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Improvement of extracellular bacterial protein production in *Pichia pastoris* by co-expression of endoplasmic reticulum residing GroEL–GroES

Pijug Summpunn,[1,2,*] Juntratip Jomrit,[3] and Watanalai Panbangred[4,5]

Food Technology and Innovation Research Center of Excellence, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80161, Thailand,[1] Center of Excellence for Shrimp, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80161, Thailand,[2] Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand,[3] Department of Biotechnology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand,[4] and Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU:CRC), Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand[5]

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Pichia pastoris **is an established host system for heterologous protein expression. However, the potential productivity of this system can be limited. In this study, the *Escherichia coli* chaperones (GroES–GroEL) were expressed from the P_{GAP} promoter and targeted to the secretory pathway through the endoplasmic reticulum (ER). The ability of the ER targeted chaperones to improve production of bacterial protein in *P. pastoris* was evaluated. The chaperones tagged with α-factor secretion- and ER retention-signal sequences were co-expressed with either extracellularly secreted phytase or intracellular D-phenylglycine aminotransferase (D-PhgAT) enzymes. The ER residing GroEL–GroES successfully increased the levels of active phytase extracellularly, 1.5–2.3-fold higher than the phytase expression alone, but did not enhance the formation of active, intracellular D-PhgAT. These results indicate that the chaperones have the potential to enhance production of active enzymes when present in the same trafficking pathway. This is the first report on the improvement of extracellular bacterial protein production through co-expression with ER residing bacterial chaperones in the *Pichia* system. The modified *P. pastoris* expression system may be beneficial for extracellular expression of other prokaryotic proteins.**

[**Key words:** Chaperone; Expression system; *Pichia*; Productivity; Protein folding]

Production of recombinant or biopharmaceutical proteins as well as bioactive metabolites has traditionally employed bacterial and eukaryotic organisms as hosts. Bacteria and yeasts share some beneficial features such as single cell growth, easy cultivation on inexpensive media, and availability of genetic manipulation systems. The methylotrophic yeast, *Pichia pastoris*, has attracted increased attention due to its efficient secretion of heterologous proteins, high biomass concentration, ability to perform post-translational modifications, and potential for metabolic engineering applications (1). However, a number of bottlenecks and stress factors limit the potential of this expression system and reduce the final yield of expressed proteins (2,3). Several approaches, including metabolic and cell engineering, have been utilized to improve the productivity in *P. pastoris* (4–7). Interestingly, Jariyachawalid et al. (4) reported the successful use of bacterial chaperones to co-express and enhance intracellular expression of a functional bacterial enzyme in *P. pastoris*. It was the first report on the use of *Escherichia coli* GroEL–GroES to enhance the yield of a functional enzyme, D-phenylglycine aminotransferase (D-PhgAT) from *Pseudomonas stutzeri*, through heterologous expression in yeast. Moreover, it was reported that increasing gene copy numbers for *groEL* and *groES* resulted in a progressive increase in D-PhgAT activity and yield. This indicated that the bacterial chaperone is an important to maximize functional expression of bacterial enzymes in *P. pastoris*. We hypothesize that in addition to increasing gene dosage, if the retention of co-expressed functional chaperones can be prolonged or recycled within the endoplasmic reticulum (ER), chaperones would be better able assist with the native folding of heterologous expressed proteins. Thus expression of chaperones in the ER may enhance the yield of functional proteins that are ultimately secreted.

Specific retention sequence (HDEL, His–Asp–Glu–Leu) (8) have been successfully used to retrieve enzymes from the Golgi apparatus to the ER through the cytosolic coat protein I (COP I) dependent pathway. This can facilitate enhanced concentrations of the enzyme in the ER which is important if activity in the ER is required (9,10). In this study, we focused on the improving the expression of functional proteins through correct protein folding in the *Pichia* expression system. Target proteins were co-expressed with chaperones, GroEL–GroES, localized to the ER with the use of HDEL retention system. The resulting modified *Pichia* expression system was evaluated as a production host for heterologous expression of either extracellular (phytase) or intracellular (D-PhgAT) proteins.

MATERIALS AND METHODS

Stains, plasmid, oligonucleotides, enzymes, media and chemicals *E. coli* DH5α was purchased from Novagen (Madison, WI, USA) and used as a cloning host

* Corresponding author at: School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80161, Thailand. Tel.: +66 7 567 2371; fax: +66 7 567 2302.
E-mail address: pijug.su@wu.ac.th (P. Summpunn).

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TABLE 1. Primers used in PCR, qPCR, and DNA sequencing reactions.

Primer	Sequence (5′ → 3′)
groEL–HDEL-F1	GTCTCCAATTTATGGCAGCTAAAGACGT
groEL–HDEL-R1	TTAAAGCTCGTCGTGCATCATGCCGCC
groEL–HDEL-F2	GTACGCGGTCTCCAATTTATGGCAGCTAAAG
groESL–HDEL-R2	ATAGTTTAGCGGCCGCTTAAAGCTCGTCGTG
groES–HDEL-F1	CTCCAATTTATGAATATTCGTCCATT
groES–HDEL-R1	TTAAAGCTCGTCGTGCGCTTCAACAAT
groES–HDEL-F2	GTACGCGGTCTCCAATTTATGAATATTCGTC
SP-HDEL-F	GTACGCGAATTCATGAGATTTCCTTCA
SP-HDEL-R	GTACGCTCTAGATTAAAGCTCGTCGTG
pGAP-Forward	GTCCCTATTTCAATCAATTGAA
5′AOX1	GACTGGTTCCAATTGACAAGC
3′AOX1	GCAAATGGCATTCTGACATCC
appA-H27_F	GCGGAATTCGCCGCGCCGGTTGCCATA
appA-H27his-R	CTAGTAGCGGCCGCTTAATGGTGATGGTGATGGTGAATATGGCAAGCAGGTTCAATC
SdpgA_F	CGGGATCCACCATGTCCATCCTGAACG
SdpgA_R	AGGAATTCTTAGCAGTGATGGTGAT
F_AppA	GTTCGCTCGCCAACCAAAC
R_AppA	TGAGCATAAATAACCGCGTCAG
F_DPhgAT	GCCTGCTCCAGGTGTCTTGC
R_DPhgAT	ACTGTCTAGCCAATTCAGCACC
F_GroEL	TGTCCGTACCATGCTCTGAC
R_GroEL	CAGGTAGCCACGGTCGAAC
F_Actin	AAAAGATCTGGCACCACACC
R_Actin	AGTGGTTCTACCGGAAGCG

for plasmid propagation. *P. pastoris* KM71 and expression vectors (pPIC3.5K, pPIC9K, and pGAPZ B) were purchased from Invitrogen (San Diego, CA, USA). Plasmid pGro7 harboring *groEL* and *groES* genes was purchased from Takara Bio Inc (Japan). Plasmid pUC57_D-PhgAT harboring *dpgA* gene encoded for D-PhgAT (4) was provided by Asst. Prof. Dr. Suthep Wiyakruttra (Mahidol University, Thailand). The phytase-encoding gene in pUC57-H27 plasmid was synthesized by Genscript (Piscataway, NJ, USA) based on the *appA* sequence of *Yersinia intermedia* strain H-27 (accession number DQ986462). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and are listed in Table 1. All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). *Pfu* and *Taq* DNA polymerases were supplied by Promega (Madison, WI, USA). QuantiTect SYBR Green PCR Kit was obtained from Qiagen (Hilden, Germany). Rabbit anti-GroEL antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. Culture media for bacteria and yeast (Luria–Bertani, Yeast extract-Peptone-Dextrose, yeast extract, peptone, biotin) were obtained from Becton Dickinson and Company (Franklin Lakes, NJ, USA). Chemicals and reagents (glycerol, potassium phosphate, sodium phosphate, methanol, phenylmethylsulfonyl fluoride and ethylenediamine tetraacetic acid) and yeast nitrogen base were purchased from Sigma–Aldrich. Ampicillin and zeocin were obtained from Bio Basic (Markham, ON, Canada).

Construction of *P. pastoris* KM71Gro for expression of ER residing GroEL and GroEL The *groEL* and *groES* genes encoding the GroEL and GroES chaperones respectively, were individually amplified from pGro7 with the in frame incorporation of HDEL-retention signals at 3′-end of the genes using two-step PCR with the *Pfu* DNA polymerase. For amplification of the *groEL* gene, PCR was first carried out using primers groEL–HDEL-F1 and groEL–HDEL-R1 (Table 1) to incorporate a *Bsa*I site at 5′-end and HDEL encoding region at the 3′ end of the amplified fragments. The amplification product was subjected to a second PCR using primers groEL–HDEL-F2 and groESL–HDEL-R2 (Table 1) to incorporate a *Not*I site at the 3′-end of the final PCR product.

For *groES* gene, pairs of oligonucleotides groES–HDEL-F1 and groES–HDEL-R1 and groES–HDEL-F2 and groESL–HDEL-R2 (Table 1) were used as primers in the first-step PCR and second-step PCR, respectively, to incorporate a *Bsa*I site at the 5′-end, HDEL encoding region, and a *Not*I site at 3′-end using the same approach as described above.

The amplicons of *groEL* and *groES* were digested with *Bsa*I and *Not*I and separately ligated into *Eco*RI/*Not*I-linearized pPIC9K, downstream of the α-factor secretion signal (*sp*) sequence. The resulting pPIC9K–groEL–HDEL and pPIC9K–groES–HD contained *sp–groEL–HDEL* and *sp–groES–HDEL* cassettes, respectively.

The DNA fragment containing the *sp–groEL–HDEL* cassette was amplified from pPIC9K–groEL–HDEL using primers SP–HDEL-F and SP–HDEL-R (Table 1). The PCR product was digested with *Eco*RI and *Xba*I, and then inserted into the corresponding sites on pGAPZ-B downstream of the strong constitutive *GAP* promoter (P_{GAP}), yielding pGAP–sp–groEL–HDEL containing the P_{GAP}*–sp–groEL–HDEL* expression cassette. The *sp–groES–HDEL* fragment was amplified from pPIC9K–groES–HDEL using the same primers (SP–HDEL-F and SP–HDEL-R) as above. After *Eco*RI and *Xba*I digestion, the fragment was also ligated into pGAPZ-B resulting in pGAP–sp–groES–HDEL which contains the P_{GAP}*–sp–groES–HDEL* expression cassette.

To combine both expression cassettes (P_{GAP}*–sp–groEL–HDEL* and P_{GAP}*–sp–groES–HDEL*) together on the same replicon, the plasmid pGAP–sp–groES–HDEL was digested with *Bgl*II and *Bam*HI and the resulting 1.5 kb fragment containing the P_{GAP}*–sp–groES–HDEL* expression cassette was ligated into the *Bam*HI-digested pGAP–sp–groEL–HDEL. The resulting recombinant plasmid pGAP_GsEL–HDEL/GsES–HDEL containing both the P_{GAP}*–sp–groEL–HDEL* and P_{GAP}*–sp–groES–HDEL* expression cassettes was subsequently transformed into *P. pastoris* KM71 by electroporation (1.5 kV, 25 μF and 200 Ω). Transformants were plated on yeast extract-peptone-dextrose (YPD, 1% yeast extract, 2% peptone, 2% dextrose) agar containing Zeocin at a final concentration of 100 μg mL^{-1}. The integration of both expression cassettes in the chromosome of KM71 was verified by PCR using primers pGAP-Forward and 3′AOX1 (Table 1). The strain was designated as KM71Gro.

Construction of *P. pastoris* for co-expressing ER residing GroEL–GroES and phytase (KM71GroApp) or D-PhgAT (KM71GroPhg) In order to evaluate the efficiency of heterologous protein production, the KM71Gro strain was used as an expression host. In this study, *groEL–HDEL* and *groES–HDEL* were co-expressed with either extracellular (phytase) or intracellular (D-PhgAT) enzymes. The phytase encoding gene (*appA*) with a hexahistidine tag coding region at the 3′-end was amplified from pUC57-H27 using *Pfu* DNA polymerase with primers appA-H27_F and appA-H27his-R (Table 1). The PCR product was digested with *Eco*RI and *Not*I and then ligated into the corresponding restriction sites of pPIC9K downstream of the α-factor secretion signal (*sp*) sequence and under the control of alcohol oxidase promoter (*AOX1*) yielding plasmid pPIC9K-appAhis. After propagation in *E. coli* DH5α, plasmid pPIC9K-appAhis was linearized with *Sac*I and transformed into KM71 and KM71Gro using electroporation. *Pichia* transformants were selected on minimal dextrose agar without histidine [MD, 1.34% yeast nitrogen base (YNB) with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 2% dextrose, 1.5% agar]. The D-PhgAT encoding gene (*dpgA*) with a hexahistidine tag coding region at 3′-end was amplified from pUC57_D-PhgAT (4) using primers SdpgA_F and SdpgA_R (Table 1). The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the corresponding restriction sites of pPIC3.5K downstream of *AOX1* promoter. The resulting recombinant plasmid, pPIC3.5K-dpgAhis, was linearized by *Sac*I and transformed into KM71 and KM71Gro to obtain KM71Phg and KM71GroPhg, respectively. *Pichia* transformants were selected on minimal dextrose (MD) agar without histidine. PCR was performed using primers 5′AOX1 and 3′AOX1 (Table 1) to establish integration of genes encoding phytase and D-PhgAT.

Quantitative real time PCR The quantitative real time PCR (qPCR) was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to obtain the copy number of the integrated genes in each strain. All reaction conditions and melting curve analysis were carried out as previously described (4). The actin gene was use as reference to normalize the copy number. The relative amount of gene copy number was calculated using standard curve method (11). All primers for qPCR (F_AppA, R_AppA, F_DPhgAT, R_DPhgAT,

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