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Metabolic Flux Distribution for γ-Linolenic Acid Synthetic Pathways in *Spirulina platensis*

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Abstract *Spirulina* produces γ-linolenic acid (GLA), an important pharmaceutical substance, in a relatively low level compared with fungi and plants, prompting more research to improve its GLA yield. In this study, metabolic flux analysis was applied to determine the cellular metabolic flux distributions in the GLA synthetic pathways of two *Spirulina* strains, wild type BP and a high-GLA producing mutant Z19/2. Simplified pathways involving the GLA synthesis of *S. platensis* formulated comprise of photosynthesis, gluconeogenesis, the pentose phosphate pathway, the anaplerotic pathway, the tricarboxylic cycle, the GLA synthesis pathway, and the biomass synthesis pathway. A stoichiometric model reflecting these pathways contains 17 intermediates and 22 reactions. Three fluxes – the bicarbonate (C-source) uptake rate, the specific growth rate, and the GLA synthesis rate – were measured and the remaining fluxes were calculated using linear optimization. The calculation showed that the flux through the reaction converting acetyl-CoA into malonyl-CoA in the mutant strain was nearly three times higher than that in the wild-type strain. This finding implies that this reaction is rate controlling. This suggestion was supported by experiments, in which the stimulating factors for this reaction (NADPH and $MgCl_2$) were added into the culture medium, resulting in an increased GLA-synthesis rate in the wild type strain.

Keywords: metabolic flux analysis, *Spirulina platensis*, γ-linolenic acid

INTRODUCTION

Spirulina (*Arthrospira*) *platensis*, a cyanobacterium, is long known to be nutritious food, and has been widely used for decades as human health food and animal feed [1-3]. It provides protein, β-carotene, vitamins, minerals, chlorophyll, sulfolipids, glycolipids, phycocyanin, enzymes, RNA, DNA, and supplies many nutrients that are lacking in most modern diets [3,4]. In addition, the essential polyunsaturated fatty acid, γ-linolenic acid (GLA), is present at a relatively high level among cyanobacteria (30% of total fatty acid or 1~1.5% of dry cell weight) [5]. This fatty acid has high pharmaceutical and nutritional values. It makes a hormone like substance called prostaglandin E1 (PGE1), which performs a variety of functions in cluding preventing heart attacks and strokes, slowing the production of cholesterol, and improving nerve functions, just to name a few. Studies have indicated that GLA also reduces high blood pressure and eases such conditions as arthritis, premenstrual pain, eczema, and other skin conditions [6-8]. Not surprisingly, GLA is widely used in food supplements, and in the pharmaceutical and cosmetic industries. However, the cost of pure GLA is high as the production of the current sources, *Mortirella* [9] and evening primrose [10], is not enough to satisfy market demand. Although *S. platensis* produces GLA at a lower level than *Mortirella* and evening primrose, it has several advantages in terms of large scale commercial cultivation and thus it is of great interest as an alternative source of GLA [11,12].

Much work has been carried out in attempts to increase the GLA-content. It has been found that many environmental conditions such as temperature, growth phase, light and nutrients, affect the level of GLA [12-15] Among these, temperature was found to be the most significant factor. A decrease in temperature from 35°C to 22°C increases the content of GLA by 30~35% [14,15]. Furthermore, Tanticharoen *et al.* [12,16] used pyradazinone SANDOZ 9785, an inhibitor of certain steps in the GLA-biosynthetic pathways. By increasing the concentration of SANDOZ 9785 in the cultivation medium, a GLA-overproducing mutant was noted. The mutant strain,

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Z19/2, was able to produce GLA up to 2.5% of dry cell weight when it was cultivated under outdoor conditions [12,16].

Metabolic flux analysis (MFA) is a powerful technique that provides insights into cell physiology, and often guides ways for the design and modification of further pathways toward the overproduction of a desired product. MFA has been extensively applied on many microorganisms for this purpose [17,21]. In this study, metabolic flux analysis was exploited to obtain flux distributions in the GLA synthetic networks of two different *S. platensis* strains: BP-wild type and the mutant, Z19/2. The flux results for all key pathways between these two strains were compared in detail to determine a key reaction that regulates the synthesis of GLA. By this means, the flux through the reaction that converts acetyl-CoA into malonyl-CoA was proposed as a rate controlling reaction, the modification of which can potentially lead to an improved GLA yield in *S. platensis*.

MATERIALS AND METHODS

Strains and Culture Conditions

The strains used were *S. platensis* strains BP (a wild type) and Z19/2 (a high GLA producing strain). BP was isolated from a stabilization pond of a tapioca starch factory, Ban Pong district, Rachaburi province, Thailand. Z19/2 is a BP mutant obtained by screening BP cells with the pyradazinone, SANDOZ 9785 [12]. The medium used was Zarrouk's medium having bicarbonate as the sole carbon source. This defined medium, whose composition is described elsewhere [22], provides a suitable condition that enables phototrophic growth. The culture was first carried out under sterile conditions in 100 mL of Zarrouk's medium with illumination by a white fluorescent light providing 20 $\mu Em^{-2}s^{-1}$. The temperature was maintained at 35°C. Then, it was transferred to 1,000~1,500 mL of Zarrouk's medium and incubated at 35°C with illumination at 100 $\mu Em^{-2}s^{-1}$ with continuous stirring. During cultivation, 100~200 mL samples were taken for further analysis. For the external metabolite addition, cells were cultured in 1,500 mL Zarrouk's medium for 2 days to attain exponential growth, after which the external metabolites, NADPH (Sigma Co.) and $MgCl_2$ (Merck Co.) were added, and samples were taken every 6 h for further analysis.

Sample Analysis

Biomass concentration was measured as dry cell weight. Each sample was first filtered through a Whatman GF/C fiberglass filter paper (47-mm diameter), dried in an oven at 80°C for 3 h, and weighed. The bicarbonate level was measured by titration with 0.1 N H_2SO_4 [23]. The GLA level was analyzed using gas chromatography (Shimadzu, GC 9A) with a 15% DEGS-column (3 m × 3 mm) at 90°C (FID, injector and flame ionization detector temperature 220°C) and integrated with a Shimadzu CR-3A integrator. The oxygen evolution rate was determined by monitoring the concentration of oxygen generated from light-reacting photosynthesis using a Clark-type oxygen electrode [24].

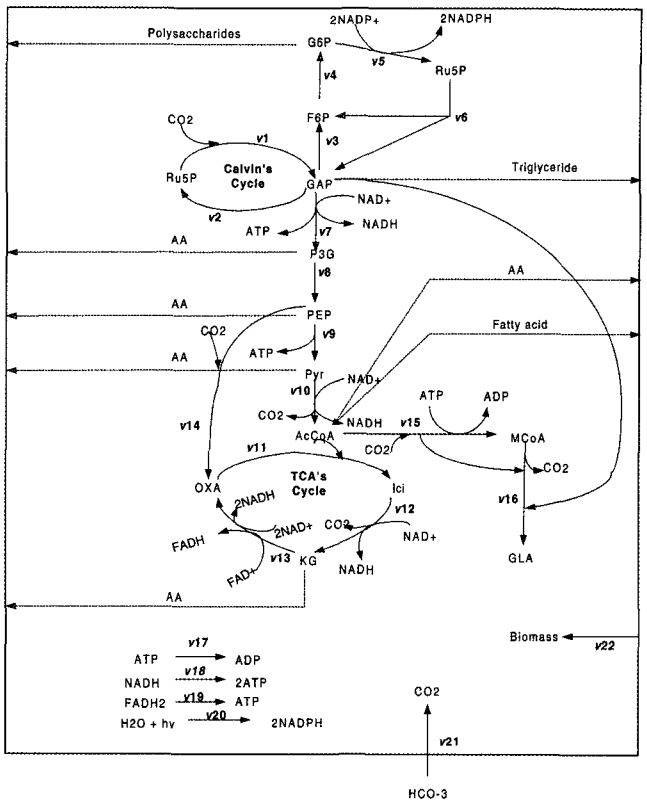

Fig. 1. Metabolic network for *S. platensis* in a medium with bicarbonate as a sole carbon source.

Biochemical Pathways of *S. platensis*

Since available information about the metabolic pathways of *S. platensis* is rather fragmented, we assumed that the pathways that lead to the synthesis of GLA are similar to those in *Synechocystis* sp. PCC 6803 whose full genome sequence is known [25,26]. The pathways of interest are depicted in Fig. 1. Hydrogen bicarbonate (HCO_3^-), the sole carbon source, is imported into the cells, then incorporated into the central metabolism through the Calvin cycle, and eventually converted to glyceraldehyde-3-phosphate (GAP) using energy from light. GAP is a precursor of the synthesis of many macromolecules including carbohydrates, lipids, and proteins through main metabolic pathways *i.e.*, gluconeogenesis, glycolysis (bottom part), the tricarboxylic cycle, and the pentose phosphate pathway. For anabolism, the synthesis of building blocks, *i.e.*, amino acids, fatty acids, and glucose, is lumped together as parts of macromolecules in biomass as performed by Hua *et al.* [27]. The GLA synthesis pathway is deliberately set as a separate pathway from other fatty acid syntheses to enable the comparison of fluxes of different strains through this pathway. The

set of reactions considered in this study is listed in Appendix A. The reaction for biomass synthesis as well as the biomass formula are assumed to be the same as those in the yeast *Torulopsis glabrata* [27]. These assumptions are valid when one only intends to compare fluxes of two different strains [28].

Metabolic Flux Analysis (MFA)

MFA involves the calculation of *in vivo* fluxes from specific substrate consumption rates as well as specific product formation rates by using a system of linear equations formulated from pathway stoichiometry. This flux calculation enables an assessment of pivotal pathways controlling the product of interest and the metabolism as a whole. A comparison of different metabolic flux distributions can show the effect of different genetic or environmental conditions on the pathways, and can indicate strategies for the selection of media, strains, and genetic engineering targets leading to a more efficient formation of desired products [29]. Detailed procedures of MFA have been well described elsewhere [29]. Briefly, the analysis is based on the measurement of extracellular rates, assuming a pseudo-steady state, and a stoichiometric model for the metabolic network under study. The stoichiometric model can be written as:

$$Av = 0$$

where A is a stoichiometric coefficient matrix of the metabolic network, the dimension of which is $m \times n$. v is a flux vector, the dimension of which is $n \times 1$. m is the number of intermediates, and n is the number of metabolic reactions. The degree of freedom (DF) of the system is the difference between n and m (n-m). The system is fully determined if the number of measured extracellular rates equals the DF, in which a unique flux vector solution is obtained. However, the system is over-determined or under-determined if the number of measured rates is higher or lower than the DF, respectively. The flux vector solutions can be obtained by the use of the Moore-Penrose pseudo-inverse approach, and the linear optimization approach, respectively [29].

RESULTS AND DISCUSSION

Flux Distributions of BP and Z19/2 Strains

The stochiometric model developed in this work comprises 17 intermediates and 22 reactions, giving a system with a degree of freedom of 5. Only three fluxes, the bicarbonate consumption rate, the GLA formation rate, and the specific growth rate were measured experimentally. Thus, the system is under-determined because the number of unknown fluxes still exceeds the number of intermediates, leading to a plurality of flux vector solutions. This can be solved by using linear optimization (linear programming by LINDO). Imposed by material balance constraints, the metabolic fluxes in the two *S. platensis* strains, wild type BP and mutant Z19/2, were calculated by having minimal fluxes in the pentose phosphate pathway as the objective function, which has been assured by Yang *et al.* [30], who reported that the flux distribution of the pentose phosphate pathway is very small for the photoautotrophic growth of *Chlorella*. The mutant Z19/2 has been previously found to produce a GLA content about 2-fold higher than the wild type under outdoor conditions [Tanticharoen *et al.*, unpublished data]. It was anticipated that a critical comparison of the flux distributions between the two strains may provide an understanding of how the metabolic reactions interact, or may help to identify the potential rate-controlling reaction for the synthesis of GLA in *S. platensis*. Fig. 2 shows the flux distributions in the GLA synthetic pathways of the wild type and mutant strains. The numerical values are normalized values in the unit mmole/gDCW/h, assuming 100 units of the carbon source (CO_2) enter the pathway through the Calvin cycle. The fluxes of photosynthesis (both dark and light reactions) are relatively close in both strains. So are the fluxes of glycolysis. The fluxes of gluconeogenesis and the pentose phosphate pathways of the mutant strain are approximately twice as much as those of the wild type strain. The reason for this is that the mutant cells prepare precursors for both cell growth and GLA-production. One part of glucose-6-phosephate is further polymerized to polysaccharides, which are major cellular components; the other part enters the pentose phosphate pathways to generate NADPH reducing power that is indispensable for fatty acid synthesis and amino synthesis. There is no flux from ribulose-5-phosphate back to fructose-6-phosphate and glyceraldehyde-3-phosphate in both strains, indicating that ribulose-5-phosphate generated in this pathway is preferably utilized to glyceraldehyde-3-phosphate *via* the Calvin cycle. This is because the model is forced to minimize the pentose phosphate pathways to agree with the results previously reported [30]. Interestingly, the flux at the reaction where acetyl-CoA is converted to malonyl-CoA of the mutant strain is nearly three times higher than that of the wild type strain. This flux difference indicates that this particular reaction plays a crucial role in controlling fatty acid synthesis. In other words, this reaction could be the bottleneck of the GLA-synthesis pathway. If we could rationally design or modify this reaction either at the enzyme or genetic level to stimulate this reaction, we would expect to see more production of GLA. It is noteworthy that more acetyl-CoA was partitioned to malonyl-CoA in the mutant than in the wild-type, giving a slightly reduced flux of acetyl-CoA through the tricarboxylic cycle. This is because cells compensate for the loss by having more flow to the tricaboxylic cycle *via* the anaplerotic pathway.

Model Verification

The stoichiometric model developed in this study was verified by comparing the predicted value of the oxygen evolution rate from the light reaction of photosynthesis ($2v20$) with the experimental values. The evolution of the oxygen in the light dependent reaction was determined

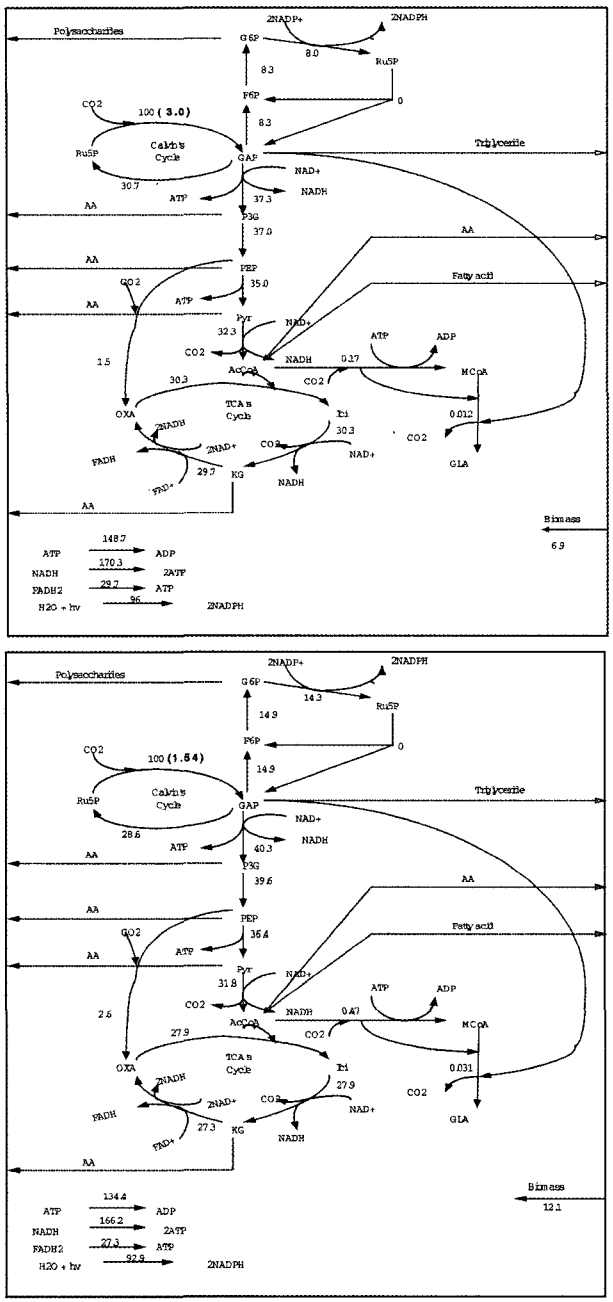

Fig. 2. Metabolic flux distribution of the *S. platens*is Wildtype BP strain (Top) and Mutant Z19 (bottom). The values in parenthesis indicate the un-normalized values of the CO_2 consumption rates in mmole/gDCW/h.

experimentally by measuring the oxygen concentration [24]. This *in vitro* measurement ensures that evoluted oxygen will not participate in other reactions, *e.g.*, oxidative phosphorylation. In addition, oxygen from the atmosphere is excluded. The results from this measurement are in μmole O_2/mg chlorophyll/h, but were further converted to mmol O_2/gDCW/h to enable the comparison with the predicted results. The predicted values for the

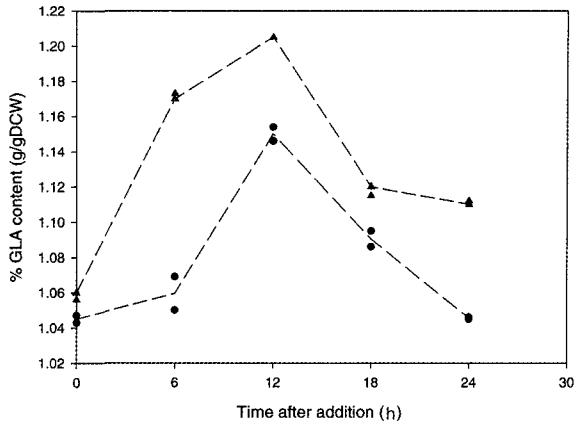

Fig. 3. Effect of NADPH addition on % GLA content of the *S. platens*is wildtype BP strain (the circle symbols) and mutant Z19 (the triangle symbols). The dash lines join averaged values from duplicate experiments.

Table 1. Comparison of oxygen evolution rates in the light reaction of photosynthesis between experimental and predicted values

Strains	O_2 evolution rate (mmole/gDCW/h)	
	Experimental	Predicted by model (2*v*20)
Wildtype BP	5.00	5.76
Mutant Z19	4.38	2.86

BP and Z19/2 strains deviate from the experimental values by 15 and 35%, respectively (Table 1). These errors may be partly due to measurement error, and partly due to the strategy used to calculate the fluxes (linear optimization by minimizing pentose phosphate pathways). However, when comparing the flux values between the two strains, the experimental and the model results do agree in that the oxygen evolution rate of the wild type strain is higher than that of the mutant strain. These results suggest that the model is reasonably valid for a comparison purpose, and the calculated flux distributions over the network can provide some understanding of how to modify the pathway towards an increased GLA-production.

Enhancement of GLA Synthesis

As suggested by the model, the reaction of acetyl-CoA to malonyl-CoA may play a vital role in controlling fatty acid synthesis. This speculation was proven by the direct addition of 1 μM of NADPH, which has been reported as a stimulator for this reaction [31] into the culture medium of the BP strain. Typically, this reaction is driven by the enzyme acetyl-CoA carboxylase having coenzyme A as a cofactor. NADPH was found to stimulate this enzyme since its structure is similar to coenzyme A [31]. It is clearly seen that NADPH addition helps increase the GLA content when compared with the control in which no NADPH was added (Fig. 3). Immediately after this

addition, GLA content sharply increased. This can be explained that external NADPH is somehow imported into the cell, and is used as a stimulator of the reaction, resulting in a higher GLA-synthesis rate. This result corresponds to what is predicted by the model. The time profiles of both cases are similar; the GLA content is highest at 12 h after the pulse, and begins to decline afterward. This decline might be due to the combination of the high growth rate, and the low GLA production rate. This enhancement of the GLA content by the NADPH addition is relatively low when compared with previous reports that used temperature change [14,15] and mutation [12,16]. The reason for the small effect of NADPH on the rate of GLA synthesis may be due to the non-specificity of NADPH. External NADPH, once imported into the cells may be involved in many reactions in the network including the light reaction of photosynthesis, the pentose phosphate pathways, and the synthesis pathways of other fatty acids. Though NADPH can increase the GLA content of a wild type strain in the laboratory, it would not be economical in practice because NADPH is more costly than GLA itself; hence, the effect of $MgCl_2$ on the GLA synthesis rate was studied further. Mg^{2+} has also been reported to activate the activity of acetyl-CoA carboxylase in plants [31]. Various concentrations of $MgCl_2$ were added into the culture medium, and it was found that low concentrations of $MgCl_2$ (0.5 and 1 mM) enhance the synthesis rate of GLA, while high concentrations of $MgCl_2$ (5 and 10 mM) inhibit the synthesis rate of GLA (data not shown). This suggests that the concentration of $MgCl_2$ in the original culture medium (Zarrouk's medium) is not yet optimal for GLA production. Zarrouk's medium contains 0.2 g/L $MgSO_4 \cdot 7H_2O$ as a source of Mg^{2+}, which is equivalent to 0.8 mM. This suggests that the suitable $MgCl_2$ concentration for GLA synthesis is in the range of 1.3~1.8 mM. Unlike NADPH, $MgCl_2$ addition is practical in the field because of its low cost and availability. Further studies should be performed to determine the optimal $MgCl_2$ concentration, and to test the production scale in the field. These two experimental studies clearly demonstrate that the results of flux analysis could provide valuable information in pathway modification. In the current study, the flux analysis showed that to enhance GLA-synthesis in a wild type strain, one needs to increase the flux flow through the acetyl-CoA/malonyl-CoA reaction. We verified this by *in vivo* studies.

Maximum Theoretical Yields of Biomass and GLA

The developed stoichiometric model was used to estimate the maximal yields of both biomass and GLA in *Spirulina* on a CO_2 source by means of linear programming having a biomass synthesis rate ($v22$), and a GLA synthesis rate ($v16$) as objective functions, respectively. In both cases, the carbon source (CO_2) is assumed to enter the network via the Calvin cycle. The flux distributions are shown in Fig. 4. The maximum theoretical yields of biomass and GLA on CO_2 were found to be 67.4 and 3.4 % mole/mole, respectively. It can be clearly seen

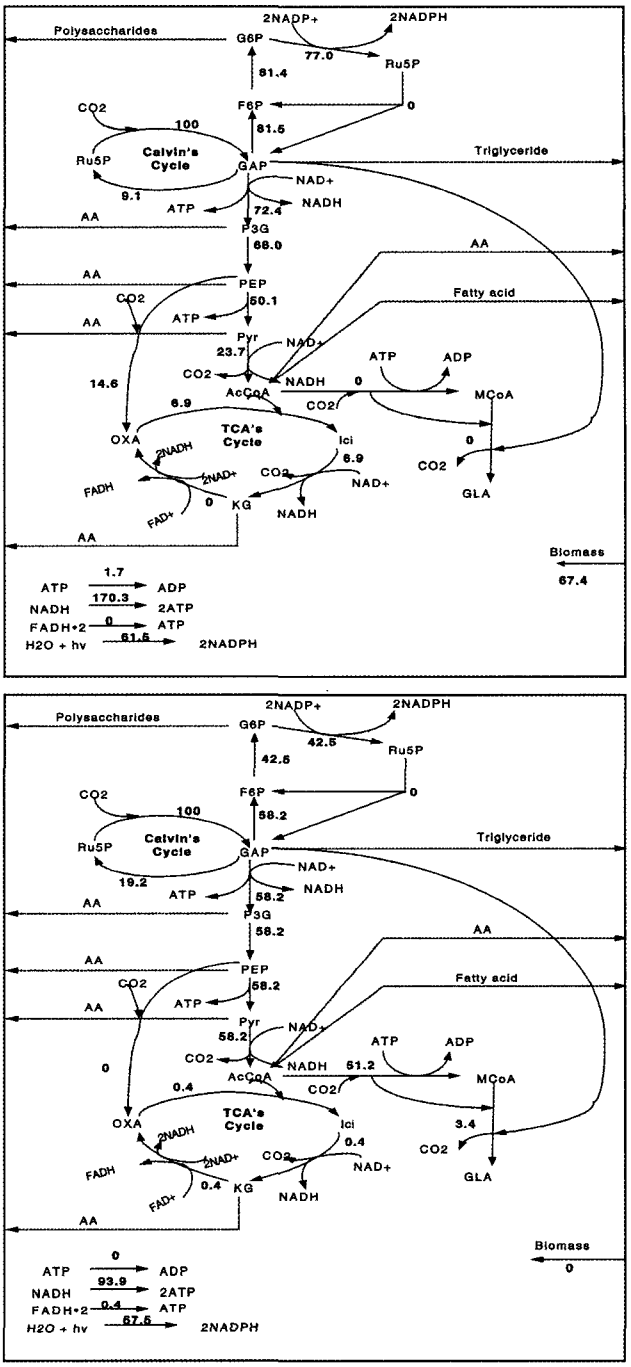

Fig. 4. Optimal flux distribution that maximizes biomass (Top) and GLA (bottom) yields of an organism on glucose

that to obtain a maximal biomass yield, the flux of the GLA synthesis rate is zero, and *vice versa*. The experimental GLA yields in the wild type and mutant strains were 0.012 and 0.031, respectively (Fig. 2). These *in vivo* values are far below the maximum theoretical value. Although it may not be possible to attain in practice, the maximum theoretical value unveils the limit to the GLA yield, and suggests that there is room to improve the GLA

yields. When analyzing the flux distribution, to obtain the maximal biomass yield, cells have very high fluxes in producing NADPH, which is very important in the synthesis of a macromolecule. These fluxes are relatively higher than those in the case of maximizing the GLA yield. It is noted that cell maintenance in both cases is close to zero. This is understandable since cells used up most of the energy for producing macromolecules. It was noticed that the flux in the tricarboxylic cycle in the case of maximizing the GLA-yield is much lower than that in the case of maximizing the biomass yield. This can be explained by the fact that cells drain the majority of acetyl-CoA into the fatty acid synthesis pathways. While the flux through the acetyl-CoA/malonyl-CoA reaction must be zero to reach a maximal biomass yield, as high as 51.2 units of this flux is required to obtain the maximal GLA-yield. Yet it is worth mentioning that zero flux in this reaction only indicates zero GLA production. Cells still need to convert acetyl-CoA to malonyl-CoA for the syntheses of other fatty acids and lipids as part of cell growth. In addition, flux distributions in the glycolysis and anaplerotic pathways are higher in the case of maximizing the biomass. This information gives us an understanding on how the carbon fluxes flow through the network, and guides us on how to modify the pathway towards a specific goal.

CONCLUSION

S. platensis is a rich source of nourishment, *e.g.*, proteins, phycocyanin, β-carotene, polysaccharides, vitamins, and fatty acids. Among fatty acids, GLA is of great importance because of its pharmaceutical properties such as preventing heart diseases. In this study, metabolic flux analysis was applied to determine the metabolic flux distributions in the GLA synthetic pathways of two *S. platensis* strains, wild type BP and the high-GLA producing mutant Z19/2. Flux distributions clearly showed that the flux through the acetyl-CoA/malonyl-CoA reaction in the mutant strain is over two times higher than that in the wild type strain. This finding implies that this reaction may play a key role in controlling the synthesis of GLA.

We found that activating this specific reaction experimentally by the addition of either NADPH or $MgCl_2$, increased the GLA-content in the wild type strain. These results are in agreement with the analysis of metabolic fluxes. This finding revealed a potential strategy in increasing the production of GLA in a large scale *Spirulina* culture; *i.e.*, adding a suitable concentration of Mg^{2+} ions into the culture medium.

The theoretical yields of biomass and GLA were estimated for the *Spirulina* culture on a CO_2 source. This study also suggested that to reach the maximal GLA yield, the flux through the acetyl-CoA/malonyl-CoA reaction has to be at the highest level.

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APPENDIX

Appendix A: Reactions considered in the stoichiometric model

Calvin's cycle (dark reaction)

$Ru5P + CO_2 + H_2O + 3ATP + 2NADPH \rightarrow 2GAP + 3ADP + 2NADP^+$

$5GAP \rightarrow 3Ru5P$

Gluconeogenesis

$GAP + ADP \rightarrow F6P + ATP$

$F6P \rightarrow G6P$

Pentose phosphate pathway

$G6P + 2NADP^+ \rightarrow Ru5P + CO_2 + 2NADPH$

$Ru5P \rightarrow 0.67\ F6P + 0.33\ GAP$

Glycolysis

$GAP + NAD^+ + ADP \rightarrow P3G + NADH + ATP$

$P3G \rightarrow PEP + H_2O$

$PEP \rightarrow Pyr + ATP$

Tricarboxylic cycle

$Pyr + NAD^+ \rightarrow AcCoA + CO_2 + NADH$

$AcCoA + OXA \rightarrow Ici$

$Ici + NAD^+ \rightarrow \alpha KG + CO_2 + NADH$

$\alpha KG + FAD + ADP + 2NAD^+ \rightarrow OXA + CO_2 + FADH_2 + ATP + 2NADH$

Anaplerotic pathway

$PEP + CO_2 + ADP \rightarrow OXA + ATP$

Fatty acid and GLA synthesis

$AcCoA + CO_2 + ATP \rightarrow MCoA + ADP$

$2AcCoA + 15\ McoA + 30\ NADPH + GAP + 7NADH + ADP \rightarrow GLA + 15CO_2 + 12H2O + 30NADP^+ + ATP + 7NAD^+$

Energetic reactions

$ATP \rightarrow ADP$

$NADH \rightarrow 2ATP$

$FADH_2 \rightarrow ATP$

Light reaction

$H_2O + h\nu \rightarrow 2NADPH + 1/2O_2$

Carbonate transfer and dissociation

$HCO_3^- \rightarrow CO_2$

Biomass synthesis

$0.067G6P + 0.064Ru5P + 0.009GAP + 0.065P3G + 0.05PEP + 0.176Pyr + 0.095OXA + 0.102\alpha KG + 0.249AcCoA + 1.142\ NADPH^+ + 0.157\ NAD + 3.82ATP \rightarrow C_{3.93}H_{7.07}O_{1.96}N_{0.79} + 1.142\ NADP + 0.157\ NADH + 3.82\ ADP + 0.127\ CO_2$

Appendix B: Metabolites for material balances

Ru5P ribulose-5-phosphate

CO_2	carbon dioxide
ATP	adenosine 5'-triphosphate
NADPH	nicotinamide adenine dinuleotide phosphate
GAP	glyceraldehyde-3-phosephate
F6P	fructose-6-phosephate
G6P	glucose-6-phosephate
P3G	3-phosphoglycerate
NADH	nicotinamide adenine dinucleotide
PEP	phosphoenolpyruvate
Pyr	pyruvate
AcCoA	acetyl coenzymeA
OXA	oxaloacetate
Ici	isocitrate
αKG	alpha ketoglutarate
$FADH_2$	flavin adenine dinucleotide
McoA	malonyl coenzyme A
GLA	gamma-linolenic acid
HCO_3^-	bicarbornate
Biomass	

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