Dihydroxyacetone production in an engineered *Escherichia coli* through expression of *Corynebacterium glutamicum* dihydroxyacetone phosphate dephosphorylase

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HIGHLIGHTS

- Dihydroxyacetone biosynthesis in engineered *Escherichia coli* is demonstrated.
- Stepwise increase in dihydroxyacetone production by rational design of mutants.
- Produced 6.60 g/l of dihydroxyacetone, the highest among engineered lab strains.

ABSTRACT

Dihydroxyacetone (DHA) has several industrial applications such as a tanning agent in tanning lotions in the cosmetic industry; its production via microbial fermentation would present a more sustainable option for the future. Here we genetically engineered *Escherichia coli* (*E. coli*) for DHA production from glucose. Deletion of *E. coli* triose phosphate isomerase (*tpiA*) gene was carried out to accumulate dihydroxyacetone phosphate (DHAP), for use as the main intermediate or precursor for DHA production. The accumulated DHAP was then converted to DHA through the heterologous expression of *Corynebacterium glutamicum* DHAP dephosphorylase (*cghdpA*) gene. To conserve DHAP exclusively for DHA production we removed methylglyoxal synthase (*mgsA*) gene in the Δ*tpiA* strain. This drastically improved DHA production from 0.83 g/l (0.06 g DHA/g glucose) in the Δ*tpiA* strain bearing *cghdpA* to 5.84 g/l (0.41 g DHA/g glucose) in the Δ*tpiA*Δ*mgsA* double mutant containing the same gene. To limit the conversion of intracellular DHA to glycerol, glycerol dehydrogenase (*gldA*) gene was further knocked out resulting in a Δ*tpiA*Δ*mgsA*Δ*gldA* triple mutant. This triple mutant expressing the *cghdpA* gene produced 6.60 g/l of DHA at 87% of the maximum theoretical yield. In summary, we demonstrated an efficient system for DHA production in genetically engineered *E. coli* strain.

Keywords: *Escherichia coli*; *Corynebacterium glutamicum*; Metabolic engineering; Dihydroxyacetone; Dihydroxyacetone phosphate dephosphorylase; Dihydroxyacetone phosphate

1. Introduction

Dihydroxyacetone (DHA) has many applications within the cosmetic industry and according to the American Academy of Dermatology; DHA is the main constituent for most effective sunless tanning products. DHA is not absorbed through the skin into the body and has no known toxicity [1]. As such DHA has been listed with the Food and Drug Administration (FDA) since 1973, and has been used in cosmetic preparations for close to 30 years [2]. In

addition DHA is also used as a versatile building block for the synthesis of fine chemicals using organic synthesis [3]. DHA can be produced via fermentation or organic synthesis route. However production of DHA via organic synthesis is often complicated with side reactions [2] and the incapability to fulfill the strict Good Manufacturing Practice (GMP) regulations for use in cosmetic industry. Hence DHA is mainly produced commercially via fermentation, dominated by few major companies at a high price of US$150/kg [4].

In nature, *Gluconobacter oxydans* can transform glycerol to DHA [5], while *Corynebacterium glutamicum* convert sugars to DHA under aerobic conditions [6]. Biotransformation of glycerol to DHA was carried out using *Escherichia coli* [7]. Surprisingly, there is only one metabolic engineering effort dedicated for DHA production to date [8], in which *S. cerevisiae* was engineered to produce 0.7 g/l DHA from 20 g/l glucose using glycerol as an intermediate. Considering the lower conversion efficiency of glycerol to DHA [9], we searched for a pathway that converts glucose to DHA without using a glycerol intermediate.

In this work, DHA is produced in a metabolically engineered *E. coli* strain through heterologous expression of *C. glutamicum* dihydroxyacetone phosphate dephosphorylase (*cghdpA*) gene [6] using glucose as the sole carbon source. This gene (*cghdpA*) has been recently identified in *C. glutamicum* [6] and its presence has not been discovered in any other organisms to the best of our knowledge. The main precursor used is dihydroxyacetone phosphate (DHAP) which is dephosphorylated to DHA using *cghdpA* in a single step biochemical reaction (Fig. 1). Our strategy was thus based on augmenting the availability of precursor (DHAP) and blocking the competitive pathways by engineering the *E. coli* strain (Fig. 1). Stepwise improvements in DHA production has been demonstrated with the highest concentration of 6.60 g/l at 87% of the maximum theoretical yield. To the best of our knowledge, the yield and concentration of DHA achieved in this study is currently the highest among the commonly used genetically amenable model organisms such as *E. coli* and *S. cerevisiae.*

2. Materials and Methods

2.1. Bacterial strains and plasmids

E. coli MC4100Δ*ara*714 strain (WT) was used as the parental strain in this study [10]. *C. glutamicum* genomic DNA, the source of dihydroxyacetone dephosphorylase (*hdpA*) gene (InterPro accession number IPR006357) was purchased from Deutsche Sammlung von

Mikroorganismen und Zellkulturen (DSMZ), Germany (DSM No.: 20300). *S. cerevisiae* INVSc1 from Invitrogen (USA) was used as the host for plasmid construction via homologous recombination. Plasmids pKD3, pKD4, pKD46 and pCP20 were utilized for gene deletion in *E. coli* [11]. pETDuet vector, Novagen (Australia) was used for the expression of *cghdpA* gene. Oligos were synthesized by AITBiotech (Singapore) and IDTDNA Technologies (Singapore) as listed in Table 1. All other chemicals were purchased from Sigma-Aldrich, unless otherwise mentioned. A list of strains and plasmids are shown in Table 1.

2.2. Mutant creation and gene expression

All the mutants were created on Lysogeny broth (LB) agar plates containing the appropriate antibiotics. The genes triose phosphate isomerase (*tpiA*), methylglyoxal synthase (*mgsA*) and glycerol dehydrogenase (*gldA*) were deleted using the phage λ Red recombinase protocol [11]. The disruption gene cassette was prepared by PCR-amplification from pKD4 (kanamycin) (for *tpiA* and *mgsA* deletion) or pKD3 (chloramphenicol) (for *gldA* deletion) using the oligo pairs listed in Table 1. The selectable marker gene was removed by transforming with pCP20. pkD46 vector which contained the λ Red recombinase gene was utilized for recombination in *E. coli*. The primers to create disruption gene cassettes and gene deletion confirmations are listed in Table 1. The strategy to delete all the genes along with gel pictures of deletion confirmations have been provided in Supplementary Figure 1 and Supplementary Figure 2.

The pETDuet vector containing yeast 2μ sequence and URA3 gene cassette was utilized to clone genes by homologous recombination in yeast [12]. *hdpA* gene was amplified from the genomic DNA of *C. glutamicum* and introduced homologous arms by the designed oligos (*cghdpA*-for and *cghdpA*-rev) in Table 1. The resulting PCR product was co-transformed with the pETDuet vector linearized by digestion with BglII into electrocompetent *S. cerevisiae* INVSc1 for homologous recombination in vivo. Yeast transformants were selected on SC-URA plate and subjected to yeast plasmid miniprep via a commercial kit from ZYMO RESEARCH (USA). The isolated yeast plasmids were re-transformed by electroporation into *E. coli* TOP10 cells for propagation and isolation. The isolated plasmids were confirmed initially by restriction digestion analysis and further by DNA sequencing of the cloned genes. The constructed plasmid

bearing the *cghdpA* gene was then transformed into individual *E. coli* mutant for expression and DHA production.

2.3. Culture medium and growth conditions

The preculture for fermentation was prepared by picking single colonies of different mutants from fresh plates and inoculating into 5 ml of Lysogeny broth (LB) medium with 100 µg/ml ampicillin. This was cultivated overnight in a shaking incubator (250 rpm) at 37°C aerobically. This overnight 5 ml cell culture was then inoculated into 50 ml of fresh LB medium containing 100 µg/ml ampicillin and grown overnight aerobically with shaking (250 rpm) at 37°C. The 55 ml resulting cell culture was then inoculated into the sterile 2.5 L bioreactor (Biostat Q Plus: Sartorius) containing 945 ml of M9 minimal medium resulting in 1 L of the working volume of fermenter. The M9 minimal medium contained 15 g/l glucose, 3 g/l Na_2HPO_4, 1.5 g/l KH_2PO_4, 0.25 g/l NaCl, 0.5 g/l NH_4Cl, 0.001 M $MgSO_4$, $5x10^{-5}$ M $CaCl_2$, 100 µg/ml ampicillin, 0.25 mM IPTG and Promocell trace element #42331 mix (Promocell).

Fermenter containing M9 minimal medium (without glucose, antibiotic, trace elements solution, $MgSO_4$, $CaCl_2$ and IPTG) was first autoclaved at 121°C for 15-20 minutes. After autoclaving, remaining components and cell culture were added under sterile condition. During fermentation, pH of the culture broth was controlled at 7.2 using 4M NaOH and at 37°C temperature with 250 rpm agitation. Aeration with 0.25 vvm atmospheric air was used throughout the fermentation and dissolve oxygen (DO) was not controlled during fermentation. Optical density (OD) was measured at the wavelength of 600nm using UV spectrophotometer (Shimadzu). OD_{600} was measured like this: 5 ml sample was withdrawn at the specified time points. The sample was diluted to bring down OD_{600} in the range of 0.1-0.9 whenever needed. After measurement of OD_{600}, the value was multiplied by the dilution factor to achieve the actual OD_{600}. After OD_{600} measurement, samples were immediately filtered using sterile 0.25 µm filter and stored in -20°C to use it later for HPLC and LC-MS analysis. All experiments were carried out in triplicate.

2.4. Analysis of metabolites formed during fermentation

Glucose, acetic acid, ethanol, succinate and lactate were analyzed by High Performance Liquid Chromatography (HPLC; Shimadzu LC-20 A series Quaternary HPLC system) using an

Aminex HPX-87H column (300 mm x 7.8 mm) and a refractive index (RI) detector. The retention time for glucose, acetic acid, ethanol, succinate and lactate were 10.8, 17.78, 24.01, 13.83 and 15.03 min respectively. The operating conditions for HPLC were: mobile phase, 12 mM H_2SO_4; column temperature, 50°C; flow rate, 0.5 ml/min. To measure glycerol and DHA, Liquid Chromatography-Mass spectrometry (LC-MS) was used with Agilent LC 1200 series and Agilent MS 6210 series. The LC-MS was carried out using a C-18 column with internal diameter of 4.6 mm, length 150 mm and film thickness 5 µm. Time of flight (TOF) method was used in MS to determine the ion's mass to charge ratio via a time measurement. The operating conditions used for TOF were: Gas temperature, 325°C; flow rate of drying gas (nitrogen), 5 l/m; nebulizer, 30 psig and fragmenter, 150 volts. The extracted ion chromatogram (M+H) for glycerol and DHA were 93.0546 and 91.0390 respectively. The HPLC and LC-MS results are provided in Supplementary Table 1.The spectrums for the LC-MS analysis for DHA and glycerol standards and a representative sample are shown in Supplementary Figure 3.

2.5 Malachite green assay for phosphate detection

Cell extract used for the assay of HdpA enzyme activity was prepared using a glass bead homogenizing method. The reaction mix to release inorganic phosphate from DHAP contained (in final volume of 100 µl) 100 mM Tris-Malate (pH7.5), 5 mM MgSO4, 5.0 mM DHAP lithium salt and cell extract to make up the final volume to 100 µl. The reaction mix was incubated at room temperature for 10 minutes and formation of inorganic phosphate was detected using the malachite green and ammonium molybdate method described earlier [13].

3. Results and discussion

3.1. Growth analysis of mutants with and without expression of DHAP dephosphorylase

Growth of WTΔ*tpiA* mutant designated "Single mutant" (SM) was hampered in M9 (but not in LB) media with glucose as sole carbon source and expression of *cghdpA* gene in SM only partially complemented the growth defect caused by *tpiA* deletion (Fig. 2). This is likely due to biosynthesis of toxic methylglyoxal [14] from DHAP using MgsA in the SM. Deletion of *mgsA* gene in the Δ*tpiA* background resulted in WTΔ*tpiA*Δ*mgsA* designated "double mutant" (DM). Growth of DM was completely abolished on transfer to M9 medium as no time course increment in OD_{600} was observed (Fig. 2). This was unlike DM growth in LB medium where growth was

possible albeit much slower as compared to SM or WT growth in LB medium (data not shown). In a previous study, the authors surmised that the no-growth phenotype of the DM in a medium containing glucose as sole carbon source is due to phosphate depletion [15]. However in current study we used media which is phosphate rich due to the addition of Na_2HPO_4 and KH_2PO_4. Therefore the no-growth phenotype of DM in M9 media with glucose as sole carbon source could be caused by accumulation of DHAP. Being a phosphorylated compound DHAP has a reduced transport/diffusion rate [16] thereby exacerbating its growth limiting characteristics. Possibility of growth of DM in LB medium (in which this mutant was created and maintained) could be due to a lower accumulation or production of DHAP as compared to M9 medium. Interestingly, expression of *cghdpA* in DM fully restored the growth up to the level of WT in M9 media (Fig. 2). We postulate that the restoration of growth of DM by *cghdpA* expression could be due to the conversion of accumulated DHAP to DHA together with its movement out of the cells. The growth phenotype of WTΔ*tpiA*Δ*mgsA*Δ*gldA* designated "triple mutant" (TM) bearing *cghdpA* gene was similar to DM containing the same gene (Fig. 2), demonstrating that further deletion of *gldA* in the competing pathway (Fig. 1) in DM expressing *cghdpA* has no negative effect on the growth.

3.2. Surplus acetate production hampers DHA production in SM

We started with the expression of *cghdpA* gene encoding DHAP dephosphorylase in WT strain for DHA production. However there was no detectable DHA production (data not shown) in WT expressing *cghdpA*. Since *in vitro* activity of *cghdpA* was confirmed using Malachite green assay, we speculated that a probable reason for no detectable DHA production in WT expressing *cghdpA* could be insufficient supply of precursor DHAP, which was efficiently transformed to glyceraldehyde-3-phosphate by the *tpiA* gene product (Fig. 1). We therefore deleted *tpiA* gene in WT thereby creating SM and expressed *cghdpA* gene in SM for DHA production. Expression of *cghdpA* in SM produced 0.83 g/l DHA (Fig. 3A, Table 2) with acetate (6.07 g/l) as the major metabolite of fermentation (Fig. 3A, Table 2). Theoretically, in SM, 1 mole of glucose through glycolysis should produce 1 mole of pyruvate thus giving rise to 1 mole total of acetate and ethanol (0.5 moles each of acetate and ethanol). However SM having *cghdpA* gene produced 1.28 moles of acetate and 0.67 moles of ethanol (calculation based on data from Table 2), giving rise to a total of almost 2 moles (acetate + ethanol) per mole of consumed

glucose. This data suggest that an additional 1 mole of pyruvate is formed probably using 1 mole of accumulated DHAP in the SM. Indeed the production of DHA from DHAP using *cghdpA* in SM is only 0.12 moles/mole of glucose which is only 12% of the maximum theoretical yield (1 mole/mole of glucose) implying that the major flux of accumulated DHAP in SM expressing *cghdpA* is converted to methylglyoxal using MgsA. Methylglyoxal being a toxic compound gets converted to pyruvate using the *E. coli* methylglyoxal degradation pathway, [17] (Fig. 1) thereby providing an additional mole of pyruvate. The total 2 moles of generated pyruvate in SM did not give rise to equimolar concentrations of acetate and ethanol as was expected. We surmise that the higher flux of pyruvate towards acetate in comparison to ethanol could be due to the need to generate extra ATP through the *pta-ackA* pathway under fermentative conditions [18]. Succinate and lactate were not detected in the fermentation broth.

3.3. Deletion of mgsA in SM drastically augmented DHA production

The high levels of acetate production in SM even after *cghdpA* expression suggests that cells are engaging in methylglyoxal production and degradation pathway by default, thus compromising in DHA production in this instance. Therefore an improvement in the biosynthesis of DHA was carried out by cutting off the accumulated DHAP flux towards acetate through methylglyoxal by deleting the *mgsA* gene. Deletion of *mgsA* in SM created DM and expression of *cghdpA* gene in DM significantly improved DHA production (7-fold) to 5.84 g/l (Fig. 3B, Table 2) as compared to the SM containing the same gene (0.83 g/l) (Fig. 3A, Table 2), demonstrating that *mgsA* gene deletion eliminated the competition for DHAP between MgsA and HdpA thereby improving the DHAP flux towards DHA. Together with the enhancement of DHA production in DM bearing *cghdpA* gene, reductions in acetate and ethanol production were observed (Fig. 3B, Table 2) with only 0.61 moles of acetate and 0.35 moles of ethanol formed per mole of glucose consumed. The lower production of these metabolites in the double mutant as compared to single mutant reveals an alleviated conversion of DHAP to methylglyoxal thereby facilitating the production of DHA from DHAP using *cghdpA*.

3.4. Deletion of gldA gene in DM further enhanced DHA production

After sorting out the issue of acetate production, focus was shifted to restrict the conversion of intracellular DHA to glycerol to further enhance DHA production. This was

carried out by an additional deletion of the *gldA* gene in DM thereby creating TM. It is earlier reported [9] that *E. coli* cells remove toxic intracellular DHA by converting DHA to glycerol using glycerol dehydrogenase (*gldA*). From their study, it was proposed that the likely role of *gldA* is to regulate the intracellular level of DHA by converting it into glycerol. Expression of *cghdpA* in TM demonstrated reduced glycerol production (Fig. 3C, Table 2) thereby further improving the titer and yield of DHA. Finally we obtained 6.60±0.2 g/l of DHA from 15.11±0.19 g/l of glucose which is equivalent to around 87% of the maximum theoretical yield of DHA from glucose.

4. Conclusion

The paper describes the engineering of *E. coli* metabolism to produce DHA. The re-modeling of the *E. coli* metabolism has allowed significant increase in DHA yield from 11.67% to 87.37% of the maximum theoretical yield. Our metabolic engineering efforts for DHA production (6.60 g/l and 0.44 g DHA/g glucose) in *E. coli* demonstrated a significant improvement from the previous study (0.7 g/l and 0.035 g DHA/g glucose) in *S. cerevisiae* [8]. Although the final titer and productivity of DHA in this study which are 6.60 g/l and 0.165 g/l/h respectively are not commercially viable, the insights generated in this study can be utilized to further improve DHA production. For example, deletion of *gldA* did not improve DHA concentration drastically, which could be due to residual glycerol production through unspecific dehydrogenases. Since *E. coli* has several such dehydrogenases which might catalyze unspecific conversion of DHA to glycerol [19], completely blocking or inhibiting the undesired conversion of DHA to glycerol can lead to even higher concentrations of DHA. Moreover codon optimizing the native sequence of *C. glutamicum* HdpA for *E. coli* might also enhance DHA production as *C. glutamicum* has different codon usages as compared to *E. coli* [20]. Initial growth of the mutants bearing HdpA might be improved by using rich media (addition of tryptone or yeast extract) or a high inoculum density. However we did not supplement the media with these nutrients in this study since our aim was to demonstrate the production of DHA using glucose as the sole carbon source. Finally fed batch fermentations can be employed to improve the overall titer of DHA. In conclusion this study highlights the successful use of *C. glutamicum* DHAP dephosphorylase in *E. coli* mutant for DHA production which has wide applications in several industries.

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Figure Captions

Fig. 1. Diagram shows manipulations in the metabolic pathways of E. coli for the biosynthesis of DHA. Genes deleted in this study such as *tpiA*, *mgsA* and *gldA* are indicated (×). Deletion of *tpiA* accumulated DHAP which was converted to DHA using heterologous expression of *cghdpA*. Deletion of *mgsA* and *gldA* was carried out to minimize the production of acetate and glycerol respectively.

Fig. 2. Time course experiment for the aerobic growth of strains used in this study. OD_{600} for WT (diamond), Δ*tpiA* mutant with empty vector (triangle), Δ*tpiA* mutant with *cghdpA* gene (cross), Δ*tpiA*Δ*mgsA* double mutant with empty vector (dash), Δ*tpiA*Δ*mgsA* double mutant with *cghdpA* gene (circle) and Δ*tpiA*Δ*mgsA*Δ*gldA* triple mutant with *cghdpA* gene (square). The data represents the averages and standard deviations of three replicates.

Fig. 3. Time course experiment for the production of metabolites in Δ*tpiA* (3A), Δ*tpiA*Δ*mgsA* (3B) and Δ*tpiA*Δ*mgsA*Δ*gldA* (3C) with *cghdpA* during a fermenter study. Consumption of glucose (square) is shown on the primary axis and production of DHA (plus), glycerol (circle), acetate (triangle) and ethanol (cross) are shown on secondary axis. The data represents the averages and standard deviations of three replicates.

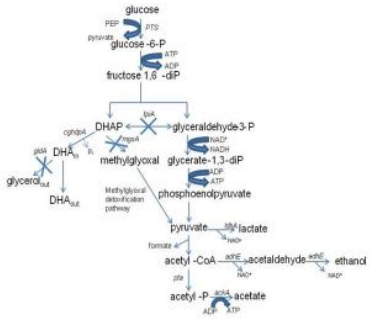

Fig. 1

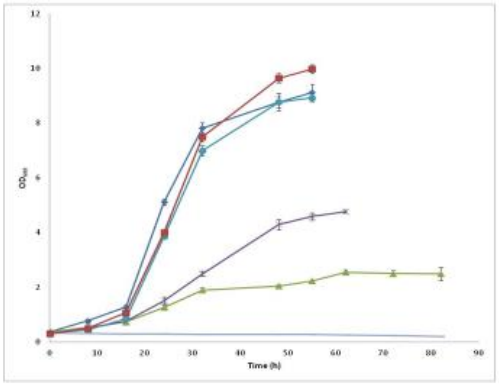

Fig. 2

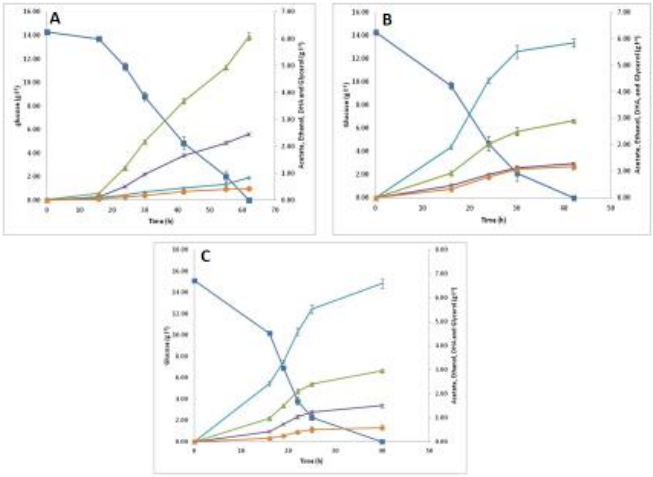

Fig. 3

Table 1. List of microbial strains, plasmids and primers used in this study.

	Reference
Strains	
WT	[10]
SM	This study
DM	This study
SM + pETDuet(*cghdpA*)	This study
DM+ pETDuet(*cghdpA*)	This study
TM + pETDuet(*cghdpA*)	This study
Plasmids	
pETDuet	Novagen
pKD3,4 and 46	[11]
pCP20	[11]
pETDuet(*cghdpA*)	This study
Primers	**Description**
Kn-gldA-for (CM)	ATTTGGCACTACTCATCTCTAAAGGAGCAATTATGGACGTGTAGGCTGGAGCTGCTTC
Kn-gldA-rev (CM)	AACTCCCGGACAAGCCGGGAGTTTGGAGTAGGTTATTCATGGGAATTAGCCATGGTCC
Conf-kn-gldA-for	GGCATTCGTACGGTTCAGGAGCTGCAAACG
Conf-kn-gldA-rev	ATGGAGAAAAAAATCACTGGATATACCACC
Kn-tpiA-for	TTTATTCGCTTATAAGCGTGGAGAATTAAAATGCGATGTAGGCTGGAGCTGCTTCG
Kn-tpiA-rev	GCCGGATATGAAATCCGGCACCTGTCAGACTTAAGCCATATGAATATCCTCCTTAG
Conf-kn-tpiA-for	CAAAGCCTTTGTGCCGATGAATCTCTATAC
Conf-kn-tpiA-rev	GGTTTGCGCGGGCATGAATACCTGGCGCGT
Kn-mgsA-for	CTGTAGGAAA GTTAACTACGGATGTACATTATGGAATGTAGGCTGGAGCTGCTTCG
Kn-mgsA-rev	AAACAGGTGGCGTTTGCCACCTGTGCAATATTACTTCATATGAATATCCTCCTTAG
Conf-kn-mgsA-for	CCTGAGATTTTTGTGCCTGTGCGCAGGCTT
Conf-kn-mgsA-rev	GCACTGTTTAACAAAGAGAATCCCTCTCCC
cghdpA-for	ATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATATGACAGTGAACATTTCA
cghdpA-rev	CCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCTAGTCAGCGAACTGCTG

Table 2. Consumption of glucose and production of various metabolites (g/l) at the end of fermentation by mutants expressing *C. glutamicum* dihydroxyacetone dephosphorylase (*cghdpA*). Yield of DHA is defined as grams of DHA formed per gram of glucose consumed. Theoretical yield of DHA from glucose is taken as 0.5 g DHA/g glucose consumed. Actual yield is calculated by dividing the DHA formed at the end of fermentation by the total glucose consumed. DHA efficiency is calculated by dividing actual yield by theoretical yield. The data represents the averages and standard deviations of three replicates.

Strains	Time (h)	glucose consumed (g/l)	Acetate (g/l)	Ethanol (g/l)	DHA (g/l)	Glycerol (g/l)	DHA efficiency (%)
*SM+*cghdpA*	62	14.11±0.21	6.07±0.14	2.45±0.05	0.83±0.03	0.43±0.02	11.67±0.29
*DM+*cghdpA*	42	14.27±0.24	2.92±0.04	1.29±0.04	5.84±0.15	1.17±0.11	81.89±3.51
*TM+*cghdpA*	40	15.11±0.19	2.96±0.06	1.50±0.06	6.60±0.2	0.58±0.11	87.37±1.68

*SM= WTΔ*tpiA*, *DM= WTΔ*tpiA*Δ*mgsA*, *TM= WTΔ*tpiA*Δ*mgsA*Δ*gldA*, WT= Wild type