consequence, starch biomass is still the most commonly used feedstock for ethanol production.

Practical ethanol production from starchy biomass such as cassava, rice, sweet sorghum, and sweet potato have been reported (Ueda et al. 1981; Lee et al. 1993; Kiran Sree et al. 1999; Roble et al. 2003); of these, corn is the most commonly used starchy feedstock for bioethanol production (Sánchez and Cardona 2008). In Japan, agricultural policies adopted by the government have resulted in the existence of extensive areas of unutilized rice fields. This has resulted in numerous initiatives being launched to produce ethanol using high-yielding rice that has been cultivated in these underutilized paddies (Matsumoto et al. 2009). Thus, in addition to developing ethanol production processes using commonly used starchy feedstocks such as corn, it is also important to develop cost effective and efficient processes using alternative starchy feedstocks like high-yielding rice which are common in the region.

Despite its efficiency as an ethanol producer, *Saccharomyces cerevisiae* cannot produce ethanol from raw starch directly because it lacks the ability to degrade raw starch into glucose. This is because conventional ethanol production from raw starch requires the following three steps: liquefaction of starch by heating and addition of α-amylase, enzymatic saccharification of the

low-molecular liquefaction products to glucose, and fermentation of glucose to ethanol. The liquefaction process, which accounts for 30-40% of the total energy used for ethanol production, combined with the large quantities of enzymes that are required to convert the raw starch into glucose (Lee et al. 1993; Lim et al. 2003), both contribute to making conventional ethanol production an expensive and complex process. However, co-utilization of commercial enzymes and/or microorganisms and use of yeast capable of degrading raw starch can be used to reduce the costs of ethanol production from raw starch (Ueda et al. 1981; Lee et al. 1993; Kiran Sree et al. 1999; Roble et al. 2003; Jeon et al. 2008). We previously constructed a high-performance, starch-degrading yeast capable of direct ethanol production from purified raw corn starch by combining δ-integration and polyploidization with high ethanol yield (Yamada et al. 2010). The polyploid characteristics of this yeast strain meant that it is an efficient ethanol producer as well as being robust in culture. In this study, we attempted to produce ethanol from high-yielding rice harvested in Japan. To our knowledge, this would be first report describing direct ethanol production from real biomass using an amylase-expressing yeast. Importantly, the low-cost and efficient ethanol production process described here was performed without supplementing the growth media with nutrients such as yeast extract or peptone.

Material and methods

Yeast strains and media

The α-amylase and glucoamylase expressing tetraploid strain of *S. cerevisiae*, MNIV/δGS, was used for ethanol production (Yamada et al. 2010). The yeast was aerobically cultured in YPS medium (10 g/l of yeast extract (Nacalai Tesque, Kyoto, Japan), 20 g/l of Bacto-peptone (Difco Laboratories, Detroit, MI, USA), 50 g/l of soluble starch (Nacalai Tesque, Kyoto, Japan)). The control yeast strain, MT8-1, which is unable to synthesize amylase, has also been used to produce ethanol (Tajima et al. 1985). The control yeast strain was also aerobically cultured in YPD medium (10 g/l of yeast extract, 20 g/l of Bacto-peptone, 20 g/l of glucose (Nacalai Tesque)). Ethanol fermentation was performed using two kinds of medium, one containing a carbon source that was supplemented with nutrients (100 g/l of carbon source, such as purified raw corn starch and high-yielding rice, 10 g/l of yeast extract, 20 g/l of Bacto-peptone), and the other medium containing only a carbon source without any nutrients (200 g/l of high-yielding brown rice).

High-yielding rice

High-yielding rice was harvested in Hyogo prefecture, Japan. The rice was either left in its harvested state (brown rice) or polished (white rice) before being ground to produce flour with a particle size of approximately 0.5 mm using a laboratory disintegrator (Sansho Industry Co., Ltd., Osaka, Japan) for use as carbon sources for ethanol fermentation. According to the analysis of the Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries (Hyogo, Japan), the high-yielding brown rice was composed of 12.8% water, 75.3% carbohydrate, 8.3% protein, 2.3% lipid, and 1.3% ash.

Ethanol fermentation from purified raw corn starch or high-yielding rice

Yeast cells were cultured aerobically in YPS medium at 30°C for 72 h. Yeast cells were harvested by centrifugation at 3,000 × g for 5 min, washed twice with water, and re-suspended in 50 ml of medium containing the carbon source supplemented with nutrients. Raw corn starch or high-yielding rice was used as a carbon source. The initial cell concentration was adjusted to 50 g of

wet cells/l, and wet cell weight was determined by weighing cells that had been pelleted by centrifuging at 3,000 × g for 5 min. The estimated dry cell weight was approximately 0.15-fold the wet cell weight (data not shown).

For the low-cost fermentation, yeast cells (initial O.D.600= 0.05) were also cultured aerobically in YPS medium at 30°C for 72 h. The culture broth was inoculated into the fermentation medium containing only a carbon source. The inoculated volume ranged between 5, 10, 20% of the volume of the fermentation medium. The total volume of the fermentation medium was adjusted to same volume in each inoculum size.

Ethanol fermentation proceeded in a working volume of 50 ml incubated at 30°C with mild agitation in 100 ml bottles equipped with a bubbling CO_2 outlet, or in a working volume of 1 L agitated at 500 rpm in 2 L jar fermenters.

Analytical methods

Ethanol concentration was 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 with a water mobile phase and flow rate of 0.6 ml/min. The ethanol concentration was determined using a refractive index detector (Shimadzu RID-10A). Culture supernatant was separated from the fermentation broth by centrifugation at 14,000 × g for 10 min and then analyzed by HPLC. The activities of the glucoamylase and α-amylase in the fermentation broth were assayed as described previously using kits (Kikkoman Corp., Chiba, Japan), respectively (Yamada et al. 2009).

Results and discussion

Fermentation of raw corn starch or high-yielding rice carbon sources supplemented with nutrients

Using an amylase-expressing tetraploid yeast strain, we performed direct ethanol fermentation tests with high-yielding rice as a carbon source.

Figure 1 shows the fluctuation in the ethanol concentration during the fermentation of raw, high-yielding rice and raw, purified corn starch. The ethanol productivity, ethanol yield, and amylase activities are summarized in Table 1. The ethanol productivity obtained over a 24 h period on brown rice (1.1 g/L/h) was about 5 fold higher than that obtained on purified raw corn starch (0.2 g/L/h). The ethanol yield obtained using brown rice (96%) was higher than the yields obtained on polished white rice or raw corn starch. In the yeast strain that did not produce amylase, the ethanol productivity and yield were 0.1 g/L/h and 10%, respectively, indicating that the expressed amylases facilitated ethanol fermentation on both corn starch and high-yielding rice.

Compared with the other carbon sources, the activities of glucoamylase and α-amylase were highest on brown rice (16.3 U/mL and 22.6 U/mL). Rice is known to contain relatively high concentrations of metal ions (Science and Technology Agency 2005), and it is likely that ions stabilized α-amylase in the presence of the high ethanol concentrations (Yamakawa et al. 2010; Stoner et al. 2005; Pelegrini 2006). Such stabilization would ensure the continued functioning of the α-amylase and may account for the relatively higher ethanol yield obtained using brown rice.

Even in the case using control yeast strain, only glucoamylase activity (2.4 U/mL) was detected after fermentation on brown rice; nonetheless, this activity was 1.6-fold higher than that obtained on raw corn starch. Taken together, these findings imply that high-yielding rice contains relatively large amounts of glucoamylase, the activity of which is likely to increase as the rice is degraded by α-amylase, resulting in high ethanol yields after fermentation.

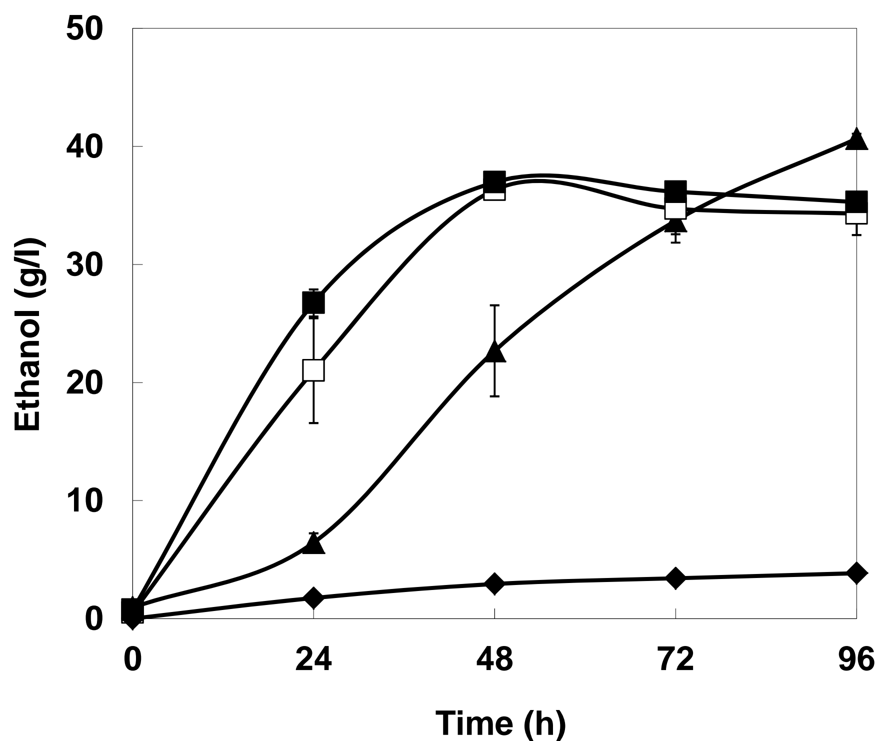

Figure 1 Ethanol production from fermentation of various carbon sources supplemented with nutrients. Raw corn starch (closed triangles), polished white rice (open squires), brown rice (closed squares), and brown rice fermented with the yeast strain that did not produce amylase (closed diamonds). Data are averages of three independent experiments.

Table 1 Fermentation of various carbon sources supplemented with nutrients.

Condition		Ethanol productivity in 24 h (g/L/h)	Ethanol yield to the theoretical yield (%)	Maximum glucoamylase activity (U/mL)	Maximum α-amylase activity (U/mL)
Biomass	Yeast				
Brown rice	MNIV/δGS	1.1	96 [a]	16.3	22.6
Polished white rice	MNIV/δGS	0.9	95 [a]	12.8	19.2
Raw corn starch	MNIV/δGS	0.2	91 [b]	1.5	1.2
Brown rice	MT8-1	0.1	10 [a]	2.4	0

[a] carbohydrate-based theoretical yield

[b] weight-based theoretical yield

Direct and cost-effective fermentation of brown rice not supplemented with nutrients

Direct inoculation of pre-cultured medium into the fermentation medium without nutrients is a simple way to achieve cost-effective ethanol fermentation. To evaluate the effect of initial inoculum size on ethanol productivity, ethanol fermentation on brown rice lacking any nutrient supplements was performed by varying the inoculum size of the pre-culture medium. Figure 2 shows the change in the ethanol concentration during the fermentation of brown rice obtained using a variety inoculum sizes. Although ethanol productivity over a 24 h period was higher for the 20% inoculum (1.8 g/L/h) compared with the 10% inoculum, the ethanol yield obtained using the 10% inoculum (86%) was higher. These results clearly show that direct and cost-effective ethanol fermentation can be achieved using high-yielding rice without any nutrients.

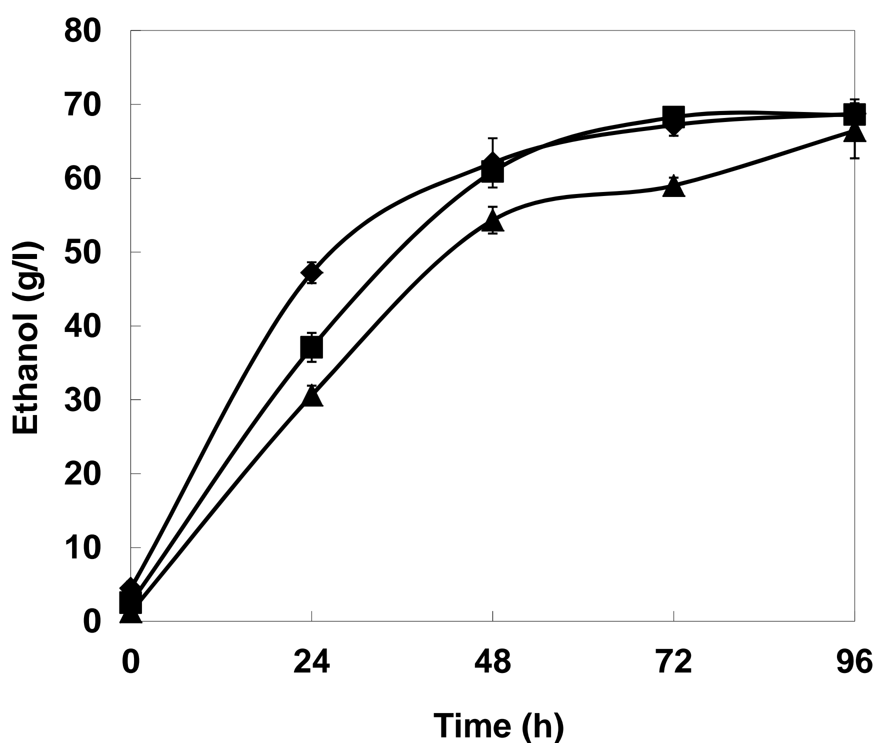

Figure 2 Ethanol production from fermentation of brown rice as the only carbon source without any nutrients. Size of inoculum relative to volume of fermentation medium: 5% (closed triangles), 10% (closed squires), and 20% (closed diamonds). Data are averages of three independent experiments.

Kadam et al reported that, to achieve low cost ethanol fermentation, at least 0.3% of corn steep liquor and 2.5 mM of $MgSO_4 \cdot 7H_2O$ are required to maintain yeast fermentation ability (Kadam and Newman 1997). According to the Standard Tables of Food Composition in Japan (Science and Technology Agency 2005), approximately 10 g/L of protein and 10 mM of magnesium derived from high-yielding rice were included in the medium. In addition to facilitating low-cost fermentation, the constituents of high-yielding rice are well suited for use as feedstock for low cost bioethanol production.

The ethanol productivity was improved by increasing the volume of the pre-culture inoculum (Table 2). In the initial stage of ethanol fermentation from starch, endo-acting α-amylase plays an important role in producing the reducing ends of starch chains which are then subsequently attacked by glucoamylase (Modena et al. 1986). It therefore appears that ethanol productivity could be improved by increasing inoculum size.

Table 2 Fermentation of brown rice as a carbon source without any nutrients.

Condition		Ethanol productivity in 24 h (g/L/h)	Ethanol yield to the theoretical yield (%)	Maximum glucoamylase activity (U/mL)	Maximum α-amylase activity (U/mL)
Inoculum size (%)	Fermenter size				
5	100 mL	1.2	84	4.7	1.8
10	100 mL	1.4	86	5.3	2.1
20	100 mL	1.8	85	4.8	2.4
10	2 L	1.2	101	3.3	1.2

As shown in Fig. 3, although ethanol productivity in a jar fermenter (1.2 g/L/h) over a 24 h period was slightly lower than that in a bottle (1.4 g/L/h), the ethanol yield (101%) obtained using the ethanol fermenter was significantly higher than that obtained in the bottle (86%). These findings suggest the ethanol production process established in this study can be scaled up relatively easily and does not require pretreatment with enzymes or nutrients. Furthermore, compared with previous reports (Ueda et al. 1981; Lee et al. 1993; Kiran Sree et al. 1999; Roble et al. 2003), the high ethanol productivity (1.2 g/L/h) and yields (101%) obtained in this study indicate that the process described here is more efficient for producing ethanol. In addition, since our amylase-expressing yeast could be used for more than 10 repeated batch fermentations (Yamakawa et al. 2010), further reductions in the cost of ethanol production could be affected by recycling yeast cells in a repeated batch fermentation.

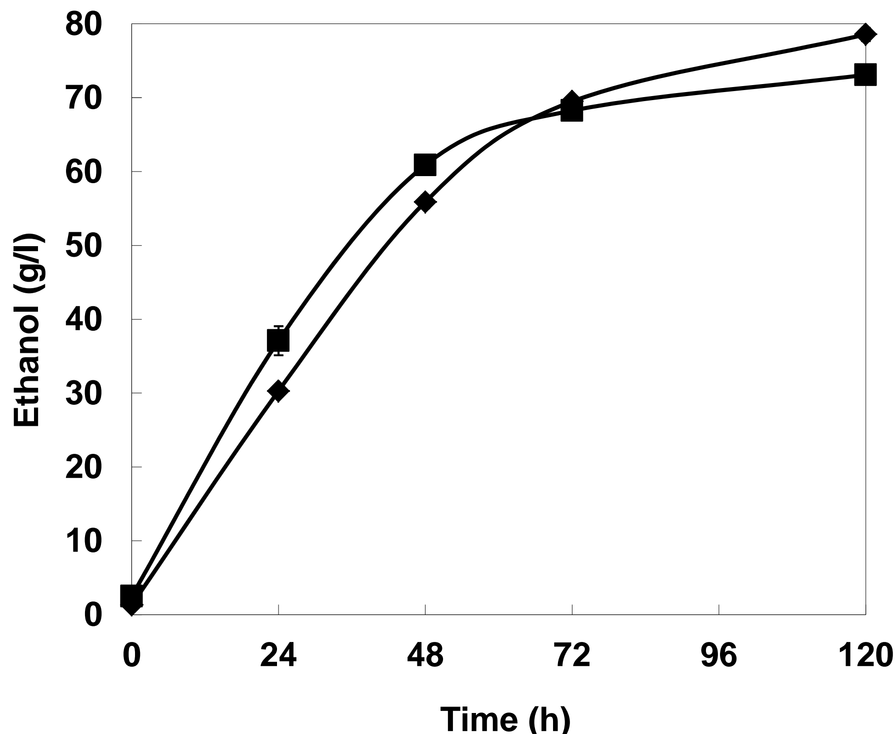

Figure 3 Ethanol production from fermentation of carbon source without any nutrients in different fermenter volumes. 100 mL glass bottle (closed squires) and 2 L jar fermenter (closed diamonds). Data are averages of three independent experiments.

In conclusion, we established a direct and efficient ethanol production process using high-yielding rice without requiring any pretreatment, addition of enzymes or nutrient supplements. The omission of any pretreatment is considered beneficial as compounds derived from such processes often inhibit fermentation (Sánchez and Cardona 2008). In addition, the high-yielding rice used in this study would be well suited for use as a feedstock of bioethanol production, and our polyploid, amylase-expressing yeast strain was sufficiently robust and capable of producing ethanol efficiently from real biomass. To our knowledge, this is first report of direct ethanol production from real biomass using amylase-expressing yeast without any pretreatment or addition of commercial enzymes.

Acknowledgments
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Part II.

Direct ethanol production from lignocellulosic biomass

Chapter 1. Cocktail delta-integration: a novel method to construct cellulolytic enzyme expression ratio-optimized yeast strains.

Introduction

Given the eventual exhaustion of fossil fuels and environmental issues such as global warming and acid rain, utilization of biomass as a source of fuels and fine chemicals has recently become an attractive option. Utilization of biomass, especially cellulosic materials, is desirable because it is abundant, inexpensive, renewable, and has favorable environmental properties. Therefore, an efficient and cost-effective method for degradation of cellulosic materials into glucose to produce alternative fuels or other fine chemicals is required.

Efficient degradation of cellulose requires a synergistic reaction of the cellulolytic enzymes endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL). Although there are many reports concerning cost reduction for cellulosic material degradation using recombinant bacteria, fungi, and yeast (Bayer et al. 2004; Ito et al. 2004; Kumar et al. 2008), cellulase degradation efficiency has not been improved enough. We previously reported direct ethanol production from phosphoric acid swollen cellulose (PASC) using a yeast strain co-displaying *Trichoderma reesei* EGII, CBHII, and *Aspergillus aculeatus* BGL1 (Fujita et al. 2004). This method however requires higher cellulase degradation for cost-effective ethanol production from cellulosic materials.

The filamentous fungus *T. reesei* degrades cellulose effectively and is known to produce various types of cellulolytic enzymes and control their expression levels simultaneously depending on the environment; the ratio of the cellulases and their synergetic effects are important for effective cellulose degradation (Dashtban et al. 2009; Stricker et al. 2008). Promoter engineering is one method to control cellulase expression levels (Maya et al. 2008; Nacken et al. 1996, Walfridsson et al. 1997). However, the variety of promoters adequate for cellulase overexpression is limited, and expression levels by each promoter vary with environmental conditions such as glucose concentration or the phase of cell growth (Maya et al. 2008). Furthermore, the optimum expression ratio of various cellulolytic enzymes for efficient cellulose degradation is unknown, and the ratio will differ depending on the content of the cellulosic material.

In this study, to construct engineered yeast with efficient cellulose degradation, we

developed a simple method to optimize cellulase expression levels, called cocktail δ-integration. The δ-integration method is known as multicopy-integration in yeast (Sakai et al. 1990). In cocktail δ-integration, several kinds of cellulase expression cassettes are integrated into yeast chromosomes simultaneously in one step, and strains with high cellulolytic activity (i.e., expressing the optimum ratio of cellulases) can be easily obtained. The goal of this study was to create a cellulase expression-optimized yeast strain for efficient degradation of cellulose using our novel cocktail δ-integration method.

Materials and methods

Strains, plasmids, and media

Table 1 summarizes the genetic properties of all strains used in this study. In brief, the host for recombinant DNA manipulation was the *Escherichia coli* strain NovaBlue (Novagen, Madison, WI, USA), and cellulolytic enzymes were expressed in the haploid yeast strain *S. cerevisiae* MT8-1 (Tajima et al. 1985).

E. coli transformants were grown in Luria-Bertani medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl) supplemented with 100 μg/ml ampicillin. Yeast transformants were screened in SD medium (6.7 g/l yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI, USA] and 20 g/l glucose) supplemented with the appropriate amino acids and nucleic acids. For cocktail δ-integration, SPASC medium (6.7 g/l of yeast nitrogen base without amino acids [Difco Laboratories] and 10 g/l PASC) supplemented with the appropriate amino acids and nucleic acids was used. Yeast cells were aerobically cultured in SD or YPD medium (10 g/l yeast extract, 20 g/l Bacto-peptone [Difco Laboratories], and 20 g/l glucose).

Table 1 Characteristics of bacterial and yeast strains used in this study.

Strains or plasmids	Relevant features	Reference
Bacterial strain		
E. coli Novablue	*endA1 hsdR17*(r_{K12}^{-} m_{K12}^{+}) *supE44 thi-l gyrA96 relA1 lac recA1/F′* [*proAB*$^{+}$ *lacI*^{q} ZΔM15::Tn*10*(Tet^{r})]	Novagen
S. cerevisiae yeast strains		
MT8-1	*ade his3 leu2 trp1 ura3*	(Tajima et al. 1985)
MT8-1/IBEC	*ade leu2* Integration of β-glucosidase, endoglucanase, and cellobiohydrolase gene	This study
MT8-1/δBEC	*ade leu2* δ-Integration of β-glucosidase, endoglucanase, and cellobiohydrolase gene	This study
MT8-1/cocδBEC1	*ade his3 leu2 ura3* cocktail δ-Integration of β-glucosidase, endoglucanase, and cellobiohydrolase gene	This study
MT8-1/cocδBEC2	*ade his3 leu2* cocktail δ-Integration of β-glucosidase, endoglucanase, and cellobiohydrolase gene	This study
MT8-1/cocδBEC3	*ade leu2* cocktail δ-Integration of β-glucosidase, endoglucanase, and cellobiohydrolase gene	This study

Plasmid construction

Table 2 and Figure 1 represent the genetic properties of all plasmids used in this study. The integrative plasmid pIHAGBGL-NotI (Yanase et al. 2009) was used for surface expression of BGL1.

The integrative plasmid for surface expression of *T. reesei* EGII was constructed as follows: The NotI-NotI DNA fragment encoding the *S. cerevisiae PGK1* promoter, a secretion signal sequence, the *T. reesei* endoglucanase gene, the 3'-half of the α-agglutinin gene, and the PGK1 terminator was amplified from pIWAGEGII (Yanase et al. 2009) by PCR with the primers pPGKF(NotI): 5'-ATGCATGCGGCCGCCGATTTGGGCGCGAATCCTT-3' and tAGR(NotI): 5'-ATAAGAATGCGGCCGCTTTGATTATGTTCTTTCTATTTGAATGAGATATG-3'. The fragment was digested with NotI and inserted into the NotI site of the plasmid pRS406 (Stratagene). The resultant plasmid was named pIU-PGAGEG.

The integrative plasmid for surface expression of *T. reesei* CBHII was constructed as

follows: The NotI-NotI DNA fragment encoding the *S. cerevisiae PGK1* promoter, a secretion signal sequence, the *T. reesei* cellobiohydrolase gene, the 3'-half of the α-agglutinin gene, and the PGK1 terminator was amplified from pIHAGCBHII (Yanase et al. 2009) by PCR with the primers pPGKF(NotI) and tAGR(NotI). The fragment was digested with NotI and inserted into the NotI site of the plasmid pRS404 (Stratagene). The resultant plasmid was named pIW-PGAGCBH.

The δ-integrative basic plasmid pδH (*HIS3* as a selective marker) was constructed as follows: The XhoI-XhoI DNA fragment encoding the large portion of the promoter-deficient *HIS3* (*HIS3d*) marker gene was amplified from pRS403 (Stratagene) by PCR using the primers *HIS3*dF (XhoI): 5'-ACCGTCGACCTCGAGCTTCGAAGAATATACTAAAA-3' and *HIS3*dR (XhoI): 5'-GGGCCCCCCCTCGAGTCGAGTTCAAGAGAAAAAAA-3'. The fragment was inserted into the XhoI site of plasmid pδseq (Yamada et al. 2010) and the resulting plasmid was named pδH.

The δ-integrative plasmid for surface expression of BGL1 was constructed as follows: The NotI-NotI DNA fragment encoding the *S. cerevisiae PGK1* promoter, a secretion signal sequence, the *A. aculeatus* β-glucosidase gene, the 3'-half of the α-agglutinin gene, and the PGK1 terminator was obtained by NotI digestion of pIHAGBGL-NotI and inserted into the NotI sites of the plasmids pδU, pδW (Yamada et al. 2010), and pδH, respectively. The resultant plasmids were named pδU-PGAGBGL, pδW-PGAGBGL, and pδH-PGAGBGL, respectively.

The δ-integrative plasmids for surface expression of EGII were constructed as follows: The NotI-NotI DNA fragment encoding the *S. cerevisiae PGK1* promoter, the *T. reesei* endoglucanase gene with a secretion signal sequence, and the 3'-half of the α-agglutinin gene with a terminator was obtained by NotI digestion of pIU-PGAGEG and inserted into the NotI sites of the plasmids pδU, pδW, and pδH to generate pδU-PGAGEG, pδW-PGAGEG, and pδH-PGAGEG, respectively.

The δ-integrative plasmids for surface expression of CBHII were constructed as follows: The NotI-NotI DNA fragment encoding the *S. cerevisiae PGK1* promoter, the *T. reesei* cellobiohydrolase gene with a secretion signal sequence, and the 3'-half of the α-agglutinin gene with a terminator was obtained by NotI digestion of pIW-PGAGCBH and inserted into the NotI sites of the plasmids pδU, pδW, and pδH to generate pδU-PGAGCBH, pδW-PGAGCBH, and pδH-PGAGCBH, respectively.

Table 2 Characteristics of plasmids used in this study.

Plasmid	Recombination type	Selection marker	Expressing cellulase gene
pIH-PGAGBGL	Integration	HIS3	β-glucosidase
pIU-PGAGEG		URA3	Endoglucanase
pIW-PGAGCBH		TRP1	Cellobiohydrorase
pδW-PGAGBGL	δ-integration	TRP1	β-glucosidase
pδU-PGAGBGL		URA3	β-glucosidase
pδH-PGAGBGL		HIS3	β-glucosidase
pδW-PGAGEG		TRP1	Endoglucanase
pδU-PGAGEG		URA3	Endoglucanase
pδH-PGAGEG		HIS3	Endoglucanase
pδW-PGAGCBH		TRP1	Cellobiohydrorase
pδU-PGAGCBH		URA3	Cellobiohydrorase
pδH-PGAGCBH		HIS3	Cellobiohydrorase

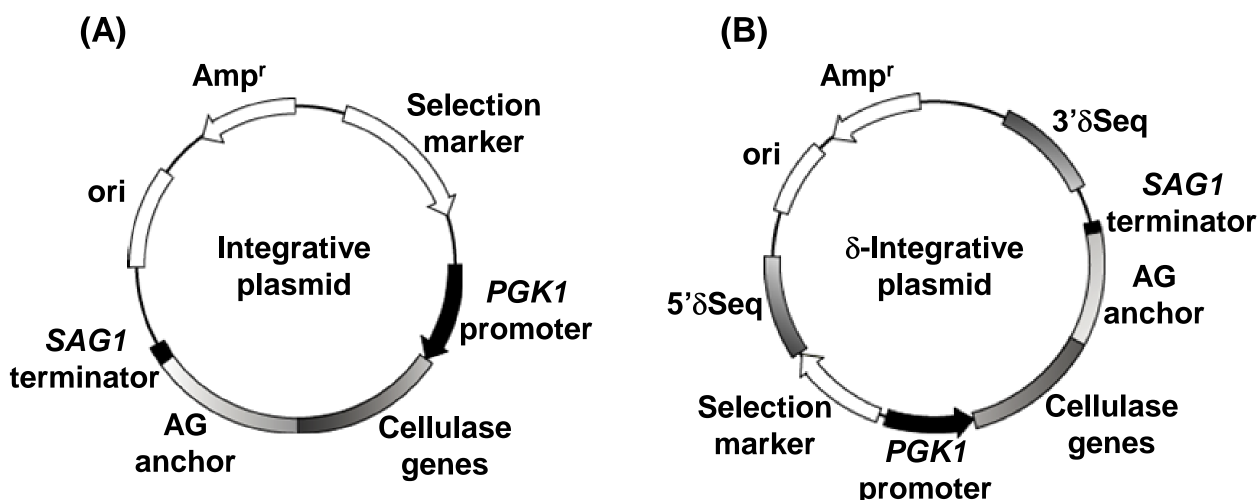

Figure 1 Genetic property of cellulase expressing plasmids.

(A) Plasmid for integration. (B) Plasmid for δ-integration.

Yeast transformation and cocktail δ-integration

In conventional integration and δ-integration, plasmids were transformed into *S. cerevisiae* MT8-1 using lithium acetate as described (Chen et al. 1992). The transformants with the highest cellulolytic activity were selected from several colonies and used in subsequent experiments.

In cocktail δ-integration, identical amounts of three co-marked δ-integrative plasmids (over 20 μg of each plasmid), pδW-PGAGBGL, pδW-PGAGEG, and pδW-PGAGCBH, were mixed and transformed simultaneously. The transformants were spread on SPASC medium, and the transformant with the highest cellulolytic activity was selected from tested over 100 numbers of large colonies. The selected transformant was named MT8-1/cocδBEC1 and used in the subsequent transformation, named repeated cocktail δ-integration. Similar to the first step, identical amounts of three co-marked δ-integrative plasmids, pδU-PGAGBGL, pδU-PGAGEG, and pδU-PGAGCBH, were mixed and transformed into MT8-1/cocδBEC1 simultaneously. The selected transformant with the highest cellulolytic activity on SPASC medium was named MT8-1/cocδBEC2. Finally, identical amounts of three co-marked δ-integrative plasmids, pδH-PGAGBGL, pδH-PGAGEG, and pδH-PGAGCBH, were mixed and transformed into MT8-1/cocδBEC2 simultaneously. The selected transformant with the highest cellulolytic activity on SPASC medium was named MT8-1/cocδBEC3.

Enzyme assay

Yeast cells cultivated in YPD medium for 72 h at 30°C and collected by centrifugation at 3,000 × *g* for 5 min at 4°C and washed with distilled water twice were used for determination of β-glucosidase and PASC degradation activity.

β-Glucosidase activity was measured in 50 mM sodium acetate buffer (pH 5.0) at 30°C with 2 mM p-nitrophenyl-β-D-glucopyranoside (Nacalai Tesque, Inc., Kyoto, Japan) as the substrate. The wet cell concentration of the reaction mixture was adjusted to 1 g-wet cell/l. After the reaction, supernatants were separated by centrifugation at 10,000 × *g* at 4°C. The amount of released p-nitrophenol was determined by measuring the absorbance at 400 nm. One unit of β-glucosidase activity was defined as the amount of enzyme producing 1 μmol/min p-nitrophenol at 30°C, pH 5.0.

PASC degradation activity was determined by hydrolysis of 1 g/l amorphous cellulose in 50 mM sodium acetate buffer (pH 5.0) at 50°C. PASC was prepared from Avicel PH-101 (Fluka Chemie GmbH, Buchs, Switzerland) as amorphous cellulose (Den Haan et al. 2007). The wet cell concentration of the reaction mixture was adjusted to 50 g-wet cell/l. After hydrolysis, the supernatant was separated by centrifugation for 5 min at 10,000 × *g* at 4°C, and the produced glucose concentration was measured using the Glucose CII test Wako (Wako Pure Chemical, Osaka). One unit of PASC degradation activity was defined as the amount of enzyme producing 1 μmol/min glucose at 50°C, pH 5.0.

Quantification of integrated copy numbers by real-time PCR

The integrated copy number of each recombinant strain was quantified using real-time PCR. Template genome DNA was isolated from yeast cells cultivated in SD medium for 72 h at 30°C using a YeaStar Genomic DNA kit (Zymo Research, Orange, CA). The 3 sets of PCR primers, BGL 761F: 5'- CTTCCAGGGCTTTGTGATGTC-3' and BGL 858R: 5'-AGGTGATATCGCCAGGCATT-3', and EGII 694F: 5'- CCACGGTCCAAGAGGTTGTAA-3' and EGII 774R: 5'- GCCAATCATTTCCAGGCAAA-3', and CBHII 571F: 5'-GGCGTCGCCAAATATAAGAACT-3' and CBHII 653R: 5'-ATAACCAGGAGGGTCCGGATA-3' were used to detect the BGL, EG, and CBH genes respectively. Quantitative real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The normalized gene copy number was calculated by the standard curve method with the *PGK1* gene as the house keeping gene.

Results

General strategy of repeated cocktail δ-integration

Cocktail δ-integration works as follows. First, three cellulase genes, BGL, EG, and CBH, were introduced into yeast genomes simultaneously by using the δ-integration method with one marker gene. As a result, a pool of recombinants with various genes having a different number of copies was constructed. Then a transformant with optimized cellulase expression was selected from the recombinant pool by its ability to form colonies on SPASC medium and its cellulolytic activity. Using the selected transformant, a 2nd round of cocktail δ-integration was carried out using a different marker gene to obtain a transformant with higher PASC degradation ability. After a 3rd round of cocktail δ-integration, the resulting transformant had almost the same PASC degradation activity as the 2nd round transformant, showing that PASC degradation activity was saturated (see the following section). This strategy is referred to as repeated cocktail δ-integration.

Construction of yeast strains

The five recombinant yeast strains constructed in this study are shown in Figure 2. The yeast strain MT8-1/IBEC has BGL, EG, and CBH genes integrated into its chromosome using a conventional integration method. The δ-integrated strain MT8-1/δBEC and cocktail δ-integrated strains MT8-1/cocδBEC1, MT8-1/cocδBEC2, and MT8-1/cocδBEC3 have the same genes integrated using the δ-integration and cocktail δ-integration method, respectively.

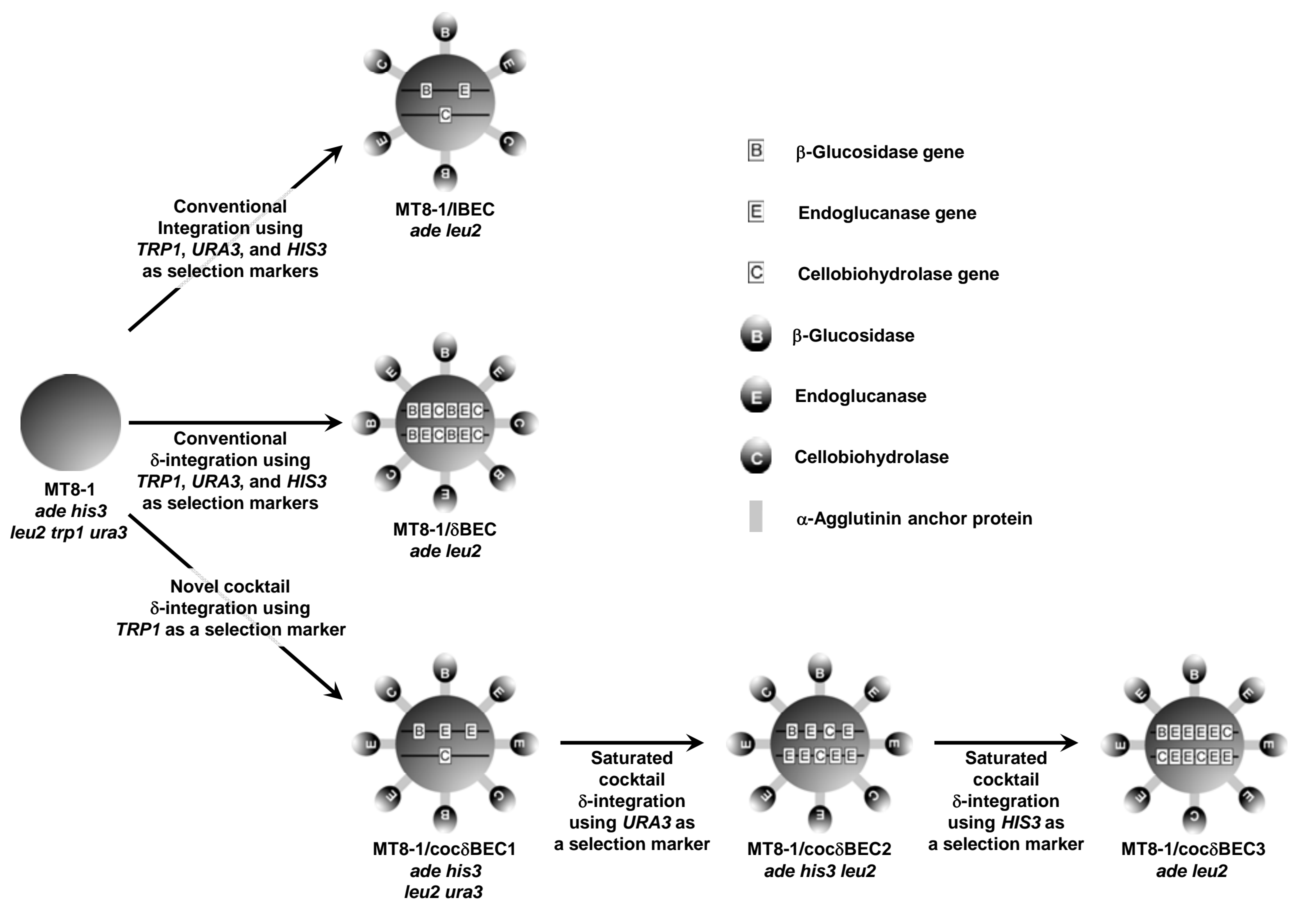

Figure 2 Strategy for constructing cellulase expressing yeast.

β-Glucosidase and PASC degradation activities

To investigate the effect of cocktail δ-integration, the BGL and PASC degradation activities of each recombinant yeast cell were measured after cultivation on YPD medium. As shown in Figure 3A, the BGL activity of the conventional δ-integrated strain MT8-1/δBEC (14.5 U/g-wet cell) was 3-fold higher than that of the conventional integrated strain MT8-1/IBEC (4.9 U/g-wet cell). The BGL activities of all cocktail δ-integrated strains (MT8-1/cocδBEC1, MT8-1/cocδBEC2, and MT8-1/cocδBEC3) were lower than that of the conventional integrated strain MT8-1/IBEC.

As shown in Figure 3B, the PASC degradation activity of the conventional δ-integrated strain MT8-1/δBEC (57.6 mU/g-wet cell) was 5-fold higher than that of the conventional integrated strain MT8-1/IBEC (11.8 U/g-wet cell). Alternatively, the PASC degradation activity of the cocktail δ-integrated strain MT8-1/cocδBEC1 was approximately 64.9 mU/g-wet cell, which was higher than that of MT8-1/δBEC. The PASC degradation activities of repeated cocktail δ-integrated strains MT8-1/cocδBEC2 and MT8-1/cocδBEC3 were respectively 75.8 and 75.1 mU/g-wet cell, which are almost the same as each other and significantly improved compared to MT8-1/δBEC.

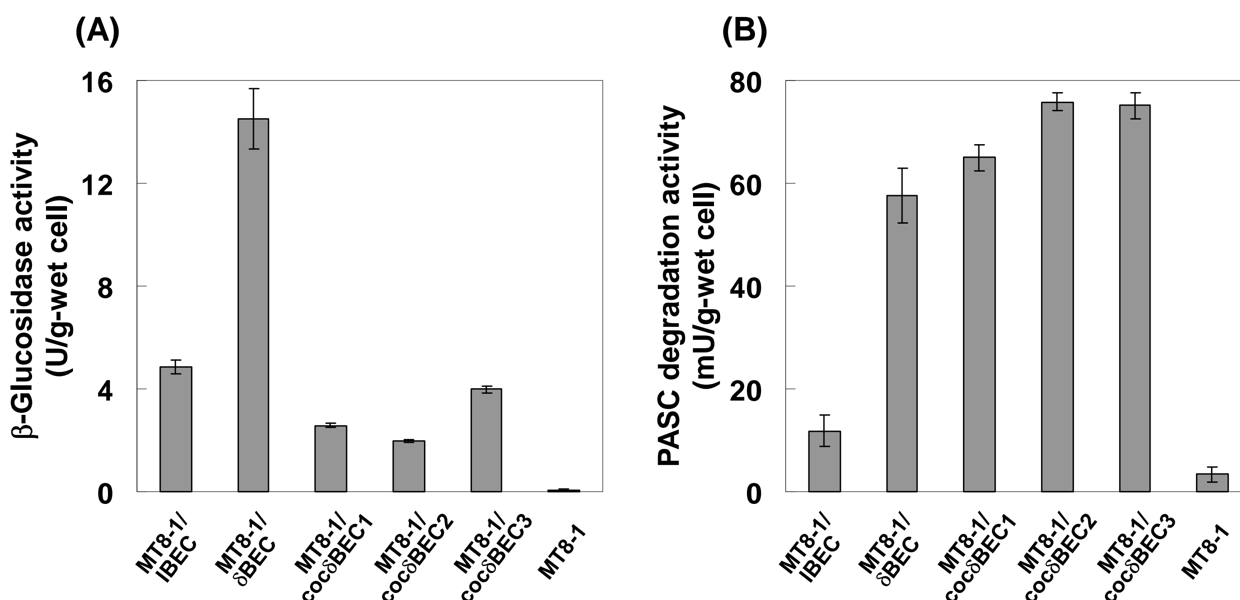

Figure 3 (A) β-Glucosidase and (B) PASC degradation activities of cellulase expressing yeast strains. MT8-1/IBEC, conventional integrated strain; MT8-1/ δBEC, conventional δ-integrated strain; MT8-1/cocδBEC1, cocktail δ-integrated strain; MT8-1/cocδBEC2 and MT8-1/cocδBEC3, repeated cocktail δ-integrated strain; MT8-1, wild-type strain. Data are averages from five independent experiments.

Integrated copy numbers of cellulolytic genes

To investigate the integrated copy number of transformants, real-time PCR was conducted using each transformant genomic DNA as the template. Figure 4 shows the copy number of cellulase integrated strains. As expected, the number of each integrated gene (BGL, EG, and CBH) in the conventional integrated strain MT8-1/IBEC was estimated to be 1. On the other hand, the number of integrated BGL, EG, and CBH genes in the conventional δ-integrated strain MT8-1/δBEC was estimated to be 6, 5, and 9, respectively. The integrated number of BGL, EG, and CBH genes in the cocktail δ-integrated strain MT8-1/cocδBEC1 was estimated to be 1, 8, and 2, respectively, and the EG copy number was increased preferentially after the 2nd and 3rd rounds of cocktail δ-integration.

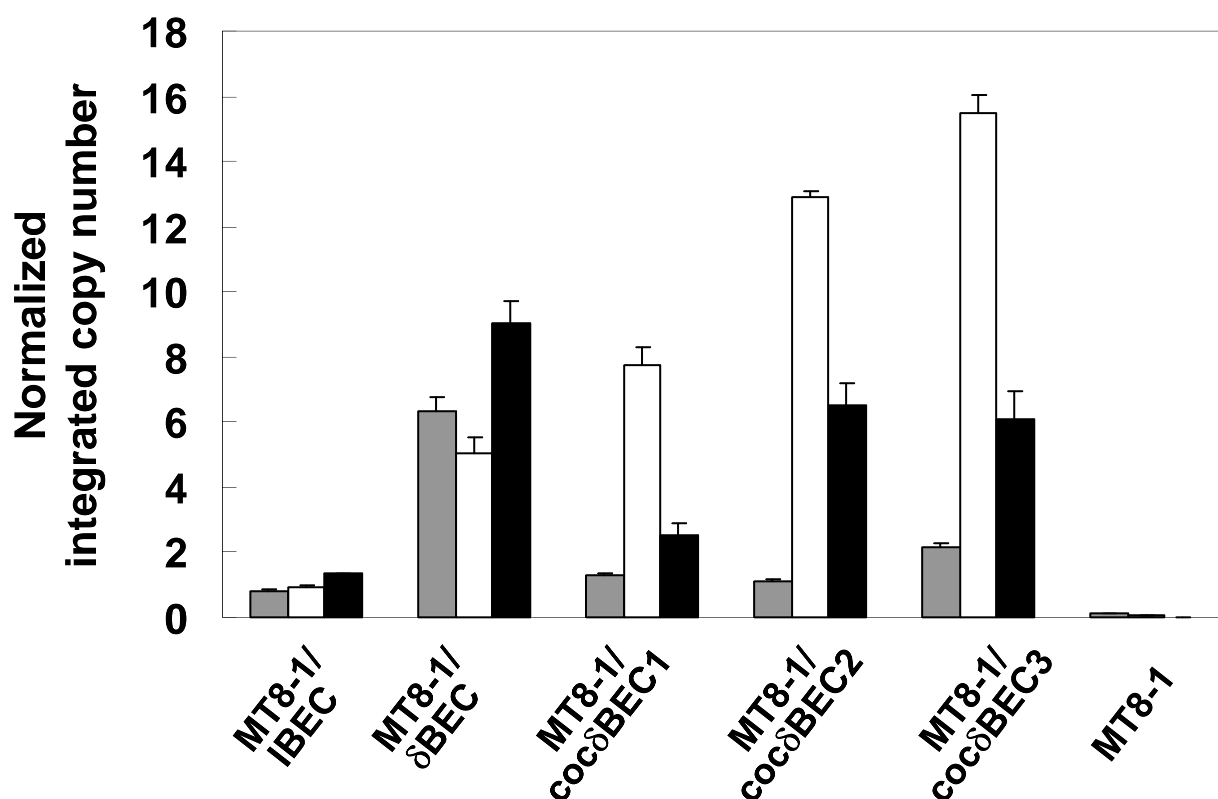

Figure 4 Determination of cellulase gene copy number of cellulase-expressing yeast strains. Gray bar, β-glucosidase; white bar, endoglucanase; black bar, cellobiohydrolase. MT8-1/IBEC, conventional integrated strain; MT8-1/ δBEC, conventional δ-integrated strain; MT8-1/cocδBEC1, cocktail δ-integrated strain; MT8-1/cocδBEC2 and MT8-1/cocδBEC3, repeated cocktail δ-integrated strain; MT8-1, wild-type strain. Data are averages from three independent experiments.

Discussion

We developed a high performance cellulolytic yeast strain via a novel δ-integration method by optimizing the expression ratio of three types of cellulase genes. To our knowledge, this is the first report concerning the expression of cellulase genes by δ-integration and optimization of various foreign genes by δ-integration in yeast.

Table 3 shows a summary of results in this study. The BGL activity of the conventional δ-integrated strain MT8-1/δBEC was the highest among all of the strains constructed in this study. Although all three cocktail δ-integrated strains (MT8-1/cocδBEC1, MT8-1/cocδBEC2, and MT8-1/cocδBEC3) have lower BGL activity, they show higher PASC degradation activity compared to MT8-1/δBEC. In addition, nearly all the produced soluble sugar from PASC in all strains was glucose, which was confirmed by the Somogyi-Nelson method (Wood et al. 1988) (data not shown). This clearly shows that BGL activity is sufficient in all strains constructed in this study; however, the EG and CBH activities are insufficient for efficient PASC degradation. These results correspond to the low copy number of BGL in optimized MT8-1/cocδBEC1, MT8-1/cocδBEC2, and MT8-1/cocδBEC3 constructed using repeated cocktail δ-integration (Figure 4).

Table 3 Summary of results.

Strain	BGL activity (U/g-wet cell)	PASC degradation activity (mU/g-wet cell)	Expected copy number of BGL	Expected copy number of EG	Expected copy number of CBH	Toal integrated copy number	Recombination number
MT8-1/lBEC	5	12	1	1	1	3	3
MT8-1/δBEC	15	58	6	5	9	20	3
MT8-1/cocδBEC1	3	65	1	8	2	11	1
MT8-1/cocδBEC2	2	76	1	13	6	20	2
MT8-1/cocδBEC3	4	75	2	16	6	24	3
MT8-1	0	3	0	0	0	0	0

Although the total integrated gene copy number of MT8-1/cocδBEC1 was about half that of MT8-1/δBEC, the PASC degradation activity was higher (Table 3). This suggests that optimization of the cellulase expression ratio improves PASC degradation more so than overexpression. Additionally, after the 2nd and 3rd rounds of cocktail δ-integration, the integrated gene copy numbers as well as PASC degradation activity reached a plateau. These results show that uses of repeated cocktail δ-integration can optimize the cellulase expression ratio.

The copy number of integrated EG in the cocktail δ-integrated yeast, MT8-1/cocδBEC3, was the highest compared to that of CBH or BGL (Table 3). This result indicates the importance of EG expression for efficient PASC degradation. Many reports have suggested that amorphous cellulose such as PASC and β-glucan can be degraded into glucose by EG and BGL activity without CBH (Den Haan et al. 2007; Fujita et al. 2002). CBH activity is more important than EG activity for efficient degradation of crystalline cellulose such as Avicel, and EG activity is more important in the degradation of amorphous cellulose such as PASC (Toda et al. 2005). The fact that the integrated EG copy number was increased preferably for PASC degradation is consistent with these previous reports (Den Haan et al. 2007; Fujita et al. 2002; Toda et al. 2005).

One advantage of our cocktail δ-integration method is that optimization of cellulase expression levels for efficient cellulose degradation can be achieved without knowing the optimum cellulase expression ratio. Although the filamentous fungus *T. reesei*, which effectively degrades cellulose, simultaneously controls the expression levels of various cellulolytic enzymes (Ilmen et al. 1997), the expression levels and activities of various individual enzymes are still unknown. In addition, the optimum expression ratio varies depending on the cellulosic material and degradation conditions. For the novel cocktail δ-integration method developed in this study, in which we only prepare δ-integrative vectors for a target substrate such as PASC, we can construct a target protein expressing strain with an optimum ratio under the desired conditions. Additionally, using this cocktail δ-integration method, several genes are introduced simultaneously with only a single recombination operation, and the strain with the highest activity can be improved by repeated cocktail δ-integration. This simple procedure not only reduces time and effort, but also facilitates the construction of recombinant industrial yeast strains because of their weak recombinant host

character such as lacking of auxotrophic marker (Akada 2002).

The conventional integration method is not adequate for the optimization of expression because of the low integrated copy number and expression level (Figures 3 and 4). Although conventional δ-integration or 2μ-based multicopy plasmid type recombination allow for overexpression of target genes, it is difficult to control and optimize the expression levels of each gene with these methods. Our cocktail δ-integration method has advantage in that it optimizes the ratio of cellulase expression levels with high cellulolytic activity.

Conclusions

We constructed three strains of cellulase gene expression-optimized yeast via a novel cocktail δ-integration method. This method should be very effective and easily applied to other multi-enzymatic systems like the degradation of hemicellulose (Katahira et al. 2004). In addition, it can also be easily applied to the construction of recombinant strains using industrial yeast because several genes are integrated simultaneously in one step.

Acknowledgments

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Chapter 2. Direct ethanol production from cellulosic materials using a cellulolytic enzymes expression optimized diploid *Saccharomyces cerevisiae* strain

Introduction

Due to growing concerns over the dwindling supply of fossil fuels and environmental problems, such as global warming and acid rain, associated with their combustion, the utilization of biomass as a source of renewable, environmentally friendly energy has attracted much attention. In particular, bioethanol produced from biomass represents a promising alternative fuel or gasoline enhancer. Currently, the main feedstock for bioethanol production is starch-rich biomass, as it is relatively easily decomposed by microbial enzymes. However, lignocellulosic biomass such as rice straw, which is one of the most abundant lignocellulosic waste materials in the world, is regarded as a promising starting material for bioethanol production, because it is abundant, inexpensive, renewable, and has favorable environmental properties (Sánchez and Cardona 2008). Despite these advantages, the use of lignocellulosic biomass as feedstock for bioethanol requires high-cost and complex hydrolyzing steps, such as pretreatment with high temperature and pressure, acid, and alkaline, and extensive cellulase treatment before fermentation (Sánchez and Cardona 2008). Therefore, efficient and cost-effective methods for the degradation and fermentation of lignocellulosic biomass to ethanol are required.

The efficient degradation of cellulose requires the synergistic action of the cellulolytic enzymes endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL). Although there are numerous reports of lower-cost ethanol production from cellulosic material by consolidating hydrolyzing and fermentation steps using recombinant *Saccharomyces cerevisiae* expressing cellulolytic enzymes (Den Haan et al. 2007; Fujita et al. 2004; Tsai et al. 2009; Wen et al. 2010), cellulose degradation efficiency has not been sufficiently improved. Several filamentous fungi capable of effective cellulose degradation have also been identified, including *Trichoderma reesei*, which produce various cellulolytic enzymes and simultaneously control their expression levels in response to the environment. The ratio and synergetic effects of the fungal cellulases are important for the efficient degradation of cellulose (Dashtban et al. 2009; Stricker et al. 2008).

We previously developed a simple method, named cocktail δ-integration, to optimize cellulase expression levels for cellulose degradation (Yamada et al. 2010c). Using this method, the phosphoric acid swollen cellulose (PASC) degradation activity of cellulase-displaying *S. cerevisiae*, which is a promising microorganism for efficient ethanol production from cellulose (Yanase et al. 2010), significantly improved (Yamada et al. 2010c). One advantage of the cocktail δ-integration method is that optimization of cellulase expression levels for cellulose degradation can be achieved without prior knowledge of the optimum ratios of the target enzymes. In addition, genes integrated into the yeast genome by cocktail δ-integration are maintained stably in non-defined inexpensive industrial media, such as molasses- and corn steep liquor (CSL)-based media (Kadam et al. 1997; Kim et al. 2007).

Diploidization is another promising strategy to improve expression levels of heterologous genes and enhance fermentation ability of *S. cerevisiae* (Yamada et al. 2009, 2010a, 2010b, 2011). As diploid yeast strains have higher cell growth rates, cell yields, and tolerances to various stresses than haploid strains, they are particularly suited for industrial applications (Yamada et al. 2010b; Martin and Jönsson 2003). In a previous study, we developed recombinant *S. cerevisiae* capable of efficient direct ethanol production from starch by combining the genome integration of amylase genes and diploidization (Yamada et al. 2009, 2010a). Using this strategy, we successfully imparted high amylolytic activity and cell growth rates to the modified diploid yeast. However, the optimization of cellulase expression ratios in a diploid yeast strain has not been reported, and may be an effective approach for improving ethanol fermentation.

In this study, we aimed to directly produce ethanol from rice straw using a cellulolytic enzyme-expressing diploid strain of *S. cerevisiae*. First, we evaluated the ethanol productivity from PASC of cellulolytic enzyme expression-optimized haploid strain. Diploidization of the haploid strain was then conducted, and direct ethanol production from rice straw was performed using the resultant diploid strain.

Materials and methods

Strains, plasmids, and media

Table 1 summarizes the genetic properties of the strains and plasmids used in this study. Briefly, the host for recombinant DNA manipulations was *Escherichia coli* strain NovaBlue (Novagen, Madison, WI, USA), and cellulolytic enzymes were expressed in the haploid yeast strains *S. cerevisiae* MT8-1 (Tajima et al. 1985) and NBRC1440ΔHUWL (Yamada et al. 2010a). The haploid and diploid *S. cerevisiae* strains 1440/cocδBEC3 and MNII/cocδBEC3, respectively, were constructed as described in next section.

E. coli transformants were grown in Luria-Bertani medium (10 g/L tryptone [Nacalai Tesque, Kyoto, Japan], 5 g/L yeast extract [Nacalai Tesque], and 5 g/L NaCl [Nacalai Tesque]) supplemented with 100 μg/mL ampicillin. Yeast transformants and fusants were screened in SD medium (6.7 g/L yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI, USA] and 20 g/L glucose [Nacalai Tesque]) or SPASC medium (6.7 g/L yeast nitrogen base without amino acids and 10 g/L PASC) supplemented with appropriate amino acids and nucleic acids. PASC was prepared from Avicel PH-101 (Fluka Chemie GmbH, Buchs, Switzerland) as amorphous cellulose (Den Haan et al. 2007).

Yeast cells were aerobically cultured in YPD medium (10 g/L yeast extract, 20 g/L Bacto-peptone [Difco Laboratories], and 20 g/L glucose) or molasses medium (5% [v/v] molasses, 0.5% [v/v] CSL [Sigma-Aldrich Japan, Tokyo, Japan], and 0.01% [v/v] antifoam SI [Wako Pure Chemical Industries, Ltd., Osaka, Japan]). The pH of molasses medium was adjusted to 5.0 by addition of sodium hydrate. Ethanol fermentation proceeded in YP medium (10 g/L yeast extract and 20 g/L Bacto-peptone) supplemented with either 20 g/L PASC or 100 g/L hot-water pretreated rice straw (purchased from Mitsubishi Heavy Industry, Tokyo, Japan). The water-insoluble fraction of hot-water pretreated rice straw was washed twice with distilled water, dried at 80 °C for 16 h, ground to a particle size of approximately 0.5 mm using a laboratory disintegrator (Sansho Industry Co., Ltd., Osaka, Japan), and then used as carbon source for ethanol fermentation. The composition of the prepared water-insoluble fraction, which was determined according to the procedure published by the National Renewable Energy Laboratory (Sluiter et al. 2004), was as follows:

44.8% (w/w) glucan, 0.3% (w/w) xylan, 0.8% (w/w) galactan, and low detectable amounts of arabinan and mannan.

Table 1 Characteristics of bacterial, yeast strains, and plasmids used in this study.

Strains or plasmids	Relevant features	Reference
Bacterial strain		
Escherichia 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)
NBRC1440ΔHUWL	*MAT*α *his3 leu2 trp1 ura3*	(Yamada et al. 2010a)
MT8-1/IBEC	*MAT*a *ade leu2*, Single-copy integration of β-glucosidase, endoglucanase, and cellobiohydrolase genes	(Yamada et al. 2010c)
MT8-1/cocδBEC3	*MAT*a *ade leu2*, Cocktail δ-integration of β-glucosidase, endoglucanase, and cellobiohydrolase genes	(Yamada et al. 2010c)
MT8-1/cocδBEC3/LEU2	*MAT*a *ade*, Cocktail δ-Integration of β-glucosidase, endoglucanase, and cellobiohydrolase genes	This study
1440/cocδBEC3	*MAT*α *leu2*, Cocktail δ-integration of β-glucosidase, endoglucanase, and cellobiohydrolase genes	This study
MNII/cocδBEC3	*MAT*a/α, Cocktail δ-integration of β-glucosidase, endoglucanase, and cellobiohydrolase genes	This study
Plasmids		
pδW-PGAGBGL	*TRP1*, Expression of β-glucosidase by δ-integration	(Yamada et al. 2010c)
pδU-PGAGBGL	*URA3*, Expression of β-glucosidase by δ-integration	(Yamada et al. 2010c)
pδH-PGAGBGL	*HIS3*, Expression of β-glucosidase by δ-integration	(Yamada et al. 2010c)
pδW-PGAGEG	*TRP1*, Expression of endoglucanase by δ-integration	(Yamada et al. 2010c)
pδU-PGAGEG	*URA3*, Expression of endoglucanase by δ-integration	(Yamada et al. 2010c)
pδH-PGAGEG	*HIS3*, Expression of endoglucanase by δ-integration	(Yamada et al. 2010c)
pδW-PGAGCBH	*TRP1*, Expression of cellobiohydrolase by δ-integration	(Yamada et al. 2010c)
pδU-PGAGCBH	*URA3*, Expression of cellobiohydrolase by δ-integration	(Yamada et al. 2010c)
pδH-PGAGCBH	*HIS3*, Expression of cellobiohydrolase by δ-integration	(Yamada et al. 2010c)
pRS405	*LEU2*, No expression	Stratagene

Yeast transformation and cocktail δ-integration

A cellulolytic enzyme expression-optimized strain that expressed the enzymes EG, CBH, and BGL was first constructed by the cocktail δ-integration method, as described previously (Yamada et al. 2010c). Briefly, identical amounts (about 20 μg of each plasmid) of three co-marked δ-integrative plasmids, pδW-PGAGBGL, pδW-PGAGEG, and pδW-PGAGCBH, which allow expression of BGL, EG, and CBH, respectively, on the cell surface, were mixed and co-transformed into NBRC1440ΔHUWL. The transformants were spread on SPASC medium for selection, and the transformants with the highest cellulolytic activity were selected. The selection was repeated three times using *TRP1*, *URA3*, and *HIS3* as selection markers, and one resultant transformant was selected and named 1440/cocδBEC3.

To screen diploid strains constructed by mating, pRS405 was integrated into MT8-1/cocδBEC3 using a conventional integration method, as previously described (Chen et al. 1992). The resultant transformant was designated MT8-1/cocδBEC3/LEU2.

Diploidization by mating

The diploid strain MNII/cocδBEC3 was constructed by mating the haploid strains MT8-1/cocδBEC3/LEU2 and 1440/cocδBEC3, as described previously (Yamada et al. 2010a). Briefly, both haploid strains were grown separately on liquid SD medium containing appropriate amino acids and nucleic acids for 24 h at 30 °C, harvested, spread together on SD plates supplemented with appropriate amino acids and nucleic acids, and the further incubated for 72 h at 30°C. The strains were then replica-plated onto SD plates and incubated for 3 days at 30°C, and the resulting isolated single colonies were selected as the diploid strain.

Cell growth and PASC degradation activity

For cell growth measurements and determination of PASC degradation activity, yeast cells were cultivated in YPD or molasses media for 48 h at 30°C (initial O.D. 600= 0.05), collected by centrifugation at 3,000 × *g* for 5 min at 4°C, and then washed twice with distilled water. Cell growth was determined by counting cell numbers microscopically in a Bürker Türk hemocytometer with appropriate dilution of cultures.

Washed cells were then added at a final concentration of 25 g wet cell/L to a 3 ml solution of 1 g/L PASC in 50 mM sodium citrate buffer (pH 5.0) and 100 mM methyl glyoxal (Nacalai Tesque), which was added to prevent assimilation of the produced glucose by yeast cells (Kalapos 1999). After the hydrolysis reaction was allowed to proceed at 50°C for 4 h, the supernatant was collected by centrifugation for 5 min at 10,000 × *g* at 4°C to remove cells and debris, and the produced glucose concentration was measured using the Glucose CII test (Wako Pure Chemical Industries, Ltd.). One unit of PASC degradation activity was defined as the amount of enzyme producing 1 μmol/min glucose at 50°C, pH 5.0.

Quantification of transcription level of cellulolytic genes by real-time PCR

The transcription levels of each cellulolytic gene were quantified by reverse transcription (RT) real-time polymerase chain reaction (PCR). Total RNA was isolated from yeast cells cultivated in YPD medium for 48 h at 30°C using a RiboPure Yeast Kit (Ambion, Austin, TX, USA), and cDNA was then synthesized from total RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). RT real-time PCR using synthesized cDNA as a template was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with three sets of PCR primers (BGL 761F and BGL 858R, EGII 694F and EGII 774R, and CBHII 571F and CBHII 653R (Yamada et al. 2010c)) and Thunderbird SYBR qPCR Mix (Toyobo). Transcription levels of the three cellulolytic genes were normalized to the house keeping gene *PGK1* using the standard curve method.

Ethanol fermentation from PASC and rice straw

Yeast cells were precultured aerobically in YPD or molasses media at 30°C for 48 h, harvested by centrifugation at 1,000 × g for 5 min, and then washed twice with distilled water. The wet cell weight was then determined by harvesting the washed cells by centrifugation at 3,000 × g for 5 min and weighing the cell pellet (the estimated dry cell weight for all strains was approximately 0.15-fold the wet cell weight). The cells were then re-suspended in 20 mL YP medium containing 20 g/L PASC or 100 g/L hot-water pretreated rice straw at an initial cell concentration adjusted to 200 g wet cell/L. Ethanol fermentation proceeded at 37°C for 72 h with mild agitation in 100-ml closed bottles equipped with a bubbling CO_2 outlet. Ethanol concentration was determined using a gas chromatograph (model GC-2010, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a DB-FFAP column (60 m x 0.25 mm id, 0.5 μm film thickness; Agilent Technologies, Palo Alto, CA, USA) using helium as a carrier gas. The column temperature was programmed at 40 to 170°C (10°C/min).

Results

Ethanol production from PASC by haploid strain

Ethanol productivity from PASC was first evaluated by performing fermentations with haploid *S. cerevisiae* strains that were previously engineered to express the cellulases BGL, EG, and CBH through either a conventional or cocktail δ-integration method. As shown in Figure 1, cellulolytic enzyme expression-optimized strain MT8-1/cocδBEC3 exhibited the highest ethanol productivity. The maximum ethanol production of MT8-1/cocδBEC3 reached 3.1 g/L from 20 g/L PASC in 72 h, which was 1.6-fold higher than that of conventional-integrated strain MT8-1/IBEC (1.9 g/L). The final ethanol yield of MT8-1/cocδBEC3 and MT8-1/IBEC from PASC after 72 h of fermentation was 30% and 19% of the theoretical yield, respectively. Notably, wild-type strain MT8-1 did not produce detectable amounts of ethanol from PASC.

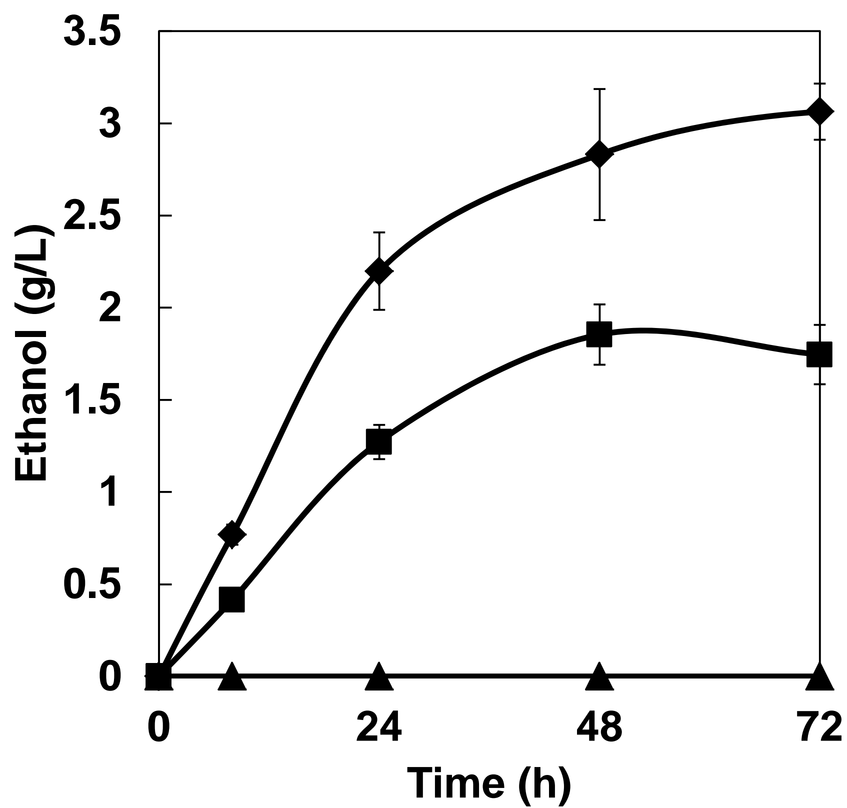

Figure 1 Time course of ethanol production from PASC by haploid strain MT8-1 (triangles), MT8-1/IBEC (squares), and MT8-1/cocδBEC3 (diamonds). Data are averages from three independent experiments.

Construction of diploid yeast strain

To improve the cellulolytic activity of the recombinant haploid strain MT8-1/cocδBEC3, the diploid strain MNII/cocδBEC3 was constructed by mating the haploid cellulolytic enzyme-expression optimized strain MT8-1/cocδBEC3, which was previously constructed by the cocktail δ-integration (Yamada et al. 2010c), with strain 1440/cocδBEC3 (Figure 2).

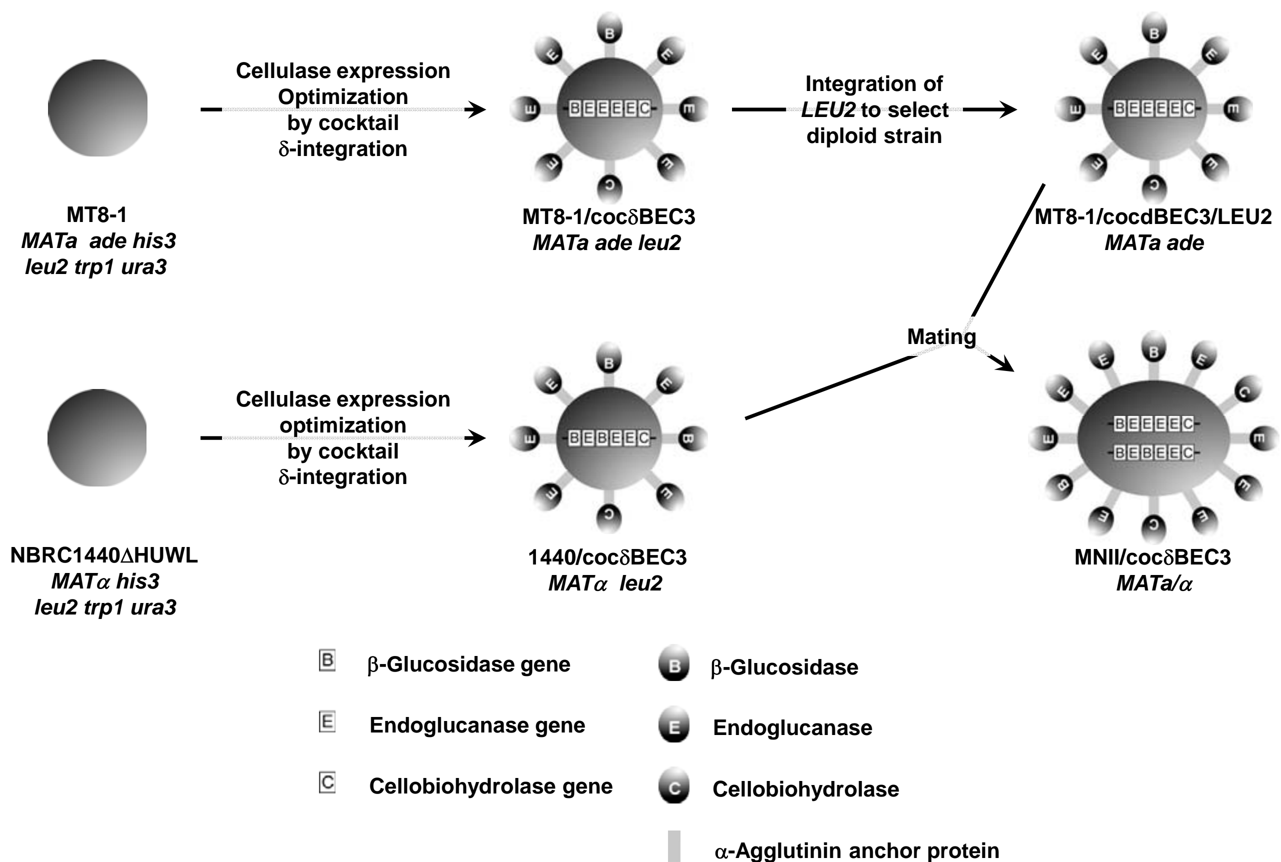

Figure 2 Strategy for constructing cellulase expressing diploid yeast strain.

Cellulolytic enzymes expression ratio

To evaluate the cellulolytic enzyme expression ratios of the haploid and diploid strains, transcription levels of the three cellulolytic enzymes were quantified by RT real-time PCR (Figure 3). Although the transcription level ratios of the three types of cellulases were nearly identical in strains MT8-1/cocδBEC3 and MNII/cocδBEC3, the EG transcription level was markedly higher than the other two enzymes.

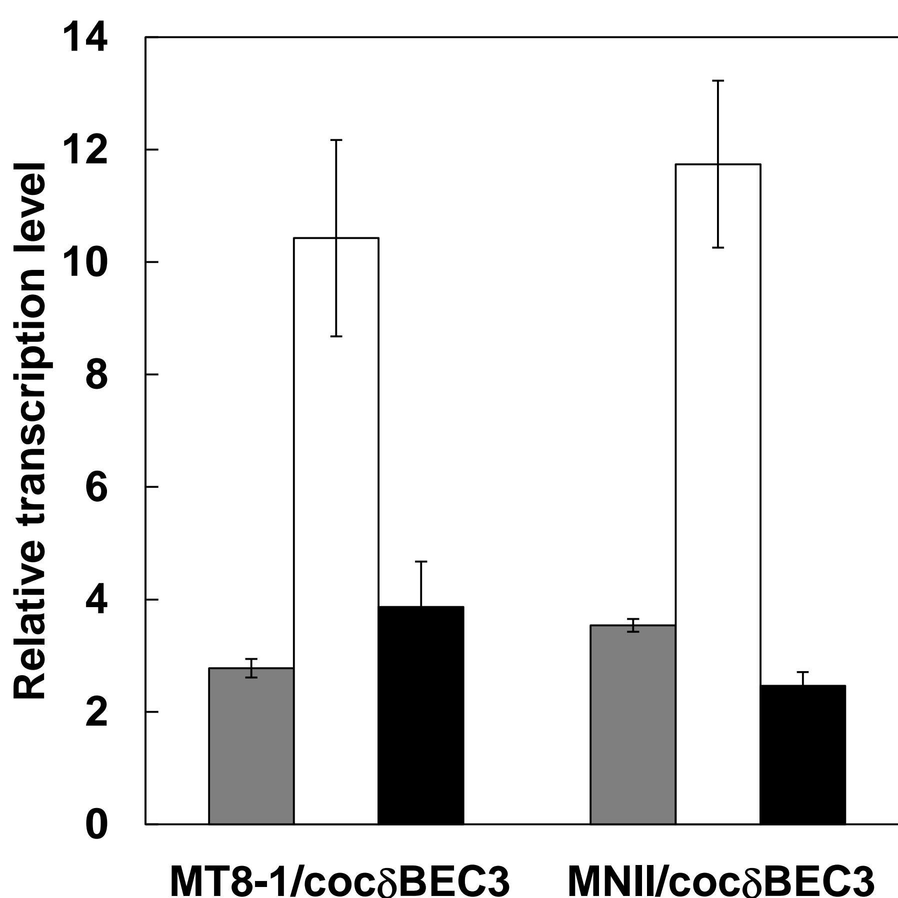

Figure 3 Transcription levels of cellulolytic enzymes of haploid strain MT8-1/cocδBEC3 and diploid strain MNII/cocδBEC3. Gray bar, β-glucosidase; white bar, endoglucanase; black bar, cellobiohydrolase. Data are averages from six independent experiments.

Cell growth and PASC degradation activity of haploid and diploid strain after cultivation in YPD or molasses medium

To examine differences in growth profiles and fermentation ability between haploid and diploid strains, cell growth and PASC degradation activity following cultivation in YPD or molasses medium were evaluated. As shown in Table 2, both haploid strain MT8-1/cocδBEC3 and diploid strain MNII/cocδBEC3 cultured using YPD medium (5.3×10^7 and 20.0×10^7 cells/mL, respectively) showed higher cell growth than when the respective strains were grown in molasses medium (1.4 and 5.3×10^7 cells/mL, respectively).

When cells of the two strains cultured in YPD and molasses media were subjected to PASC hydrolysis reactions, haploid strain MT8-1/cocδBEC3 showed higher PASC degradation activity when precultured in YPD medium (180.1 mU/g wet cell) than in molasses medium (63.5 mU/g wet cell). In contrast, diploid strain MNII/cocδBEC3 prepared using YPD medium (234.1 mU/g wet cell) displayed lower PASC degradation activity than cells prepared using molasses medium (381.4 mU/g wet cell). Despite this difference, diploid strain MNII/cocδBEC3 showed higher cell growth and PASC degradation activity than haploid strain MT8-1/cocδBEC3 after cultivation in both YPD and molasses media.

Table 2 PASC degradation activity and cell growth.

Medium	Strain	PASC degradation activity (mU/g-wet cell)	Cell growth ($\times 10^7$ cells/mL)
YPD	MT8-1/cocδBEC3	180.1 ± 5.7	5.3 ± 0.15
	MNII/cocδBEC3	234.1 ± 7.2	20.0 ± 0.51
Molasses	MT8-1/cocδBEC3	63.5 ± 6.2	1.4 ± 0.08
	MNII/cocδBEC3	381.4 ± 6.6	5.3 ± 0.13

Ethanol production from cellulose by diploid strain

Finally, to evaluate ethanol productivity of diploid strain MNII/cocδBEC3, ethanol fermentations from PASC and pretreated rice straw were performed with cells precultured in either YPD or molasses media (Figure 4). After 72 h of fermentation at 37 °C, maximum ethanol production from PASC reached 4.1 and 7.6 g/L by the diploid strain prepared using YPD and molasses media, respectively, representing an approximately 2-fold higher increase for cells precultured with molasses as a carbon source. The final ethanol yields from the initial sugars reached 40% and 75% of the theoretical yield in 72 h for the diploid strain precultured in YPD and molasses medium, respectively.

Figure 5 shows the time course of ethanol fermentation from rice straw by the diploid strain prepared using molasses medium. The maximum ethanol concentration and ethanol yield from the initial glucan reached 7.5 g/L and 33% of the theoretical yield in 72 h, respectively.

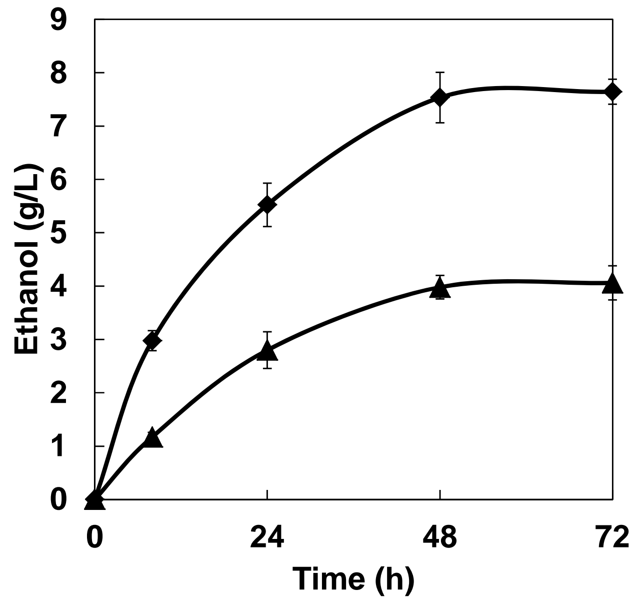

Figure 4 Time course of ethanol production from PASC by diploid strain MNII/cocδBEC3 prepared using YPD medium (triangles) and molasses medium (diamonds). Data are averages from three independent experiments.

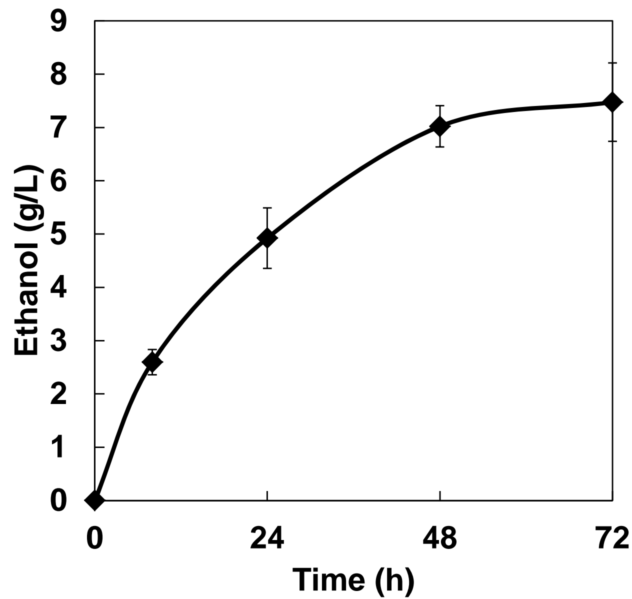

Figure 5 Time course of ethanol production from pretreated rice straw by diploid strain MNII/cocδBEC3 prepared using molasses medium. Data are averages from three independent experiments.

Discussion

Here, we developed a cellulolytic enzyme expression-optimized diploid *S. cerevisiae* strain that was able to directly produce ethanol from PASC and pretreated rice straw with relatively high yields. To our knowledge, this is the first report of ethanol production from agricultural biomass using recombinant yeast expressing cellulolytic enzymes without the addition of exogenous enzymes.

PASC degradation activity of MT8-1/cocδBEC3 (75 mU/g wet cell) was approximately six-fold higher than that of MT8-1/IBEC3 (12 mU/g wet cell) (Yamada et al. 2010c), which corresponded to two-fold higher ethanol productivity by the expression-optimized MT8-1/cocδBEC3 strain generated by cocktail δ-integration (Figure 1). These results clearly show that optimization of cellulolytic enzyme expression is effective for enhancing ethanol production from PASC, as well as for improving PASC degradation ability; however, the total ethanol yield remained low (30% of the theoretical yield). Therefore, to improve PASC degradation activity and increase ethanol production, we constructed a cellulolytic enzyme expressing diploid strain with optimized cellulase expression (Figure 2).

PASC degradation activity was improved by diploidization (Table 2), which was due to the increased copy number of cellulase genes integrated into the genome (Yamada et al. 2010b). Notably, the transcription level of the EG gene was markedly higher than the other two integrated cellulase genes, BGL and CBH, in the cellulolytic enzyme expression-optimized haploid strain MT8-1/cocδBEC (Figure 3). This result correlates well with the observation that the EG gene preferably integrates into yeast genomes in high copy number compared with BGL and CBH genes during the optimization process (Yamada et al. 2010c). In addition, the transcription levels of the three cellulolytic genes in the haploid host strain 1440/cocδBEC3 were nearly identical with those of MT8-1/cocδBEC3 (data not shown) and MNII/cocδBEC3 (Figure 3), suggesting that total amount of gene expression could be improved by diploidization retaining optimized gene expression of parental strains. Hence, performing diploidization after optimization of multi-gene expression could be an effective strategy for constructing cellulose degrading yeast.

To reduce yeast preparation costs for industrial applications, we evaluated cell growth of

the haploid and diploid strains using molasses medium (Table 2). One advantage of the cocktail δ-integration method is that cultivation of recombinant strains under non-selective conditions is available because foreign genes integrated into the genome are stably maintained (Yamada et al. 2010b). Cell growth of both MT8-1/cocδBEC3 and MNII/cocδBEC3 in molasses medium decreased compared to growth in YPD medium, which was likely caused by nutrient richness of YPD medium and susceptibility to inhibitory factors in molasses and/or CSL (Lee et al. 2000; Park et al. 2000; Shoda and sugano 2005; Vu and Kim 2009). Surprisingly, however, the PASC degradation activity of MNII/cocδBEC3 after precultivation in molasses medium was significantly higher compared to that following growth in YPD medium. One possible explanation is that metal ions present in molasses and/or CSL improved the stability of cellulolytic enzymes (Elvana et al. 2010; Saha 2004; Wang 1998).

As expected from the PASC hydrolysis reaction results, high ethanol production and yield (7.6 g/L and 75% of the theoretical yield, respectively) from PASC was achieved using the diploid strain prepared in molasses medium (Figure 4). From the comparison of different cellulase expression systems of *S. cerevisiae* shown Table 3, our diploid strain clearly achieved the highest ethanol production and yields among previous reports. In addition to these promising findings, the cellulolytic enzyme-expressing diploid yeast strain was also able to produce ethanol from pretreated rice straw (Figure 5). To our knowledge, this represents the first report of ethanol production from agricultural lignocellulosic biomass using only a cellulolytic enzyme-expressing yeast without any exogenous cellulases added to the fermentation. Although the ethanol production rate from rice straw was nearly identical to that from PASC, the ethanol yield from rice straw was relatively low (33% of the theoretical yield). As this result suggests that highly crystalline regions of cellulose in rice straw were not effectively degraded, reducing these regions by improving the efficiency of pretreatment or further optimizing cellulolytic enzyme expression ratios in recombinant diploid yeast generated by the cocktail δ-integration method may lead to improved bioethanol yields from agricultural waste biomass (Yamada et al. 2010c).

Table 3 Comparison of the ethanol productivity from PASC.

Strain	Cellulase expression	Initial PASC concentration (g/L)	Maximum ethanol production (g/L)	Maximum ethanol reaching time (h)	Ethanol yield of the theoretical yield from initial cellulose (%)*	Reference
S. cerevisiae	Cell surface display	10	2.9	40	57	(Fujita et al. 2004)
S. cerevisiae	Secretion	10	1	192	20	(Den Haan et al. 2007)
S. cerevisiae and *E. coli*	Minicellulosome constructed by *E. coli* produced cellulases	10	3.5	48	69	(Tsai et al. 2009)
S. cerevisiae	Minicellulosome	10	1.8	70	35	(Wen et al. 2010)
S. cerevisiae	Cell surface display in optimum ratio	20	3.1	72	30	This study
S. cerevisiae	Cell surface display in optimum ratio with diploidization	20	7.6	72	75	This study

*Calculated from each result.

Conclusion

We developed a cellulolytic enzymes expression optimized diploid yeast strain that has high ethanol productivity from cellulosic materials. To our knowledge, this is the first report of ethanol production from agricultural waste biomass using a recombinant cellulolytic enzyme-expressing diploid yeast without the addition of exogenous enzymes. In addition, the diploid strain can be cultivated in inexpensive industrial medium while retaining high PASC degradation activity. Multi-gene expression optimization in yeast using our cocktail δ-integration method appears applicable for improving the efficiency of ethanol production from various types of readily available lignocellulosic biomass.

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GENERAL CONCLUSION

This study was carried out to developed high performance yeast strain to achieve efficient and cost-effective bioethanol production from starch-rich or lignocellulosic real biomass by CBP.

In Part I, Chapter 1, to construct a stable and efficient process for the direct production of ethanol from raw starch using a recombinant *S. cerevisiae*, three recombinant yeast strains were constructed, two haploid strains (MT8-1SS and NBRC1440SS) and one diploid strain (MN8140SS). The recombinant strains were constructed by integrating the glucoamylase gene from *Rhizopus oryzae* fused with the 3'-half of the α-agglutinin gene as the anchor protein, and the α-amylase gene from *Streptococcus bovis,* respectively, into their chromosomal DNA by homologous recombination. The diploid strain MN8140SS was constructed by mating these opposite types of integrant haploid strains in order to enhance the expression of integrated amylase genes. The diploid strain had the highest ethanol productivity and reusability during fermentation from raw starch. Moreover, the ethanol production rate of the integrant diploid strain was maintained when batch fermentation was repeated three times (0.67, 0.60, and 0.67 g/l/h in each batch). These results clearly show that a diploid strain developed by mating two integrant haploid strains is useful for the establishment of an efficient ethanol production process.

In Part I, Chapter 2, to improve levels of amylase gene expression and the practical potential of yeast, novel recombinant strategy combining δ-integration and polyploidization through cell fusion were developed. *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 10-fold, 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.

In Part I, Chapter 3, to achieve cost-effective and efficient ethanol production by CBP from starch-rich biomass, the ethanol productivity from real biomass high-yielding rice was evaluated using the polyploid yeast strain. Ethanol productivity from high-yielding brown rice (1.08 g/L/h) was 4.7 fold higher than that obtained from purified raw corn starch (0.23 g/L/h) when nutrients were added. Using an inoculum volume equivalent to 10% of the fermentation volume without any nutrient supplementation resulted in ethanol productivity and yield reaching 1.21 g/L/h and 101%, respectively, in a 24-hour period. High-yielding rice was demonstrated to be a suitable feedstock for bioethanol production. In addition, our polyploid amylase-expressing yeast was sufficiently robust to produce ethanol efficiently from real biomass. This is first report of direct ethanol production on real biomass using an amylase-expressing yeast strain without any pretreatment or commercial enzyme addition.

In Part II, Chapter 1, to construct engineered yeast with efficient cellulose degradation, simple method to optimize cellulase expression levels named cocktail δ-integration was developed. The filamentous fungus *T. reesei* effectively degrades cellulose and is known to produce various cellulolytic enzymes such as β-glucosidase, endoglucanase, and cellobiohydrolase. The expression levels of each cellulase are controlled simultaneously, and their ratios and synergetic effects are important for effective cellulose degradation. However, in recombinant *Saccharomyces cerevisiae*, it is difficult to simultaneously control many different enzymes. To construct engineered yeast with efficient cellulose degradation, we developed a simple method to optimize cellulase expression levels, named cocktail δ-integration. In cocktail δ-integration, several kinds of cellulase expression cassettes are integrated into yeast chromosomes simultaneously in one step, and strains with high cellulolytic activity (i.e., expressing an optimum ratio of cellulases) are easily obtained. Although the total integrated gene copy numbers of cocktail δ-integrant strain was about half that of a conventional δ-integrant strain, the phosphoric acid swollen cellulose (PASC) degradation activity (64.9 mU/g-wet cell) was higher than that of a conventional strain (57.6 mU/g-wet cell). This suggests that optimization of the cellulase expression ratio improves PASC degradation activity more so than overexpression. To our knowledge, this is the first report on the expression of cellulase genes by δ-integration and optimization of various foreign genes by δ-integration in yeast. This

method should be very effective and easily applied for other multi-enzymatic systems using recombinant yeast.

In Part II, Chapter 2, to produce ethanol by CBP from rice straw, one of the most abundant lignocellulosic biomass, cellulolytic enzymes expression optimized diploid strain was developed. The diploid strain prepared using molasses medium (381.4 mU/g-wet cell) showed about 6-fold higher PASC degradation activity than haploid strain (63.5 mU/g-wet cell). In ethanol fermentation from PASC, the diploid strain produced 7.6 g/L of ethanol in 72 h, and the ethanol yield from initial sugar reached 75% of the theoretical yield. Moreover, the diploid strain could produce 7.5 g/L of ethanol from pretreated rice straw in 72 h. To our knowledge, this is the first report about ethanol production from agricultural waste biomass using cellulolytic enzymes expressing yeast without any exogenous enzyme addition.

In this study, to realize cost-effective and efficient ethanol production by CBP from starch-rich and/or lignocellulosic real biomass, innovative recombinant yeast strains were developed by novel strategies such as combining multi gene integration and polyploidization and gene expression optimization. These achievements should be the next big step for industrial cost-effective and efficient ethanol production by CBP from various biomasses.

PUBLICATION LIST

Part I.

Chapter 1.

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Part II.

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