Biotechnology and Bioprocess Engineering 20: 1088-1098 (2015)
DOI 10.1007/s12257-015-0607-x

RESEARCH PAPER

Cyclohexanone-induced Stress Metabolism of *Escherichia coli* and *Corynebacterium glutamicum*

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Received: 18 September 2015 / Revised: 23 September 2015 / Accepted: 15 October 2015
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Abstract Solvent stress occurs during whole-cell biocatalysis of organic chemicals. Organic substrates and/or products may accumulate in the cellular membranes of whole cells, causing structural destabilization of the membranes, which leads to disturbances in cellular carbon and energy metabolism. Here, we investigate the effect of cyclohexanone on carbon metabolism in *Escherichia coli* BL21 and *Corynebacterium glutamicum* ATCC13032. Adding cyclohexanone to the culture medium (*i.e.*, glucose mineral medium) resulted in a decreased specific growth rate and increased cellular maintenance energy in both strains of bacteria. Notably, carbon metabolism, which is mainly involved to increase cellular maintenance energy, was very different between the bacteria. Carbon flux into the acetic acid fermentation pathway was dominantly enhanced in *E. coli*, whereas the TCA cycle appeared to be activated in *C. glutamicum*. In fact, carbon flux into the TCA cycle in *E. coli* appeared to be reduced with increasing amounts of cyclohexanone in the culture medium. Metabolic engineering of *E. coli* cells to maintain or improve TCA cycle activity and, presumably, that of the electron transport chain, which are involved in regeneration of cofactors (*e.g.*, NAD(P)H and ATP) and formation of toxic metabolites (*e.g.*, acetic acid), may be useful in increasing solvent tolerance and biotransformation of organic chemicals (*e.g.*, cyclohexanone).

Keywords: *Escherichia coli*, *Corynebacterium glutamicum*, cyclohexanone, solvent stress, constraints-based flux analysis

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1. Introduction

The number of chemicals that can be prepared *via* whole-cell biocatalysis is increasing steadily due to rapid development of enzyme screening and engineering, metabolic engineering, and systems and synthetic biology [1-8]. For example, ε-caprolactone, which is widely used to produce poly-ε-caprolactone and other chemical products, can be produced from cyclohexanone by microbes (*e.g.*, *Escherichia coli* and *Corynebacterium glutamicum*) expressing a gene encoding cyclohexanone monooxygenase [9,10]. The chemical was also reported to be synthesized from cyclohexanol by recombinant *E. coli*-based catalysts expressing $NADP^+$-dependent alcohol dehydrogenase and an NADPH-dependent cyclohexanone monooxygenase gene [11,12]. Metabolic engineering of recombinant *E. coli* expressing the NADPH-dependent cyclohexanone monooxygenase of *Acinetobacter calcoaceticus* NCIMB 9871 to improve NADPH regeneration activity [13] or oxygen transport [14] resulted in a significant increase in biocatalytic performance. Besides, ε-caprolactone, which is produced from cyclohexanol *via* a whole-cell reaction, is converted into oligo-ε-caprolactone *in situ* through subsequent direct ring-opening oligomerization using lipase A from *Candida antarctica* [15]. These results suggest that enzymatic synthesis of poly-ε-caprolactone will soon be possible.

One of the limiting factors influencing whole-cell productivity and final product concentrations is the toxic effects of organic substrates and/or products (*e.g.*, styrene, styrene oxide, cyclohexanone) to the host organisms [16-18]. Organic chemicals can accumulate in cellular membranes, causing structural destabilization and reducing metabolic activities [19-22] critical for regenerating cofactors (NAD(P)H, ATP) and protein turnover. Therefore, it is essential to understand adaptation and tolerance mechanisms of microbes

toward the toxic or inhibitory effects of organic chemicals.

We investigated the metabolic responses of *E. coli* BL21 and *C. glutamicum* ATCC13032, which are extensively used to produce organic chemicals, on the toxic effects of cyclohexanone. Our results will contribute to characterizing engineering targets to improve the solvent tolerance of bacteria, which is important for cofactor-dependent whole-cell biotransformation activities.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

E. coli BL21 was cultivated in LB medium and M9 mineral medium [23] supplemented with 4 g/L glucose for the seed and main cultures, respectively. *C. glutamicum* ATCC 13032 was grown in brain-heart infusion medium and CGXII minimal medium [24] containing 40 g/L glucose for the seed and main cultures, respectively. The *E. coli* and *C. glutamicum* cultures were incubated in a shaking incubator at 37 and 30°C, respectively (200 rpm). When the *E. coli* and *C. glutamicum* cultures (100 mL) reached the exponential growth phase at about 4 and 7.5 h, respectively, after initiating the main culture, they were split into 20 mL subcultures, to which different concentrations of cyclohexanone (*i.e.*, 0 ~ 10 g/L) were added.

For the incubation experiments of *E. coli* in the absence and the presence (5 g/L) of cyclohexanone under non-growth conditions, the *E. coli* cultures were harvested at the exponential growth phase, centrifuged, and washed twice with sodium-potassium phosphate buffer (pH 7.0). Then, they were resuspended in the same buffer containing 5 g/L glucose in side-arm flasks. The headspace was replaced with 100% O_2, and the flasks were incubated at 37°C at 200 rpm.

2.2. Analytical methods

Glucose and organic acid concentrations in the culture medium were quantified by high performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) using an HPX-87H column (Bio-Rad, Hercules, CA, USA). A refractive index detector and UV dual absorbance detector (Waters) were utilized to detect the metabolites. The column was eluted with 5 mM sulfuric acid at a constant rate of 0.6 mL/min at 45°C. The culture samples were centrifuged at 14,000 × *g* and 4°C for 5 min, and the supernatant was analyzed. Gas-phase side-arm flasks were sampled with a syringe and analyzed using a 6890N gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) equipped with a 19095P-MS6 column and thermal conductivity detector to determine CO_2 concentrations, as described previously [18].

2.3. Constraints-based flux analysis

In silico carbon flux analyses were conducted using the *E. coli i*AF1260 genome-scale metabolic model [25], which includes 1,260 reactions. *In silico* carbon flux analyses of *C. glutamicum* was carried out with a metabolic network model, which consisted of 31 metabolites (including external metabolites) and 32 biochemical reactions (including transport reactions). The *C. glutamicum* model was constructed by modifying the model published in a previous study [26] by adding ATP to adjust for non-growth-associated maintenance energy. We performed constraint-based flux analysis subjected to stoichiometric and capacity constraints to determine the metabolic fluxes. The biomass reaction was maximized to simulate the intracellular carbon flux distribution of the cells growing at the exponential growth phase, as in previous studies [27-29]. ATP regeneration was maximized to estimate the intracellular carbon flux distribution for the *in silico* carbon flux analysis of the *E. coli* cells incubated under non-growth conditions. The exchange fluxes of oxygen and other minerals were unconstrained to provide basic nutrients for cell growth. The measured glucose and products (acetate, lactate, and CO_2) were constrained at specific consumption or production rates. Carbon flux into the pentose phosphate pathway was assumed to correlate with specific growth rate. The COBRA toolbox [30] was utilized to investigate the constraints-based flux analysis.

3. Results and Discussion

3.1. Growth and carbon metabolism of *E. coli* in the presence of cyclohexanone

E. coli BL21 was cultivated in a glucose mineral medium containing different cyclohexanone concentrations (Fig. 1). The bacteria grew by consuming glucose and produced acetic acid as a major fermentation product. The specific growth rate of the cells reached 0.40/h during the exponential growth in the absence of cyclohexanone. The specific glucose uptake rate and the specific acetic acid production rate were 7.7 and 2.0 mmol/g_{CDW}/h, respectively (Figs. 2 and 3). Increasing the cyclohexanone concentration to 5 g/L led to a significant change in specific growth rate and carbon metabolism; the specific growth rate decreased linearly with increasing cyclohexanone concentration, whereas the specific acetic acid production rate increased markedly with increasing cyclohexanone concentration (Fig. 2). The glucose uptake rate remained rather unchanged. A further increase in the cyclohexane concentration to 10 g/L appeared to cause a great deal of stress, resulting in a substantial reduction in specific glucose uptake and specific growth rate.

The *in silico* carbon flux analysis was carried out with an

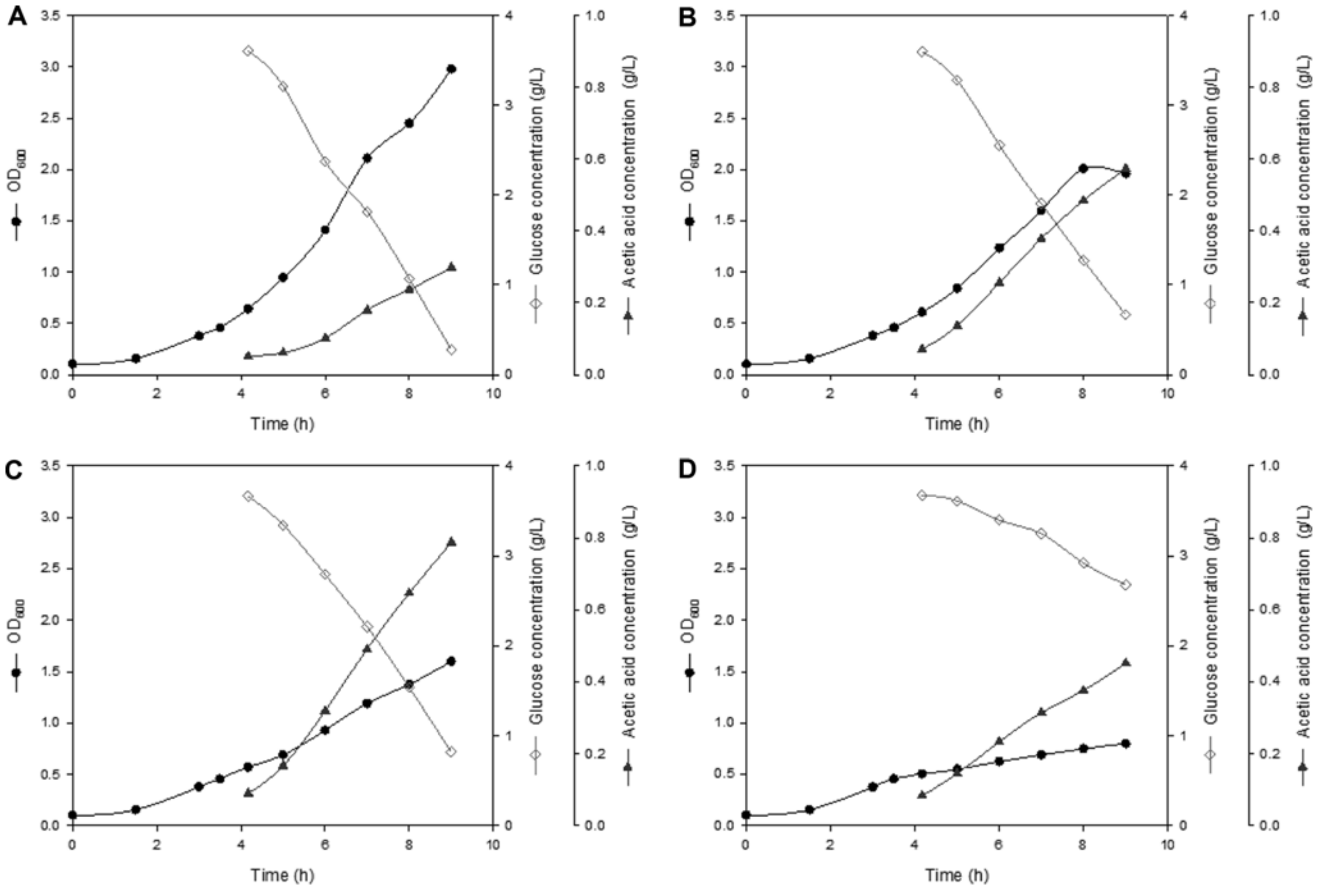

Fig. 1. Fermentation profiles of *Escherichia coli* BL21. Fermentation was carried out in M9 mineral medium containing 4 g/L glucose in the absence (A) and presence (B–D) of cyclohexanone. When the bacterial cultures (100 mL) reached the exponential growth phase, after about 4 h of cultivation, they were split into 20 mL subcultures, to which different concentrations of cyclohexanone (2 g/L (B), 5 g/L (C), and 10 g/L (D)) were added.

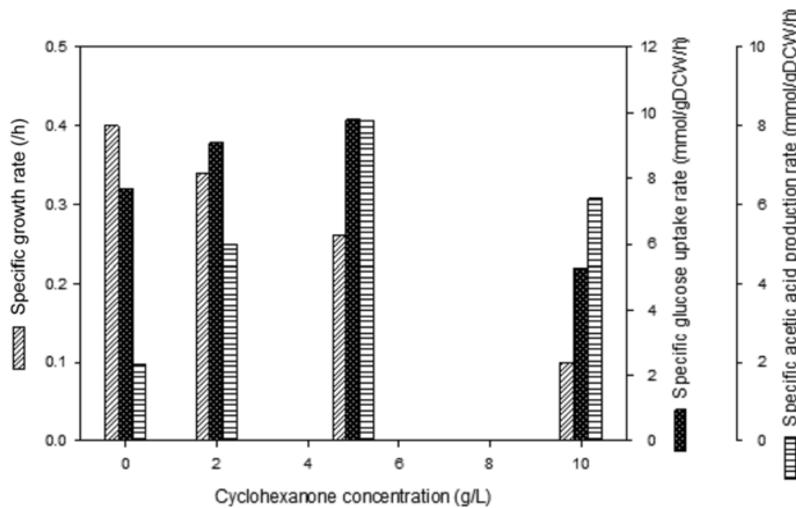

Fig. 2. Specific growth rate, specific glucose uptake rate, and specific acetic acid production rate during the exponential growth phase of the fermentation shown in Fig. 1.

E. coli metabolic model [25] to investigate the effect of cyclohexane concentration on *E. coli* carbon and cofactor metabolism. Increasing the cyclohexane concentration from 0 to 5 g/L directed more carbon into the acetic acid

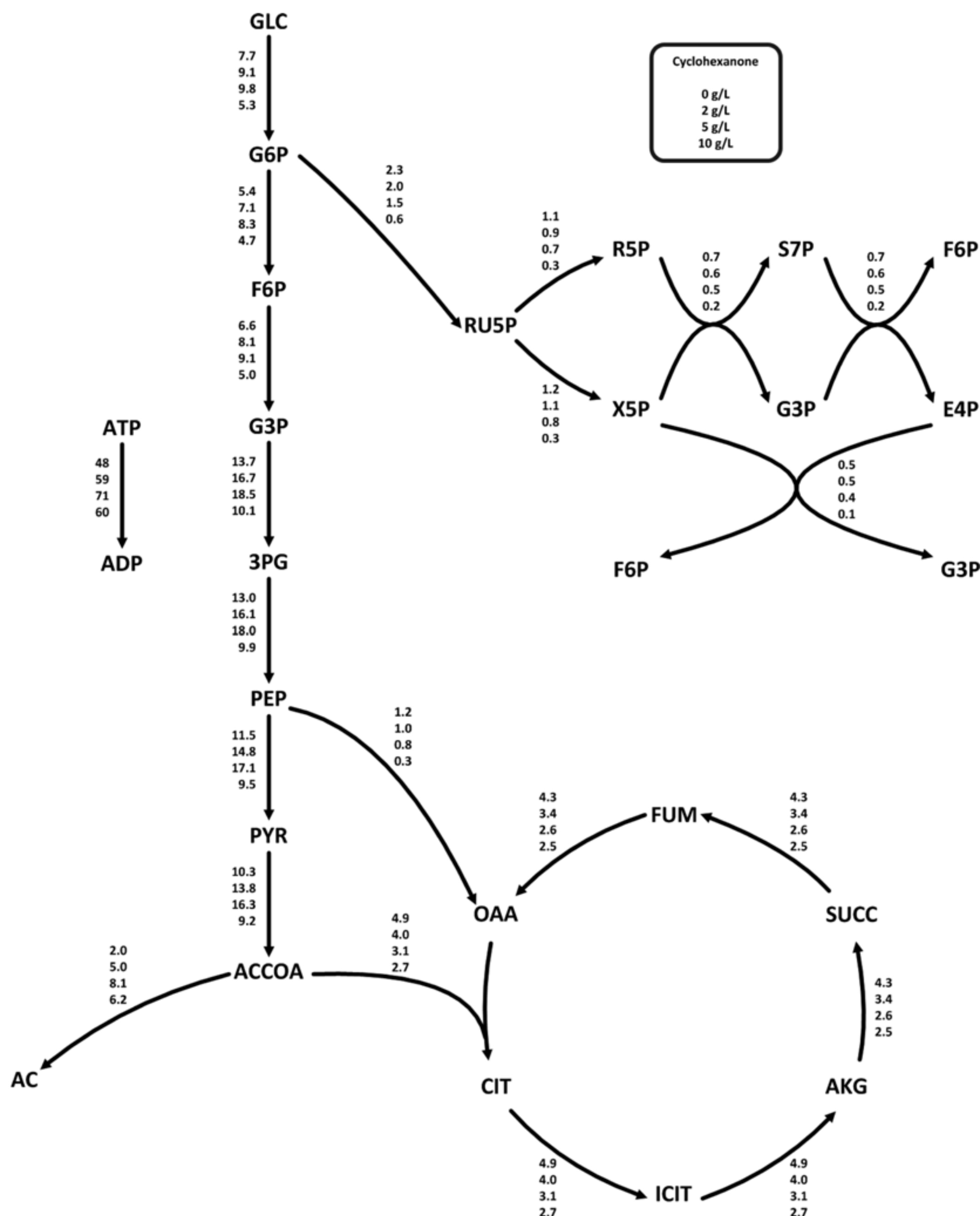

Fig. 3. Internal carbon flux distribution in *Escherichia coli* BL21 growing at the exponential growth phase in the absence (upper values) and presence (2 g/L (second upper values), 5 g/L (third upper values), and 10 g/L (lower values)) of cyclohexanone shown in Fig. 1. The carbon flux distribution was estimated based on stoichiometric constraints using a genome-scale metabolic model of *E. coli* *i*AF1260 [25].

fermentation pathway; carbon flux through the acetic acid fermentation pathway increased significantly from 2.0 to 8.1 mmol/g_{CDW}/h (Fig. 3). In contrast, carbon flux into the TCA cycle decreased with increasing cyclohexanone concentration (Figs. 3 and 4). These results indicate that the fermentation pathway rather than the TCA cycle was stimulated in *E. coli* upon exposure to high cyclohexanone concentrations. The increased carbon flux into the fermentation pathway suggests that more glucose was oxidized to speed up regeneration of energy molecules (*i.e.*, ATP and NAD(P)H). The cellular energy required for maintenance was calculated according to previous studies [29,31,32] and was positively correlated with the increase in cyclohexanone concentration (Fig. 4). A larger amount of energy for cellular maintenance may be required to overcome cyclohexanone-induced stress.

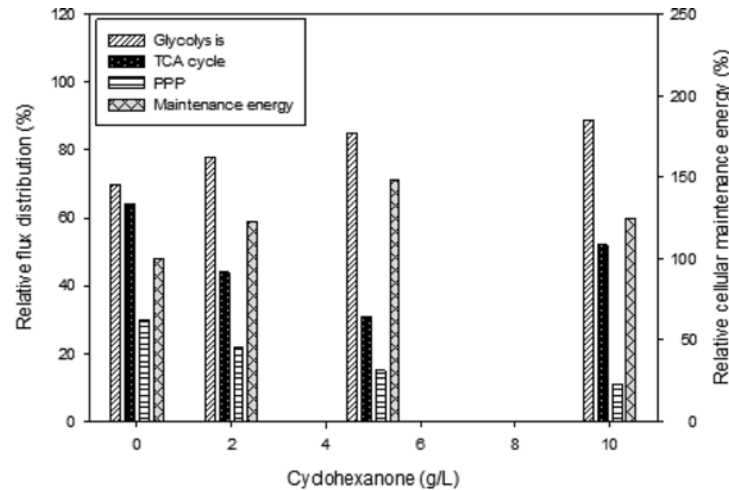

Fig. 4. Carbon flux into the TCA cycle and the acetic acid fermentation pathway and the amount of cellular maintenance energy used during the exponential growth phase of the cultivation shown in Fig. 1. Cellular maintenance energy was calculated according to previous studies [29,31,38].

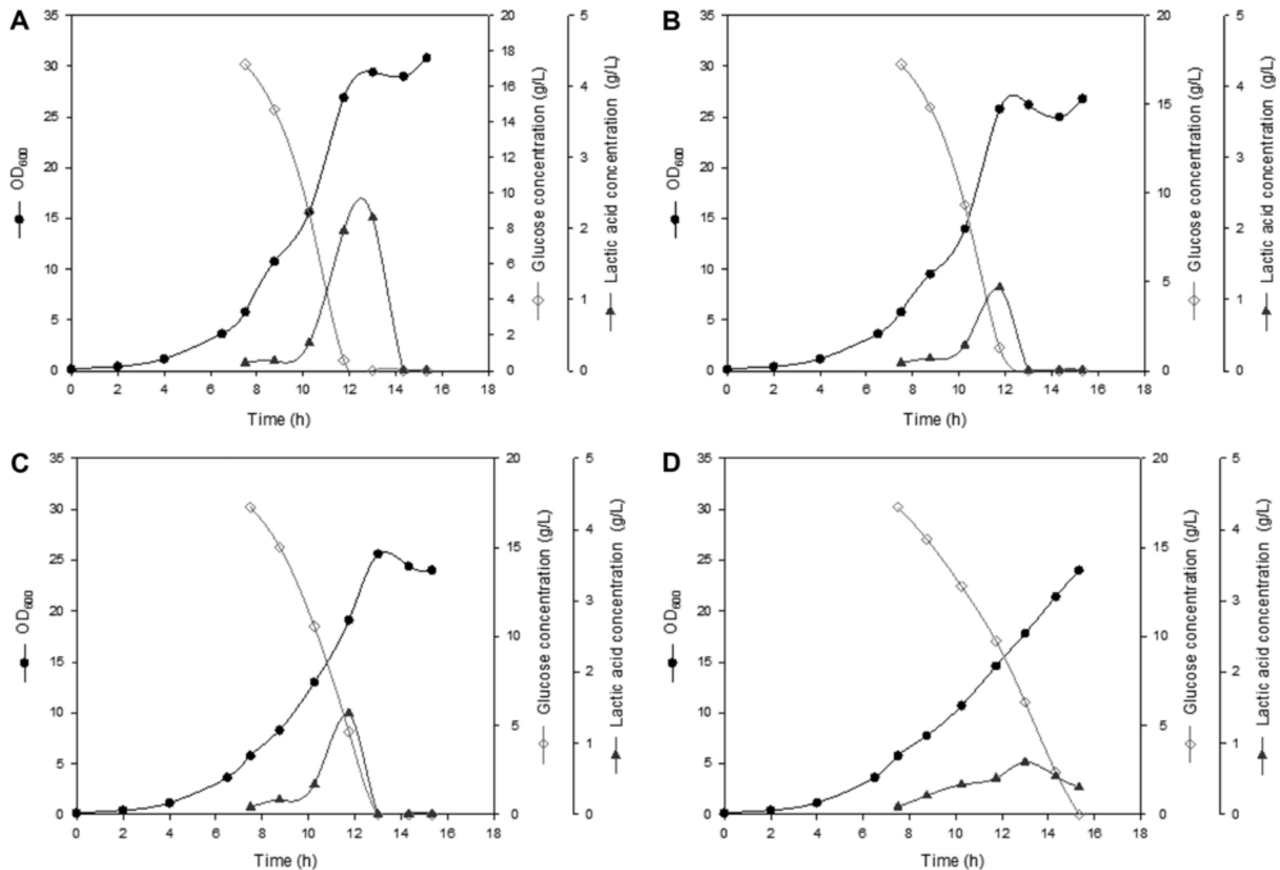

Fig. 5. Fermentation profiles of *Corynebacterium glutamicum* ATCC13032. Fermentation was carried out in CGXII mineral medium containing 40 g/L glucose in the absence (A) or presence (B–D) of cyclohexanone. When the bacterial cultures (100 mL) reached the exponential growth phase, after about 7.5 h of cultivation, they were split into 20 mL subcultures, to which different concentrations of cyclohexanone (2 g/L (B), 5 g/L (C), and 10 g/L (D)) were added.

3.2. Growth and carbon metabolism of *C. glutamicum* in the presence of cyclohexanone

The growth profiles of *C. glutamicum* ATCC13032 in glucose mineral medium-containing cyclohexanone were similar to those of *E. coli* BL21 (Fig. 5). The specific growth rate decreased linearly as the cyclohexanone

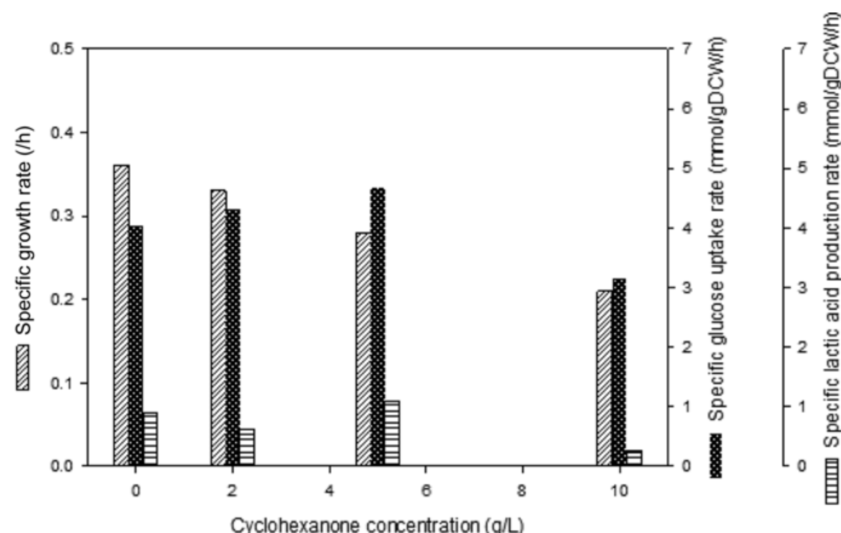

Fig. 6. Specific growth rate, specific glucose uptake rate, and specific lactic acid production rate during the exponential growth phase of the cultivation shown in Fig. 5.

concentration increased from 0 to 10 g/L (Figs. 5 and 6). However, the reduction in specific growth rate in the presence of cyclohexanone was much lower than that of *E. coli* BL21. Adding cyclohexanone at 5 g/L resulted in a 22% decrease in specific growth rate compared to that in the absence of cyclohexanone, whereas a 35% reduction in specific growth rate was observed in *E. coli* BL21 cells under the same conditions. This result indicates that *C. glutamicum* ATCC13032 is more tolerant to cyclohexanone stress. In addition, the formation profiles of fermentation products (e.g., lactic acid) were very different. The highest lactic acid concentration in the culture medium and the specific lactic acid production rate decreased gradually with increasing cyclohexanone concentration (Figs. 5 and 6).

We carried out *in silico* carbon flux analysis during the exponential growth phase to examine the influence of cyclohexanone on *C. glutamicum* ATCC13032 carbon metabolism (Fig. 7). Remarkably, carbon flux into the TCA cycle significantly increased, from 2.2 to 4.1 mmol/g_{CDW}/h, as the cyclohexanone concentration added was increased from 0 to 5 g/L (Figs. 7 and 8). In contrast, carbon flux through the lactic acid fermentation pathway remained approximately the same (Fig. 7). These results indicate that the TCA cycle rather than the fermentation pathway was stimulated when *C. glutamicum* was exposed to high concentrations of cyclohexanone. Relative cellular maintenance energy also increased linearly with increasing cyclohexanone concentration in the culture medium (Fig. 8), suggesting that increased cellular maintenance energy was supplied via TCA cycle metabolism rather than *via* the fermentation pathway.

3.3. Carbon metabolism of *E. coli* under non-growth conditions

Whole-cell biocatalysis is often conducted in a buffer solution without a nitrogen source [33,34]. Therefore, we investigated the effect of cyclohexanone on *E. coli* BL21 carbon metabolism under non-growth conditions. Adding cyclohexanone to sodium-potassium phosphate buffer (pH 7.0) containing 5 g/L glucose had no significant impact on carbon metabolism (Table 1). The *in silico* carbon flux analysis suggested that most glucose in the reaction medium was catabolized *via* glycolysis and the TCA cycle in the absence and presence of cyclohexanone (Fig. 9). The acetic acid fermentation pathway appeared to play a minor role in carbon metabolism under these conditions. These results indicate that the TCA cycle rather than the fermentation pathway is activated in *E. coli* upon exposure to high cyclohexanone concentrations under non-growth conditions. Although we do not understand the reason for the difference in carbon metabolism between growing and non-growing cells, the high TCA cycle activity in the non-growing cells appeared to allow high catalytic activity with respect to NAD(P)H-dependent biotransformation of organic compounds. For the NADH-dependent stereoselective epoxidation of styrene to (*S*)-styrene oxide, resting *E. coli* cells containing the styrene monooxygenase StyAB of *Pseudomonas* sp. showed significantly higher activity in short-term assays (80 ~ 100 U/g_{CDW}) than the same cells growing under optimized conditions (40 ~ 50 U/g_{CDW}) [35,36]. The recombinant *E. coli* biocatalyst also exhibited twofold higher specific styrene epoxidation activity in a two-liquid phase setup as compared to growing cells in a similar setup [33].

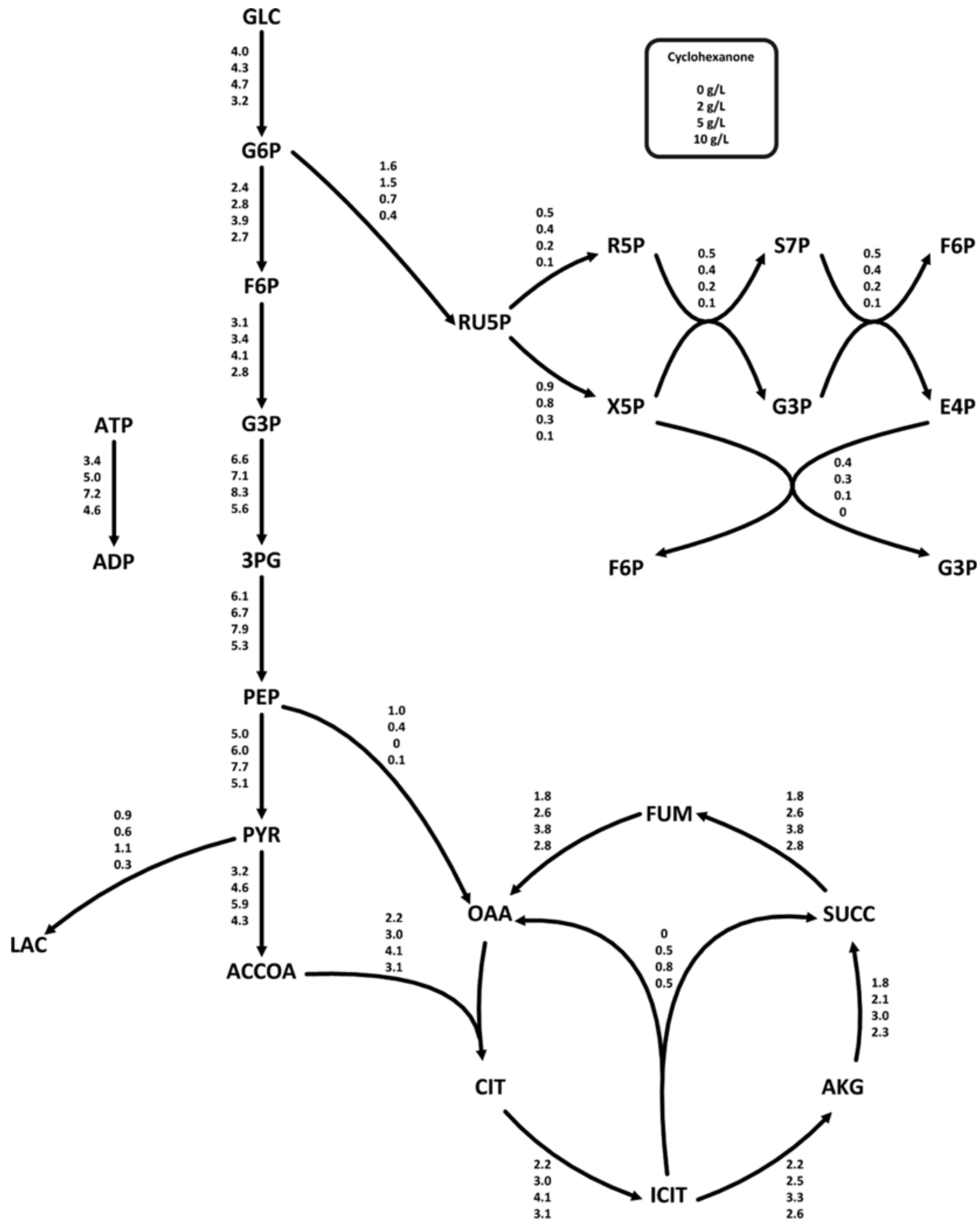

Fig. 7. Internal carbon flux distribution in *C. glutamicum* growing at the exponential growth phase of the fermentation in the absence (upper values) and presence (2 g/L (second upper values), 5 g/L (third upper values), and 10 g/L (lower values)) of cyclohexanone shown in Fig. 5. Carbon flux distribution was estimated based on stoichiometric constraints using a modified *C. glutamicum* metabolic network model (see Materials and Methods for details).

3.4. General discussion

The metabolic responses of *E. coli* to cyclohexanone stress appeared to be similar to those in isobutyl alcohol-induced stress. The specific growth rate of *E. coli* decreased linearly with increasing isobutyl alcohol concentrations in the culture medium [37]. Expression of TCA cycle enzymes was repressed significantly, indicating that carbon flux into the TCA cycle was reduced in the presence of isobutyl alcohol. The authors [37] postulate that the solvent disturbed the quinone/quinol balance in the cytoplasmic membrane, as quinol was not appropriately oxidized into quinone, resulting in quinol accumulation in cellular membranes.

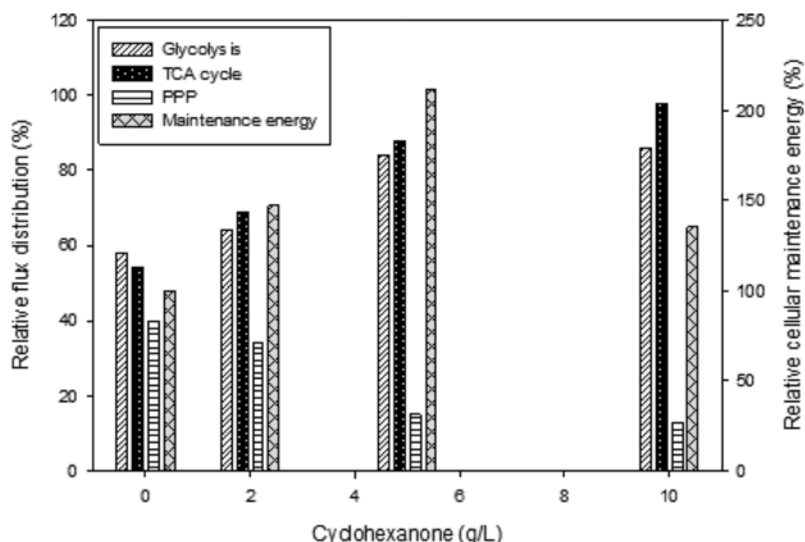

Fig. 8. Carbon flux into the TCA cycle and the lactic acid fermentation pathway and amount of cellular maintenance energy used during the exponential growth phase of the cultivation shown in Fig. 5. Cellular maintenance energy was calculated according to previous studies [29,31,38].

Table 1. Glucose uptake and product formation rates of *E. coli* under non-growth conditions

Cyclohexanone concentration (g/L)	Specific glucose uptake rate (mmol/g_{CDW}/h)	Specific acetate production rate (mmol/g_{CDW}/h)	Specific CO_2 evolution rate (mmol/g_{CDW}/h)
0	2.2	0.1	10.7
5	2.4	0.3	11.1

This led to activation of the ArcAB two-component regulatory system, which, in turn, repressed the expression of the genes encoding TCA cycle enzymes. We assume that cyclohexanone may also accumulate in cytoplasmic membranes and disturb the quinone/quinol balance because the hydrophobicity of cyclohexanone is comparable to that of isobutyl alcohol. Thus, the substantial reduction of carbon flux into the TCA cycle by *E. coli* in the presence of cyclohexanone may be driven by activation of the ArcAB regulatory system.

Destabilization of the lipid bilayer by organic solvents may also lead to proton leakage and reduced proton motive force across the cytoplasmic membrane, which, in turn, influences ATP regeneration *via* oxidative phosphorylation in the electron transport chain [19-22]. The disturbance of ATP regeneration *via* oxidative phosphorylation together with deactivation of the TCA cycle may have caused the *E. coli* cells to suffer from low ATP availability. As a result, the acetic acid fermentation pathway was stimulated in *E. coli* to increase ATP regeneration. Overall, we postulate that carbon flux into the acetic acid fermentation pathway was increased significantly to increase ATP regeneration in the presence of cyclohexanone (Fig. 3).

In contrast, cyclohexanone did not activate a fermentation pathway in the Gram positive bacteria *C. glutamicum*. Instead, carbon flux into the TCA cycle increased with increasing cyclohexanone concentration in the culture medium (Fig. 7). This result indicates that organic solvent stress metabolism including the regulatory systems in *C. glutamicum* is significantly different from those of *E. coli*. The reason for the difference remains to be investigated.

C. glutamicum appears to be promising as a whole-cell biocatalyst, if the catalytic enzymes could be overexpressed in a functional form, as discussed previously [10]. First, *C. glutamicum* did not produce any toxic metabolites when exposed to cyclohexanone, indicating that toxic metabolites, which may inhibit whole-cell catalytic activities, would not be produced during biocatalysis of organic chemicals. Second, the specific growth rate of *C. glutamicum* was better maintained in the presence of cyclohexanone compared to that of *E. coli* BL21 (Figs. 2 and 6). This suggests that carbon metabolism and metabolic activity of *C. glutamicum* is less influenced by cyclohexanone stress. This may allow for cellular maintenance of cofactor regeneration and protein turnover activity during whole-cell biocatalysis.

The solvent tolerance of *E. coli* cells could be improved *via* maintaining or enhancing activity of the TCA cycle

Fig. 9. Internal carbon flux distribution in *Escherichia coli* BL21 incubated under non-growth conditions in the absence (upper values) and presence (5 g/L (lower values)) of cyclohexanone. Carbon flux distribution was estimated based on stoichiometric constraints using a genome-scale metabolic model of *E. coli* *i*AF1260 [25].

and, presumably, the electron transport chain, which appeared to be repressed under solvent stress. Since the TCA cycle and electron transport chain are involved in regeneration of cofactors (*e.g.*, NAD(P)H and ATP) and formation of toxic metabolites (*e.g.*, acetic acid), maintaining or enhancing activity of both pathways could be critical for whole-cell biotransformation activity.

4. Conclusion

We investigated the metabolic responses of *E. coli* BL21 and *C. glutamicum* ATCC13032 during aerobic culture in glucose mineral medium containing cyclohexanone using *in silico* carbon flux analysis. Adding cyclohexanone to the culture medium decreased specific growth rate and increased

the cellular maintenance energy required by both cells. However, carbon metabolism, which was mainly used to afford increased cellular maintenance energy, was very different between the two bacteria. The acetic acid fermentation pathway rather than the TCA cycle was stimulated in *E. coli* upon exposure to high concentrations of cyclohexane. In contrast, the TCA cycle rather than the fermentation pathway was activated in *C. glutamicum* under comparable conditions. Metabolic engineering to increase solvent tolerance of *E. coli* and biocatalytic performance appears to include improving the activities of the TCA cycle and presumably the electron transport chain, which are involved in formation of toxic metabolites (*e.g.*, acetic acid) and regeneration of cofactors (*e.g.*, NAD(P)H and ATP).

Acknowledgements

This study was supported by a Marine Biomaterials Research Center grant from the Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, Korea.

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