J. Microbiol. Biotechnol. (1998), **8**(5), 441 – 448

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Development of Cellobiose-utilizing Recombinant Yeast for Ethanol Production from Cellulose Hydrolyzate

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Received: March 25, 1998

Abstract A cellobiose-utilizing recombinant yeast having β-glucosidase activity was developed for ethanol production from a mixture of glucose and cellobiose. Using δ-sequences of Ty1 transposon of yeast as target sites for homologous recombination, a heterologous gene of β-glucosidase was integrated into the chromosome of *Saccharomyces cerevisiae*. The δ-integrated recombinant yeast, *Saccharomyces cerevisiae* L2612 (pδ-BGL), showed perfect mitotic stability even in nonselective media and showed ca. 1.5 fold higher β-glucosidase activity than the recombinant yeast harboring the 2μ-based plasmid vector system. A mathematical model was developed to describe the β-glucosidase formation and ethanol production from the *Saccharomyces cerevisiae* L2612 (pδ-BGL). The model newly described that the heterologous β-glucosidase production mediated by *ADH1* promoter is regulated by glucose and repressed by ethanol.

Key words: *Saccharomyces cerevisiae*, β-glucosidase, δ-integration, cellobiose, ethanol, modeling, *ADH1* promoter

Ethanol production from cellulosic materials has received increasing attention as an alternative energy source, because cellulose is the most abundant and low-cost biomass material in nature [14]. The ethanol production process is composed of saccharification of cellulose and fermentation of glucose [8]. For the efficient degradation of crystalline cellulose, the cooperative action of cellulolytic enzymes such as exoglucanase, endoglucanase, and β-glucosidase is needed [6]. In order to increase the efficiency of cellulose hydrolysis, the SSF (simultaneous saccharification and fermentation) process where the product inhibition to cellulolytic enzymes can be avoided has been suggested [27]. However, the cost of the cellulolytic enzymes is very high and thus is one of the most critical bottlenecks hindering the feasibility of this process [21]. Much research has been done to resolve this problem.

As an approach to reduce the cost of the enzymes, the SSF process has been conducted with co-cultivation of cellulolytic organisms with ethanologenic organisms [22]. In addition to various trials with co-cultivation, cellulolytic enzymes have been isolated and their structural genes have been introduced to ethanologenic organisms, including yeast which do not possess all of the cellulolytic genes. *Saccharomyces cerevisiae*, one of the yeasts known for ethanol production, has been used as a host strain for constructing a recombinant microorganism which can hydrolyze and utilize cellulosic materials as a carbon source [1, 2, 15, 16, 20, 26]. The recombinant *Saccharomyces cerevisiae* produced cellulolytic enzymes and supplied the enzymes required to carry out the saccharification. However, it is still difficult to apply these recombinant *Saccharomyces cerevisiae* to the ethanol production process from cellulosic materials, because there are many genetic problems in elevating the expression level of the cellulolytic enzymes and stably maintaining their activities during fermentation.

Once cellulose is hydrolyzed, a mixture of glucose, cellobiose, and other oligosaccharides are formed. For efficient ethanol production, cellobiose and oligosaccharides as well as glucose in the mixture must be utilized. Thus, β-glucosidase, which improves the saccharification yield by hydrolyzing cellobiose to glucose and reducing cellulase inhibition [4], was required for the saccharification reaction. In this study, we constructed a cellobiose-utilizing recombinant *Saccharomyces cerevisiae* which can be applied to ethanol fermentation from the cellulose hydrolyzate (a mixture of mainly glucose and cellobiose). To increase and maintain the expression level of β-glucosidase, a heterologous gene of β-glucosidase was integrated into the chromosome of *Saccharomyces*

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cerevisiae by using δ-sequences of the Ty1 transposon of yeast as target sites for homologous recombination. The usage of the δ-integrative vector enables a heterologous gene to be integrated into the chromosome in multicopies, and to be highly and stably expressed [18, 23]. For the development of a process using cellobiose-utilizing yeast, a mathematical model was suggested to describe β-glucosidase expression and ethanol production. This model newly described that the heterologous protein production mediated by *ADH1* promoter is regulated by glucose and is repressed by ethanol.

MATERIALS AND METHODS

Strain and DNA

Saccharomyces cerevisiae L2612 (α *leu2-3 leu2-112 ura 3-52 trp1-289 can1 cyn1 gal*$^+$) was used as a host strain for transformation. Plasmid pδ-BGL was constructed by introducing a β-glucosidase gene into the δ-integrative vector, pδ-neo, containing a bacterial *neo*^{r} gene originally from plasmid pGH54. The expression cassettes of the gene consists of an *ADH1* promoter, a killer toxin signal sequence, a structural gene cloned from *Bacillus circulans* sub sp., and a *PGK* transcription terminal sequence. δ-Integration vector, pδ-BGL, was transformed into *S. cerevisiae* L2612 by the electroporation method. δ-Integrated transformants were selected by their resistance to an aminoglycoside antibiotic G418 (0.5 g/l). Restriction enzyme digestion, electrophoresis, DNA ligation transformation, and DNA preparation from *E. coli* were performed by the standard methods according to Sambrook *et al.* [24].

Media and Cultivation Condition

YPD medium was composed of glucose (20 g/l), yeast extract (10 g/l), and bacto-peptone (20 g/l). YPDC medium was composed of glucose (10 g/l), cellobiose (10 g/l), yeast extract (10 g/l), and bacto-peptone (20 g/l). For the modeling experiment, a semi-synthetic medium which was composed of glucose (5-20 g/l), yeast nitrogen base w/o amino acid (6.7 g/l), casamino acid (6 g/l), and yeast extract (5 g/l) was used.

Batch culture of recombinant yeast was conducted in a 500-ml flask containing 100 ml medium at 30°C, 280 rpm. Seed culture was performed in a cap tube containing 5 ml YPD medium by inoculating a single colony of each recombinant strain. After cultivating for 24 h at 30°C, 280 rpm, the seed culture was transferred to main flasks.

Assays

The copy number of δ-integrated transformants was calculated from Southern hybridization using an ECL (Enhanced Chemo Luminescence, Amersham) kit. Total yeast chromosomal DNA was prepared according to Sambrook *et al.* [24]. DNA fragment digested by appropriate restriction enzymes were separated by electrophoresis in a 0.8% agarose gel, and then transferred onto nylon membrane (Hybond-N$^+$, Amersham). URA3 was used as a standard probe for the calculation of the copy number. The β-glucosidase gene probe was a 792 bp *Pvu*II fragment of pSK-BGL. A laser densitometer was used to scan the relative densities of each blot for the calculation of the copy number of the integrated genes.

The expressed and secreted protein was verified using SDS-polyacrylamide gel electrophoresis. The extracellular proteins were obtained using solvent precipitation according to Bollag and Edelstein [3]. The proteins separated by 10% SDS-PAGE were stained with Coomassie blue.

The mitotic stability was checked as follows: The transformants of yeast were kept growing in liquid YPD medium by transferring 100 μl of culture medium on nonselectable YPD plates at each sampling time. After incubation for 2 days, 100 colonies of each plate were transferred onto YPD-G418 (0.5 g/l) selection plates. The percentages of resistant colonies on the YPD-G418 plate were determined as mitotic stability.

The β-glucosidase activity was determined by measuring the pNP (*p*-nitrophenol) concentration derived from pNPG (*p*-nitrophenyl-β-D-glucose). The reaction mixtures consisted of 450 μl of 10 mM potassium phosphate buffer (pH 5.8), 50 μl of 10 mM pNPG, and 100 μl of cultured supernatant after centrifugation. They were incubated at 45°C for 20 minutes. One unit of pNPGase activity was defined as the amount of enzyme required for releasing total reducing sugar equivalent to 1 μmol of pNP per minute. The pNP concentration was measured by a spectrophotometer (Pharmacia LKB-Ultraspec III) at 420 nm.

Cell growth was monitored with a spectrophotometer (Pharmacia LKB-Ultraspec III) at 600 nm. Residual glucose concentration was measured with a glucose analyzer (YSI 2700, Yellow Springs, OH). Residual cellobiose concentration was determined with HPLC (Waters 510, Waters). The LC column was purchased from Alltech Econoshere and a RI detector (Waters 410, Waters) was used. Ethanol concentration in the culture medium was measured with GC (Shimadzu GC-14B)-FID. The GC column was 2 feet long and 1/8 inch diameter packed with Porapak Q (80-100 mesh). The temperature of the detector was maintained at 280°C, injector temperature was at 250°C, and column temperature was kept at 220°C. The flow rates of N_2, air, and H2 were 200, 50, 50 ml/min, respectively. Isopropanol (10 g/l) was used as an internal standard for ethanol.

RESULTS AND DISCUSSION

Development of Cellobiose-utilizing Yeast

Construction of Cellobiose-utilizing Yeast *S. cerevisiae* L2612 (pδ-BGL). The δ-sequences of the Ty-1 transposon of yeast was used as target sites for recombination, because δ-integration enables a heterologous gene to be integrated into a chromosome in multicopies, and to be highly and stably expressed [18, 23]. For the subcloning of the β-glucosidase expression cassette from plasmid pSK-BGL into pδ-neo, pSK-BGL and pδ-neo were digested by *Sal*I and *Bam*HI, respectively. To make blunt ends, the digested plasmids were treated with Klenow enzymes. Then, they were digested with *Sac*I. The expression cassette fragment from pSK-BGL was ligated with linearized pδ-neo using T4 DNA ligase. The resulting plasmid was named pδ-BGL. The schematic diagram for the construction of integrative vector pδ-BGL is shown in Fig. 1. After cutting with *Sal*I, linearized pδ-BGL was transformed into *Saccharomyces cerevisiae* using electroporation. The conditions of transformation and selection such as DNA concentration, electric capacitance, and G418 concentration could be optimized to get a good recombinant yeast strain [5]. In this study, 5 mg vector DNA was used per transformation and 1.4 kV, 25 MF, 180 Ω was set in the electroporator. The transformant which survived on the 0.5 g/l YPD-G418 plate showed the highest β-glucosidase activity. This new yeast strain was named *S. cerevisiae* L2612 (pδ-BGL).

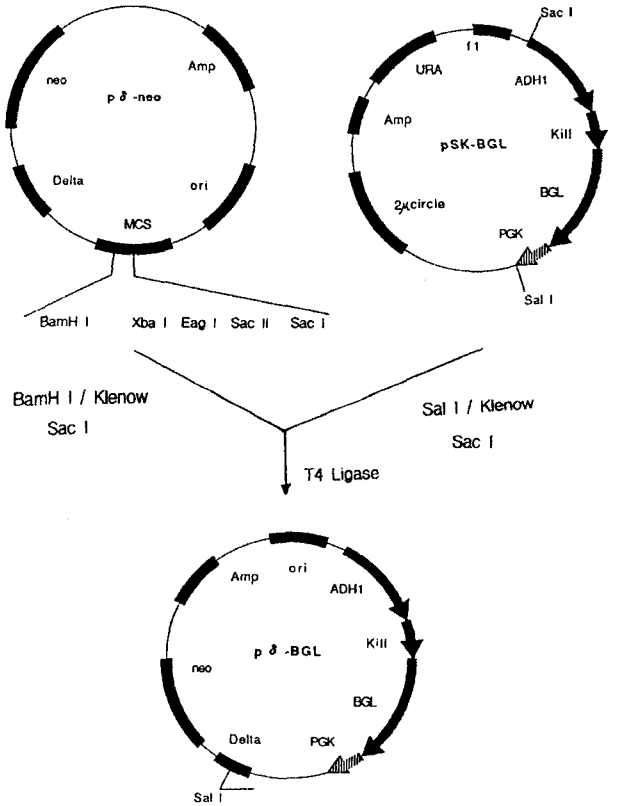

Fig. 1. Schematic diagram for the construction of the integrative vector, pδ-BGL.

By using this δ-integrative vector, multiple copies of the β-glucosidase gene could be integrated into yeast chromosomes. It was reported that the integrated copy number must be elevated to enhance the activity of the enzyme introduced into the yeast strain [5, 16]. To investigate the relation between the δ-integrated copy number of the gene and the expression level of the cloned gene, Southern hybridization was conducted. Several colonies of *S. cerevisiae* L2612 (pδ-BGL) were cultivated in YPD for 40 h and the total chromosomal DNA of the cells were prepared. Chromosomal DNA was digested by *Sac*I and the fragments were separated by electrophoresis. The strain having higher integrated copy numbers showed a higher expression level of β-glucosidase.

To investigate the expression and secretion of β-glucosidase, SDS-PAGE was conducted. The newly constructed yeast, *S. cerevisiae* L2612 (pδ-BGL), successfully expressed and secreted β-glucosidase.

Batch Culture and Characterization of *S. cerevisiae* L2612 (pδ-BGL). *S. cerevisiae* L2612(pδ-BGL) was cultured in YPD and YPDC media (the equivalent amount of glucose) to examine the usage of cellobiose in

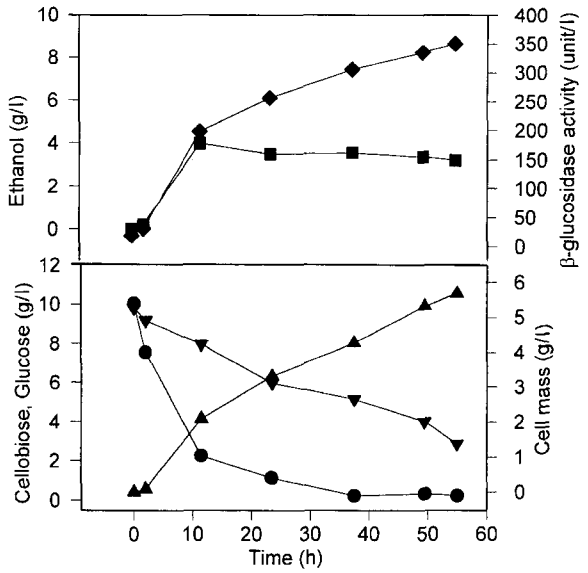

Fig. 2. Time courses of the cell mass, β-glucosidase activity, glucose concentration, cellobiose concentration, and ethanol concentration of L2612 (pδ-BGL) in the YPDC medium.

Initial glucose conc. 10 g/l; initial cellobiose conc. 10 g/l; ▲, cell mass; ◆, β-glucosidase activity; ■, ethanol; ●, glucose; ▼, cellobiose.

the medium. The glucose and cellobiose concentrations were measured, and the cell growth, the ethanol production, and the recombinant protein expression of *S. cerevisiae* L2612 (pδ-BGL) were compared for the different media. Using YPDC medium, cellobiose was utilized by the recombinant yeast, *S. cerevisiae* L2612 (pδ-BGL), as shown in Fig. 2.

The expression levels were measured and compared for the recombinant yeasts using different vector systems. The β-glucosidase activity of δ-integrated *S. cerevisiae* L2612 (pδ-BGL) was about 1.5~1.7 times higher than that of *S. cerevisiae* L2612 (pSK-BGL), which harbored 2 μ-multicopy plasmid (pSK-BGL) as shown in Fig. 3. The δ-integration system makes the gene expression level stable and high.

The mitotic stability of integrated genes was determined using YPD-G418 plates. While the segregational stability of the plasmid harboring yeast, *S. cerevisiae* L2612 (pSK-BGL), was significantly decreased with cultivation, the δ-integrated yeast, *S. cerevisiae* L2612 (pδ-BGL), showed perfect stability even in nonselectable media as shown in Fig. 4.

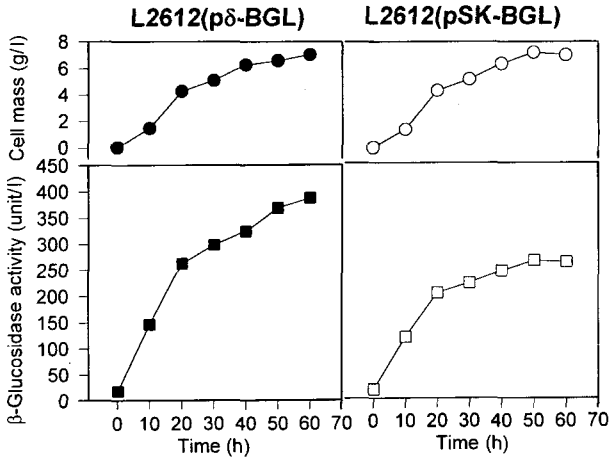

Fig. 3. Comparison of the cell mass and β-glucosidase activity between δ-integrated recombinant yeast [L2612 (pδ-BGL), left] and plasmid-harboring recombinant yeast [L2612 (pSK-BGL), right] in the YPDC medium.

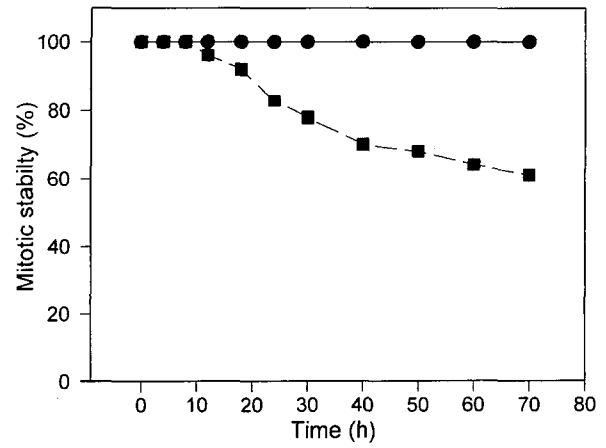

Fig. 4. Comparison of mitotic stability between δ-integrated recombinant yeast [●, L2612 (pδ-BGL)] and plasmid-harboring recombinant yeast [■, L2612 (pSK-BGL)].

The cellobiose-availability of *S. cerevisiae* L2612 (pδ-BGL) was compared with that of the wild-type (host, *S. cerevisiae* L2612) in a glucose and cellobiose mixture fermentation. The results in various media showed that the recombinant yeast utilized cellobiose for the cell growth and ethanol production. More cell mass and ethanol were produced with *S. cerevisiae* L2612(pδ-BGL) than with the wild-type using YPDC medium.

Modeling and Simulation

Model Development for Heterologous Protein Biosynthesis and Ethanol Production. Ollis and Chang [17] developed a mathematical model to describe the behavior of plasmid-haboring cells and growth-associated product formation. The proposed model was based on the framework reported by Imanaka and Aiba [7]. The growth-associated production rate was assumed to be proportional to the specific growth rate [17]. It is common to associate the production of heterologous proteins with plasmid instability and the cell growth. Since the chromosome-integrated *S. cerevisiae* shows perfect mitotic stability, as shown in Fig. 4, the plasmid instability need not be considered for the development of a model to describe the production of heterologous recombinant proteins. In addition, the recombinant protein is deactivated in the media. After all, the production rate of heterologous proteins was composed of two parts. One part is the production rate of the δ-integrated cell, and the other the deactivation of produced extracellular protein.

However, we investigated that the expression level mediated by the *ADH1* promoter is regulated by glucose. It is true that *ADH1* is constitutively expressed, but the expression level is regulated at the transcription stage by glucose [7]. In addition, as shown in Fig. 5, the specific β-glucosidase production rate, $(1/X)(dE/dt)$ was regulated by ethanol as well as glucose. The fact is explained as follows; alcohol dehydrogenase 1 (ADH1) in the yeast catalyzes the regeneration of NAD^+ from NADH with concomitant production of ethanol from acetaldehyde. In the conditions where the glucose concentration is high, the yeast generates many NADHs. Because the regeneration of NAD^+ from NADH is required to balance the ratio of NADH to NAD^+, the expression level of ADH1 is elevated in the yeast. When glucose concentration is low and ethanol concentration is high, the regeneration of NAD^+ from NADH is not required any longer but the

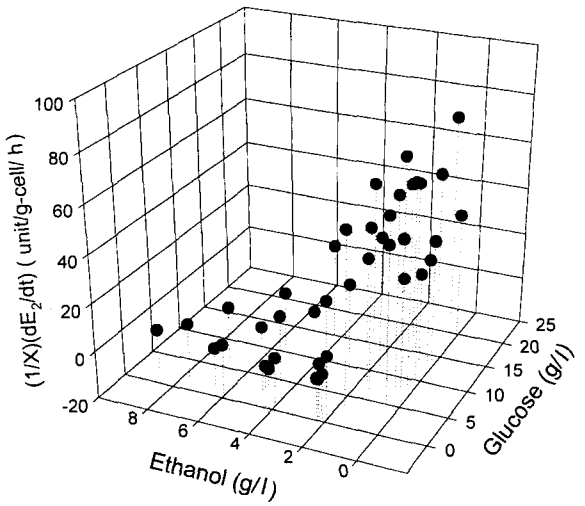

Fig. 5. Variation of the specific β-glucosidase production rate with ethanol and glucose concentration.

generation of energy with concomitant conversion of ethanol to acetaldehyde is required. Thus, the expression level of ADH1 is regulated by the glucose and ethanol concentration. Because the *ADH1* promoter originates from alcohol dehydrogenase 1, the promoter follows the features mentioned above.

It has been reported that the *ADH1* promoter has the regulational sequence in the upstream [10, 12, 25]. Ethanol showed noncompetitive inhibition to the *ADH1* promoter-mediated production. Thus, on the basis of these reports and experimental data, a model equation of recombinant protein production could be suggested. The production of heterologous protein mediated by *ADH1* promoter is no longer constitutive. In other words, the proportional constant in the model proposed by Ollis and Chang is expressed as a function of glucose and ethanol concentration. The glucose effect on the production of heterologous protein using the *ADH1* promoter can be described as a modified Monod model using glucose as substrate [9].

The effect of ethanol on the production of the heterologous protein using the *ADH1* promoter can be considered as a noncompetitive inhibition.

$$\frac{dE}{dt} = \gamma X - K_d E = \left(\frac{\gamma_{max} G}{K_\gamma + G}\right)\left(\frac{K_I}{P + K_I}\right) X - K_d E, \quad (1)$$

where E is the activity of β-glucosidase (unit/l), X is the cell mass (g/l), G is the glucose concentration (g/l), K_d is the deactivation constant for β-glucosidase (h^{-1}), γ is the specific production rate (unit/g-cell·h), γ_{max} is the maximum specific production rate (unit/g-cell·h), K_γ is the glucose saturation constant for gene expression, K_I is the ethanol inhibition constant for gene expression (g/l), and P is the ethanol concentration (g/l).

Since cellobiose was hydrolyzed to glucose by β-glucosidase, the equation of cellobiose-hydrolysis was necessary. As shown in Eq. (2), it is represented by a Michelis-Menten type equation considering glucose inhibition [19].

$$\frac{dB}{dt} = -\frac{k_2 E B}{K_m(1 + G/K_{G2}) + B} \quad (2)$$

where B is the cellobiose concentration (g/l), k_2 is the reaction constant (g/unit·h), K_{G2} is the glucose inhibition constant (g/l), and K_m is the Michelis-Menten constant (g/l).

The glucose consumption rate is expressed by the saccharification rate of cellobiose, the glucose consumption by the cell, and the maintenance energy used by the cell. The constant 1.053 is the hydrolysis constant of cellobiose to two glucose [19]. The equation is represented by

$$\frac{dG}{dt} = 1.053\frac{k_2 E B}{K_m(1 + G/K_{G2}) + B} - \frac{1}{Y}\frac{dX}{dt} - mX \quad (3)$$

where Y is the cell yield from glucose (g-cell/g-glucose), and m is the maintenance constant (h^{-1}).

The cell growth and the ethanol production models were represented as shown in Eqs. (4) and (5), respectively. In these models, Monod type models were used and the ethanol inhibition to the cell growth and ethanol production [13] were also considered.

$$\frac{dX}{dt} = \mu X = \left(\frac{\mu_{max} G}{K_G + G}\right)\left(1 - \frac{P}{P_m}\right) X \quad (5)$$

$$\frac{dP}{dt} = \mu X = \left(\frac{\pi_{max} G}{K_P + G}\right)\left(1 - \frac{P}{P_m^\circ}\right) X \quad (6)$$

where μ is the specific cell growth rate (h^{-1}), μ_{max} is the maximum specific cell growth rate (h^{-1}), π is the specific ethanol production rate (h^{-1}), π_{max} is the maximum specific ethanol production rate (h^{-1}), K_G is the saturation constant for glucose, K_P is the saturation constant for ethanol (g/l), P_m is the ethanol inhibition constant for growth (g/l), and P_m° is the ethanol inhibition constant for ethanol production (g/l).

Parameter Estimation and Simulation

The cell (X) ,the glucose (G), the ethanol (P) concentrations, and the β-glucosidase activity (E) were measured from the batch experiments using the chromosome-integrated yeast L2612 (pδ-BGL) to obtain the kinetic parameters of the proposed model. The parameters for the heterologous protein production [Eq. (1)] were estimated from experimental data and a regression analysis. At first, the deactivation constant (K_d) was determined by the graphical method. The slope of the logarithm plot of β-glucosidase activity was obtained as the value of K_d.

Then, parameters γ_{max}, K_γ, and K_I were estimated using the Simplex method.

The parameters for the cell growth and the ethanol production were determined from the batch experiment. The ethanol inhibition constants P_m and P_m^o in Eqs. (4) and (5) were determined from experimental results [13]. The specific cell growth rate, μ_{max} and the saturation constant for glucose, K_G, were determined by Lineweaver-Burk plotting. Also, the specific ethanol production rate, π_{max} and the saturation constant for ethanol, K_P, were determined by Lineweaver-Burk plotting.

The enzyme kinetic parameter k_2, and K_m in Eqs. (2) and (3) were determined by Lineweaver-Burk plotting using experimental data. K_{G2} was obtained from the reported experimental value [19]. The cell yield coefficient Y was taken by obtaining the average values of the cell mass yield from the glucose consumption in each of the batch cultures. The values of the cell growth, the ethanol production, and the cell yield in this study are similar to those calculated in other studies. The values of the parameters are summarized in Table 1.

Experimental data for a number of batch cultures are presented with simulation results of the proposed model. The simulation results (lines) and the experimental data (symbols) are shown in Fig. 6, in which the initial glucose concentration was 15 g/l. The simulation results using different models of β-glucosidase production were compared. The simulation results using the model [(Eq. (1)] fitted experimental data better than those using the conventional model [9, 17]. As the initial glucose concentration was increased, the simulation results of the conventional model deviated from the experimental data. However, the results of the proposed model follow the trend of experimental data. Under the condition of low ethanol concentration, both of the models predict well the production of heterologous protein mediated by the *ADH1* promoter. However, under the high ethanol concentration conditions, the model proposed in this study can predict the trend of the production of heterologous protein well. Experimental data (symbols) and simulation results (lines) for the proposed model were compared in

Table 1. Parameter values of fermentation and gene expression.

Parameter	Value	Method
μ_{max}	0.481 h^{-1}	experiment
K_G	0.338 g/l	experiment
π_{max}	1.42 h^{-1}	experiment
K_P	0.536 g/l	experiment
Y	0.155	experiment
γ_{max}	98.2 unit/g-cell·h	estimated
K_γ	0.512 g/l	estimated
K_I	1.22 g/l	estimated
K_d	0.015 h^{-1}	experiment
k_2	0.902 mg/unit·h	experiment
K_m	0.913 g/l	experiment
K_{G2}	0.610 g/l	[19]
P_m	82.5 g/l	experiment
P_m^o	120 g/l	experiment
m	0.001 h^{-1}	experiment

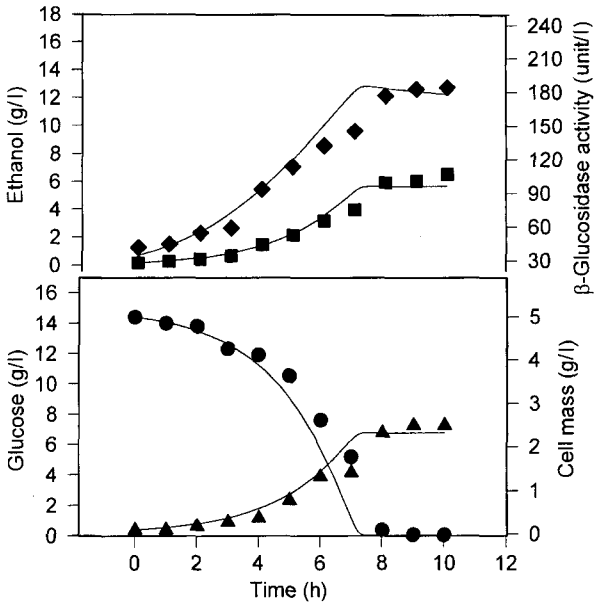

Fig. 6. Experimental data (symbols) and simulation results (lines) of δ-integrated recombinant yeast [L2612 (pδ-BGL)] fermentation and β-glucosidase activity on glucose.

Initial glucose conc. 15 g/l; ▲, cell mass; ◆, β-glucosidase activity; ■, ethanol; ●, glucose.

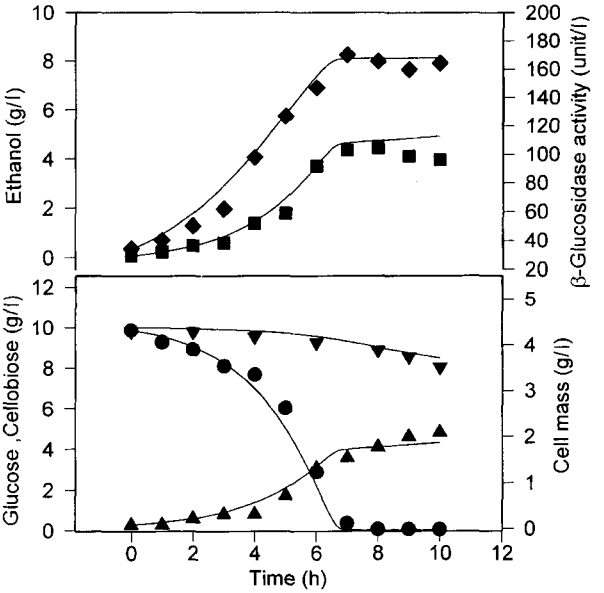

Fig. 7. Experimental data and simulation results of δ-integrated recombinant yeast L2612 (pδ-BGL) fermentation and β-glucosidase activity on glucose and cellobiose.

Initial glucose conc. 10 g/l; initial cellobiose conc. 10 g/l; ▲, cell mass; ◆, β-glucosidase activity; ■, ethanol; ●, glucose; ▼, cellobiose.

the mixture of 10 g/l glucose and 10 g/l cellobiose as shown in Fig. 7 and they fit well. Using the mathematical model, the behavior of *S. cerevisiae* L2612 (pδ-BGL) can be understood. The model can be applied to the development of efficient ethanol production from cellulose-hydrolyzate using the recombinant yeast developed in the present study.

CONCLUSION

Our new recombinant yeast, *S. cerevisiae* L2612 (pδ-BGL), expressed heterologous β-glucosidase and utilized cellobiose as a carbon source. It showed perfect mitotic stability and a higher expression level compared to the recombinant yeast using Yep-type plasmid vector. Thus, there can be merits in using the new strain for the production of ethanol from the glucose and cellobiose mixture. In addition, an unstructured mathematical model was developed to describe the β-glucosidase formation, the cell growth, and the ethanol production of the *S. cerevisiae* L2612 (pδ-BGL). We newly described that the heterologous β-glucosidase production mediated by the *ADH1* promoter is regulated by glucose and repressed by ethanol. The developed recombinant yeast and its mathematical model can be used for the development of an efficient ethanol production process from a cellulose-hydrolyzed mixture.

Acknowledgments

This work was supported by a grant from Korea Institute of Energy and Resources.

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