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Aspergillus oryzae-based cell factory for direct kojic acid production from cellulose

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Abstract

Background: Kojic acid (5-Hydroxy-2-(hydroxymethyl)-4-pyrone) is one of the major secondary metabolites in *Aspergillus oryzae*. It is widely used in food, pharmaceuticals, and cosmetics. The production cost, however, is too high for its use in many applications. Thus, an efficient and cost-effective kojic acid production process would be valuable. However, little is known about the complete set of genes for kojic acid production. Currently, kojic acid is produced from glucose. The efficient production of kojic acid using cellulose as an inexpensive substrate would help establish cost-effective kojic acid production.

Results: A kojic acid transcription factor gene over-expressing the *A. oryzae* strain was constructed. Three genes related to kojic acid production in this strain were transcribed in higher amounts than those found in the wild-type strain. This strain produced 26.4 g/L kojic acid from 80 g/L glucose. Furthermore, this strain was transformed with plasmid harboring 3 cellulase genes. The resultant *A. oryzae* strain successfully produced 0.18 g/L of kojic acid in 6 days of fermentation from the phosphoric acid swollen cellulose.

Conclusions: Kojic acid was produced directly from cellulose material using genetically engineered *A. oryzae*. Because *A. oryzae* has efficient protein secretion ability and secondary metabolite productivity, an *A. oryzae*-based cell factory could be a platform for the production of various kinds of bio-based chemicals.

Keywords: *Aspergillus oryzae*, Kojic acid, Cellulose, Cellulase, Starch

Background

Kojic acid (5-Hydroxy-2-(hydroxymethyl)-4-pyrone) is widely used in the food industry, pharmaceutical industry, and in cosmetics [1,2]. Furthermore, kojic acid is also used as a building block for biodegradable plastics [3,4]. Plastics have phenolic hydroxyl groups that are derived from kojic acid. Thus, it has some unique properties. Futamura et al. [5] reported that the production cost of kojic acid is about \$10/kg. It must be decreased to less than \$2/kg before it could be used in many applications. Thus, an efficient and cost-effective kojic acid production process would be desirable.

The utilization of biomass, particularly cellulosic materials, is desirable because it is abundant, inexpensive, renewable, and has favorable environmental properties.

However, an efficient and cost-effective method for the degradation of cellulosic materials into glucose would be required before it could be used for the production of bio-based chemicals.

Cellulose has a structure that is very rigid with a high degree of crystallinity. Thus, the degradation of cellulose requires large amounts of various types of cellulase. The degradation of cellulose requires a synergistic reaction of at least three types of cellulase: endoglucanase (EG), cellobiohydrolase (CBH), and β -glucosidase (BGL). Although many studies have reduced the cost of cellulosic material degradation using recombinant bacteria, fungi, and yeast [6], cellulase degradation efficiency has not been sufficiently improved. The filamentous fungus *Trichoderma reesei* degrades cellulose effectively and is known to produce more than several dozen g/L of cellulase in the medium [7], therefore, it is known for its high cellulose degradation ability. *Aspergillus oryzae* is known as a high protein-secreting microorganism and its genetic recombination procedures are well developed. There are

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many reports concerning the production of a large amount of protein by *A. oryzae* [8]. Thus, *A. oryzae* shows promise as a host strain that could produce a large amount of cellulase and bio-based chemicals from cellulose. There have been reports concerning a single cellulase gene expression by *A. oryzae*, but no studies have focused on the cultivation of the 3 types of cellulase expressing *A. oryzae* on cellulose as a sole carbon source.

Kojic acid is one of the major secondary metabolites in *A. oryzae*. In the 100 years since it was discovered, there has been no elucidation of the entire pathway of kojic acid biosynthesis in *A. oryzae*. Recently, some genes regarding the production of kojic acid in *A. oryzae* were reported [9]. According to the report, 14 genes from AO090113000132 to AO090113000145 including a transcription factor gene (*kojR*; AO090113000137), an enzyme gene (*kojA*, AO090113000136), and a transporter gene (*kojT* AO090113000138) form a cluster. These genes are engaged in kojic acid production. The *kojR* gene encodes a fungal-specific Zn(II)₂Cy₆ transcription factor that is located between *kojA* (upstream 743 bp) and *kojT* (downstream 383 bp). The *kojR* regulates the transcription of *kojA* and *kojT*. A strain with a disrupted transcription factor gene could not produce kojic acid at all [10]. Thus, *kojR* seems to be a key factor in kojic acid production.

The goal of the present study was to construct an *A. oryzae*-based cell factory for direct kojic acid production from cellulose. First, *A. oryzae* over-expressing the transcription factor gene *kojR* was constructed, and kojic acid production from glucose and starch by the resultant strain was carried out. Then, *A. oryzae* co-expressing the transcription factor and 3-types of cellulase genes was constructed. Finally, direct kojic acid production from cellulose by the resultant strain was carried out.

Methods

Strains and media

Table 1 summarizes the genetic properties of all strains used. The *A. oryzae* strain RIB40 [11] was used as the wild-type strain. The strain NSPID1 [12] derived from *A. oryzae* RIB40 was used as a transformation host strain. Potato dextrose agar (PDA; Nissui Pharmaceutical, Tokyo, Japan) media and GPY medium (3% glucose, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 1% peptone, and 0.5% yeast extract, pH 6.0) were used for the cultivation of *A. oryzae*. Czapek-Dox (CD) medium (2% glucose, 0.3% NaNO₃(CD-NO₃), 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, and 0.8 M NaCl, 1.5% agar, pH 6.0) with a required supplement (0.0015% methionine, 20 mM uridine, 0.2% uracil) was used for the *niaD*-based plasmid transformation. CD medium was used for *sC*-based plasmid transformation.

GY medium (8% glucose, 0.25% yeast extract, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, pH 6.0), SY medium (8%

soluble starch, 0.25% yeast extract, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, pH 6.0), and CY medium (1% phosphoric acid swollen cellulose (PASC), 0.25% yeast extract, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, pH 6.0) was used for kojic acid production. PASC was prepared from Avicel PH-101 (Fluka Chemie GmbH, Buchs, Switzerland) as amorphous cellulose [13]. The transformant and wild-type strains used for kojic acid production with GY medium and SY medium were cultivated in 500 mL Erlenmeyer flasks containing 100 mL medium at 30°C that was shaken at 150 rpm using an orbital shaker with inocula of 1 × 10⁵ spores/mL. The transformant and wild-type strain used for kojic acid fermentation in the CY medium was pre-cultivated in a 200 mL Erlenmeyer flask with baffles containing 100 mL GPY medium at 30°C that was shaken at 150 rpm using an orbital shaker with inocula of 1 × 10⁵ spores/mL. The mycelium in culture medium was separated from the culture medium by filtration using Miracloth (Calbiochem, Darmstadt, Germany) and was washed by distilled water. The washed mycelium was recultivated in 500 mL Erlenmeyer flasks with baffles containing 100 mL CY medium at 30°C that were shaken at 150 rpm using an orbital shaker.

E. coli NovaBlue (Novagen, Inc., Madison, WI, USA) was used as the cloning host for recombinant DNA manipulations. The bacterium was grown in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 0.1 mg/mL of ampicillin.

Construction of plasmids

The primers used in the present study are summarized in Table 2. The PCR amplification of DNA fragments was performed using KOD plus neo DNA polymerase (Toyobo, Osaka, Japan).

The transcription factor gene (*kojR*, AO090113000137) expression plasmid was constructed as follows. The *kojR* was amplified by PCR using primers *ascI*-*kojR*-F and *notI*-FLAG-*kojR*-R from *A. oryzae* genome DNA as a template. The amplified fragment was inserted into the *AscI*/*NotI* sites of the digested pISI, which contained the *sodM* promoter and *glaB* terminator from *A. oryzae* [17]. The resultant plasmid was named pISI-*kojR*.

The *CBHI* gene from *Trichoderma* sp. expressing plasmid pISI-*svaA*-*CBHI* was constructed as follows. The DNA fragment encoding the *Trichoderma* sp. *CBHI* gene (GenBank accession no. X69976) was amplified by PCR using primers *CBHI*-F and *CBHI*-R from a synthesized gene (GENEWIZ, South Plainfield, NJ, USA). The fragment was inserted into the *AscI*/*NotI* site of the plasmid pISI using an In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan). The resultant plasmid was named pISI-*CBHI*. Then, the *CBHI* gene was amplified by PCR using primers *psvaA*-*ascI*-*CBHI*-F and *tsvaA*-*notI*-*CBHI*-R with pISI-*CBHI* as a template. The *svaA* promoter [17] and

Table 1 Strains and plasmid used in this study

Strains and plasmids	Relevant features	Reference
<i>Escherichia coli</i> strain		
NovaBlue	<i>endA1 hsdR17(r K12-m K12+) supE44 thi-I gyrA96 relA1 lac recA1/F[proAB + lacIq ZΔM15::Tn10(Tetr)]</i>	Novagen
<i>Aspergillus oryzae</i> strains		
RIB40	Wild type	Machida et al. [11]
NSPID1	<i>niaD⁻ sC⁻ adeA⁻ΔargB::adeA⁻ ΔligD::argB ΔpyrG::adeA</i>	Maruyama and Kitamoto [12]
NSPID1/pIS1-kojR	<i>niaD⁻::pIS1-kojR [P-sodM::kojR::T-glaB] sC⁻ adeA⁻ ΔargB::adeA⁻ ΔligD::argB ΔpyrG::adeA</i>	This study
NSPID1/pIS1-kojR/pIS1sC-CBHI-EGI-BGLI	<i>niaD⁻::pIS1-kojR [P-sodM::kojR::T-glaB] sC⁻::pIS1sC-CBHI-EGI-BGLI [P-svaA::CBHI::T-svaA, P-hlyA::EGI::T-hlyA, P-sodM::CutL-BGLI::T-glaB] adeA⁻ ΔargB::adeA⁻ ΔligD::argB ΔpyrG::adeA</i>	This study
Plasmids		
pIS1-CBHI	CBHI gene from <i>T. reesei</i> containing plasmid	This study
pIS1-svaA-CBHI	Vector for expression of CBHI from <i>T. reesei</i> [<i>P-svaA::CBHI::T-glaB</i>]; <i>niaD</i> marker	This study
pIS1-EG	EGI (<i>T. reesei</i>) gene containing plasmid	This study
pIS1-hlyA-EGI	Vector for expression of EGI from <i>T. reesei</i> [<i>P-hlyA::EG::T-hlyA</i>]; <i>niaD</i> marker	This study
pIS1-BGLI	Vector for expression BGLI from <i>A. aculeatus</i> [<i>P-sodM::CutL-BGLI::T-glaB</i>]; <i>niaD</i> marker	This study
pIS1-CBHI-EGI-BGLI	Vector for expression of CBHI, EGI, and BGLI [<i>P-svaA::CBHI::T-svaA, P-hlyA::EGI::T-hlyA, P-sodM::CutL-BGLI::T-glaB</i>]; <i>niaD</i> marker	This study
pIS1sC-CBHI-EGI-BGLI	Vector for expression CBHI, EGI, and BGLI [<i>P-svaA::CBHI::T-svaA, P-hlyA::EGI::T-hlyA, P-sodM::CutL-BGLI::T-glaB</i>]; sC marker	This study
pIS1-kojR	Vector for expression of transcription factor (AO090113000137) [<i>P-sodM::kojR::T-glaB</i>]; <i>niaD</i> marker	This study

svaA terminator were amplified by PCR using primers pIS1-psvaA-F and CBHI-ascI-psvaA-R, and CBHI-notI-tsvaA-F and pIS1-tsvaA-R, respectively, with *A. oryzae* genome DNA as a template. These PCR-amplified DNA fragments were simultaneously inserted into the XbaI and PstI sites of pIS1 using an In-Fusion PCR Cloning kit. The resultant plasmid was named pIS1-svaA-CBHI.

An EGI gene from the *T. reesei*-expressing plasmid pIS1-hlyA-EGI was constructed. The EGI-expressing plasmid was constructed as follows. The DNA fragment encoding the *T. reesei* EGI gene was amplified by PCR using the primers EGI-F and EGI-R with *T. reesei* cDNA as a template. The fragment was inserted into the AscI/NotI site of the plasmid pIS1 using an In-Fusion HD Cloning Kit. The resultant plasmid was named pIS1-EG. The *T. reesei* EGI gene was then amplified by PCR using the primers phlyA-ascI-EGI-F and thlyA-notI-EGI-R with pIS1-EG as a template. The hlyA promoter [18] and hlyA terminator were amplified by PCR using primers pIS1-phlyA-F and EGI-ascI-phlyA-R, and EGI-notI-thlyA-F and pIS1-thlyA-R, respectively, using the *A. oryzae* genome DNA as a template. These PCR-amplified DNA fragments were simultaneously inserted into the XbaI and PstI

sites of pIS1 using an In-Fusion PCR Cloning kit. The resultant plasmid was named pIS1-hlyA-EGI.

The BGL1 gene from *A. aculeatus* expressing plasmid pIS1-BGL1 was constructed as follows. The DNA fragment encoding the secretion signal from *A. oryzae* cutinase, the 28 amino acids from the N-terminal region of *Rhizopus oryzae* lipase, and the *A. aculeatus* BGL gene were amplified by PCR using the primers sCutL-F and sCutL-R, N28-F and N28-R, and BGL-F and BGL-R, respectively, from the genome DNA of *A. oryzae*, pIS1-GFP [14], and pδU-PGAGBGL [15], respectively. These fragments were simultaneously inserted into the AscI/NotI site of the plasmid pIS1 using an In-Fusion HD Cloning Kit. The resultant plasmid was named pIS1-BGL.

The pIS1-CBHI-EGI-BGL1 plasmid was constructed as follows. The pIS1-svaA-CBHI was linearized by PCR using the primers pIS1-svaA-CBHI-F and pIS1-svaA-CBHI-R. The EGI- and BGL1-expressing cassettes were amplified by PCR using primers phlyA-into-pIS1-svaA-CBHI-F and connect-thlyA-and-psodM-R, and connect-psodM-and-thlyA-F and tglab-into-pIS1-svaA-CBHI-R, respectively, from pIS1-hlyA-EGI and pIS1-BGL1, respectively. These fragments were fused using an In-Fusion PCR

Table 2 Polymerase chain reaction primers used in this study

Primers	Sequence (5'-3')	Template DNA
ascl-kojR-F	AAAAAGGCGGCCATGTCGTTGAATACCGACGATTCC	<i>A. oryzae</i> genome DNA
notI-FLAG-kojR-R	AAAGCGGCGCTTACTTGTATCATCATCGCTTATAGTCTCTATATCTCTGACCACCTGC	
CBHI-F	CCAAACCACCCAAAGGGCGGCCATGTATCGGAAGTTGGCCGTCATC	synthesized gene
CBHI-R	GAAAGTACATGTCGAGCGCCGCTTACAGGCACTGAGAGTAGTAAG	
psvaA-ascl-CBHI-F	GGCGCGCCAAAAAATGTATCGGAAGTTGGCCGTCATC	pSI-CBHI
tsvaA-notI-CBHI-R	AAATGATGCGGCCGCTTACAGGCACTGAGAGTAGT	
pSI-psvaA-F	CACAACACTCTCGACTGCAGCGGTTACACCGAAGACCGG	<i>A. oryzae</i> genome DNA
CBHI-ascl-psvaA-R	ATTTTTTGGCGCGCCCTTGCAGCAGGGGGATAAT	
CBHI-notI-tsvaA-F	GCGGCCGCATATTTTCCCGCTTGTATCTGTGCGGTTCCC	<i>A. oryzae</i> genome DNA
pSI-tsvaA-R	GGTACCCGGGGATCCTCTAGATCAAGCATAACTACAACAGGGCAAGGAATACGG	
EGI-F	CCAAACCACCCAAAGGGCGGCCATGGCGCCCTCAGTTACTACTGC	<i>T. reesei</i> cDNA
EGI-R	GAAAGTACATGTCGAGCGCCGCTTAAAGGCATTGCGAGTAGTCTG	
phlyA-ascl-EGI-F	GGCGCGCCAAAAAATGGCGCCCTCAGTTACTACTGC	pSI-EG
thlyA-notI-EGI-R	AGAGAGAGCGGCCGCTTAAAGGCATTGCGAGTAGT	
pSI-phlyA-F	CACAACACTCTCGACTGCAGTACAGCATGGTCTGGATTCCAATCCACGCAGC	<i>A. oryzae</i> genome DNA
EGI-ascl-phlyA-R	ATTTTTTGGCGCGCCGTTGTTGTGGTGTGAAGGGTATTGATGTGAGACC	
EGI-notI-thlyA-F	GCGGCCGCTCTCTCCCTATACGTGATACCGTA	<i>A. oryzae</i> genome DNA
pSI-thlyA-R	GGTACCCGGGGATCCTCTAGAGATGCAAATTTGAGTTAAAT	
sCutL-F	CCAAACCACCCAAAGGGCGGCCATGCATCTTGTATCAAGTCTC	<i>A. oryzae</i> genome DNA
sCutL-R	TCACTAGTTCTCTCAACCAGAGCATTGTGTTGG	
N28-F	TTGAGAGAACTAGTGATGACAACCTGGTTGGTGGCATG	pSI-GFP (Adachi et al. [14])
N28-R	AGTTCATCCTCGACACGCTTGGCGCTGTTGG	
BGL-F	GTGTGAGGATGAACTGGCGTTCTCTCCTC	pDU-PGAGBGL (Yamada et al. [15])
BGL-R	GAAAGTACATGTCGAGCGCCGCTTACTTGTATCATGCATCCTTG	
pSI-svaA-CBHI-F	GGCTTTCCCGTCAAGCTCT	pSI-svaA-CBHI
pSI-svaA-CBHI-R	GGCGAACGTGGCGGAGAAAGG	
phlyA-into-pSI-svaA-CBHI-F	CTCGCCACGTTTCGCTACAGCATGGTCTGGATTCCAATCCACGCA	pSI-hlyA-EGI
connect-thlyA-and-psodM-R	TATTTAAATATCGATGATGCAAATTTGGAGTTAAATATTAATAAC	
connect-psodM-and-thlyA-F	ATCGATATTTAAATATTATGTACTCCGTAAGTTGATTATTA	pSI-BGL1
tglaB-into-pSI-svaA-CBHI-R	TTGACGGGGAAAGCCGGATGTAGTATGTACTTAGTTTATTGTC	
hlyA-EGI-svaA-CBHI-sodM-BGLI-F	TTTATATCCAAGATCACCTGCAGCGGTTACACCGAAGACCGGTA	pSI-CBHI-EGI-BGL1
hlyA-EGI-svaA-CBHI-sodM-BGLI-R	TGCCAAGAGAAGCTTGGCGTAATCATGGTCATAGCTGTTT	
sC-F	AAGCTTCTCTTGGAATAGTGCC	pUSC (Yamada et al. [16])
sC-R	GATCTTGGATATAAAAAATCAAATATGGCTCCTCG	
kojR-RT-PCR-F	TGGTGCAATCAGCGAAGGA	wild type and engineered <i>A. oryzae</i> cDNA
kojR-RT-PCR-R	AGACTACTCTCCTGCATCATGCC	
kojA-RT-PCR-F	ATCCGAAGGCGAATGGTTT	wild type and engineered <i>A. oryzae</i> cDNA
kojA-RT-PCR-R	ATGAACCCAGCGTCGTATT	
kojT-RT-PCR-F	CATGGTGCCGCATATTTACTTC	wild type and engineered <i>A. oryzae</i> cDNA
kojT-RT-PCR-R	AATGGACACAATGGGTTGCC	
β -Tubulin-RT-PCR-F	GCCAGTGTGGTAACCAAATAGTT	wild type and engineered <i>A. oryzae</i> cDNA
β -Tubulin-RT-PCR-R	TAAACCCGGAGCCGTC	

Cloning kit. The resultant plasmid was named pIS1-CBHI-EGI-BGL1.

The pIS1sC-CBHI-EGI-BGL1 plasmid was constructed as follows. The CBHI-, EGI-, and BGL1-expressing cassettes were amplified by PCR using primer (hlyA-EGI-svaA-CBHI-sodM-BGL1-F and hlyA-EGI-svaA-CBHI-sodM-BGL1-R). The *sC* gene was amplified by PCR using primer (sC-F and sC-R) from a pUSC plasmid [16]. These amplified fragments were fused using an In-Fusion PCR Cloning System. The resultant plasmid was named pIS1sC-CBHI-EGI-BGL1.

Transformation of *A. oryzae*

The transformation of *A. oryzae* was carried out according to the previously described method with minor modifications [19].

The *niaD*-based pIS1-*kojR* plasmids were introduced into the NSPID1 strain. The resultant strain was named NSPID1/pIS1-*kojR*. The *sC*-based plasmid pIS1sC-CBHI-EGI-BGL1 was introduced into the NSPID1/pIS1-*kojR* strain. The resultant strain was named NSPID1/pIS1-*kojR*/pIS1sC-CBHI-EGI-BGL1.

RNA extraction and reverse transcription

The RNA was isolated from fungus bodies cultivated in GY medium for 6 days at 30°C using NucleoSpin RNA II (Takara Bio Inc.) according to the manufacturer's protocol. The cDNA synthesis was carried out using a ReverTra Ace qPCR RT Kit (Toyobo) according to the manufacturer's protocol.

Real-time PCR analysis

The transcription level of Kojic-acid-producing genes (*kojR*, *kojA*, *kojT*) was quantified by real-time PCR. Quantitative real-time PCR was performed using an Mx3005P Real-Time QPCR System (Agilent Technologies, Santa Clara, CA, USA) with a Thunderbird SYBR qPCR Mix (Toyobo). The normalized transcription level was calculated using the standard curve method with β -tubulin as the house-keeping gene [20]. All primers used for real-time PCR analysis are summarized in Table 2.

Enzyme assay

A wild-type strain and 3-types of cellulase gene-expressing strains were cultivated in GY medium for 6 days at 30°C, and the culture supernatant was used for the determination of PASC degradation activity. PASC degradation activity was measured in 25 mM sodium acetate buffer (pH 5.0) at 30°C with 5 g/L of PASC. After hydrolysis, the supernatant was separated by centrifugation for 5 min at 10,000 \times g and 4°C, and the produced glucose concentration was measured. One unit of PASC degradation activity was defined as the amount of enzyme producing 1 μ mol/min glucose at 30°C, pH 5.0.

Analytical methods

The kojic acid concentration was determined using a colorimetric method described previously [21]. The total sugar concentration was determined by a colorimetric phenol-sulfuric acid method described previously [22]. The glucose concentration was determined using a Wako Glucose CII-Test kit (Wako Pure Chemical Industries, Osaka, Japan).

Results

Kojic acid production from glucose using the engineered *A. oryzae*

To confirm the effect of the over-expression of the transcription factor for kojic acid production, the kojic acid productivity from the transcription factor over-expressing strain NSPID1/pIS1-*kojR* from glucose was evaluated. As shown in Figure 1 the wild-type strain produced 16.4 g/L of kojic acid after 14 days of fermentation. By contrast, the transcription factor over-expressing strain NSPID1/pIS1-*kojR* produced 26.4 g/L of kojic acid after 14 days of fermentation and it was 1.6-fold higher than that of the wild-type strain.

Quantification of the transcription level of kojic-acid-producing genes by real-time PCR

To confirm the effect of the over-expression of transcription factor at the level of the kojic-acid-producing genes, the transcription level of the transcription factor, the kojic-acid-producing enzyme, and the transporter gene were evaluated by real-time PCR. As shown in Figure 2, the transcription factor of the over-expressing strain NSPID1/pIS1-*kojR* showed a 1.8, 1.8, and 5.4-fold increases in the transcription level of transcription factor, enzyme, and

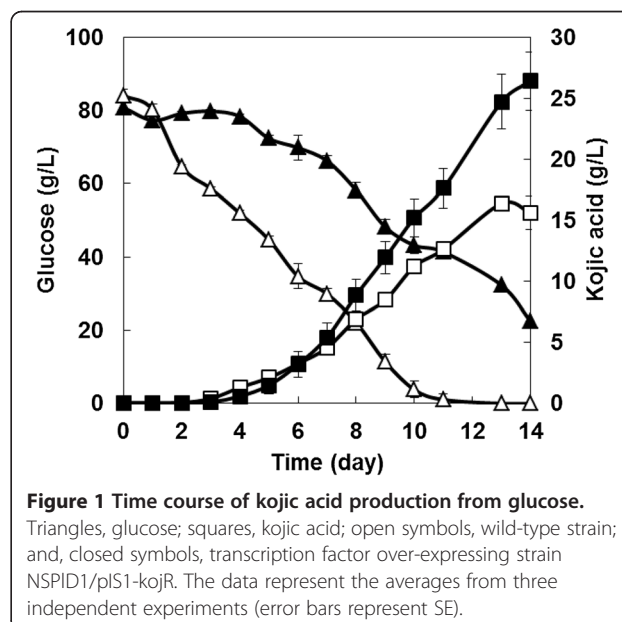
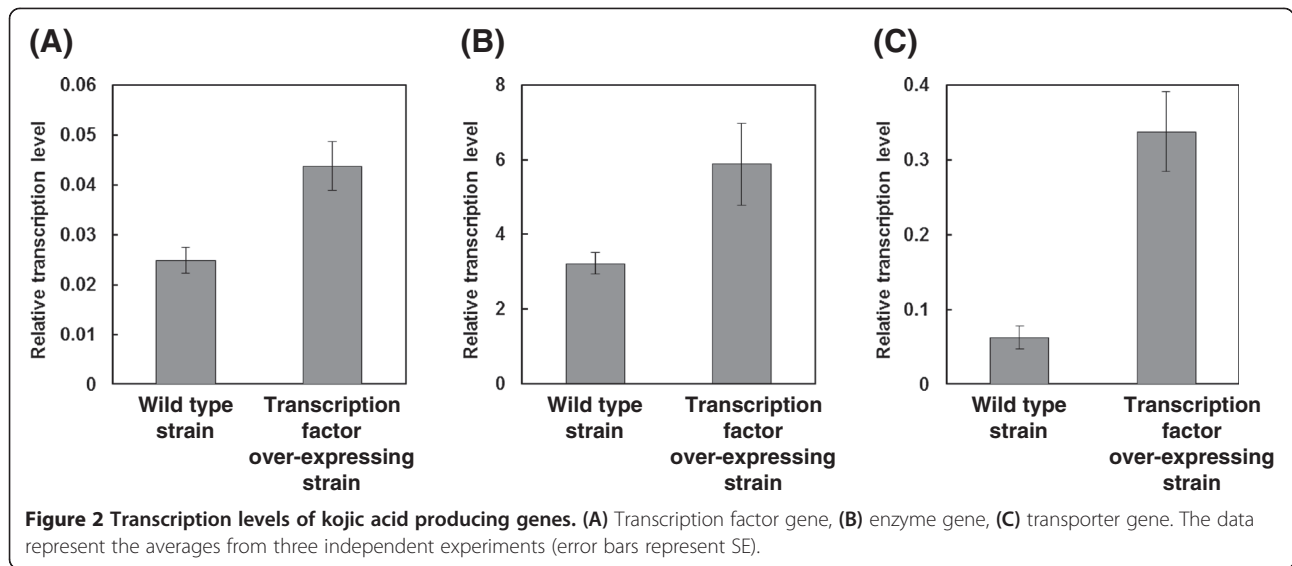


Figure 1 Time course of kojic acid production from glucose. Triangles, glucose; squares, kojic acid; open symbols, wild-type strain; and, closed symbols, transcription factor over-expressing strain NSPID1/pIS1-*kojR*. The data represent the averages from three independent experiments (error bars represent SE).



transporter gene, respectively, compared with the wild-type strain.

Kojic acid production from soluble starch by recombinant *A. oryzae*

Since *A. oryzae* can assimilate starch as a sole carbon source intrinsically, direct kojic acid production from starch using the transcription factor over-expressing strain NSPID1/pIS1-kojR was carried out. As shown in Figure 3, the wild-type strain produced 6.2 g/L of kojic acid after 18 days of fermentation. In contrast, the transcription factor over-expressing strain produced 19.4 g/L of

kojic acid after 18 days of fermentation, i.e. 3.1-fold gain.

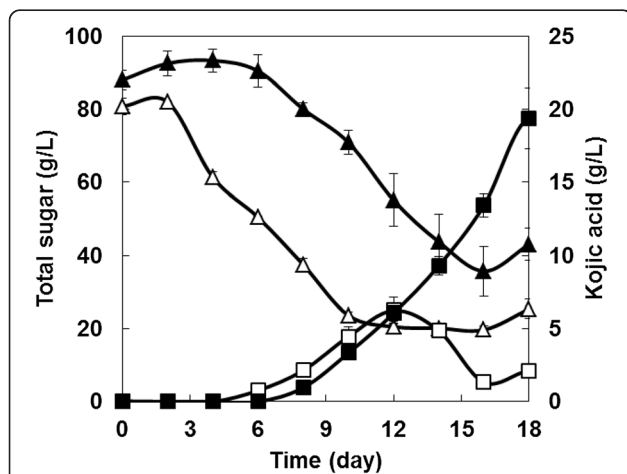


Figure 3 Time course of kojic acid production from starch. Triangles, total sugar; squares, kojic acid; open symbols, wild-type strain; and, closed symbols, transcription factor over-expressing strain NSPID1/pIS1-kojR. The data represent the averages from three independent experiments (error bars represent SE).

Evaluation of cellulase activity

To confirm the cellulase expression of engineered *A. oryzae*, the PASC degradation activity of 3-types of cellulase were evaluated. As shown in Figure 4, the 3-types of cellulase gene-expressing strains NSPID1/pIS1-kojR/pIS1sC-CBHI-EGI-BGLI showed 29.9 U/L after 6 days of

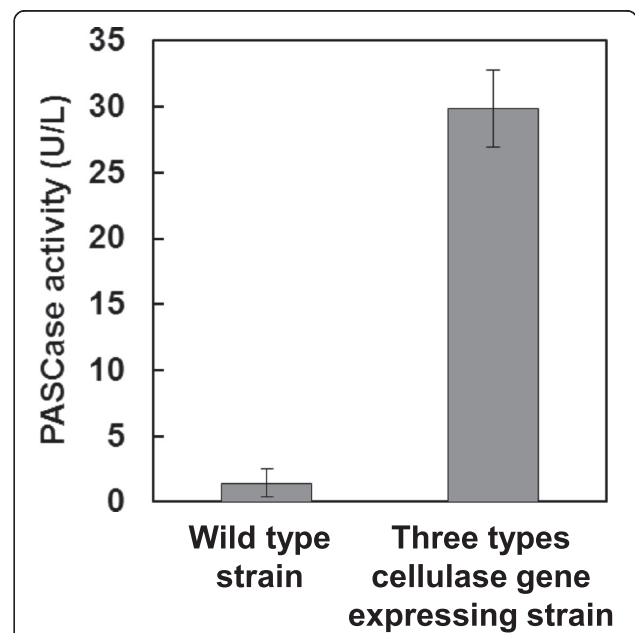


Figure 4 PASCase activity of 3-types of cellulase gene-expressing strains. The data represent the averages from three independent experiments (error bars represent SE).

cultivation. In contrast, the wild-type strain showed significantly lower PASCase activity (1.4 U/L).

Direct kojic acid production from PASC

To confirm direct kojic acid productivity from cellulose, kojic acid production from PASC was carried out. As shown in Figure 5, there was 0.18 g/L of kojic acid produced after 6 days of fermentation when using a transcription factor gene and the 3-types of cellulase genes co-expressing strain NSPID1/pIS1-kojR/pIS1sC-CBHI-EGI-BGLI. In contrast, the wild-type strain did not produce a detectable amount of kojic acid.

Discussion

In the present study, a transcription factor gene over-expressing *A. oryzae* NSPID1/pIS1-kojR was developed by gene recombination strategy. Furthermore, the transcription factor gene and 3-types of cellulase genes co-expressing *A. oryzae* NSPID1/pIS1-kojR/pIS1sC-CBHI-EGI-BGLI was constructed and the strain successfully produced kojic acid directly from PASC.

The transcription factor gene *kojR*, which is responsible for kojic acid production, was over-expressed in this study, and as a result, kojic acid productivity was improved. As shown in Figure 2, the transcription levels of both the kojic acid-producing enzyme *kojA* and the exporting transporter *kojT* were enhanced. This was because the kojic acid productivity was improved. Terabayashi et al. [9] reported that the *kojR*, *kojA*, and *kojT* genes would encode the transcription factor, the kojic acid-producing enzyme, and the transporter, respectively. In the present

study, the transcription level of the enzyme and the transporter were enhanced by an over-expression of the transcription factor, which correlates well with that report. Although there are reports of improved kojic acid productivity through fermentation engineering strategies [1,5,23], to our knowledge, this study is the first to report an improvement in kojic acid productivity via genetic engineering. Kojic acid productivity could be improved even more by combining fermentation engineering and genetic engineering in future work.

Direct kojic acid production from cellulose was successfully demonstrated by *A. oryzae* expressing 3-types of cellulase genes. However, the yield of kojic acid remained low. This was due to the low levels of cellulase production. In previous reports, some microorganisms such as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Bacillus subtilis*, and *E. coli* were used for bio-based chemical production from cellulose by conferring cellulolytic activity [6]. Although most reports did not indicate the amount of cellulase production, Zhang et al. [24] reported that about 14 mg/L of endoglucanase was produced by recombinant *Bacillus subtilis*. In any case, because *T. reesei* produces several dozen g/L of cellulase [7], the amount of cellulase expression from cellulolytic activity conferred microorganisms has not yet reached the level of *T. reesei*. Although *A. oryzae* has an extremely high protein secreting ability, cellulase expression of *T. reesei* is still higher than that of *A. oryzae*. Because the maximum protein production is approximately 20 g/L in *Aspergillus* organisms [25], the cellulase productivity could be improved with genetic engineering.

Three types of cellulase genes and specific transcription factors were successfully over-expressed simultaneously in recombinant *A. oryzae*. To this point, only a few reports have focused on the co-expression of more than 3-kinds of genes in *A. oryzae*. This is because the selection marker for gene recombination is limited in *A. oryzae* compared with other microorganisms such as yeast *S. cerevisiae* [26]. In the present study, a 3-cassette plasmid with different promoters was constructed and the simultaneous overexpression of 4 genes was successful. Thus, the simultaneous multi gene expression technique, which is an important technique for the construction of a cell factory in *A. oryzae*, has been well established. By using this technique, an *A. oryzae*-based cell factory could be a promising strategy for producing various bio-based chemicals.

Conclusions

The productivity of kojic acid from *A. oryzae* was made more efficient by the over-expression of specific transcription factors. To accomplish this, recombinant *A. oryzae* expressing 3 types of cellulase genes was constructed. Direct kojic acid production from PASC by genetically engineered *A. oryzae* was successfully demonstrated.

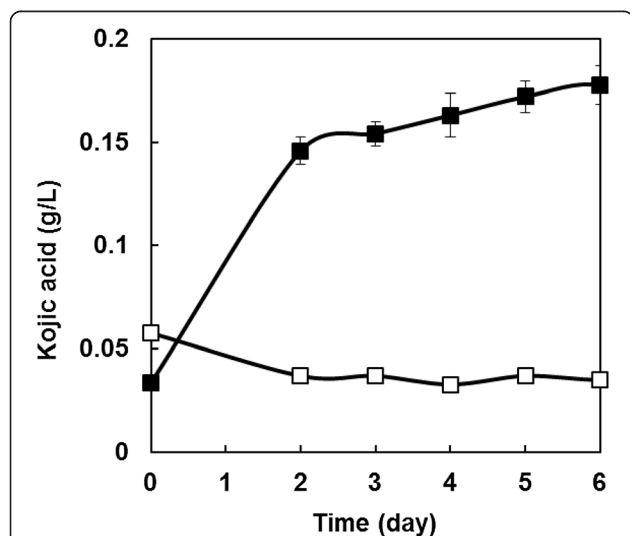


Figure 5 Time course of kojic acid production from PASC. Open symbols, wild-type strain; closed symbols, transcription factor and 3-types of cellulase gene over-expressing strains NSPID1/pIS1-kojR/pIS1sC-CBHI-EGI-BGLI. The data represent the averages from three independent experiments (error bars represent SE).

Although this research was conducted as the proof of a concept, the results showed that an *A. oryzae*-based cell factory could be established. Because, *A. oryzae* has efficient protein secretion ability and secondary metabolite productivity, an *A. oryzae*-based cell factory could be a promising platform for producing various kinds of bio-based chemicals.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TY designed and performed the experiments. NAN performed the experiments. TY and RY wrote the paper. SW, FO, CO, HH, HT, YH and AK commented and supervised on the manuscript. All the authors approved the final manuscript.

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