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ORIGINAL RESEARCH PAPER

Construction of a direct starch-fermenting industrial strain of *Saccharomyces cerevisiae* producing glucoamylase, α-amylase and debranching enzyme

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Abstract To develop a strain of *Saccharomyces cerevisiae* that produces ethanol directly from starch, two integrative vectors were constructed to allow the simultaneous multiple integration of the *Aspergillus awamori* glucoamylase gene (*GA1*) and the *Debaryomyces occidentalis* α-amylase gene (*AMY*) and glucoamylase with debranching activity gene (*GAM1*) into the chromosomes of an industrial strain of *S. cerevisiae*. The *GA1* and *AMY* genes were constitutively expressed under the *ADC1* promoter in *S. cerevisiae* using the double δ-integration system. The *GAM1* gene was constitutively expressed under the corresponding promoter using the double 18S rDNA-integration system. The recombinant industrial strain secreting biologically active α-amylase, glucoamylase and debranching enzyme was able to ferment starch to ethanol in a single step. The new strain produced 8% (v/v) ethanol (62.8 g l^{-1}) from 20% (w/v) soluble starch after 2 days, fermentation.

Keywords Amylase · *Aspergillus awamori* · *Debaryomyces occidentalis* · Glucoamylase · *Saccharomyces cerevisiae* · Starch

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Introduction

The conversion of starchy biomass to glucose, which is an important renewable biological resource for industrial and fuel ethanol production, entails liquefaction and saccharification using α-amylase, glucoamylase and debranching enzyme (Janse and Pretorius 1995; Eksteen et al. 2003). Traditionally, ethanol production has been conducted almost exclusively by *Saccharomyces cerevisiae* because of its high fermentation rate and ethanol tolerance. Unfortunately, *S. cerevisiae* cannot degrade starch naturally. Manipulation of *S. cerevisiae* to synthesize and secrete amylolytic enzymes would contribute to the direct conversion of starch-rich materials to ethanol (Marin et al. 2001). Many genes from filamentous fungi and yeasts that encode amylolytic enzymes have been expressed in *S. cerevisiae* (Kim and Kim 1996; Shigechi et al. 2004). For example, *Aspergillus awamori* glucoamylase capable of degrading raw starch is widely used to hydrolyze both α-1,4- and α-1,6-glucosidic bonds of starch for saccharification.

However this glucoamylase exhibits low debranching activity toward the α-1,6-linkages which could lead to incomplete hydrolysis of starch (Ma et al. 2000).

Of the more than 150 starch-assimilating yeast species, *Debaryomyces occidentalis*, which secretes both α-amylase and glucoamylase, expresses significant debranching activity as a part of the glucoamylase (Dohmen et al. 1990). Industrial strains of *S. cerevisiae* that could express the *A. awamori* glucoamylase gene (*GA1*), *D. occidentalis* α-amylase gene (*AMY*) and glucoamylase with debranching activity gene (*GAM1*) would be capable of efficiently producing ethanol from starch (Janse and Pretorius 1995). Transformation of industrial *S. cerevisiae* with *GA1*, *AMY* and *GAM1* genes can be achieved by their integration into the genome via homologous recombination (Lee and Da Silva 1997; Nieto et al. 1999). In this study, we constructed two linearized integrative vectors to develop an amylolytic industrial strain of *S. cerevisiae* that expresses high glucoamylase, α-amylase and debranching enzyme activities via *δ*-integration of the *GA1* and *AMY* genes and 18S rDNA-integration of the *GAM1* genes. The growth, substrate utilization and enzyme activity of the recombinant strain were then examined. We also analyzed the fermentative activity of the new strain.

Materials and methods

Strains and plasmids

Escherichia coli DH5α was used for the transformation and plasmid construction. An industrial polyploid strain of *S. cerevisiae* ATCC 9763 distillery yeast (Ness et al. 1993) was used as the host for the yeast transformation experiment. Plasmid YIpAURG1 and YIp*δ*AURSG*δ* (Ghang et al. 2005, 2007) were used to clone the *GA1* gene and *GAM1* gene, respectively. YIp*δ*AURDpSA*δ* and YIpGB2 (Choi et al. 2002; Lim et al. 2008) served as the backbones of the *δ*-integrative system and the 18S rDNA-integrative system, respectively.

Media and culture conditions

The YPD medium [1% (w/v) yeast extract, 1% (w/v) Bacto-peptone and 2% (w/v) glucose] was used for the propagation of *S. cerevisiae*. Yeast transformants were grown on YPD plates containing aureobasidin A (1 μg ml^{-1}, TaKaRa) and/or G418 (300 μg ml^{-1}, Sigma) and then transferred onto YPDS3 plates [YPD containing 3% (w/v) soluble starch] and incubated for 4 days at 30°C, after which they were incubated at 4°C for 2 days. Buffered YPS medium (BYPS) containing 2% (w/v) soluble starch and 0.1 M sodium phosphate buffer (pH 6.0) was used to assay the activity of amylase secreted by the yeast transformants. The presence of residual starch was assayed using a starch-iodine reaction to measure the loss of iodine staining capacity (Ghang et al. 2007). The mitotic stability of the *AMY* or *GAM1* gene was determined using the method described by Nieto et al. (1999).

DNA manipulation and yeast transformation

All DNA manipulations and the transformation of *E. coli* were conducted as described by Sambrook and Russell (2001). Integrative transformation of yeast was carried out using the lithium acetate method described by Gietz et al. (1992).

Construction of integrative plasmids

To construct the double *δ* system containing the *GA1* and *AMY* genes, a 2.1 kb fragment harboring the *GA1* gene was excised from YIpAURG1 and inserted into the *Sma*I–*Xba*I sites downstream of the *ADC1* promoter (*ADC1p*) in YIp*δ*AURDpSA*δ* lacking the *phytDc* gene, thereby generating YIp*δ*AGSA*δ* (Fig. 1). A 3.3 kb fragment harboring the *ADC1p* and *GAM1* gene was excised from YIp*δ*AURSG*δ* and inserted into the *Sph*I–*Spe*I sites in rDNA-integration vector, YIpGB2, lacking the *ADC1p* and *βAMY* gene to obtain YIpSGrD. To construct the double rDNA system, a 1.8 kb 18S rDNA fragment was ligated with the linearized YIpSGrD that had been treated with *Spe*I, thereby generating YIpSG2rD (Fig. 1).

Real-time PCR and enzyme assays

Real-time PCR was performed on the Roter-Gene 3000 System (Corbett Research) using a mixture of *D. occidentalis* genomic DNA or *S. cerevisiae* transformant genomic DNA as the template DNA, *GAM1* or *AMY*, *β*-actin primers and the reagents in a SYBR Green PCR Master Mix Reagent Kit (Qiagen).

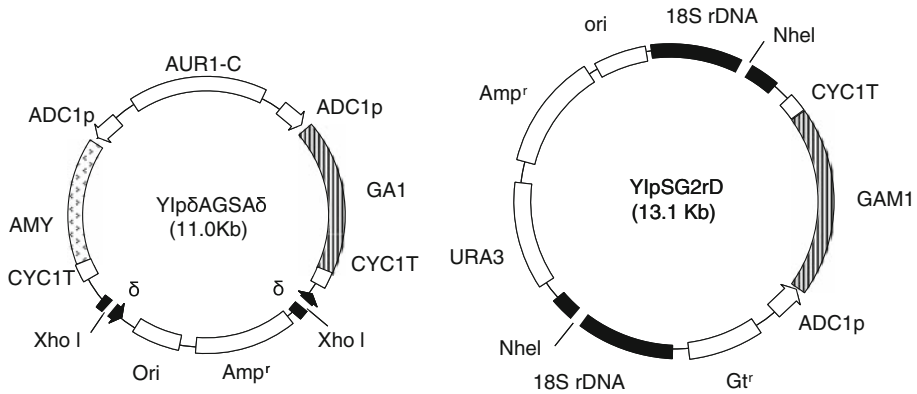

Fig. 1 Plasmid maps of YIpδAGSAδ and YIpSG2rD showing the relative size, restriction sites and locations of the insert DNA. YIpδAGSAδ and YIpSG2rD were linearized by digestion with *Xho*I and *Nhe*I, respectively

The primers used were as follows: *GAM1*, 5′-AA CATGCAAGGTGACTCCGA-3′ and 5′-GCCCAAT CAGCAGTGTTGTC-3′; *AMY*, 5′-CGATCTGGATC TCCCCAGTT-3′ and 5′-TTGAACCATCTCCGTT CCAA-3′; β-actin, 5′-GATCTGGCACCACACCT TCT-3′ and 5′-GGGGTGTTGAAGGTCTCAAA-3′. The glucoamylase and α-amylase activities were quantified at pH 5.5 and 40°C using the PGO/ODAD assay (Sigma) and dinitrosalicylic acid method, respectively (Ghang et al. 2007). All assays were repeated three times and the means were calculated. The enzymatic reaction products were analyzed by TLC using silica gel plates (Merck). SDS-PAGE was conducted on 10% (w/v) gels and the proteins were visualized by Coomassie Blue staining.

Ethanol fermentation and assay

The evaluation of ethanol production via fermentation was conducted using the method described by Ma et al. (2000). Briefly, a single colony of *S. cerevisiae* transformant was inoculated and aerobically grown in 10 ml of YPS medium at 30°C in a shaking incubator at 200 rpm for 24 h. The culture was inoculated into 100 ml of YPS containing 2% (w/v) yeast extract, 2% (w/v) Bacto-peptone and 5–30% (w/v) soluble starch (Difco) and then incubated at 30°C for 7 days. The culture was also inoculated into 100 ml of corresponding medium in a closed bottle equipped with a bubbling CO_2 outlet at 30°C for 7 days. The supernatants were assayed for ethanol content using a QuantiChrom ethanol assay kit (BioAssay Systems). Conversions: 1% (v/v) ethanol is equivalent to 7.85 g l^{-1} or 170 mM.

Results and discussion

Integration of the *GA1*, *AMY* and *GAM1* genes into *S. cerevisiae*

To enable multiple-copy stable integration of the *GA1* and *AMY* genes into the chromosomes of *S. cerevisiae*, YIpδAGSAδ was linearized by digesting δ sequences with *Xho*I (Fig. 1) and the unneeded sequences related to bacterial DNA and ampicillin resistance marker (2.8 kb) were removed to produce a smaller linear vector prior to transformation. These resulting fragments (8.2 kb) containing the *ADC1p-GA1* and *ADC1p-AMY* gene cassettes flanked by δ sequences could be integrated into δ sequences dispersed throughout the *S. cerevisiae* genome to produce multicopy integrants (Ghang et al. 2007; Lim et al. 2008). The *GAM1* gene was separately introduced into *S. cerevisiae* targeted to the rDNA sequences since δ-integrative system containing *GA1*, *AMY* and *GAM1* gene cassettes (13 kb) could exhibit lower amylolytic activity and mitotic stability, possibly because of low copy integrations by long integrating constructs (data not shown). YIpSG2rD was linearized by digesting 18S rDNA sequences with *Nhe*I (Fig. 1) and the unnecessary *URA3* and bacterial DNA sequences except for the G418 resistance marker (5.0 kb) were excised prior to transformation. These resulting fragments (8.1 kb), containing the *ADC1p-GAM1* cassette

flanked by 18S rDNA sequences, could be integrated into the rDNA cluster on chromosome XII via homologous recombination to introduce multiple copies of the *GAM1* gene into the integrants (Nieto et al. 1999). Real-time PCR reactions were conducted using two primers specific for the *AMY* gene or two primers specific for the *GAM1* gene and genomic DNAs from *D. occidentalis* and *S. cerevisiae* transformants exhibiting high *AMY* and *GAM1* activities as a template. The *AMY* and *GAM1* DNAs from one *S. cerevisiae* transformant were increased 46-fold and 12-fold relative to *D. occidentalis*, respectively. It was assumed that these increases were due to the presence of multiple copies of the *AMY* and *GAM1* genes integrated at the δ sequences and rDNA locus of *S. cerevisiae*. No amplified *AMY* or *GAM1* genes were detected from the wild-type *S. cerevisiae* ATCC 9763.

Expression and secretion of *GA1*, *AMY* and *GAM1* in an industrial strain of *S. cerevisiae*

Industrial *S. cerevisiae* ATCC 9763 distillery yeast was transformed with YIpδAGSAδ, generating ATCC 9763/YIpδAGSAδ expressing the *GA1* and *AMY* genes. To obtain *S. cerevisiae* transformants expressing the *GA1*, *AMY* and *GAM1* genes, YIpSG2rD was integrated into ATCC 9763/YIpδAGSAδ, thereby generating ATCC 9763/YIpδAGSAδ/YIpSG2rD. Both *S. cerevisiae* transformants could utilize starch in the medium whereas the parental wild-type could not. SDS-PAGE revealed that the recombinant *GA1* band (83 kDa), α-amylase band (55 kDa) and *GAM1* band (145 kDa) were present in the culture supernatant of ATCC 9763/YIpδAGSAδ/YIpSG2rD (data not shown). The α-amylase and glucoamylase activities were examined in culture supernatants from transformants grown in the BYPS media. The clones showing the highest activities among the transformants were selected for further analysis.

As shown in Table 1, the activities of the α-amylase and glucoamylase produced by ATCC 9763/YIpδAGSAδ/YIpSG2rD were 7.6-times and 4.1-times higher than those of *D. occidentalis* (Ghang et al. 2007). The multicopy integration of the *AMY*, *GA1* and *GAM1* genes may be correlated with the high-level expression of the corresponding genes (Lee and Da Silva 1997; Nieto et al. 1999). Moreover, the glucoamylase activity of ATCC 9763/YIpδAGSAδ/YIpSG2rD, which expressed both the *GA1* and *GAM1* genes, was 1.8-times higher than that of ATCC 9763/YIpδAGSAδ, which only expressed the *GA1*. The increase in glucoamylase activity was likely due to a synergistic effect of the sequential δ-*GA1* integration and rDNA-*GAM1* integration (Choi et al. 2002).

As shown in Table 2, the *GAM1* of ATCC 9763/YIpδAGSAδ/YIpSG2rD could hydrolyze isomaltose and pullulan to glucose whereas the *GA1* of ATCC 9763/YIpδAGSAδ could not. This result was confirmed by the analysis of enzymatic reaction products using TLC (Fig. 2). Ma et al. (2000) reported that the *GA1*-encoded glucoamylase has limited α-1,6 activity. The co-expression of *GAM1* with significant debranching activity (α-1,6 activity) leads to the complete hydrolysis of starch for the production of more ethanol (Janse and Pretorius 1995). Time course analyses of the α-amylase and glucoamylase activities

Table 1 α-Amylase and glucoamylase activities in cell-free culture supernatants of *S. cerevisiae* transformants

Yeast strains	α-Amylase activity[a] (U ml^{-1})	Glucoamylase activity (U ml^{-1})
S. cerevisiae ATCC 9763	ND[b]	ND
ATCC 9763/YIpSG2rD	ND	0.79[c]
ATCC 9763/YIpδAGSAδ	5.82	0.56
ATCC 9763/YIpδAGSAδ/YIpSG2rD	5.94	1.02
D. occidentalis CBS 2863	0.78[d]	0.25

[a] Yeast cells were grown in BYPS media containing 0.1 M sodium phosphate buffer (pH 6.0) at 30°C for 4 days

[b] Not detected

[c] Values were the means of the results of triplicate experiments and were expressed in amylolytic activities present in the culture supernatants

[d] Ghang et al. (2007)

Table 2 Hydrolysis of isomaltose and pullulan by the action of glucoamylase from ATCC 9763/YIpδAGSAδ and ATCC 9763/YIpδAGSAδ/YIpSG2rD

Yeast strains	Substrates	Glucoamylase activity (U ml^{-1})
ATCC 9763/YIpδAGSAδ	Isomaltose	ND[a]
	Pullulan	ND
ATCC 9763/YIpδAGSAδ/YIpSG2Rd	Isomaltose	0.51[b]
	Pullulan	0.12

[a] Not detected

[b] Glucoamylase activity was determined using 0.5% (w/v) isomaltose and pullulan as substrates instead of soluble starch. Values were the means of the results of triplicate experiments

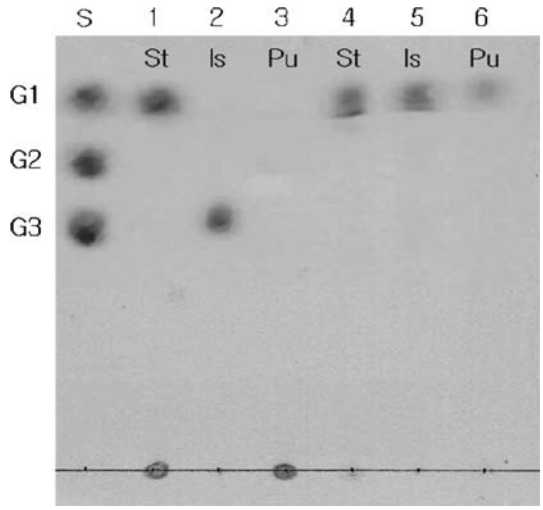

Fig. 2 Thin-layer chromatography of the enzymatic products from soluble starch, isomaltose and pullulan by glucoamylases from *S. cerevisiae* transformants. Substrates: *St* soluble starch, *Is* isomaltose, *Pu* pullulan. *Lanes*: *S* standards (G1: glucose, G2: maltose, G3: maltotriose); *1–3 GA1* from ATCC 9763/YIpδAGSAδ; *4–6 GA1* and *GAM1* from ATCC 9763/YIpδAGSAδ/YIpSG2rD

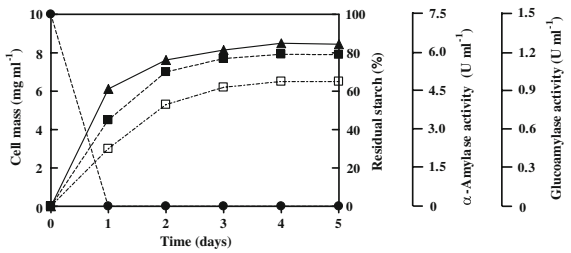

Fig. 3 Growth curve, time courses of starch hydrolysis and extracellular glucoamylase activities and α-amylase activities produced by ATCC 9763/YIpδAGSAδ/YIpSG2rD in the BYPS medium. Growth was measured on different days based on the cell dry weight and glucoamylase and α-amylase activities were measured in the culture supernatants. The remaining starch results were presented as percentages taking the starch in the uninoculated medium as 100%. Each point represents the means of three independent measurements with a standard deviation of ±5%. *Filled triangle*, mg ml^{-1} cell mass; *filled square*, U ml^{-1} α-amylase activities; *open square*, U ml^{-1} glucoamylase activities; *filled circle*, % residual starch

and cell growth for ATCC 9763/YIpδAGSAδ/YIpSG2rD over 5 days are shown in Fig. 3.

α-Amylase and glucoamylase were growth-associated and reached maximal activities after 4 days of growth. ATCC 9763/YIpδAGSAδ/YIpSG2rD utilized 100% of the soluble starch in a culture medium that contained 2% (w/v) soluble starch during 24 h growth. To examine the mitotic stability, ATCC 9763/YIpδAGSAδ and ATCC 9763/YIpSG2rD were cultivated in non-selective YPD media for 50 generations. On the YPS plate, 100% of the integrant colonies still exhibited halos. These results suggest that the δ-integrated *GA1* and *AMY* genes and rDNA-integrated *GAM1* gene were stably maintained in the chromosomes of the integrants (Nieto et al. 1999; Ghang et al. 2007).

Ethanol production from starch

The ability of *S. cerevisiae* ATCC 9763 and ATCC 9763/YIpδAGSAδ/YIpSG2rD to produce ethanol directly via the fermentation of 20% (w/v) soluble starch was examined (Fig. 4). As expected, the wild-type *S. cerevisiae* ATCC 9763 could not produce any ethanol, whereas ATCC 9763/YIpδAGSAδ/YIpSG2rD fermented starch to ethanol directly. After 2 days of fermentation, 8% (v/v) ethanol (62.8 g l^{-1}) was produced, and the maximum ethanol concentration was attained after 7 days. Additionally, the starch content decreased drastically during fermentation as

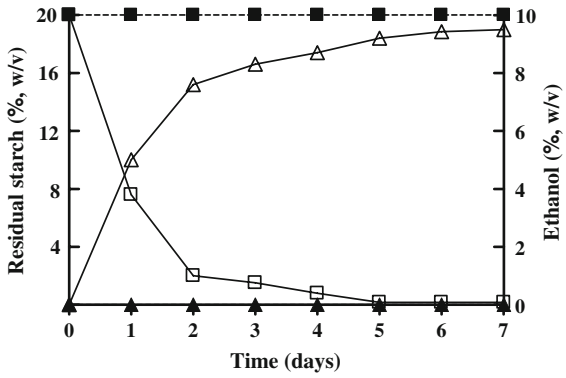

Fig. 4 Time courses of direct ethanol production via fermentation from starch by *S. cerevisiae* ATCC 9763 and ATCC 9763/YIpδAGSAδ/YIpSG2rD. Each point represents the means of three independent measurements with a standard deviation of ±5%. *Filled triangle*, ethanol concentration in *S. cerevisiae* ATCC 9763; *open triangle*, ethanol concentration in ATCC 9763/YIpδAGSAδ/YIpSG2rD; *filled square*, residual starch concentration in *S. cerevisiae* ATCC 9763; *open square*, residual starch concentration in ATCC 9763/YIpδAGSAδ/YIpSG2rD

the ethanol concentration increased to 9.5% (v/v) (74.6 g l^{-1}). The residual starch was 0.16% (w/v) after 7 days of fermentation indicating that the starch was almost completely degraded and converted to ethanol (Shigechi et al. 2004). Kim and Kim (1996) reported that an industrial *S. cerevisiae* strain secreting *S. diastaticus* glucoamylase and mouse salivary α-amylase produced 7.5% (v/v) ethanol in 20% (w/v) starch-containing medium after 8 days of fermentation, and the residual starch content after fermentation was 1.68% (w/v). These results indicate that the introduction of *GA1* and *GAM1* with α-1,4 and α-1,6 activity resulted in increased starch hydrolysis rates and ethanol production when compared to *S. diastaticus* glucoamylase with α-1,4 activity (Janse and Pretorius 1995; Ma et al. 1999).

When ethanol production in YPS medium containing soluble starch of over 5% (w/v) was examined, the final concentration of ethanol produced by this strain after 7 days of fermentation was 5.9% (v/v) (46.3 g l^{-1}) and 10.2% (v/v) (80.1 g l^{-1}) in medium containing 10% (w/v) and 30% (w/v) starch, respectively. In the present study, ethanol production increased gradually as the starch concentration of the medium increased although the yield of ethanol produced per gram of starch did not increase in proportion to starch concentration due to exhaustion of other nutrient components. Birol et al. (1998) reported that a haploid laboratory strain of *S. cerevisiae* strain secreting *A. awamori* glucoamylase and *Bacillus subtilis* α-amylase produced 43.8 g ethanol l^{-1} in 10% (w/v) starch-containing medium. However, the haploid laboratory strain does not have fermentation characteristics as good as the industrial strain due to its genetic instability (Kim and Kim 1996). In this study, a direct and efficient starch-fermenting industrial polyploid yeast strain was produced by multiple integrations of the *GA1*, *AMY* and *GAM1* genes. The results presented here may lead to the development of various industrial strains of *S. cerevisiae* for the production of commercially important products, including ethanol, from starch in a one-step process (Lin et al. 1998; Eksteen et al. 2002; Shigechi et al. 2004).

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References

Birol G, Onsan ZI, Kirdar B, Oliver SG (1998) Ethanol production and fermentation characteristics of recombinant *Saccharomyces cerevisiae* strains grown on starch. Enzym Microb Technol 22:672–677

Choi EY, Park JN, Kim HO, Shin DJ, Im SY, Lee HB, Chun SB, Bai S (2002) Construction of an industrial polyploid strain of *Saccharomyces cerevisiae* containing *Saprolegnia ferax* β-amylase gene and secreting β-amylase. Biotechnol Lett 24:1785–1790

Dohmen RJ, Strasser AWM, Dahlems UM, Hollenberg CP (1990) Cloning of the *Schwanniomyces occidentalis* glucoamylase gene (*GAM1*) and its expression in *Saccharomyces cerevisiae*. Gene 95:111–121

Eksteen JM, van Renseburg P, Cordero Otero RR, Pretorius IS (2003) Starch fermentation by recombinant *Saccharomyces cerevisiae* strains expressing the α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. Biotechnol Bioeng 84:639–646

Ghang DM, Lee SA, Chun YH, Chin JE, Ko HM, Lee HB, Bai S (2005) Expression of *Aspergillus awamori* glucoamylase gene in an industrial strain of *Saccharomyces cerevisiae*. Korean J Microbiol 41:146–151

Ghang DM, Yu L, Lim MH, Ko HM, Im SY, Lee HB, Bai S (2007) Efficient one-step starch utilization by industrial strains of *Saccharomyces cerevisiae* expressing the glucoamylase and α-amylase genes from *Debaryomyces occidentalis*. Biotechnol Lett 29:1203–1208

Gietz D, St. Jean A, Woods R, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425

Janse BJ, Pretorius IS (1995) One-step enzymatic hydrolysis of starch using a recombinant strain of *Saccharomyces cerevisiae* producing α-amylase, glucoamylase and pullulanase. Appl Microbiol Biotechnol 42:876–883

Kim TG, Kim K (1996) The construction of a stable starch-fermenting yeast strain using genetic engineering and rare-mating. Appl Biochem Biotechnol 59:39–51

Lee FWF, Da Silva NA (1997) Improved efficiency and stability of multiple cloned gene insertions at the δ sequences of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 48:339–345

Lim MH, Lee OH, Chin JE, Ko HM, Kim IC, Lee HB, Im SY, Bai S (2008) Simultaneous degradation of phytic acid and starch by an industrial strain of *Saccharomyces cerevisiae* producing phytase and α-amylase. Biotechnol Lett 30: 2125–2130

Lin LL, Ma YJ, Chien HR, Hsu WH (1998) Construction of an amylolytic yeast by multiple integration of the *Aspergillus awamori* glucoamylase gene into a *Saccharomyces cerevisiae* chromosome. Enzym Microb Technol 23:360–365

Ma Y, Lin LL, Chien HR, Hsu WH (2000) Efficient utilization of starch by a recombinant strain of *Saccharomyces cerevisiae* producing glucoamylase and isoamylase. Biotechnol Appl Biochem 31:55–59

Ness F, Lavallee F, Dubourdieu D, Aigle M, Dulau L (1993) Identification of yeast strains using the polymerase chain reaction. J Sci Food Agric 62:89–94

Nieto A, Prieto JA, Sanz P (1999) Stable high-copy number integration of *Aspergillus orizae* α-amylase cDNA in an industrial baker's yeast strain. Biotechnol Prog 15: 459–466

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NY

Shigechi H, Koh J, Fujita Y, Matsumoto T, Bito Y, Ueda M, Satoh E, Fukuda H, Kondo A (2004) Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and α-amylase. Appl Environ Microbiol 70:5037–5040