Rewiring of glycerol metabolism in *Escherichia coli* for effective production of recombinant proteins

Chung-Jen Chiang¹, Yi-Jing Ho², Mu-Chen Hu² and Yun-Peng Chao²,³,⁴*

**Abstract**

**Background:** The economic viability of a protein-production process relies highly on the production titer and the price of raw materials. Crude glycerol coming from the production of biodiesel is a renewable and cost-effective resource. However, glycerol is inefficiently utilized by *Escherichia coli*.

**Results:** This issue was addressed by rewiring glycerol metabolism for redistribution of the metabolic flux. Key steps in central metabolism involving the glycerol dissimilation pathway, the pentose phosphate pathway, and the tricarboxylic acid cycle were pinpointed and manipulated to provide precursor metabolites and energy. As a result, the engineered *E. coli* strain displayed a 9- and 30-fold increase in utilization of crude glycerol and production of the target protein, respectively.

**Conclusions:** The result indicates that the present method of metabolic engineering is useful and straightforward for efficient adjustment of the flux distribution in glycerol metabolism. The practical application of this methodology in biorefinery and the related field would be acknowledged.

**Keywords:** Metabolic engineering, Crude glycerol, Recombinant protein

**Background**

The advent of recombinant DNA technology has revolutionized the biotechnology industry [1]. In particular, cells can be reprogrammed and instructed to express the protein of interest. This approach is acknowledged by its power for overproduction of pharmaceutical and industrial proteins in an efficient way. The annual market value of recombinant proteins reaches billions of dollars, and the global market still continues to grow [2]. Recent progress in identification of microbes with specific functions has accelerated the accumulation of genomic information [3–5], which facilitates exploration of more proteins applicable in industry and biorefinery. Accordingly, there arises a pressing need for an economically viable method to efficiently produce recombinant proteins.

*Escherichia coli* is recognized as the biotechnology workhorse because it has several advantages including easy manipulation with genetics, culturability with the cost-effective medium, and scalable production scheme [6]. Employment of *E. coli* that harbors the gene-born plasmid has been commonly applied for production of recombinant proteins based on glucose [7]. However, many problems associated with protein overproduction occur. For instance, the presence of a plasmid negatively affects cell proteins and ribosome components [8]. The gratuitous overexpression of β-galactosidase (β-Gal) was reported to cause the ribosome destruction in cells [9]. The folding process is usually afflicted with an abnormal level of the expressed protein, consequently forming inclusion bodies [10]. It is known that the physiological function of central metabolism provides precursor...
metabolites and energy to fuel the cellular activity. Most of cellular energy is utilized for the synthesis of proteins [11]. To force the production of a gene-encoding product perturbs cell physiology as a result of energy drainage and imbalanced carbon flux in central metabolism. This in turn triggers the global stress response which further restricts the cellular growth and protein production [12]. Moreover, the metabolic response of <em>E. coli</em> to protein overproduction is complicated and depends on the strain type as well as culture compositions [13]. Taken together, the task to produce a large amount of recombinant proteins remains challenging.

The production cost of a protein-production process is closely linked to the production titer and the price of raw materials. Crude glycerol exists in the waste stream from the production of biodiesel and is a renewable and cost-effective resource. Many studies have been focused on refinement of crude glycerol for the microbial production of value-added chemicals thus far [14]. Apparently, it would be biotechnologically sound to produce recombinant protein based on crude glycerol. The aerobic growth of <em>E. coli</em> on glycerol relies on metabolic pathways for dissimilation, gluconeogenesis, and glycolysis which are under the control of global regulators involving cAMP receptor protein (CRP), catabolite repressor/activator protein (Cra), and aerobic respiration control protein (ArcA) [15]. However, glycerol is less effectively utilized than glucose and liable to induce the carbon stress response in <em>E. coli</em> [16]. To address this issue, this study was aimed to effectively produce d-hydantoinase (HDT) by manipulation of key steps in metabolic pathways of glycerol to re-distribute the metabolic flux. Consequently, the engineered strain enabled efficient utilization of crude glycerol and greatly increased production of HDT. The result indicates the useful application of metabolic engineering to the field of recombinant protein production.

**Results**

**Enhancement of the dissimilation pathway**

This study was initiated with BAD-5 strain. It was previously constructed with the l-arabinose (l-Ara)-inducible T7 expression system [17]. Upon induction with l-Ara, this strain grown on glucose enabled production of the recombinant protein in an efficient and homogenous manner. It bears a genomic copy of T7 gene<sub>1</sub> (encoding T7 RNA polymerase) under the control of the <em>araBAD</em> promoter (<em>P<sub>BAD</sub></em>). To ensure the persistent inducibility of l-Ara, l-Ara catabolism of the strain is nullified by removal of <em>araBAD</em> operon. The additional deletion of <em>ptsG</em> and <em>araFGH</em> operon in the strain aims to decouple the glucose-mediated catabolite repression of <em>P<sub>BAD</sub></em> and the l-Ara transport-induction loop, respectively. Finally, the enhanced expression of <em>araE</em> facilitates l-Ara uptake for the strain.

Figure 1 reveals that glycerol metabolism is generally fractionated into 3 module pathways: (1) the dissimilation pathway of glycerol, (2) the gluconeogenesis pathway of dihydroxyacetone phosphate (DHAP), and (3) the glycolysis pathway of DHAP. We attempted to manipulate the dissimilation pathway. The strain’s<em> glpF</em> was first fused with the<em> trc</em> promoter (<em>P<sub>trc</sub></em>). Two catabolic pathways of glycerol exist in <em>E. coli</em>. The oxidative

---

**Fig. 1** Central metabolic pathways of <em>E. coli</em> grown on glycerol. The metabolic pathways of enhancement and of the proposed acetate recycling were highlighted in blue and green, respectively. Genes involved in metabolic pathways include as follows: <em>aceA</em>, isocitrate lyase; <em>aceB</em>, malate synthase; <em>aceEF-ldp</em>, pyruvate dehydrogenase; <em>acs</em>, acetyl-CoA synthase; <em>citA</em>, citrate synthase; <em>dhaKLM</em>, dihydroxyacetone kinase; <em>fbaA</em>, fructose-biphosphatase aldolase; <em>gldA</em>, glycerol dehydrogenase; <em>glpD</em>, glycerol 3-phosphate dehydrogenase; <em>glpK</em>, glycerol kinase; <em>glpF</em>, glycerol facilitator; <em>pckA</em>, PEP carboxykinase; <em>pgl</em>, lactonase; <em>pgi</em>, isomerase; <em>pykA</em>, pyruvate kinase; <em>poxB</em>, pyruvate oxidase; <em>pnc</em>, PEP carboxylase; <em>zwf</em>, glucose-6-phosphatase dehydrogenase. Abbreviations of metabolites: Ac-CoA, acetyl-CoA; ACE, acetate; CIT, citrate; DHAP, dihydroxyacetone phosphate; F6P, fructose-6-phosphate; FDP, fructose-diphosphate; FUM, fumarate; G6P, glucose-6-phosphate; Gly, glycerol; GOL, glyoxylate; Iso-CIT, isocitrate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceraldehyde; PYR, pyruvate; SUC, succinate

---
cleavage of glycerol proceeds via the glpK–glpD route while the gldA–dhaKLM route is utilized for the anaerobic fermentation of glycerol [18]. The anaerobic degradation pathway involves the phosphorylation reaction by phosphoenolpyruvate (PEP) and produces more NADH. The protein production is recognized to consume most of cellular energy. Therefore, the glycerol flux was directed to the gldA–dhaKLM pathway.

Endogenous gldA and the dhaKLM operon in BAD-5 strain were fused with the λP₁ promoter (PλP₁) to activate the pathway activity without anaerobiosis. This genetic construction resulted in N31 strain which displayed an eightfold and threefold increase in the GldA (ca. 0.96 U/mg protein) and DhaKLM activity (ca. 0.015 U/mg protein), respectively.

HDT was chosen for illustration because it has no function interfering with cell physiology. This enzyme is of industrial importance for its usefulness to prepare D-β-hydroxyphenylglycine which is required for the synthesis of amoxicillin, a β-lactam antibiotic [19]. The performance of N31 strain was investigated for production of HDT by induction with L-Ara. Pure glycerol was used during the course of strain development. Plasmid pET-TrChHDT was transformed into the strain to obtain N31/pET-TrHDTCH strain which was cultured in a shake flask containing glycerol. BAD-5 strain bearing plasmid pET-TrChHDT (BAD-5/pET-TrChHDT) was employed as a control. As compared to BAD-5/pET-TrChHDT strain, N31/pET-TrChHDT strain consumed more glycerol and produced soluble HDT with a 1.2-fold increase in the activity (Fig. 2).

Enhancement of the gluconeogenic pathway

NADPH is required for biosyntheses. The gluconeogenic pathway of DHAP is linked to the oxidative pentose phosphate (OPP) pathway which provides NADPH (Fig. 1). It was previously illustrated to produce more reducing equivalents in the strain by redirection of carbon flux into the OPP pathway as a result of enhancing the expression level of zwf and pgl [20]. Glucose 6-phosphate (G6P) dehydrogenase encoded by zwf catalyzes the first step in the OPP pathway. N31 strain derived from E. coli B which lacks pgl (encoding lactonase) [21]. Therefore, N31 strain was engineered by fusion of PAP₁ with zwf and recruitment of the PAP₁-driven pgl. The construction resulted in N31-5 strain which showed a 2- and 4.1-fold increase in Zwf (ca. 8.2 U/mg protein) and Pgl activity (ca. 1.6 U/mg protein), respectively. To remain stable, N31 and N31-5 strains were equipped with a genomic copy of the P₇₇-driven HDT to give N31(HDT) and N31-5(HDT) strains, respectively. The resulting strains were cultured and induced for the HDT production. As shown in Fig. 3, N31-5(HDT) strain displayed better growth and consumed more glycerol than N31(HDT) strain. The activity of soluble HDT in N31-5(HDT) strain was increased by sixfold relative to that for N31(HDT) strain.

Forced direction of the glycolytic flux

The flux from the glycolysis pathway of DHAP enters the tricarboxylic acid (TCA) cycle, an indispensible pathway which provides reducing equivalents and precursors for biosyntheses (Fig. 1). Its operation mode consists of the oxidative or reductive route in response to oxygen
availability [22]. Citrate synthase (encoded by gltA) catalyzes the synthesis of citrate from acetyl-CoA and oxaloacetate (OAA). The TCA cycle is regulated through the control of GltA [23]. As previously illustrated, the production of reducing equivalents was reduced in the TCA cycle by lowering the GltA activity [24]. To increase the activity of the TCA cycle, the PλP L-driven gltA of Corynebacterium glutamicum CCRC 11384 (CggltA) was introduced into N31-5(HDT) strain. The construction gave N31-5AK(HDT) strain which gained a 2.1-fold increase in the GltA activity. The shake-flask culture of N31-5AK(HDT) strain was carried out for the protein production. The result showed that the strain performed equally well in either glycerol or crude glycerol (Table 1).

As shown in Fig. 4a, the strain consumed all crude glycerol and enabled production of soluble HDT with the activity reaching 2.84 U/mL. Its HDT production reached 12% of total cell proteins (Fig. 4b). In comparison with BAD-5/pET-TrChHDT strain, N31-5AK(HDT) strain displayed an increase in the intracellular NADH/NAD⁺ (ca. 0.38) and NADPH/NADP⁺ ratios (ca. 1.64) by 25% and 30%, respectively.

**Discussion**

Glucose is commonly employed for the production of recombinant proteins in *E. coli*. It has an advantage of easy dissimilation through central metabolism which provides precursor metabolites and energy to fuel cellular activities. Therefore, considerable efforts have been devoted to achieve the efficient production of target proteins based on glucose. Acetate overflow appears to be a frequently encountered event in the protein-overproducing *E. coli* which is incapable of properly coordinating glucose metabolism. This phenomenon is generally ascribed to the lower activity of the TCA cycle and respiratory chain relative to the uptake rate of glucose [25]. Metabolic engineering, thus, emerges as an enabling technology to address this issue. As successfully illustrated, acetate could be reduced in *E. coli*

### Table 1 Summary of the production kinetics for strains with engineered traits

| Strain         | ∆S     | OD (T₀,h) | HDT    | Y      | Manipulated gene |
|----------------|--------|-----------|--------|--------|------------------|
|                |        |           |        |        | arcA  zwf  glpF |
| Pure glycerol  |        |           |        |        |                  |
| BAD-5          | 0.04 ± 0.00 | 0.28 ± 0.01 (1.54) | 0.09 ± 0.01 | 225 | −     −     −     −     −     −     −     |
| N31            | 0.13 ± 0.01 | 0.43 ± 0.02 (1.35) | 0.19 ± 0.01 | 216 | −     −     −     −     −     −     +     −     |
| N31(HDT)       | 0.13 ± 0.01 | 0.53 ± 0.02 (1.32) | 0.22 ± 0.01 | 244 | −     −     −     −     +     +     −     −     |
| N31-5(HDT)     | 0.32 ± 0.02 | 1.55 ± 0.05 (0.91) | 1.68 ± 0.13 | 672 | −     +     +     +     −     −     +     −     |
| N31-Arc(HDT)   | 0.37 ± 0.02 | 1.61 ± 0.06 (0.91) | 2.59 ± 0.19 | 948 | △+  +     +     +     +     +     +     +     |
| N31-5AK(HDT)   | 0.40 ± 0.03 | 1.71 ± 0.08 (0.84) | 2.83 ± 0.22 | 969 | −     +     +     +     +     +     +     +     |
| Crude glycerol |        |           |        |        |                  |
| N31-5AK(HDT)   | 0.40 ± 0.03 | 1.76 ± 0.09 (0.84) | 2.84 ± 0.22 | 969 | −     +     +     +     +     +     +     +     |

Note that BAD-5 and N31 strains harbored plasmid pET-TrChHDT for expression of HDT. Glycerol consumption rate (∆S) in g/L h, final biomass (OD) in g dry cell weight (DCW)/L, the protein production (HDT) in U/mL and the protein yield based on glycerol (Y) in U/g were calculated based on experimental data. Numbers in parentheses indicate the doubling time (T₀,h) in h of strains.

Symbols for manipulated genes: +, enhancement; −, wild type, △, deletion.
over-expressing the anaplerotic enzyme (i.e., PEP carboxylase) to direct more carbon flux to OAA and deletion of fadR for activation of the glyoxylate shunt [26]. A similar approach was conducted with the expression of heterologous pyruvate carboxylase, and the β-Gal production improved by 68% [27]. Replacement of the phosphotransferase system-based glucose transport by galactose permease lowered glucose uptake and reduced acetate formation, consequently leading to fourfold higher production of green fluorescent protein [28]. In response to oxygen availability, the two-component system involving arcA and arcB mediates the anaerobic repression of enzymes in the TCA cycle, glyoxylate shunt, and others [29]. Inactivation of ArcA and/or ArcB reduced acetate yield and increased the β-Gal production by 30% [30]. Global regulators function to control metabolic pathways in connection with the physiological status of bacteria. Interestingly, mutant strains carrying a single knockout in the global regulator, including arcA, arcB, cra, crp, cya, fnt, or mlc, were shown to produce less acetate at the expense of glucose uptake [31].

Glycerol dissimilation is less efficient than glucose in E. coli. There are few efforts investigating the bacterial production of proteins based on glycerol. Overproduction of poorly folded proteins (i.e., inclusion bodies) is prone to induction of the stress response. To address this issue, the supplement of glycerol in the culture medium was proven to be useful for improving solubility of recombinant proteins [32, 33]. In addition, the application of glycerol as the glucose substitute for the recombinant protein production enabled reduction of acetate formation in E. coli [34]. As compared to E. coli K12, E. coli B exhibits a higher activity in the TCA cycle and glyoxylate shunt due to the lower expression level of arcA and iclR [35]. This metabolic characteristic renders B strain less efficient in the production of acetate when grown on glucose. Alternatively, a study reported that an unusually high level of poxB, acs, pckA, and genes in glyoxylate shunt (aceA and aceB) was observed in E. coli during aerobic growth on glycerol [16]. These induced genes mediate the “carbon stress-based acetate-recycling mechanism” [16]. Acetate is synthesized by PoxB and then re-assimilated via the catabolic pathway consisting of acs, glyoxylate shunt genes, and pckA, consequently leading to an undetectable level of acetate.

In contrast to these previous studies, this work was conducted by rewiring glycerol metabolism of E. coli B strain to improve the protein production. Aerobic glycerol metabolism is limited by the GlpK activity [36]. Nevertheless, the gldA–dhaKLM route responsible for the oxygen-limited glycerol metabolism was targeted for improvement. As compared to its parent strain (BAD-5/ pET-TrChHDT), N31/pET-TrChHDT strain exhibited a 2.3-fold increase in glycerol consumption. The increased glycerol flux was directed more to HDT (i.e., 100% more activity) than to biomass (i.e., 40% more) (Table 1). The result indicates that the engineered gldA–dhaKLM pathway is functional to augment the ability of the strain for aerobic utilization of glycerol, which produces more NADH (Fig. 1). Interestingly, the performance of N31 strain remained roughly unaffected when bearing either the multicopy plasmid (ColE1 origin) containing HDT (i.e., N31/pET-TrChHDT) or a genomic copy of HDT (i.e., N31(HDT)) (Table 1). Metabolic burden occurs in the cell overexpressing the plasmid-encoded gene [37, 38]. Therefore, the approach by integration of the target gene into the cell genome may alleviate the plasmid-caused adverse effect. The previous study reported that the level of fbaA and pgI increased in the strain grown on glycerol [16], which suggests that gluconeogenesis metabolism is necessary for provision of fructose 6-phosphate (F6P) and G6P from DHAP. The OPP pathway provides precursor metabolites and produces more reducing equivalents. Therefore, zwf and pgl were overexpressed to direct the flux through G6P into the OPP pathway. The resulting strain (N31-5(HDT)) metabolized 1.4-fold more glycerol than its parent strain (N31(HDT)). The increased consumption of glycerol was utilized to increase the synthesis of biomass and HDT by 2.1- and 6.6-fold (Table 1), respectively. Expression levels of genes in the OPP pathway remained unaffected in E. coli irrespective of glycerol or glucose [16, 39]. Nevertheless, the current result suggests that the OPP pathway presents to be a bottleneck in the aerobic metabolism of glycerol. Note that the engineered dissimilation of glycerol via the gldA–dhaKLM route diverts PEP to pyruvate. An elevated level of pykA encoding pyruvate kinase occurs in E. coli during growth on glycerol [16, 39]. Accordingly, PEP produced in the glpK–glpD route is in part converted to pyruvate by PykA. Pyruvate is further oxidized to acetyl-CoA by the reaction of pyruvate dehydrogenase and via the “acetate-recycling pathway”. E. coli grown on glycerol displays an unusually high level of pckA encoding PEP carboxykinase that converts OAA to PEP and serves to complete the cycling pathway for acetate reuse [16]. Therefore, available OAA and acetyl-CoA were directed into the TCA cycle by enhanced expression of citrate synthase in N31-5(HDT) strain. The resulting strain (N31-5AK(HDT)) enabled consumption of 25% more glycerol, contributing to 70% more HDT activity and 10% more biomass (Table 1). In a parallel study, the HDT production could also be improved in N31-5(HDT) strain deprived of arcA (i.e., N31-Arc(HDT)) but was inferior to that for N31-5AK(HDT) strain (Table 1). Derepression of genes in the TCA cycle by null arcA can improve the cycle activity [29]. Taken together, the current result suggests that GltA
is a limiting step of the TCA cycle in the aerobic metabolism of glycerol. Finally, Table 1 revealed that enhancement of the OPP pathway greatly improved biomass, glycerol utilization, and the HDT production, while additional enhancement of the TCA cycle further improved the HDT production.

**Conclusion**

In this work, key steps in the glycerol dissimilation pathway, the PP pathway, and the TCA cycle were identified and manipulated to rewire glycerol metabolism. This approach improves the production of biomass by 4.5-fold and of the HDT activity by 30-fold (N31-5AK(HDT) vs. BAD-5/pET-TrChHDT strain) (Table 1). The result clearly indicates that re-distribution of glycerol flux in central metabolism is very efficient for production of recombinant proteins (refer to Y in Table 1). In particular, specific genes instead of global regulators are targeted for engineering. This is in sharp contrast to the method of reprogramming global regulators as commonly employed for the production of recombinant proteins using glucose. Modification of global regulators usually leads to a pleiotropic effect on cell physiology, which is complex and unpredictable. In contrast, the present method of metabolic engineering is useful and straightforward for efficient adjustment of the flux distribution in glycerol metabolism. In summary, a modern biorefinery is composed of network and cascade processes aimed at production of value-added bioproducts (including enzymes, chemicals, and antibiotics) and biofuels [40]. This crude glycerol-based production platform for recombinant proteins would have a potential application in biorefinery.

**Methods**

**Genetic manipulation**

Plasmid pET-TrChHDT carried the fusion gene consisting of *trxA*, HDT gene, and the chitin-binding domain (ChBD) under the control of the T7 promoter (P<sub>T7</sub>), and was constructed as follows. The DNA containing HDT-ChBD was amplified from plasmid pChHDT [41] by PCR with primers (atctggagatccatatcagcactgcga and acctgggaattcagcttatcagaagc). The BamHI-treated PCR DNA was incorporated into plasmid pET-32a by digestion with EcoRI and SmaI sites of plasmid pLoxTrc (Lab collection) to obtain plasmid pLoxTrc-GltA. Treated with the BamHI–NdeI cut, the DNA containing GltA was recovered from plasmid pLoxTrc and then ligated into plasmid pLoxKm-PR (Lab collection) to obtain plasmid pLoxKm-GltA. Treated with NdeI–SacI cut and then incorporated into plasmid pET32a (Novagen Co.) to give plasmid pET-TrChHDT. The fusion gene was recovered from plasmid pET32-TrChHDT treated with the SacI–XbaI cut and then incorporated into plasmid pET20b (Novagen Co.) to obtain plasmid pET-TrChHDT. Note that TrxA improves the solubility of HDT and ChBD facilitates the immobilization of HDT [41].

The DNA containing the fusion gene linked to P<sub>T7</sub> was recovered from plasmid pET-TrChHDT subjected to the NdeI–SacI digestion. The recovered DNA was incorporated into plasmid pHil80-Km [42], resulting in plasmid pHil-TrHDTC. This plasmid was applied for insertion of the P<sub>T7</sub>-driven fusion gene into the strain's genome following the reported protocol [42].

The DNA containing P<sub>trc</sub> was amplified from plasmid pTrc99A by PCR with Ta1–Ta2 primers (ctggaatcagcttatcagcactgcga and atctgggaattcagcttatcagaagc). By digestion with EcoRI, the PCR DNA was incorporated into the EcoRI and SmaI sites of plasmid pPL-Kan [24]. The construction gave plasmid pTrc-kan which carried the LE*-kan-RE*-P<sub>trc</sub> cassette. The passenger DNA contained LE*-kan-RE*-P<sub>trc</sub> with two homologous regions of glpF and was obtained from plasmid pTrc-kan by PCR with Ta3–Ta4 mega primers (acaacccggtacgaggaattcagcaatgcactgcccaccgagtgttttagcactatatgataacctctcttggaggtccgtgactttcacgcatacaacaaacaactctctcagattcctttcagagttttgctgattccttgtcttcggaattcagcttatcagaagc). The BamHI-treated PCR DNA was incorporated into the strain's genome at λ attachment locus following the previous report [24]. Passenger DNAs were then electroproporated into the strain and underwent homologous recombination by the act of λRed. Meanwhile, passenger DNAs were amplified from plasmid pPR-zwf and plasmid pSPL-pgl with primers RC11417/RC11418 and RC13034/RC13035, respectively [20]. By a similar approach, zwf and pgl were fused with P<sub>AP<sub>L</sub></sub>. The integrated kan marker was later removed by Cre.

The P<sub>AP<sub>L</sub></sub>-driven gltA of *C. glutamicum* CCRC 11384 was constructed in several steps. The first step was amplified by PCR with primers (catacatggacttgtagtctctcttgagga and gttgactggtgactggtggtg). The PCR DNA was treated by *Apal–NdeI* cut and then incorporated into plasmid pLoxTrc (Lab collection) to obtain plasmid pLoxTrc-GltA. Treated with the BamHI–NdeI cut, CggtLA was recovered from plasmid pLoxTrc and then ligated into plasmid pLoxKm-PR (Lab collection) to give plasmid pPR-CggtLA. Finally, the DNA containing the fusion of P<sub>AP<sub>L</sub></sub> with CggtLA was amplified by PCR with primers (ctggaatcagcttatcagcactgcga and actatggatccatatcagcactgcga). The BamHI-treated PCR DNA was spliced into plasmid pLam-LoxKm [43] which was digested by BamHI and NruI to obtain plasmid pLam-P<sub>AP<sub>L</sub></sub>CggtLA. The P<sub>AP<sub>L</sub></sub>-driven CggtLA was integrated into the strain's genome at λ attachment locus following the reported protocol [43].

The deletion of arcA was carried out by λRed-mediated homologous recombination of the PCR DNA (ArcA:: FRT-kan-FRT) after electroproporation. This DNA fragment was amplified from JW4364-1 strain [44] with
the primers (gaaagtacctcgacaagc and tgacccgtaatatgacctg). The kan marker flanked by FRT was later removed using Flp.

**Bacterial culturing**

The cell density was measured turbidimetrically at 550 nm (OD$_{550}$). The recombinant strain was grown on LB medium (10-g/L Tryptone, 5-g/L yeast extract, and 5-g/L NaCl) with or without ampicillin (30 μg/mL) overnight. The overnight culture was seeded into Erlenmeyer flasks (125 mL) containing 15-mL M9Y medium (6-g/L Na$_2$HPO$_4$, 3-g/L KH$_2$PO$_4$, 0.5-g/L NaCl, 1-g/L NH$_4$Cl, 1-mM MgSO$_4$, 0.1-mM CaCl$_2$, 2-g/L yeast extract) plus 3-g/L glycerol or crude glycerol (Great Green Technology Co., Taiwan). According to the manufacturer, the main composition of crude glycerol was 82% (w/w) glycerol, 1.7% fatty acids, and 4.6% ash. Ampicillin (15 μg/mL) was additionally supplemented in the medium for the strain that harbored the gene-born plasmid. The initial cell density was maintained at OD$_{550}$ of 0.08. The bacterial culture was incubated at 37 °C in a rotary shaker set at 200 rpm. Upon reaching around 0.3 at OD$_{550}$ bacteria were induced for the protein production by adding of l-Ara (30 μM) to the culture medium. The bacterial growth was monitored along the time course and the protein production was terminated after the induction was administrated at 6 h. Glycerol was analyzed using High-Performance Liquid Chromatography (HPLC) based on the previous report [45]. The intracellular NADH/NAD performance liquid chromatography; ΗDP: Dihydroxyacetone phosphate; F6P: Fructose 6-phosphate; G6P: Glucose 6-phosphate; β-Gal: β-galactosidase; HDT: l-hydantoinase; HPLC: High-performance liquid chromatography; HPH: dl-hydroxyphenyl hydantoin; OPP: Oxidative pentose phosphate; OAA: Oxaloacetate; PEP: Phosphoenolpyruvate; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA: Tricarboxylic acid.

**Protein analysis**

The bacterial culture (1 mL) was harvested by centrifugation, and cell pellets were resuspended in 0.1-M Tris-HCl buffer (0.2 mL) at pH 8.0. Cells were disrupted by sonication, followed by centrifugation. The supernatant was recovered and saved as the cell-free extract (CFX). The total protein content of CFX per culture volume (mg/mL) was estimated based on Bio-Rad Protein Assay reagent with bovine serum albumin (BSA) as a standard. CFX (20 μL) was applied for the protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Using Image Analyzer GAS9000 (UVIttech), the resolution of SDS-PAGE was analyzed to estimate the amount of HDT. The HDT activity was assayed based on the previous report [46]. CFX (10 μL) was added to the reaction solution (1 mL) consisting of 0.1-M Tris-HCl buffer (pH 8.0), 6-mM dl-hydroxyphenyl hydantoin (HPH), and 0.5-mM MnCl$_2$. The reaction proceeded at 40 °C for 15 min and was terminated by heating the solution at 100 °C for 10 min. The concentration of un-reacted HPH was determined by HPLC and used to calculate the activity of HDT based on the protein (U/mg protein) in the reaction solution, where U was defined as μmol/min. The total activity per culture volume (i.e., volumetric activity (U/mL)) of HDT was obtained by multiplying the activity (U/mg) with the total protein content (mg/mL).

**Enzyme assay**

Activities of endogenous proteins were determined essentially following the previous report [24]. In brief, harvested cells were resuspended in 1-mL saline solution and CFX was prepared in a similar way. The measurement of enzyme activities was conducted by adding CFX (100 μL) to each reaction solution (1 mL) at 30 °C. The GldA and DhaKLM activities were determined by monitoring the reduction of NAD$^+$ from glycerol at 340 nm. The activity of G6P dehydrogenase (encoded by zwf) and of lactonase (encoded by pgl) were determined by monitoring the reduction of NADP$^+$ from G6P at 340 nm. Moreover, the activity of citrate synthase (encoded by gltA) was measured by conversion of acetyl-CoA and OAA to citrate.

**Abbreviations**

- l-Ara: l-arabinose; CFX: Cell-free extract; ChBD: Chitin-binding domain; DHAP: Dihydroxyacetone phosphate; F6P: Fructose 6-phosphate; G6P: Glucose 6-phosphate; β-Gal: β-galactosidase; HDT: l-hydantoinase; HPLC: High-performance liquid chromatography; HPH: dl-hydroxyphenyl hydantoin; OPP: Oxidative pentose phosphate; OAA: Oxaloacetate; PEP: Phosphoenolpyruvate; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA: Tricarboxylic acid.

**Acknowledgements**

We like to acknowledge Dr. Saini M for the technical support.

**Authors’ contributions**

CJC designed the study and drafted the manuscript; YJH constructed bacterial strains; MCH performed experiments; YPC completed the manuscript. All authors read and approved the final manuscript.

**Funding**

This work is supported by China Medical University (CMU-108-MF-108) and Ministry of Science and Technology (MOST 108-2221-E-035-052-MY3), Taiwan.

**Availability of supporting data**

Not applicable.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

All authors agree to submit the work to the journal.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

1 Department of Medical Laboratory Science and Biotechnology, China Medical University, No. 91, Hseuh-Shih Road, Taichung 40402, Taiwan. 2 Department of Chemical Engineering, Feng Chia University, 100 Wenhuw Road, Taichung 40724, Taiwan. 3 Department of Medical Research, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan.
University Hospital, Taichung 40447, Taiwan. 4 Department of Food Nutrition and Health Biotechnology, Asia University, Taichung 41354, Taiwan.

Received: 13 March 2020 Accepted: 3 December 2020

Published online: 14 December 2020

References

1. Khan S, Ullah MW, Siddique R, Nabi G, Manan S, Yousaf M, Hou H. Role of recombinant DNA technology to improve life. Int J Genomics. 2016;2016:2406954.

2. Puetz J, Wurm FM. Recombinant proteins for industrial versus pharma-ceutical purposes: a review of process and pricing. Processes. 2019;7:476.

3. Ash C. Year of the genome. Trends Microbiol. 1997;5:135–9.

4. Ogawa T, Shimizu S. Microbial enzymes: new industrial applications from traditional screening methods. Trends Biotechnol. 1999;17:13–20.

5. Rehm BAH. Bioinformatic tools for DNA/protein sequence analysis, functional assignment of genes and protein classification. Appl Microbiol Biotechnol. 2001;57:579–92.

6. Makrides S. Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev. 1996;60:512–38.

7. Waegeman H, Soetaert W. Increasing recombinant protein production in Escherichia coli through metabolic and genetic engineering. J Ind Microbiol Biotechnol. 2011;38:1891–908.

8. Bimbbaum S, Bailey JE. Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant Escherichia coli. Biotechnol Bioeng. 1991;37:736–45.

9. Dong H, Nilsson L, Kurland C. Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction. J Bacteriol. 1995;177:1497–504.

10. Banex F, Mujacic M. Recombinant protein folding and misfolding in Escherichia coli. Nat Biotechnol. 2004;22:1399–408.

11. Neidhardt F, Ingrum J, Schaechter M. Physiology of the bacterial cell: a molecular approach. Sunderland: Sinauer Associates Inc; 1990.

12. Gill RT, Valdes JJ, Bentley WE. A comparative study of global stress gene regulation in response to overexpression of recombinant proteins in Escherichia coli. Metab Eng. 2000;2:178–89.

13. Oh MK, Liao JC. DNA microarray detection of metabolic responses to protein overproduction in Escherichia coli. Metab Eng. 2000;2:201–9.

14. Almeida JR, Fávaro LC, Quirino BF. Biodiesel biorefinery: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. Biotechnol Biofuels. 2012;5:48.

15. Iuchi S, Cole ST, Lin EC. Multiple regulatory elements for the gpaA operon encoding anaerobic glycerol-3-phosphate dehydrogenase and the gldP operon encoding aerobic glycerol-3-phosphate dehydrogenase in Escherichia coli: further characterization of respiratory control. J Bacteriol. 1990;172:719–84.

16. Martínez-Gómez N, Flores N, Castañeda HM, Martínez-Batallar G, Hernández-Chávez G, Ramírez OT, Gosset G, Encarnación S, Bolivar F. New insights into Escherichia coli metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. Microb Cell Fact. 2012;11:46.

17. Wang ZW, Lai CB, Chang CH, Chiang CJ, Chao YP. A glucose-insensitive T7 expression system for fully-induced expression of proteins at a substrat-ing level of α-arabinose. J Agri. Food Chem. 2011;59:6534–42.

18. Gonzalez R, Murarka A, Dharmadi Y, Yazdani SS. A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in Escherichia coli. Metab Eng. 2008;10:234–45.

19. Oliveri R, Faschi E, Angeli L, Degen L. Microbial transformation of racemic hydantoin to α-amino acids. Biotechnol Bioeng. 1981;23:2173–83.

20. Sañi M, Li SY, Chiang CJ, Chao YP. Systematic engineering of the central metabolism in Escherichia coli for effective production of n-butanol. Biotechnol Biofuels. 2016;9:69.

21. Meier S, Jensen PR, Duus JØ. Direct observation of metabolic differ-ences in living Escherichia coli strains K-12 and BL21. ChemBioChem. 2012;13:308–10.

22. White D. The physiology and biochemistry of prokaryotes. 3rd ed. New York: Oxford University Press; 2007.

23. Walsh K, Koshland DEJ. Characterization of rate-controlling steps in vivo by use of an adjustable expression vector. Proc Natl Acad Sci USA. 1985;82:3577–81.

24. Saini M, Wang ZW, Chiang CJ, Chao YP. Metabolic engineering of Escherichia coli for production of n-butanol from crude glycerol. Biotechnol Biofuels. 2017;10:173.

25. El-Mansy E, Holms W. Control of carbon flux to acetate excretion during growth of Escherichia coli in batch and continuous cultures. J Gen Microbiol. 1989;135:2875–83.

26. Farmer W, Liao J. Reduction of aerobic acetate production by Escherichia coli. Appl Environ Microbiol. 1997;63:3205–10.

27. March J, Eiteman M, Altman E. Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in Escherichia coli. Appl Environ Microbiol. 2002;11:5620–4.

28. Anda RD, Lara AR, Hernández V, Hernández-Montalvo V, Gosset G, Bolivar F, Ramirez OT. Replacement of the glucose phosphotransferase transport system by galactose permease reduces acetate accumulation and improves process performance of Escherichia coli for recombinant protein production without impairment of growth rate. Metab Eng. 2006;8:281–90.

29. Salmon KA, Hung S, Steffen NR, Krupp R, Hatfielddeek GW, Gun-salius RP. Global gene expression profiling in Escherichia coli K12: effects of oxygen availability and ArcA. J Biol Chem. 2005;280:15084–96.

30. Vemuri GN, Eiteman MA, Altman E. Increased recombinant protein production in Escherichia coli strains with overexpressed water-forming NADH oxidase and a deleted ArcA regulatory protein. Biotechnol Bioeng. 2006;94:538–42.

31. Perrenoud A, Sauer U. Impact of global transcriptional regulation by ArcA, ArcB, Crc, Crp, Cya, Fnr, and Mlc on glucose catabolism in Escherichia coli. J Bacteriol. 2005;187:3171–9.

32. Strandberg L, Enfors SO. Factors influencing inclusion body formation in the production of a fused protein in Escherichia coli. Appl Environ Microbiol. 1991;57:1669–74.

33. Leandro P, Lechner MC, de Almeida IT, Konecki D. Glyceraldehyde increases the yield and activity of human phenylalanine hydroxylase mutant enzymes produced in a prokaryotic expression system. Mol. Gen. Metabol. 2001;73:173–8.

34. Holmes MH. The central metabolic pathways of Escherichia coli: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. Curr Top Cell Regul. 1986;26:109–13.

35. Waegeman H, Beauprez J, Moens H, Maertens J, De Mey M, Fouquie-Moreno MR, Heijnen JJ, Charlier D, Soetaert W. Effect of icdR and orcA knockouts on biomass formation and metabolic fluxes in Escherichia coli K12 and its implications on understanding the metabolism of Escherichia coli BL21 (DE3). BMC Microbiol. 2011;11:70.

36. Applebee MK, Joyce AR, Conrad TM, Pettigrew DW, Nilsson BØ. Func-tional and metabolic effects of adaptive glycerol kinase (GLPK) mutants in Escherichia coli. J Biol Chem. 2011;286:23150–9.

37. Neubauer P, Lin HY, Mathiszik B. Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in Escherichia coli. Biotechnol Bioeng. 2003;83:53–64.

38. Hoffmann F, Rinas U. On-line estimation of the metabolic burden result-ing from the synthesis of plasmid-encoded and heat-shock proteins by monitoring respiratory energy generation. Biotechnol Bioeng. 2001;76:333–40.

39. Oh MK, Liao JC. Gene expression profiling by DNA microarrays and meta-bolic fluxes in Escherichia coli. Biotechnol Prog. 2000;16:278–86.

40. Escamilla-Alvarado C, Perez-Pimentia JA, Ponce-Noyola T, Poggi-Valorado HM. An overview of the enzyme potential in bioenergy-producing biofer-mineries. J Chem Technol Biotechnol. 2017;92:906e24.

41. Cermak JT, Chao YP. Chitin-binding domain-based immobilization of D-Hydantoinase. J Biotechnol. 2005;117:267–75.

42. Chiang CJ, Chao YP. Reciplon-free and markerless methods for genomic insertion of DNAs in phase attachment sites and controlled expression of chromosomal genes in Escherichia coli. Biotechnol Bioeng. 2008;101:985–95.

43. Chiang CJ, Saini M, Lee HM, Wang ZW, Chen PT, Chao YP. Genomic engi-neering of Escherichia coli by the phase attachment site-based integration system with mutant loxP sites. Proc Biochem. 2012;17:2246–54.
44. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol. 2006;2006(2):0008.
45. Wang ZW, Saini M, Lin LJ, Chiang CJ, Chao YP. Systematic engineering of *Escherichia coli* for D-lactate production from crude glycerol. J Agri Food Chem. 2015;63:9583–9.
46. Chiang CJ, Lin LJ, Wang ZW, Lee TT, Chao YP. Design of a noncovalently linked bifunctional enzyme for whole-cell biotransformation. Proc Biochem. 2014;49:1122–8.

**Publisher’s Note**
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.