Toward more efficient ergothioneine production using the fungal ergothioneine biosynthetic pathway

Zhihui Chen1,2†, Yongzhi He1†, Xinyu Wu1, Li Wang1, Zhiyang Dong1* and Xiuzhen Chen1*

Abstract

Background: Ergothioneine (ERG) is a potent histidine-derived antioxidant that confers health-promoting effects. Only certain bacteria and fungi can biosynthesize ERG, but the ERG productivity in natural producers is low. ERG overproduction through genetic engineering represents an efficient and cost-effective manufacturing strategy.

Results: Here, we showed that Trichoderma reesei can synthesize ERG during conidiogenesis and hyphal growth. Co-expression of two ERG biosynthesis genes (tregt1 and tregt2) from T. reesei enabled E. coli to generate 70.59 mg/L ERG at the shaking flask level after 48 h of whole-cell biocatalysis, whereas minor amounts of ERG were synthesized by the recombinant E. coli strain bearing only the tregt1 gene. By fed-batch fermentation, the extracellular ERG production reached 4.34 g/L after 143 h of cultivation in a 2-L jar fermenter, which is the highest level of ERG production reported thus far. Similarly, ERG synthesis also occurred in the E. coli strain engineered with the two well-characterized genes from N. crassa and the ERG productivity was up to 4.22 g/L after 143 h of cultivation under the above-mentioned conditions.

Conclusions: Our results showed that the overproduction of ERG in E. coli could be achieved through two-enzymatic steps, demonstrating high efficiency of the fungal ERG biosynthetic pathway. Meanwhile, this work offers a more promising approach for the industrial production of ERG.

Keywords: Ergothioneine, Biosynthesis, Heterologous expression, Trichoderma reesei, Neurospora crassa, Escherichia coli

Background

Ergothioneine (ERG) is a thiol-containing histidine betaine derivative, that protects cells against oxidative damage caused by excess reactive oxygen species (ROS). Uniquely, ERG exists predominately in the thione tautomer at physiological pH [1] and possesses relatively high reduction potential (−60 mV), making it more stable and resistant to autooxidation than other thiol-containing antioxidants such as glutathione [2]. As a powerful antioxidant and cytoprotectant, ERG has been suggested to confer effective and beneficial roles on human health, such as anti-inflammation [3], anti-aging [4], and antidepressant properties and the ability to prevent ultraviolet damage [5–7]. Recently, ERG has been assessed as safe to use in the food and cosmetics industries [8–10], which will increase the market demand for ERG and exploration of methods to produce ERG [11].

ERG biosynthesis occurs only in certain bacteria and fungi, typically actinobacteria [12], cyanobacteria [13], methylobacteria [14], and various fungi including Neurospora crassa [15], the fission yeast Schizosaccharomyces

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revealed that there are far more bacterial species and fun-
genes has substantially increased the ERG productivity of
Likewise, recombinant expression of ERG biosynthetic
genes (\textit{E. coli}) from \textit{ERG} biosynthetic genes (\textit{E. coli}) after 216 h in a 3-L jar fermenter by expressing five
note, high production of ERG (1.31 g/L) was achieved in
\textit{E. coli} with the \textit{Grifola frondosa} \textit{egt1/egt2} genes [22] or with the combined use of \textit{N. crassa} \textit{egt}1 and \textit{Claviceps purpurea} \textit{egt}2 [23]. Of
high production of ERG (1.31 g/L) was achieved in
\textit{E. coli} after 216 h in a 3-L jar fermenter by expressing five
\textit{ETG} biosynthetic genes (\textit{egtABCDE}) from \textit{M. smegmatis}, enhancing l-cysteine (l-Cys) production, knocking out \textit{met}1 and optimizing the fermentation medium [24].
Likewise, recombinant expression of \textit{ETG} biosynthetic
gen genes has substantially increased the \textit{ETG} productivity of
natural \textit{ETG} producers such as \textit{S. pombe} [16] and \textit{Aspergillus oryzae} [25]. Therefore, this approach represents
an efficient and cost-effective means for the industrial
production of \textit{ETG} [21]. However, the output of \textit{ETG} is
still relatively low, resulting in insufficient supply of \textit{ETG}
and high price. Sequence-based phylogenies of the key
gen genes (\textit{egtB, egtD in M. smegmatis} and \textit{egt}1 in \textit{N. crassa})
revealed that there are far more bacterial species and fun-
gal phyla capable of producing \textit{ETG} than the number of
\textit{ETG}-producing microorganisms discovered thus far [12].
Exploring the potential of these microorganisms may be
the key toward high-level \textit{ETG} production, particularly
for the fungal \textit{ETG} biosynthetic pathway represented by
\textit{N. crassa} that requires only two genes and may be more
effective in \textit{ETG} biosynthesis. Until now, only a few fun-
gal \textit{ETG} biosynthesis genes have been characterized, and
in terms of the \textit{ETG} productivity, their potential does not
seem to be fully realized in the fungal systems reported
previously [22, 25]. Hence, it will be a valuable attempt to
investigate the efficiency of various fungal \textit{ETG} biosyn-
thesis genes of synthesizing \textit{ETG} in bacterial systems like
\textit{E. coli} that is a model microorganism commonly used for
synthetic biology and industrial applications.

The filamentous fungus \textit{Trichoderma reesei} is the work-
horse for the industrial production of lignocellulolytic
enzymes [26, 27]. Moreover, \textit{T. reesei} is an attractive host
for the production of recombinant proteins due to its
extraordinary ability to secrete proteins and its (GRAS)
Generally Regarded as Safe status approved by the US
Food and Drug Administration [28–30]. Although the
physiological roles of \textit{ETG} in \textit{T. reesei} remain unknown,
the presence of putative \textit{ETG} biosynthetic genes raises
the possibility that \textit{T. reesei} has the potential to produce
\textit{ETG}.

Here, we determined that \textit{T. reesei} can synthesize \textit{ETG}.
Through heterologous expression in \textit{E. coli}, we examined
the role of the two putative \textit{ETG} biosynthesis genes from
\textit{T. reesei} and investigated the possibility of synthesizing
\textit{ETG} in \textit{E. coli} using the fungal \textit{ETG} biosynthetic genes
from \textit{T. reesei} and \textit{N. crassa}. Our research showed that
high-level of \textit{ETG} production in \textit{E. coli} can be achieved
by using only two genes from fungi. This work offers a
more practical and promising approach for the industrial
production of \textit{ETG}.

**Results and discussion**

**Cloning of \textit{ETG} biosynthesis genes from \textit{T. reesei}**

Prior to cloning the \textit{ETG} biosynthesis genes, we extracted
\textit{ETG} from the conidia and mycelia to determine whether
\textit{T. reesei} has evolved the ability to synthesize \textit{ETG}. HPLC
analysis showed that the extracted samples displayed a
predominant peak at a retention time of 10–10.5 min,
which was the same as that of the \textit{ETG} standard (Fig. 1).
The predominant peak was further confirmed by LC–MS
analysis (Additional file 1: Fig. S1). These results clearly
demonstrated that \textit{T. reesei} can synthesize \textit{ETG}, which
provides a basis for cloning of functional \textit{ETG} biosyn-
thetic genes in \textit{T. reesei}.

BLASTP search with \textit{N. crassa} Ncegt1 ([NCBI Reference
Sequence: XP_956324] and Ncegt2 ([NCBI Reference
Sequence: XP_001728131]) as query
sequences revealed that two hypothetical pro-
teins designated Tregt1 ([NCBI Reference
Sequence: XP_006968620] and Tregt2 ([NCBI Reference
Sequence: XP_006968735], respectively, were probably

![Fig. 1](https://example.com/fig1.png)
involved in ERG biosynthesis in T. reesei. The coding sequences of the cloned tregt1 and tregt2 genes were 2502 bp and 1413 bp (Additional file 1: Data S1, S2), respectively. Tregt1 (Additional file 1: Data S3) shared 61.28% (97% coverage) amino acid sequence identity with NcEgt1 and contained an S-adenosylmethionine (SAM)-dependent methyltransferase domain, a DinB_2 domain, and a sulfoxide synthase domain (Fig. 2A), implying that Tregt1 may catalyze the first two steps of the ERG biosynthetic pathway.

Tregt2 (Additional file 1: Data S4), a putative selenocysteine lyase-like protein, displayed 53.77% homology (98% coverage) with NcEgt2 and included the pyridoxal phosphate (PLP)-dependent cysteine desulfurase domain present in NcEgt2 that catalyzes the conversion of hercynylcysteine sulfoxide to ergothioneine by cleaving the C–S bond. It is probably that like N. crassa [15], T. reesei synthesizes ERG through two enzymes instead of five-enzymatic catalysis for ERG biosynthesis in M. smegmatis [19] (Fig. 2B).

**ERG biosynthesis by a whole-cell biocatalyst**

To test whether Tregt1 and Tregt2 have the ability to convert l-histidine into ERG, we constructed recombinant E. coli strains harbouring the expression plasmids pBAD, pBAD-tregt1, pBAD-tregt2, and pBAD-tregt1-tregt2 (Fig. 3A). After 48 h of whole-cell biocatalyst reaction, the strain bearing pBAD-tregt1-tregt2 produced 70.59 mg/L extracellular ERG. However, ERG was not detected from the strain with pBAD-tregt2 (Fig. 3B), although tregt2 was successfully expressed in this recombinant E. coli strain (Fig. 3C). Of note, the strain with pBAD-tregt1 was able to synthesize ERG, although its production was lower than that of recombinant strain co-expressing tregt1 and tregt2, suggesting that functions of Tregt2 may be performed by other yet unknown enzymes with weak cleavage activity of hercynylcysteine sulfoxide, which was also observed in Saccharomyces cerevisiae [22].

To test whether ERG biosynthesis in E. coli can also be achieved by using two genes from other fungi, we constructed the recombinant E. coli strain bearing egt1 and egt2 genes from N. crassa. Similarly, we found that the
co-expression of egt1 and egt2 from *N. crassa* also enabled *E. coli* to produce ERG (Additional file 1: Fig.S2), showing that it is practical to synthesize ERG in *E. coli* only using two genes originating from fungi.

**ERG production by high-cell-density fermentation**

To evaluate the potential of the recombinant *E. coli* strain co-expressing *tregt1* and *tregt2* for the industrial production of ERG, we performed high-cell-density fermentation in a 2-L jar fermenter with the fed-batch strategy. During the whole fermentation process, the recombinant strain grew well, and the OD$_{600}$ of the cultures reached 105 at 60 h and 130 at 130 h. Extracellular ERG was detected at 30 h and continued to increase until 143 h, with the concentrations of ERG in the supernatant of 0.89 g/L at 48 h, 1.43 g/L at 72 h, 2.91 g/L at 94 h and 4.34 g/L at 143 h (Fig. 4A), which is the highest ERG production level reported thus far. Similarly, high level of ERG production (4.22 g/L) was achieved in the *E. coli* strain bearing the two genes responsible for *N. crassa* ERG biosynthesis (Fig. 4B). In addition to the contribution of the fermentation conditions to ERG production, another important reason for the high yield of ERG is probably due to the utilization of the genes associated with the fungal ERG biosynthesis. Compared to that from *M. smegmatis*, the fungal ERG biosynthetic pathway represented by *N. crassa* is more effective since it requires only two genes and l-Cys rather than γ-glutamylcysteine (γGC) as a sulfur donor, which facilitates hercynylcysteine sulfoxide synthesis and avoids competition with the glutathione synthesis pathway [15, 19]. From our results, the effectiveness of the fungal ERG synthesis pathway can be achieved not only in fungi but also in bacteria; therefore, it is practical to overproduce ERG through heterologous expression of ERG biosynthetic genes from fungi in *E. coli*. To maximize the ERG productivity of the recombinant strain, we will conduct closer inspection of the gene expression level, intermediate product accumulation, ERG precursor supply and proportion, and further optimizations will be made.

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Fig. 3 Plasmid profiles, protein expression and ERG biosynthesis. A Schematic drawing of plasmids expressing *tregt1* and/or *tregt2* used for transforming *E. coli* BW25113. B The production of ERG by 48-h whole cell catalysis using the recombinant strains BW-*tregt1*, BW-*tregt2* and BW-*tregt1-*tregt2*. Data in the figure are mean values (n = 3 biological replicates). C Detection of *Tregt1* and/or *Tregt2* expression in recombinant *E. coli* by SDS-PAGE. M. Protein marker; 1. BW-pBAD (control); 2. BW-*tregt1*; 3. BW-*tregt2*; 4. BW-*tregt1-*tregt2.
Conclusions
Here, we demonstrated that *T. reesei* can synthesize ERG. By bioinformatics analysis and reconstruction of the *T. reesei* ERG synthetic pathway in *E. coli*, we found that ERG biosynthesis in *E. coli* can be achieved by using only two genes from *T. reesei*, with Tregt1 being the key enzyme in this process. In addition, the recombinant *E. coli* strain co-expressing egt1 and egt2 genes from *N. crassa* also can synthesize ERG. Through fed-batch cultivation, the highest level of ERG production was achieved after 143 h of cultivation. To the best of our knowledge, this is the first report to overproduce ERG in *E. coli* with fungal biosynthetic genes.

Materials and methods
Strains and media
*Trichoderma reesei* strain QM9414 (ATCC 26,921) was cultivated on potato dextrose agar (PDA) or in liquid minimal medium (MM) with 5 g/L glucose and 40 g/L lactose as the carbon source. MM without peptone was prepared as described previously [31]. *E. coli* strain Trans1-T1 (TransGen Biotech, China) was used for standard cloning. *E. coli* K12/BW25113 (rrnB3 ΔlacZΔ787 hsdR514Δ(arAB)567 Δ(rhaBAD)568 rph-1) [32] was used as the host strain for the heterologous expression of ERG biosynthetic genes from *T. reesei* and ERG production. Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and ZYM auto-induction medium [33] was used to grow *E. coli* cells and to express enzyme, respectively. A defined medium (DM) was used for whole-cell biocatalysis and fed-batch fermentation, which contained (per L) 10 g of glucose, 8 g of (NH₄)₂HPO₄, 13.3 g of KH₂PO₄, 1.2 g of MgSO₄·7H₂O, 1.7 g of citric acid and 10 mL of a trace metal solution. The trace metal solution (per litre of 5 M HCl) consisted of 10 g of FeSO₄·7H₂O, 2.25 g of ZnSO₄·7H₂O, 1 g of CuSO₄·5H₂O, 0.5 g of MnSO₄·5H₂O, 0.23 g of Na₂B₄O₇·10H₂O, 2 g of CaCl₂·2H₂O and 0.1 g of (NH₄)₆Mo₇O₂₄. When necessary, the antibiotic ampicillin (100 mg/L) or the inducer L-arabinose (2 g/L) was added.

Extraction of ERG from *T. reesei*
*Trichoderma reesei* QM9414 was cultivated on PDA at 28 °C for 10 days, and then the conidia were suspended in 1.1 M sorbitol solution and centrifuged at 13,300 rpm for 3 min. The lower conidia pellet was frozen in liquid nitrogen and ground into a powder. After that, the conidial powder was added to an 85% methanol solution and vortexed for 1 min, and the suspension was centrifuged at 13,300 rpm for 3 min. The supernatant was collected and diluted tenfold with 70% acetonitrile solution, and then filtered through a 0.22 μm filter for ERG detection.

The conidia of *T. reesei* QM9414 were inoculated into liquid MM containing 0.5% (w/v) glucose and 4% (w/v) lactose and cultivated on a rotary shaker (200 rpm) at 28 °C for 6 days, and the mycelia were collected to extract ERG. The method of extracting ERG from mycelium was the same as that for the conidia.

Construction of the recombinant BW25113 strains
The expression vectors were constructed with the plasmid pBAD/His (Invitrogen, USA) as the backbone, which includes the pBR322 origin, araBAD promoter induced by arabinose and rrnB terminator. Primer pairs FpBAD/RpBAD were designed to amplify plasmid pBAD/His.
to obtain linearized pBAD/His for subsequent vector construction.

The entire open reading frame of the tregt1 and tregt2 genes were amplified using the primer pairs Ftregt1/Rtregt1 or Ftregt2/Rtregt2 with T. reesei QM9414 cDNA [34] as a template. The amplified tregt1 or tregt2 products were ligated with linearized pBAD/His through a Clone Express® MultiS One Step Cloning Kit (Vazyme Biotech Co, China) and then transformed into E. coli Trans1-T1 for cloning and sequencing. The resulting plasmids pBAD-tregt1 and pBAD-tregt2, in which tregt1 or tregt2 was under the control of the araBAD promoter and rrnB terminator, was used to transform BW25113 to obtain the recombinant strains BW-tregt1 and BW-tregt2. To construct the recombined E. coli strain co-expressing tregt1 and tregt2, the T7 RBS sequence (ttgtaacttaagaaggagatacct) was used to link the two genes. Briefly, primer pairs Ftregt1/RpBAD-tregt1 were designed to amplify the plasmid pBAD-tregt1 to obtain linearized pBAD-tregt1 with the T7 RBS sequence attached to the 3' end of tregt1. Additionally, tregt2 with the T7 RBS sequence at its 5' end was amplified by PCR using the plasmid pBAD-tregt2 as template and the primer pairs FpBAD-tregt2/Rtregt2. Subsequently, plasmid pBAD-tregt1-tregt2 was constructed by ligating T7 RBS-containing tregt2 to pBAD-tregt1 harbouring T7 RBS, which was transformed into BW25113 to create the recombinant strain BW-tregt1-tregt2.

We adopted the same strategy to construct the expression plasmid pBAD-ncegt1-ncegt2 harbouring egt1 (XM_951231) and egt2 (XM_001728079.2) responsible for ERG biosynthesis in N. crassa, except that the entire open reading frames of the two genes were synthesized by Tsingke Biotechnology Co., Ltd (China). The resulting plasmid (Additional file 1: Fig.S2A) was transformed into BW25113 to obtain recombinant E. coli strain BW-ncegt1-ncegt2, in which ncegt1 and ncegt2 were also successfully expressed (Additional file 1: Fig.S2C).

All primers used in this study are listed in Table S1 in additional file 1.

Protein expression and identification

BW25113 and the engineered strains derived from it were cultivated in LB medium with ampicillin at 37 °C on a rotary shaker (200 rpm) until the optical density of the cultures reached 0.6. Expression was induced by the addition of arabinose at a final concentration of 0.2% (w/v). After 24 h of induction at 30 °C and 200 rpm, the cells were collected by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 7.0). The cell suspension was sonicated and centrifuged (12,000×g, 10 min). The supernatant was used for SDS-PAGE analysis.

Whole-cell catalysis conditions

BW25113 and the engineered strains derived from it were grown in LB medium overnight at 37 °C on a rotary shaker (200 rpm). Five hundred microliter of the overnight cultures were inoculated into 50 mL of ZYM auto-induction medium containing 2 g/L of arabinose. After 24 h of induction at 30 °C and 200 rpm, the cell cultures were harvested by centrifugation at 5,000 rpm for 5 min. The resulting cell pellets were resuspended in the reaction mixture (100 mM PBS, 50 mM glucose, 1 g/L L-histidine, 1 g/L L-methionine, 1 g/L L-cysteine, 20 mg/L FeSO₄·7H₂O, pH 7.0) to form a cell suspension (OD600 = 10). The whole-cell catalysis reaction was conducted in a 100-mL Erlenmeyer flask containing 30 mL of cell suspension on a rotary shaker (200 rpm) at 30 °C for 48 h. The extracellular and intracellular ERG content was subjected to high performance liquid chromatography (HPLC) analysis.

Fed-batch cultivation

Precultures of the recombinant strains co-expressing egt1 and egt2 from T. reesei and N. crassa were prepared with ampicillin-containing LB medium in Erlenmeyer flasks at 37 °C at 200 rpm overnight. One hundred millilitres of the precultures were transferred into 900 mL of DM in 2-L jar fermenter, and cultivation was continued at 37 °C, agitated with turbine impellers. When the OD₆₀₀ of the cultures reached 30 (approximately 12 h), the inducer l-arabinose was added at a final concentration of 0.2%(w/v) to induce the expression of egt1 and egt2 at 30 °C with mixing for 12 h. After that, the amino acid mixture (40 g/L of each L-histidine, L-methionine and L-cysteine), which are the precursors of ERG biosynthesis, was constantly fed at a flow rate of 4 mL/h/L. During the whole fermentation, feeding solution (50% glucose, w/v) was periodically added after glucose depletion. The dissolved oxygen was kept above 20% air saturation by adjusting the agitation intensity and aeration rate. The pH was maintained at approximately 7.0 by automatic addition of 2.7 M ammonia solution or 1 M H₃PO₄. At the indicated time points, the cell cultures were sampled and used to extract extracellular ERG and E. coli growth as indicated by the optical density at 600 nm (OD₆₀₀).

HPLC analysis of ERG

ERG samples were diluted tenfold with a 70% acetonitrile solution. ERG standards (Std) were dissolved in a 70% acetonitrile solution. HPLC (Agilent 1200 infinity series 1260, Agilent Technologies) was performed with an Agilent ZORBAX NH₃ column (4.6×250 mm, 5 μm). A mobile phase of acetonitrile/deionized water (70:30, v/v) was used at a flow rate of 1.0 mL/min. The produced ERG was detected at 254 nm and identified by comparison.
with the retention time of the analytical ERG standard (Sigma). Quantification was conducted by dividing the slope of the standard curves by the peak area.

Liquid chromatography-mass spectrometry (LC–MS) analysis of ERG
HPLC-purified ERG was identified by LC–MS (Agilent 1260/6460LC/Triple Quadrupole MS, Agilent Technologies) with Agilent ZORBAX NH$_2$ column (4.6 × 250 mm, 5 μm). Analysis was performed with a mobile phase of acetonitrile/4 mmol/L ammonium acetate (70:30, v/v).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12934-022-01807-3.

Additional file 1: Table S1 Primers used in this study. Fig. S1 LC–MS analysis of ERG. A ERG standards (10 ppm), B the ERG sample extracted from mycelia. Fig. S2 Plasmid profiles, protein expression and ERG production of BW-ncegt1-ncegt2. A Schematic drawing of plasmids expressing Ncegt1 and Ncegt2 used in E. coli BW25113 transformation. B The production of ERG by 48-hour whole cell catalysis using the recombinant strains BW-ncegt1-ncegt2. Data in the figure are mean values (n = 3 biological replicates). C Detection of Ncegt1 and Ncegt2 expression in recombinant E. coli by SDS-PAGE. M. Protein marker; 1. BW-pBAD (control); 2. BW-ncegt1-ncegt2. Data S1 Nucleotide sequences of Tregt1 from T. reesei (2502 bp). Data S2 Nucleotide sequences of tregt2 from T. reesei (1413 bp). Data S3 Amino acid sequences of Tregt1 from T. reesei (833 aa). Data S4 Amino acid sequences of Tregt2 from T. reesei (720 aa).

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Author contributions
XZC and ZHC conceived the project and designed the experiments. ZHC, YZH and XYW performed the experiments. XZC and ZHC analyzed the data and wrote the manuscript. LW prepared the Additional file 1: Figure S1. ZDY provided resources and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Declarations
Ethics approval and consent to participate
Not applicable.

 Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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