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Ergothioneine production with *Aspergillus oryzae*

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Ergothioneine production with *Aspergillus oryzae*

To establish a reliable and practical ergothioneine (ERG) supply, we employed fermentative ERG production using *Aspergillus oryzae*, a fungus used for food production. We heterologously overexpressed the *egt-1* and *-2* genes of *Neurospora crassa* in *A. oryzae* and succeeded in producing ERG (231.0 mg/kg of media, which was 20 times higher than the wild type).

Keywords: ergothioneine; molecular breeding; heterologous expression; *Aspergillus oryzae*; *Neurospora crassa*

Ergothioneine (ERG), a histidine (His) betaine derivative with a thiol group at the C2 position of the imidazole ring (Figure 1), is known as a very stable antioxidant. ¹⁾ ERG is a natural compound and is found in the human body such as in red blood cells, the liver, the kidneys, and semen at high concentrations. However, humans cannot biosynthesize ERG and ingest it from diet via an organic cation transporter specific for ERG (OCTN1). ²⁾

ERG was first isolated from an ergot fungus, *Claviceps purpurea*, more than a century ago, and mushrooms, fungi, fission yeast, actinobacteria, cyanobacteria, and a methylobacterium have recently been shown to synthesize ERG. ^{3,4,5,6,7)} Mushrooms are major dietary sources but their slow growth, low contents, and time-consuming purification procedures lead to a high manufacturing cost. Therefore, alternative and sustainable sources of ERG are required.

A reliable and practical method for ERG production is a fermentative process using ERG-producing microorganisms, but the productivities are reported to be very low. We recently succeeded in heterologous production of ERG in *Escherichia coli* utilizing ERG biosynthetic genes identified in *Mycobacteria smegmatis*. ⁸⁾ In this study,

we tried to produce ERG in a fungus with fungal biosynthetic genes. The biosynthetic pathway in *Neurospora crassa* was recently reported (Figure 1).^{9,10)} Egt-1 is a bifunctional enzyme catalyzing successive reactions; the formation of hercynine (HER) with L-His and S-adenosylmethionine (SAM), followed by synthesis of hercynylcysteine-sulfoxide (Cys-HER) with HER, L-cysteine (L-Cys), and O₂. Egt-2, a pyridoxal phosphate-dependent C–S lyase, catalyzes ERG formation from Cys-HER with concomitant release of pyruvate and ammonia as side-products. This pathway would be better for ERG production than that of *M. smegmatis* because it requires less genes and Cys as a sulfur source.

Aspergillus oryzae is a filamentous fungus used for traditional Japanese fermented beverages and seasonings, such as sake, soy sauce, and soybean paste, and is considered to be “generally recognized as safe” by the U.S. Food and Drug Administration.¹¹⁾ In addition, genetic engineering tools for *A. oryzae* have been established and utilized for heterologous expression of various foreign genes to produce many useful compounds.¹²⁾ Therefore, we tried to heterologously produce Egt-1 and Egt-2 in *A. oryzae*.

There were no reports on ERG production by *A. oryzae* even though it has *egt-1* and -2 orthologs, AO090012000265 and AO090026000291, which have 47% and 45% amino acid sequence identities, respectively (https://www.genome.jp/kegg-bin/show_organism?org=aor). Therefore, we first examined whether *A. oryzae* produces ERG in a solid medium containing 20 g of “Nanatsuboshi” rice (polishing ratio of 90%) steeped in deionized water for 6 h, 10 mg of adenine, and 4 mL of deionized water in a 50 mL glass petri dish. After sterilization, mycelia of *A. oryzae* NSAR1¹³⁾ (Table 1) were inoculated into the solid medium and then cultured at 30°C for 5 days. After the whole culture was extracted with 100 mL methanol, an aliquot (1.2 mL) of the extract was centrifuged at 20,000×*g* for 10 min to remove insoluble components. The

supernatant (1 mL) was dried in vacuo and dissolved in 300 μ L of water. Part of the solution (20 μ L) was mixed with 0.05% (v/v) heptafluorobutyric acid (HFBA) solution (180 μ L) and used for liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis. Analytical conditions were as follows: UPLC, Waters ACQUITY system equipped with a photodiode array and a SQ Detector2 (Tokyo, Japan); column, XBridge BEH C18 XP (150 mm L $\times$ 2.0 mm; ID, 2.5 μ m; Waters); flow rate, 0.15 mL/min; temperature, 35°C; mobile phase, water containing 0.05% HFBA and 7% methanol; injection volume, 2 μ L; detection, 258 nm for ERG.⁸⁾ ERG productivity was estimated on the basis of the raw material (i.e. 20 g of steeped rice). As shown in Figure 2, only a small amount of ERG production (11.5 $\pm$ 1.0 mg/kg of media) was detected. Therefore, we attempted to enhance the ERG productivity using a genetic engineering approach.

We first attempted to overexpress the *N. crassa egt-1* gene in *A. oryzae*. DNA fragments encoding *egt-1* were amplified by PCR with *N. crassa* JCM 19069 genomic DNA as the template, the primers 5'-AACAAACTAGTATGCCGAGTGCCGAATCCATGACCCCAAG-3'/5'-TCACACTAGTCAACGACTCACAAATCCCTAACAACCTCTCGC-3' (*Spe*I restriction sites are underlined), and Tks Gflex DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR products (3,491 bp) were cloned into the same restriction site in the expression vector pUARA2¹⁴⁾ to express the *egt-1* gene under the control of the *amyB* promoter. The plasmid thus constructed, pUARA2-Nc1 (11,546 bp, Table 1), was introduced into *A. oryzae* according to a method described previously.¹⁵⁾ After the transformants were subcultured twice on a selection medium, integration of the *Ncegt-1* gene was confirmed by PCR with genomic DNA of the NSA-Nc1 strain and the same primers used for gene cloning (Figure S1A). Moreover, Southern blot analysis using a

DNA fragment carrying *Ncegt-1* as the probe showed only one strong band at approximately 11 kbp after digestion of the genomic DNA with *XhoI*, which has a unique restriction site in the *Ncegt-1* gene (Figure S1B). In addition, only one band was detected by digestion with *KasI* or *BplI*, which have unique restriction sites in the vector. Taking these results together, the *Ncegt-1* gene was suggested to be multiply integrated into the genomic DNA in a similar manner as previously reported.¹⁶⁾ ERG productivity was then examined by the same methods as described above. As shown in Figure 2, the ERG productivity of the transformant (strain NSA-Nc1) drastically increased to 124.5 ± 5.0 mg/kg of media. This result suggested that the *egt-1* gene was efficiently expressed in *A. oryzae*.

Therefore, we next introduced the *N. crassa egt-2* gene into the strain NSA-Nc1. DNA fragments were amplified by PCR with *N. crassa* genomic DNA and the primers 5'-AACAAACTAGTATGGTCGCCACCACCGTCGAGCTGCCTCTG-3'/5'-CAAATACTAGTTCAGGCGCTCTCCTTGTACTCCCCCTTAGCCAC-3' (*SpeI* restriction sites are underlined). The amplicon (1,498 bp) was digested with *SpeI* and inserted into the *SpeI* site of pAdeA2¹⁷⁾ to construct pAdeA2-Nc2 (8,086 bp, Table 1). After the plasmid was introduced into the strain NSA-Nc1, integration of the *Ncegt-2* gene into the genomic DNA was confirmed by PCR and Southern blot analysis (Figure S2). In this case, genomic Southern blot analysis with DNA fragment carrying the *Ncegt-2* gene as the probe showed at least three major bands after digestion of the genomic DNA with *NsiI*, which exists at 159 bp from 5' of the *Ncegt-2* gene. Considering that random integrations are usually dominant rather than homologous recombination in fungi¹⁸⁾, the detection of more than three hybridized bands suggested that the *Ncegt-2* was integrated into at least two different loci of the genome. Accordingly, two major bands were detected by digestion with *HindIII* or *Sall*, which

could cut the vector at unique sites. The ERG productivity of the transformant (strain NS-Nc12) was then investigated by the same methods as described above. As shown in Figure 2, ERG productivity was further increased up to 231.0 ± 1.1 mg/kg of media, suggesting that the *egt-2* gene was also efficiently expressed in *A. oryzae*.

However, significant amounts of HER (32.7 ± 1.1 mg/kg) were accumulated in the culture, suggesting Egt-1 reaction was a bottleneck. Egt-1, the bi-functional enzyme, uses L-His, SAM, and L-Cys as the substrates. Considering that ERG productivity was drastically enhanced by the integration of *egt-1* gene, their substrates were suggested to be sufficiently supplied. Indeed, the ERG productivity with *A. oryzae* were higher than that with *E. coli*⁵⁾. Therefore, the HER accumulation might be caused by a low Cys-HER synthetic activity of Egt-1.

In conclusion, fermentative ERG production by a fungus was carried out to establish a reliable and practical method for ERG production. By heterologous overexpression of the *egt-1* and -2 genes of *N. crassa* in *A. oryzae*, we achieved 20-fold higher ERG production (231.0 ± 1.1 mg/kg of media) than the wild type, which is comparable to mushrooms (16 to 417 mg/kg of wet weight)³⁾. To date, screening of ERG producers from fungi¹⁹⁾ and production of ERG by engineered fungi²⁰⁾ have been carried out. Besides these approaches, our system will also be an alternative method for ERG supply.

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References

- [1] Hartman PE. Ergothioneine as antioxidant. *Methods Enzymol.* 1990;186:310–318
- [2] Gründemann D, Harlfinger S, Golz S, Geerts A, Lazar A, Berkels R, Jung N, Rubbert A, Schömig E. Discovery of the ergothioneine transporter. *Proc. Natl. Acad. Sci. USA* 2005;102:5256–5261.
- [3] Kalaras MD, Richie JP, Calcagnotto A, Beelman RB. Mushrooms: A rich source of the antioxidants ergothioneine and glutathione. *Food Chem.* 2017;233:429–433.
- [4] Genghof DS. Biosynthesis of ergothioneine and hercynine by fungi and *Actinomycetales*. *J. Bacteriol.* 1970;103:475–478.
- [5] Pluskal T, Ueno M, Yanagida M. Genetic and metabolomic dissection of the ergothioneine and selenoneine biosynthetic pathway in the fission yeast, *S. pombe*, and construction of an overproduction system. *PLoS One* 2014;9:e97774.
- [6] Pfeiffer C, Bauer T, Surek B, Schömig E, Gründemann D. Cyanobacteria produce high levels of ergothioneine. *Food Chem.* 2011;129:1766–1769.
- [7] Alamgir KM, Masuda S, Fujitani Y, Fukuda F, Tani A. Production of ergothioneine by *Methylobacterium* species. *Front. Microbiol.* 2015;6:1185.
- [8] Osawa R, Kamide T, Satoh Y, Kawano Y, Ohtsu I, Dairi T. Heterologous and high production of ergothioneine in *Escherichia coli*. *J. Agric. Food Chem.* 2018;66:1191–1196
- [9] Bello MH, Barrera-Perez V, Morin D, Epstein L. The *Neurospora crassa* mutant *NcΔEgt-1* identifies an ergothioneine biosynthetic gene and demonstrates that ergothioneine enhances conidial survival and protects against peroxide toxicity during conidial germination. *Fungal Genet. Biol.* 2012;49:160–172.
- [10] Hu W, Song H, Sae Her A, Bak DW, Naowarojna N, Elliott SJ, Qin L, Chen X, Liu P. Bioinformatic and biochemical characterizations of C–S bond formation and cleavage enzymes in the fungus *Neurospora crassa* ergothioneine biosynthetic pathway. *Org. Lett.* 2014;16:5382–5385.

- [11] Barbesgaard P, Heldt-Hansen HP, Diderichsen B. On the safety of *Aspergillus oryzae*: a review. Appl. Microbiol. Biotechnol. 1992;36:569–572.
- [12] Wakai S, Arazoe T, Ogino C, Kondo A. Future insights in fungal metabolic engineering. Bioresour. Technol. 2017;245:1314–1326.
- [13] Jin FJ, Maruyama J, Juvvadi PR, Arioka M, Kitamoto K. Development of a novel quadruple auxotrophic host transformation system by *argB* gene disruption using *adeA* gene and exploiting adenine auxotrophy in *Aspergillus oryzae*. FEMS Microbiol. Lett. 2004; 239:79–85.
- [14] Tagami K, Minami A, Fujii R, Liu C, Tanaka M, Gomi K, Dairi T, Oikawa H. Rapid reconstitution of biosynthetic machinery for fungal metabolites in *Aspergillus oryzae*: total biosynthesis of aflatrem. ChemBioChem 2014;15:2076–2080.
- [15] Liu C, Tagami K, Minami A, Matsumoto T, Frisvad JC, Suzuki H, Ishikawa J, Gomi K, Oikawa H. Reconstitution of biosynthetic machinery for the synthesis of the highly elaborated indole diterpene penitrem. Angew. Chem. Int. Ed. 2015;54:5748–5752
- [16] Gomi K, Iimura Y, Hara S. Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. Agric. Biol. Chem. 1987;51:2549–2555
- [17] Ugai T, Minami A, Fujii R, Tanaka M, Oguri H, Gomi K, Oikawa H. Heterologous expression of highly reducing polyketide synthase involved in betaenone biosynthesis. Chem. Commun. 2015;51:1878–1881.
- [18] Lubertozzi D, Keasling JD. Developing *Aspergillus* as a host for heterologous expression. Biotechnol. Adv. 2009;27:53–75.
- [19] Fujitani Y, Alamgir KM, Tani A. Ergothioneine production using *Methylobacterium* species, yeast, and fungi. J. Biosci. Bioeng. 2018; DOI: 10.1016/j.jbiosc.2018.05.021
- [20] Hara S, Hirokawa K, Ichikawa K. Transformed fungus having enhanced ergothioneine productivity and method for producing ergothioneine. Patent, WO2016121285.

Author contributions

T. Dairi conceived and designed the experiments. S.T., Y.S., and I.O. performed the experiments and analyzed the data. Y.S. and T.D. wrote the paper.

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Abbreviations used

ERG, ergothioneine; HER, hercynine; Cys-HER, hercynylcysteine-sulfoxide; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; L-His, L-histidine; L-Cys, L-cysteine; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry.

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Description	Source
Strains		
<i>A. oryzae</i>		
NSAR1	<i>sC⁻, niaD⁻, ΔargB, adeA⁻</i>	13
NSA-Nc1	NSAR1 transformed with pUARA2-Nc1; <i>Ncegt-1⁺</i>	This study
NS-Nc12	NSA-Nc1 transformed with pAdeA2-Nc2; <i>Ncegt-1⁺, Ncegt-2⁺</i>	This study
<i>N. crassa</i>		
JCM 19069	ERG producer	JCM
<i>E. coli</i>		
XL-1 Blue	<i>hsdR17, recA1, endA1, gyrA96, thi-1, supE44, relA1, lac[F', proAB, lacI^qΔM15, Tn10(Tc^R)]</i>	Nippon Gene
Plasmids		
pUARA2	Vector; PamyB, TamyA, Ap ^R , <i>argB</i>	14
pUARA2-Nc1	pUARA2 derivative with <i>Ncegt1</i>	This study
pAdeA2	Vector; PamyB, TamyA, Ap ^R , <i>adeA</i>	17
pAdeA2-Nc2	pAdeA2 derivative with <i>Ncegt2</i>	This study

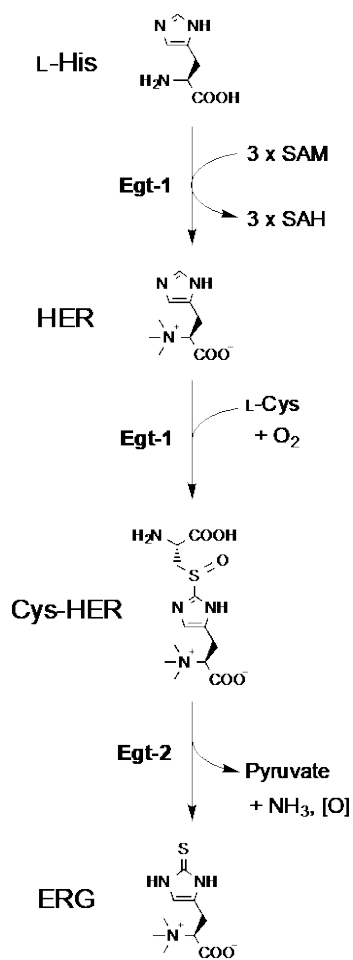
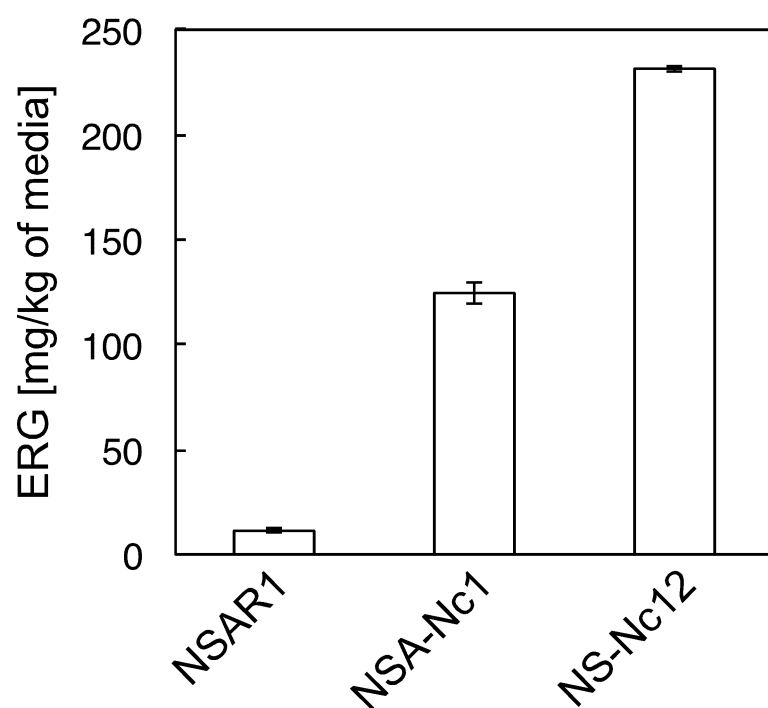


Figure 1. The ARG biosynthetic pathway in fungi.

SAH, S-adenosylhomocysteine



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251 Figure 2. ERG production by *A. oryzae* NSAR1 and its recombinants.

252 Data are presented as mean values with standard errors from three independent
253 experiments.

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