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Short Communication

A new platform host for strong expression under *GAL* promoters without inducer in *Saccharomyces cerevisiae*

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ABSTRACT

The *gal80* mutant of yeast *Saccharomyces cerevisiae* is used for the constitutive expression under strong *GAL* promoters without galactose induction. To enhance productivity of *gal80* mutant, an alternative strain, allgal, was developed by removing all galactose-utilizing genes that consume significant cellular resources in the *gal80* strain when cultured in non-galactose conditions. The efficacy of the allgal mutant (*gal80, gal1, gal2, gal7*, and *gal10*) was verified by assessing the secretory expression of three recombinant proteins, *Candida antarctica* lipase B (CalB), human serum albumin (HSA), and human epidermal growth factor (hEGF), using the *GAL10* promoter. The growth of the allgal mutant was enhanced by 15–38% compared to the *gal80* mutant, and the secretion of recombinant proteins also increased by 16–22% in fed-batch fermentation. Thus, the expression of recombinant proteins using *GAL10* promoter in the allgal mutant is suitable for the economical production of recombinant proteins in *S. cerevisiae*.

The yeast *Saccharomyces cerevisiae* is widely used as a host for recombinant protein production due in part to its high cell density culture and excellent secretion of higher eukaryotic proteins compared to other expression systems [1]. Selection of an appropriate promoter is one of the most important factors affecting recombinant protein production. Not only the constitutive promoters (*GAPDH, PGK, ADH, ENO,* and *TEF*) from the genes of the glycolytic pathway and translational elongation factor, but also the inducible promoters (*GAL1* and *GAL10*) of genes related to galactose utilization are widely used in *S. cerevisiae.* The transcription of *GAL* genes is induced by more than 1000-fold when galactose is the preferred carbon source [2,3]. When the strength of these promoters was compared using green fluorescent protein (*GFP*), the GAL promoter showed highest expression of *GFP* in the induced state [4]. Therefore, the *GAL1* and *GAL10* promoters are preferred for recombinant protein production in *S. cerevisiae* [5–7].

The expression of *GAL* genes is regulated by the transcription activator Gal4p and the transcription repressor Mig1p, which bind to the upstream activator sequence and the upstream repressor sequence of the *GAL* promoters, respectively. In non-galactose conditions, the negative regulator Gal80p directly binds to Gal4p and inhibits Gal4p function. This inhibition of Gal4p by Gal80p is suppressed by Gal3p in the presence of galactose [2]. Therefore, to induce *GAL* genes, a certain amount of galactose should be maintained as an inducer. However, the concentration of galactose continuously decreases during fermentation because it is consumed by cells as a carbon source. This is not economical for large-scale fermentation because galactose is much more expensive than glucose. Therefore, a *gal1* strain unable to metabolize galactose was developed for the production of recombinant proteins using a minimal amount of galactose, which can induce *GAL* promoters [8–10]. Similarly, a *gal80* mutant that expresses *GAL* promoters without galactose was also developed and successfully employed for the production of recombinant proteins [11,12]. In the *gal80* mutant, the expression under *GAL1* promoter increased approximately seven-fold under non-inducing conditions, whereas the expression under *GAL1* promoter increased 2.4-fold by deletion of *GAL1* under inducing conditions, compared to the wild-type strain [11]. The expression level of recombinant lipase B from *Candida antarctica* (CalB) of the *gal80* mutant without galactose was 1.6-fold higher than that of the *gal1* mutant in the presence of a minimal amount of galactose [13]. Thus, the *gal80* mutant is a promising host for the production of recombinant proteins using *GAL* promoters without using expensive galactose. However, the *gal80* mutant showed approximately 20%–30% retarded cell growth in fed-batch fermentation compared to that of the wild-type strain. It has been reported that the amount of induced *GAL* mRNA is between 1% and

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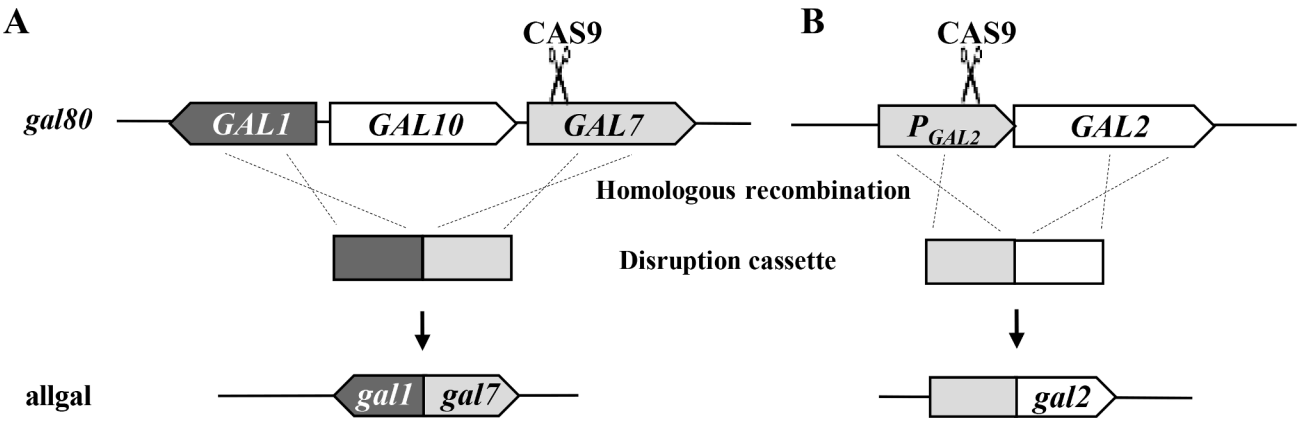

Fig. 1. Schematic diagram for targeted disruption of all *GAL* genes using CRISPR-Cas9 system. (A) One-step disruption of *GAL1, GAL10* and *GAL7*, (B) Disruption of *GAL2*. The pCAS-gGAL7 vector expresses the Cas9 nuclease and sgRNA containing a 20-nucleotide guide sequence targeted to *GAL7* (5′-GATTG-TAACGTCTATGGGAA-3′), and the disruption cassette contains 400 bp of *GAL1* and *GAL7* fragments to act as a template for homology-directed repair. The *GAL2* gene was disrupted separately using a pCAS-gGAL2 vector that expresses sgRNA containing a guide sequence targeting the *GAL2* promoter (5′-CAATTCG-GAAAGCTTCCTTC-3′) and a linear disruption cassette containing 400 bp of *GAL2* promoter and *GAL2* ORF fragments. *gal1, gal7* and *gal2* represent inactive *GAL1, GAL7* and *GAL2* gene.

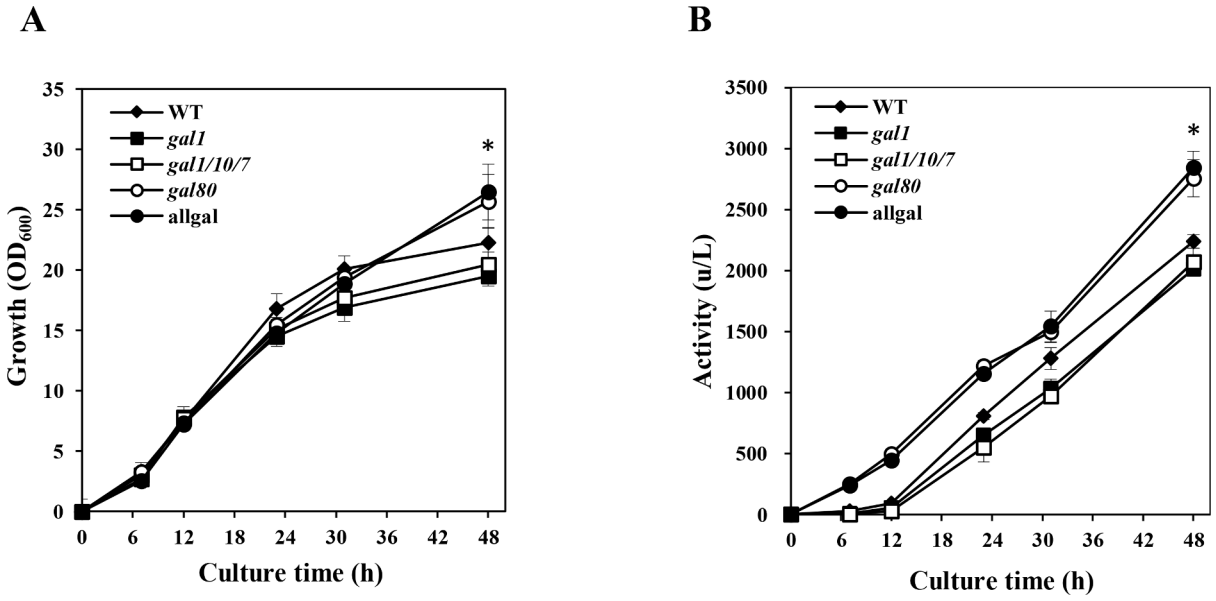

Fig. 2. Comparison of cell growth and lipase activity between gal mutants in batch culture. (A) Cell growth, (B) lipase activity. The *gal80*, allgal strains were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The wild-type was cultured in YPDG medium (1% yeast extract, 2% peptone, 1% glucose, and 1% galactose) and *gal1,* and *gal1/10/7* mutants were cultured in YPDg medium (1% yeast extract, 2% peptone, 2% glucose, and 0.05% galactose) to induce the *GAL10* promoter. The lipase activity of the culture supernatants was determined using *p*-nitrophenyl palmitate as a substrate. Mean values and standard deviations of triplicates are shown. $*p < 0.03$.

2.5% of the total mRNA within the cell [14] and the proteins for the galactose metabolism comprise ~5% of the total cellular mass [15]. Although the expression of *GAL* genes in the *gal80* mutant under non-inducing conditions was lower than that under inducing conditions, a significant amount of *GAL* mRNA was detected [16]. In fact, the products of *GAL* genes are of no use, and so are burdens for the *gal80* mutant in a non-galactose medium. Therefore, we assumed that growth retardation might be caused by the exhaustion of resources for the unnecessary expression of *GAL* metabolism genes.

In the present study, we constructed a mutant strain (allgal) in which *GAL80* and all *GAL* structural genes were disrupted and verified its effect on the growth and protein expression by *GAL10* promoter using three recombinant proteins as reporters by fed-batch fermentation. As the *GAL1, GAL10,* and *GAL7* genes are contiguously located on chromosome II, these genes, including promoter regions, are disrupted by the co-transformation of a pCAS-gGAL7 vector (Addgene plasmid # 60,847) [17] and a disruption cassette in one fell swoop (Fig. 1A). The pCAS vector expresses the Cas9 nuclease and sgRNA targeted to *GAL7*. The *GAL2* gene was disrupted separately using a pCAS-gGAL2 vector targeting the *GAL2* promoter. Because of the complete removal of the promoters of *GAL* genes, together with parts of the coding region, the allgal mutant contains no Gal4p binding sites.

The recombinant protein expression capacity of this allgal mutant was compared with that of wild-type, and the mutants *gal1, gal1/gal10/gal7* and *gal80* by using a variant of lipase B from *C. antarctica* (CalB1422) containing a 6-histidine tag at the C-terminus as a reporter. These strains were transformed with the YGaT3CalB1422 vector for the secretory production of CalB1422 using *CIS3* signal peptide under the control of the *GAL10* promoter. The *gal80* and allgal transformants were cultivated under non-inducing conditions and the wild-type, *gal1* and

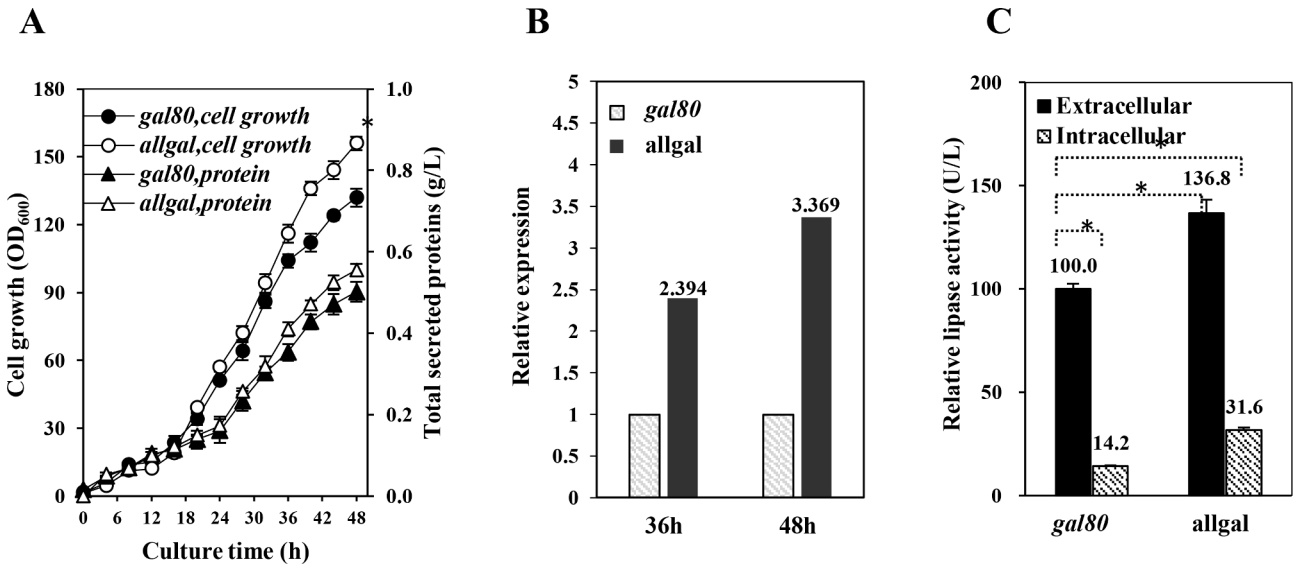

Fig. 3. Comparison of cell growth and lipase expression between *gal80* and allgal strains in fed-batch fermentation. (A) Cell growth and total secreted proteins of *gal80* and allgal harboring YGaT3CalB1422, (B) Relative expression levels of CalB1422 determined by qPCR after 36-h and 48-h fermentation, (C) Relative lipase activity of the extracellular and intracellular fractions of each strain after 48-h fermentation. Mean values and standard deviations of triplicates are shown. *$p < 0.03$.

gal1/gal10/gal7 mutants were cultivated under inducing conditions. Although the composition of the carbon source (glucose and galactose) in the media differed, the total amount of carbon sources was identical. The growth and secreted lipase activity of *gal1* and *gal1/gal10/gal7* mutants were inferior to those of the wild-type; however, the *gal80* and allgal mutants surpassed the wild-type strain in growth and lipase activity after 48-h cultivation (Fig. 2A, B). Although the medium used for cultivation of the *gal80* and allgal strains contained twice as much glucose as that used for cultivation of the wild-type, glucose repression did not occur in the *gal80* and allgal strains, unlike other strains (Fig. 2B). Because the *GAL10* promoter (500 bp) used in YGaT3-CalB1422 vector contains only one putative Mig1p binding site (Fig. S1), contrary to the *GAL1* promoter that contains two Mig1p binding sites [18], glucose repression mediated by Mig1p does not affect the expression of CalB1422 in the *gal80* and allgal strains. We confirmed this by comparison of *GAL10* promoter and *GAL10* promoter with deleted putative Mig1p binding site (Fig. S1). The secreted lipase activities of *gal80* and allgal mutants were 20% higher than that of the wild-type (Fig. 2B). However, there was no significant difference between the *gal80* and allgal mutants in batch cultures because expression of *GAL4* is repressed 4–5 fold by glucose mediated Mig1p, eventually lowering the expression of *GAL* genes [19]. Therefore the effect of *GAL* genes disruption on the growth of the host strain appear to not be prominent in batch cultures.

To minimize glucose repression of *GAL4* expression, the *gal80* and allgal strains harboring the YGaT3CalB1422 vector were compared by fed-batch fermentation maintaining low concentration of glucose. Growth of the allgal mutant expressing CalB1422 was found to be increased by approximately 20% (OD_{600}=159 vs. 132) compared to the *gal80* strain (Fig. 3A). Accordingly, the amount of total secreted proteins was also enhanced by approximately 16% (Fig. 3A). When the transcription of CalB1422 of 36-h culture was compared by qPCR, the allgal mutant showed 2.39 fold higher mRNA level than the *gal80* strain and the difference was further increased to 3.37 fold after 48-h fermentation (Fig. 3B). To confirm the effects of allgal mutation on the secretion of CalB1422, the culture supernatants and the intracellular fractions were analyzed using SDS-PAGE and western blot (Fig.S2) and the intracellular and extracellular lipase activity of the 48-h culture were compared (Fig. 3C). Because the culture broth contains various proteins in addition to CalB1422, the increase in lipase activity of the allgal strain compared to the *gal80* mutant was higher than that of the total secreted proteins (36% vs. 16%). With the enhanced expression of CalB1422 in allgal stain, intracellular lipase activity of the allgal strain was more than two-fold higher than that of the *gal80* mutant (31% vs. 14%).

To exclude recombinant protein-specific effects, human serum albumin (*HSA*) and human epidermal growth factor (*hEGF*) were also tested as passenger proteins. YGaHSA and YEGα-HL28-EGF vector [6] that express *HSA* and *hEGF* fusion proteins under the control of the *GAL10* promoter were transformed into *gal80* and allgal strains, respectively.

Cell growth was found to be affected by the type of recombinant protein, but the final cell mass and the total secreted proteins of the allgal strains were higher than those of the *gal80* mutants, regardless of recombinant proteins (growth: 15%–38% and proteins: 18%–22%) (Fig. 4). When we tested other proteins, the allgal mutant always showed higher performance than the *gal80* mutant as a recombinant protein expression host.

In conclusion, the allgal strain developed in this study demonstrated improved cell growth and protein secretion compared to the *gal80* mutant during fed-batch fermentation; this difference is likely due to the removal of the burden of expression of galactose-metabolizing genes. Consequently, the allgal strain can be used for the economical production of recombinant proteins with *GAL* promoters in *S. cerevisiae*.

Author contributions

J.H.B. and J.H.S. designed the experiments. M.J.K engineered the yeast strains. H.J.P did the fermentation. B.H.S analyzed the data. J.H.B, M.J.K and J.H.S. drafted the manuscript, which was edited by all authors.

Data statement

All data reported in the paper are available from the corresponding author upon reasonable request. Materials and Methods in this study are described in the Supplementary information.

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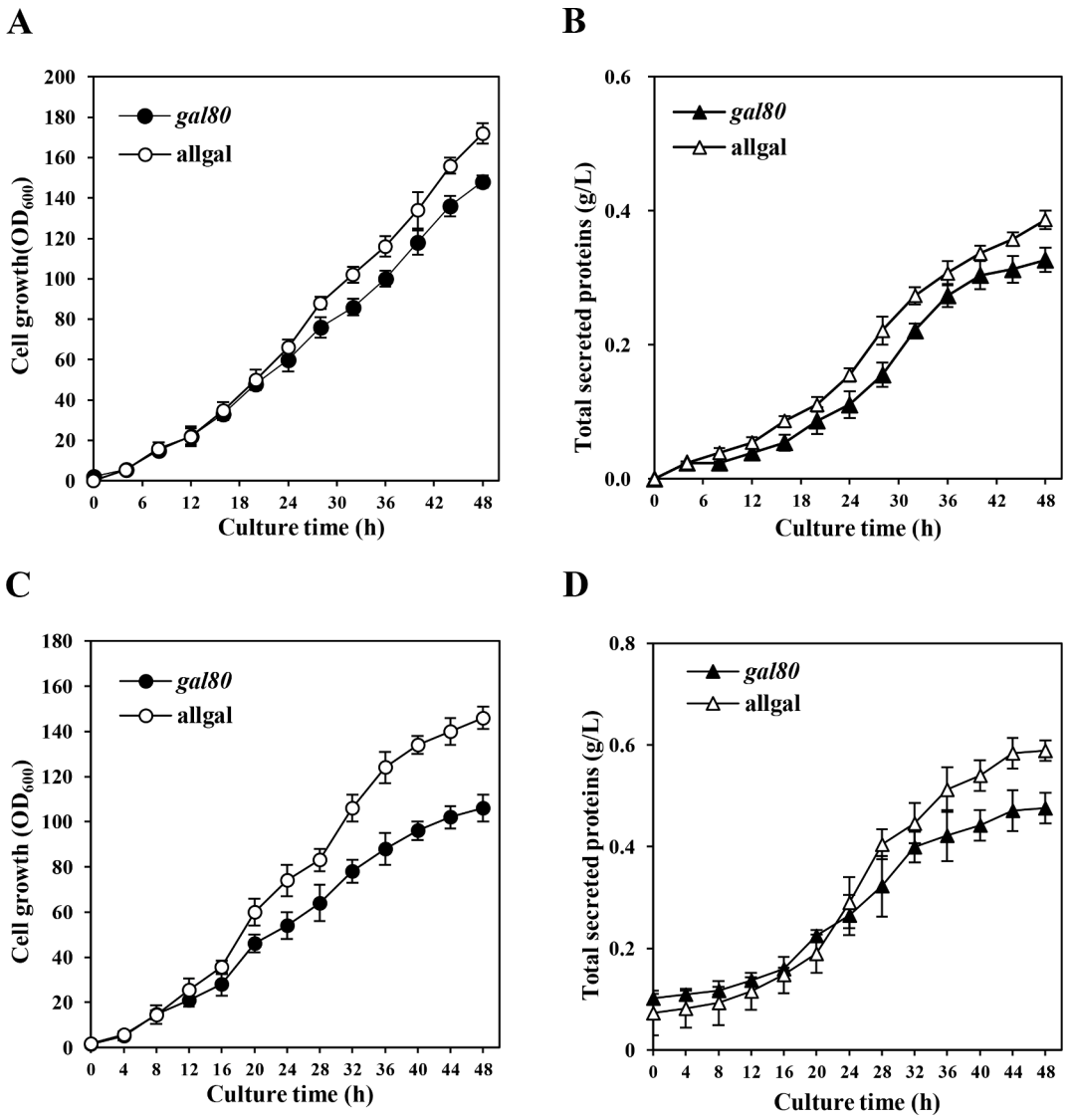

Fig. 4. Comparison of *gal80* and allgal strains by fed-batch fermentation of various proteins. Cell growth (A) and total secreted proteins (B) of *gal80* and allgal harboring YGaHSA. Cell growth (C) and total secreted proteins (D) of *gal80* and allgal harboring YEGaHL28-EGF. Mean values and standard deviations of triplicates are shown. $*p < 0.03$.

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Declaration of Competing Interest

The authors have no competing interests to declare.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00763.

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