

## Construction of Amyolytic Industrial Strains of *Saccharomyces cerevisiae* for Improved Ethanol Production from Raw Starch

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## 생전분으로부터 에탄올 생산이 증진된 전분 분해성 산업용 *Saccharomyces cerevisiae*의 개발

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To construct amyolytic industrial strains of *Saccharomyces cerevisiae* which produce ethanol efficiently from raw starch, the *Bacillus amyloliquefaciens*  $\alpha$ -amylase genes (*Amy*) or *Aspergillus awamori* glucoamylase genes (*GAI*) was separately introduced into the ribosomal DNA loci in the chromosomes of the raw starch fermenting-parental strain (ATCC 9763/YIp $\delta$ AGSA $\delta$ ), using double 18S rDNA-integration system. Ethanol production after 3 days of fermentation by the strain that produced ethanol most efficiently from raw starch (ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD) among the transformant strains was 1.5-times higher than that by the parental strain. This new strain generated 9.2% (v/v) ethanol (72 g/L) from 20% (w/v) raw corn starch and consumed 75% of the raw starch content during the same period.

**Keywords:** ethanol production, industrial *Saccharomyces cerevisiae*, raw starch

The yeast *Saccharomyces cerevisiae*, which has traditionally been used for industrial ethanol production cannot utilize starch as a carbon source because of its lack of endogenous amyolytic enzymes. Starch biomass for ethanol production needs a two-step pretreatment process, namely, liquefaction and saccharification. Raw starch also needs to be gelatinized by cooking at a high temperature, and then liquefied by  $\alpha$ -amylase (Eksteen *et al.*, 2003). Raw starch degrading enzymes (RSDE) refer to amylases that can hydrolyze raw starch directly, without an additional high energy-consuming process, such as gelatinization (Shigechi *et al.*, 2004). Many genes of filamentous fungi and yeasts that encode for RSDE have been previously expressed in *S. cerevisiae* (Sun *et al.*, 2010). A recombinant *S. cerevisiae* that could synthesize and secrete

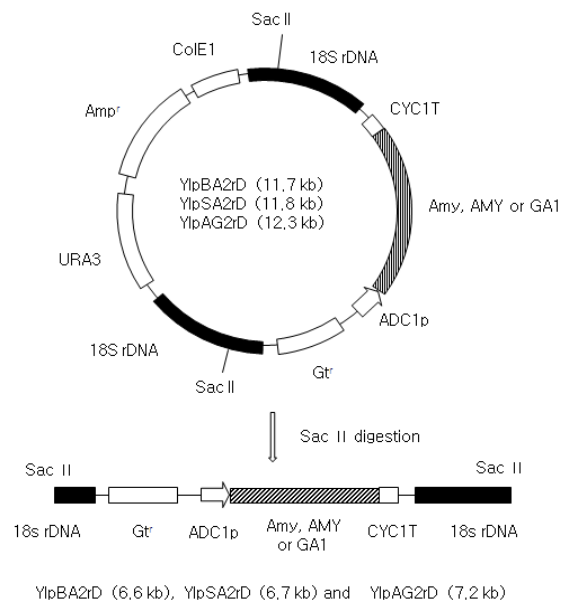
RSDE efficiently might be used to simplify the process and to reduce the costs of ethanol production from raw starch. For industrial-scale fermentation, ethanol productivity from raw starch depends on the improvement of amylase activities (Yamada *et al.*, 2009, 2010). Manipulation of *S. cerevisiae* to expresses high RSDE activities, via  $\delta$ -integration and rDNA-integration of the RSDE genes, can be useful for the improvement of ethanol productivity from raw starch (Kim *et al.*, 2011; Yamada *et al.*, 2012). We recently constructed  $\delta$ -integrative and rDNA-integrative vectors to allow for the multiple integration of amylase genes into the chromosomes of industrial strains (Ghang *et al.*, 2007; Kim *et al.*, 2010). In this study, we constructed 18S rDNA-integrative vectors containing RSDE genes, such as *Bacillus amyloliquefaciens*  $\alpha$ -amylase (*Amy*) and *Aspergillus awamori* glucoamylase genes (*GAI*), and integrated them into the RSDE-producing parental strain with  $\delta$ -integrated *GAI* and *Debaryomyces occidentalis*  $\alpha$

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-amylase (*AMY*) genes (ATCC 9763/YIp $\delta$ AGSA $\delta$ ) in an effort to develop industrial strains of *S. cerevisiae* with higher RSDE activities. We investigated the improvement of enzyme activities and of ethanol production from raw starch, using amylolytic yeast strains expressing combinations of *GAI*, *Amy*, or *AMY*, which were constructed by rDNA integration.

*Escherichia coli* DH5 $\alpha$  was used as the host for transformation and plasmid construction. *Bacillus amyloliquefaciens* ATCC 23842 was the source of the *Amy* gene (Gangadharan *et al.*, 2010). ATCC 9763/YIp $\delta$ AGSA $\delta$  was constructed from the industrial strain of *S. cerevisiae* ATCC 9763 (Kim *et al.*, 2011) and was used as the host for the yeast transformation experiment. Plasmids YIp $\delta$ AURAG $\delta$  and YIp $\delta$ AURSA $\delta$  (Ghang *et al.*, 2007) were used as sources of *GAI* and *AMY* genes, respectively. Plasmid YIpSG2rD (Kim *et al.*, 2010) was employed as the backbone of the rDNA-integrative system. The YPD medium [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose] was employed for the propagation of *S. cerevisiae*. Yeast transformants were grown on YPD plates containing aureobasidin A (1  $\mu$ g/ml, TaKaRa, Japan) and G418 (300  $\mu$ g/ml, Sigma, USA), and were then transferred onto YPDS3 plates [YPD containing 3% (w/v) soluble starch] and incubated for 2 days at 30°C, after which they were incubated for 2 days at 4°C. Yeast cells were grown in YPS media [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) soluble starch] at 30°C for 4 days to assay the amylolytic activities present in the culture supernatants. The hydrolysis of raw corn starch was evaluated via the phenol sulfuric acid method as the glucose equivalent (Shigechi *et al.*, 2004). For ethanol fermentation, YPP medium [2% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 0.05% (w/v) potassium disulfite] containing 20% (w/v) raw corn starch (Sigma) was used (Yamada *et al.*, 2009). Standard DNA manipulations and the *Escherichia coli* transformation were performed as previously described by Sambrook and Russell (2001). Yeast transformation was carried out via the lithium acetate method as described by Gietz *et al.* (1992). The *Amy* gene was amplified by PCR using the oligonucleotides 5'-ACTCCCGGGATGATTCAAAAACGAAAGCGG-3' and 5'-TGTCTAGATTATTTCTGAACATAAATGGAGAC-3'. These primers were designed using the published nucleotide sequences of the *B. amyloliquefaciens* ATCC 23842 *Amy* gene (GenBank accession no. GU591658). PCR resulted in 1.5 kb amplified DNA fragments of the whole open reading frame from genomic DNA of *B. amyloliquefaciens* ATCC 23842, which underwent digestion with *Sma*I and *Xba*I, and was then inserted into the same site of YIpSG2rD with deleted *GAM1* gene, generating YIpBA2rD (Fig. 1). *S. cerevisiae* ATCC 9763, transformed with YIpBA2rD, was then screened for  $\alpha$ -amylase activity on YPDS3 plate. To construct the double 18S

rDNA system containing the *GAI* and *AMY* genes, 2.1 kb *GAI* gene excised from YIp $\delta$ AURAG $\delta$  and 1.6 kb *AMY* genes excised from YIp $\delta$ AURSA $\delta$  were inserted into the *Sma*I-*Xba*I sites downstream of the *ADC1* promoter (*ADC1p*) in YIpSG2rD lacking the *GAM1* gene, thereby generating YIpAG2rD and YIpSA2rD, respectively (Fig. 1). The glucoamylase and  $\alpha$ -amylase activities were quantified at pH 6.0 and 40°C using the PGO/ODAD assay (Sigma) and dinitrosalicylic acid method, respectively (Ghang *et al.*, 2007). All assays were repeated in triplicate and the mean values were calculated. Ethanol fermentation was performed using the method described by Ma *et al.* (2000). In brief, Yeast cells were grown aerobically in 5 ml of YPS medium at 30°C in a shaking incubator at 200 rpm for 48 h. The culture was inoculated in 50 ml of YPP containing 20% (w/v) raw starch in a 100 ml closed flask equipped with a bubbling CO<sub>2</sub> outlet, and was incubated at 30°C for 6 days with mild agitation at 100 rpm. The culture supernatants were then assayed for ethanol content using a high performance liquid chromatography (HPLC, Waters, USA) using a Rezex ROA organic acid column (300  $\times$  7.8 mm, Phenomenex). The operating conditions were 65°C, water mobile phase, flow rate of 0.6 ml/min, and ethanol concentrations were monitored using a refractive index detector (Waters). Conversion used: 1% (v/v) ethanol is equivalent to



**Fig. 1.** Plasmid maps of linearized rDNA-integration vectors showing the relative size, restriction sites and locations of the insert DNA. Each rDNA-integration vector was linearized via digestion with *Sac*II. The bacterial vector DNA sequences harboring the ampicillin resistance marker gene and *ColE1* (pUC) origin, and *URA3* gene (5.1 kb) were excised

**Table 1.** Glucoamylase and  $\alpha$ -amylase activities in cell-free culture supernatants of *S. cerevisiae* transformants

Yeast strains	Glucoamylase activity <sup>a</sup> (U/ml)	$\alpha$ -Amylase activity (U/ml)
<i>S. cerevisiae</i> ATCC 9763	ND <sup>b</sup>	ND
ATCC 9763/YIp $\delta$ AGSA $\delta$ ( <i>GAI</i> , <i>AMY</i> )	0.56 $\pm$ 0.04 <sup>c</sup>	5.82 $\pm$ 0.25
ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpBA2rD ( <i>GAI</i> , <i>AMY</i> , <i>Amy</i> )	0.56 $\pm$ 0.08	6.04 $\pm$ 0.15
ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpSA2rD ( <i>GAI</i> , <i>AMY</i> , <i>AMY</i> )	0.56 $\pm$ 0.07	6.96 $\pm$ 0.2
ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD ( <i>GAI</i> , <i>AMY</i> , <i>GAI</i> )	1.08 $\pm$ 0.05	5.8 $\pm$ 0.15

<sup>a</sup> Yeast cells were grown in YPS medium at 30°C for 4 days

<sup>b</sup> Not detected

<sup>c</sup> Kim *et al.* (2010)

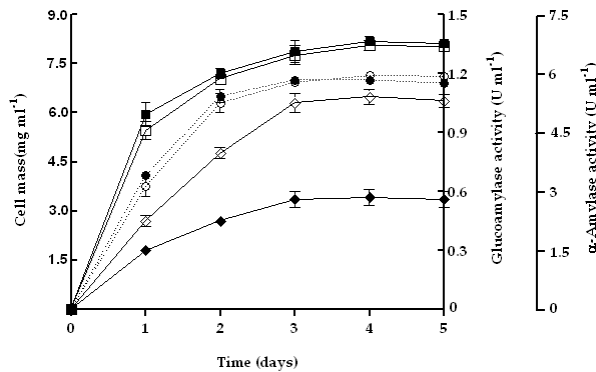
7.85 g/L or 170 mM.

Three vectors containing various amylase genes were linearized by digesting the 18S rDNA sequences with *SacII*, after which the resultant fragments harboring the *ADC1p-GAI*, *ADC1p-Amy* or *ADC1p-AMY* gene cassettes flanked by the 18S rDNA sequences could be integrated into rDNA sequences dispersed throughout the ATCC 9763/YIp $\delta$ AGSA $\delta$  genome to generate multicopy integrants (Nieto *et al.*, 1999). The integrative cassette (YIpBA2rD)-harboring yeast was named ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpBA2rD. The double 18S rDNA system generates a smaller fragment (5.1 kb) containing unnecessary bacterial sequences excised by digesting *SacII* before transformation (Fig. 1). The integrative cassettes (YIpAG2rD and YIpSA2rD)-harboring yeasts were designated ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD and ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpSA2rD, respectively (Fig. 1). Recently, Kim *et al.* (2010) reported  $\delta$ -integrated and rDNA-integrated copy numbers of 46 and 12 for the  $\alpha$ -amylase and glucoamylase genes, respectively in the recombinant yeast strain exhibiting high  $\alpha$ -amylase and glucoamylase activity, as determined by real-time PCR analysis. Therefore, the accumulation of sequential  $\delta$ -integration and rDNA-integration of RSDE genes can result in increasing the copy number of the corresponding genes and improving RSDE activities (Choi *et al.*, 2002). Such stable integrations of DNA containing *GAI*, *Amy*, and *AMY* genes is expected to facilitate industrial-scale ethanol production from starchy materials in the bioenergy industry (Eksteen *et al.*, 2003).

The recipient parental yeast strain for introducing the linearized 18S rDNA cassettes was ATCC 9763/YIp $\delta$ AGSA $\delta$ , which produces ethanol from raw starch due to its relatively strong raw starch-degrading glucoamylase activity (Kim *et al.*, 2011). To examine the effect of rDNA-integration on amylase activities,  $\alpha$ -amylase and glucoamylase activities were measured in the culture supernatants from transformants grown in YPS media. The clones evidencing the highest levels of activity among the transformants were selected for further analysis. The results (Table 1) show that the  $\alpha$ -amylase activity of transformant ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpBA2rD (secreting the glucoamylase encoded by *ADC1p-GAI* and both  $\alpha$ -amylases encoded by

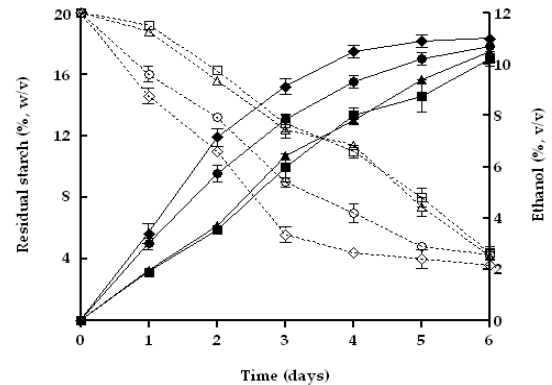
*ADC1p-AMY* and *ADC1p-Amy*) was slightly higher than that of the parental strain (ATCC 9763/YIp $\delta$ AGSA $\delta$ ). This result suggests that the secretion of raw starch degrading  $\alpha$ -amylase from *B. amyloliquefaciens* under its own signal sequences occurred at relatively low levels (Southgate *et al.*, 1993). The activities of  $\alpha$ -amylase and glucoamylase improved with increasing copy numbers via rDNA-integration (Kim *et al.*, 2010), reaching 6.95 U/ml and 1.08 U/ml, respectively in ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpSA2rD and ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD. These values were 1.2-times and 1.9-times higher than the respective activities of the parental strain (Kim *et al.*, 2011). The additional multicopy rDNA-integration of the *GAI* or *AMY* genes can be correlated with the high-level expression of the corresponding genes (Nieto *et al.*, 1999; Choi *et al.*, 2002). The increase in activities of glucoamylase in addition to  $\alpha$ -amylase is necessary for the more complete degradation of raw starch into glucose units, and consequently, more efficient ethanol production (Shigechi *et al.*, 2004; Sun *et al.*, 2010). Time courses of the cell growth and activities of  $\alpha$ -amylase and glucoamylase from ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD were compared with those of the parental strain. As shown in Fig. 2, the activities of  $\alpha$ -amylase and glucoamylase were growth-associated, reaching maximal levels after 4 days of growth, and no distinct differences in the growth rate and  $\alpha$ -amylase activity were observed between both strains. However, the rate of increase in glucoamylase activity of ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD was even higher than that of the parental strain.

Different recombinant yeast transformants were used for direct ethanol fermentation from 20% (w/v) raw corn starch under anaerobic conditions. The ethanol productivity from raw starch was improved by increasing the amylase activity, as shown in Fig. 3. ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpSA2rD, which showed 1.2-times higher *AMY* activity than the parental strain, generated 7.9% (v/v) ethanol after 3 days of fermentation. This value was about 1.3-times higher than the ethanol concentration produced by the parental strain during the same period [6.2% (v/v)] (Kim *et al.*, 2011). In contrast, the ability of ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpBA2rD to directly ferment ethanol from raw starch, which co-produces *GAI*, *AMY*, and *Amy*, was not



**Fig. 2.** Growth curve, time courses of extracellular glucoamylase and  $\alpha$ -amylase activities produced by ATCC 9763/YIp $\delta$ AGSA $\delta$  and ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD in the YPS medium. Growth was measured on different days based on the cell dry weight, and glucoamylase and  $\alpha$ -amylase activities were measured in the culture supernatants. Each point represents the means of three independent measurements. (■) mg/ml cell mass of ATCC 9763/YIp $\delta$ AGSA $\delta$ ; (□) mg/ml cell mass of ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD; (●) U/ml  $\alpha$ -amylase activities of ATCC 9763/YIp $\delta$ AGSA $\delta$ ; (○) U/ml  $\alpha$ -amylase activities of ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD; (◆) U/ml glucoamylase activities of ATCC 9763/YIp $\delta$ AGSA $\delta$ ; (◇) U/ml glucoamylase activities of ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD

distinctly different from that of the parental strain. Low *Amy* encoded  $\alpha$ -amylase activity could not contribute to the improvement of ethanol productivity, although *Amy* has been identified as RSDE (Gangadharan *et al.*, 2010; Sun *et al.*, 2010). This result indicates that increasing the amylase activity is crucial to efficiently produce ethanol from raw starch (Yamada *et al.*, 2010). On the other hand, ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD generated 9.2% (v/v) ethanol (72 g/L) after 3 days of fermentation and the ethanol concentration increased gradually and reached a maximum level after 6 days of fermentation. Most of the ethanol was generated after 3 days of fermentation. At this time, the amount of ethanol produced by this strain was 1.5-times higher than that produced by the parental strain. The raw starch content was reduced drastically in inverse proportion to the increase in ethanol concentration during fermentation. Residual starch was determined to be 5% (w/v) after 3 days of fermentation and 3.6% (w/v) after 6 days of fermentation, respectively. This result indicates that the increase in the activity of glucoamylase via rDNA integration of the *GAI* genes results in the improvements of raw starch degradation to glucose and of ethanol fermentation (Nieto *et al.*, 1999). Yamada *et al.* (2010) has reported that a tetraploid strain of *S. cerevisiae* that secretes *Streptococcus bovis*  $\alpha$ -amylase to hydrolyze raw starch and *Rhizopus oryzae* glucoamylase produced 70 g ethanol/L after 3 days of fermentation. The reduction in energy requirements during the



**Fig. 3.** Time course of ethanol production from raw corn starch by ATCC 9763/YIp $\delta$ AGSA $\delta$  (squares), ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpBA2rD (triangles), ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpSA2rD (circles) and ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD (diamonds). Open and filled symbols show residual starch and ethanol concentrations, respectively. Data are averages from three independent experiments

gelatinization process achieved by using RSDE has been currently exploited for the commercial production of ethanol (Sun *et al.*, 2010). To reduce the costs of ethanol production from raw starch, it may prove necessary to improve the rate of ethanol fermentation by increasing the activities of both raw starch degrading glucoamylase and raw starch degrading  $\alpha$ -amylase (Yamada *et al.*, 2012). Concerted efforts by multi-national research teams can be attainable to construct recombinant yeast strains which are capable of converting raw starch to glucose within 48–72 h, to a final ethanol concentration in excess of 10% in the near future (van Zyl *et al.*, 2012). Further studies are currently underway to increase raw starch-degrading  $\alpha$ -amylase activity in our expression vector systems for the improvement of ethanol production from raw starch.

## 적요

생전분으로부터 에탄올을 효율적으로 생산하는 전분 분해성 산업용 *Saccharomyces cerevisiae*를 제조하기 위해 생전분을 발효하는 모균주(ATCC 9763/YIp $\delta$ AGSA $\delta$ )의 염색체내 ribosomal DNA loci에 double 18S rDNA-integration 시스템을 이용하여 *Bacillus amyloliquefaciens*  $\alpha$ -amylase 유전자(*Amy*) 혹은 *Aspergillus awamori* glucoamylase 유전자(*GAI*)를 다중도입시켰다. 얻어진 형질전환 균주들 중 생전분으로부터 가장 효율적으로 에탄올을 생산하는 균주(ATCC 9763/YIp $\delta$ AGSA $\delta$ /YIpAG2rD)의 발효 3일째 에탄올 생산은 모균주에 비해 1.5배 높았다. 이 새로운 균주는 생옥수수 전분이 20% (w/v) 함유된 배지에서 3일간 발효를 통해 에탄올 9.2% (v/v) (72 g/L)를 생산하였고, 생전분 함유량의 75%를 소비하였다.

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