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# Increasing Nutritional Value of Cyanobacteria by Engineering Valine, Phenylalanine, and Fatty Acid Production

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**ABSTRACT:** In 2020, the United Nations estimated that 2.37 billion people globally were without food or unable to eat a healthy balanced diet. The number of people with insufficient nutrition has increased in the short term due to COVID-19 pandemic and longer-term climate change is leading to shifts in arable land and water availability leading to a continued need to develop scalable sources of nutrition. One of the options that can yield high food mass per square foot of land use is the high-density culture of microalgae or other photosynthetic microorganisms. While photosynthetic microorganisms may provide high amounts of biomass with a small land footprint, the nutritional value of unmodified microorganisms may be limited. This mini-review presents the base nutritional value in terms of macro- and micronutrients of several cyanobacteria (*Nostoc*, *Anabaena*, *Spirulina*) in relation to established human nutritional requirements as a starting point for better utilization of cyanobacteria as nutritional supplements. It also discusses synthetic biology approaches that have been implemented in different organisms to increase the production of L-valine, L-phenylalanine, and fatty acids demonstrating some common genetic engineering design approaches and some approaches that are organism-specific.

**Keywords:** Nutrition; L-valine; L-phenylalanine; Fatty acid; Photosynthetic microbe



## 1. Introduction

Globally, food insecurity affects the health and well-being of an increasingly large number of people. In 2020, the United Nations estimated that 2.37 billion people globally were without food or unable to eat a healthy balanced diet [1], and the number of undernourished individuals is increasing despite efforts to address food insecurity (607 million in 2014 growing to 720–811 million in 2020) [1]. In addressing food insecurity there is a need to re-evaluate existing approaches and to develop new sustainable food systems incorporating diverse social, economic, political, and technological aspects.

When considering food production systems, there has been growth in developing nutritional food alternatives (including synthetic and *in vitro*) that can be used to replace or supplement traditional food sources to minimize land usage, reduce greenhouse gas emissions, increase shelf stability, and reduce cost. One research focus with high potential to address a number of sustainability and food nutrition issues is the use of cyanobacteria as food supplements. In theory, cyanobacteria can be grown to high density with small space requirements with low operational costs (sequestering carbon dioxide and sunlight as inputs) while producing edible biomass that can meet a range of nutrient requirements. In practice, a closer look at productivity and nutritional content is required if cyanobacteria are to become a viable food supplement. Here, we consider the baseline nutritional requirements for humans, compare human nutritional requirements to native nutrient content in several cyanobacteria (*Nostoc*, *Anabaena*, *Spirulina*), and suggest some targeted genetic engineering approaches that could increase macro- and micro-nutrient levels to improve the food nutrition content in cyanobacteria.

## 2. Cyanobacteria Exemplars

A wide spectrum of photosynthetic microorganisms and cyanobacteria are found worldwide. Instead of attempting to compile an exhaustive list of different species, we chose *Nostoc*, *Anabaena*, and *Spirulina* as examples for comparing the nutritional content of these organisms against human daily nutritional requirements with *Nostoc* and *Anabena* being common research species and

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*Spirulina* already having high interest and use in the food industry. The commercial potential for these organisms is high if we consider for reference that 32.67 Mt of algae biomass was produced in 2016 [2].

*Spirulina* (also commonly known as *Athrospira*) is a commonly-occurring microorganism, found in many different areas from the tropics to hot springs in the North Sea and it is a bluish-green nutrient-dense cyanobacterium. The prevalence of this organism has led Spirulina to become a well-studied organism [3]. Currently, *Spirulina* is being produced in tropical countries such as Thailand and India and is being used as feed for animals and in niche circumstances used as a nutritional supplement [4]. *Anabaena* is a filamentous cyanobacterium that forms heterocysts. Due to these heterocysts, *Anabaena* excels in nitrogen and $CO_2$ fixation as a means of producing its micronutrients and amino acids [5]. This makes it an ideal food and nutrient source that can help reduce carbon footprint. *Nostoc* is closely related to the *Anabaena* genus. *Nostoc* is a cyanobacterium commonly found at the bottom of lakes and bases of trees [6]. Like *Anabaena*, *Nostoc* also excels at nitrogen and $CO_2$ fixation and is filamentous in shape. *Nostoc* is rich in essential proteins and is consumed as a delicacy in certain eastern Asian countries.

As a starting point for considering the nutritional value of *Spirulina*, *Nostoc*, and *Anabaena*, the essential amino acid and macronutrient composition of each are shown in Table 1 as a percentage of the daily recommended requirement (daily value, DV). Percentages are based off a 2000 Kcal diet and the mass of each species was adjusted to provide 100% of the daily threonine to ease comparisons between species. Threonine was chosen as the basis for normalizing all values as all three organisms contain high levels of threonine. In terms of macronutrients, *Nostoc*, *Anabaena*, and *Spirulina* have higher relative protein content and lower content of carbohydrates and fat. Mineral content varies greatly for each organism. In terms of essential amino acids, L-valine and L-histidine content were often not detected or not reported and L-phenylalanine content was low. The dense protein content of these cyanobacteria makes them ideal for supplementation use.

**Table 1.** Percent Essential Amino Acid and Macronutrient Analysis *Nostoc, Anabaena, Spirulina*.

| **Amino Acid and Macronutrient** | **Percent DV (*Nostoc*)** | **Percent DV (*Anabaena*)** | **Percent DV (*Spirulina*)** |
|---|---|---|---|
| Lysine | 57.37 [7,8] | 58.37 [8,9] | 56.17 [8,9] |
| Leucine | 86.44 [7,8] | 78.83 [7,8] | 46.47 [8,9] |
| Isoleucine | 81.27 [7,8] | 74.05 [7,8] | 88.15 [8,9] |
| Threonine | 100.00 [7,8] | 100.00 [7,8] | 100.00 [8,9] |
| Valine | ND [7,8] | ND [7,8] | 129.63 [8,9] |
| Histidine | ND [7,8] | ND [7,8] | ND [8,9] |
| Phenylalanine | 41.73 [7,8] | 38.44 [7,8] | 62.96 [8,9] |
| Iron | 182.43 [7,10] | 68.02 [7,10] | 188.54 [9,10] |
| Potassium | 6.09 [7,10] | 13.73 [7,37] | 6.80 [9,10] |
| Sodium | 54.86 [7,10] | 4.30 [7,10] | 2.17 [9,10] |
| Zinc | 179.11 [7,10] | 164.17 [7,10] | 12.70 [9,10] |
| Carbohydrates | 7.44 [7,10] | 5.38 [7,10] | 1.38 [9,10] |
| Fats | 0.63 [7,10] | 0.41 [7,10] | 3.07 [9,10] |
| Protein | 29.30 [7,10] | 22.62 [7,10] | 24.35 [9,10] |
| Calories (Kcal) | 8.05 [7,10] | 5.65 [7,10] | 4.03 [9,10] |
| Total Grams | 46.91 [7,10] | 30.61 [7,10] | 19.96 [9,10] |

Values expressed as a percent of daily value (DV). Based on a standard of filling most abundant Essential Amino Acid. ND: Not Detected.

## 3. Potential Engineering Targets

The essential amino acids L-valine and L-phenylalanine along with fat content are disproportionately low in the native macro- and micro-nutrient content of *Nostoc, Anabaena*, and *Spirulina* and will be the focus of this review. Mechanisms for increasing the synthesis of these nutrients have been studied for varying purposes in other organisms and can potentially be utilized to increase the nutrient content in these photosynthetic microorganisms. An overview of the metabolic pathways and branchpoints for the production of L-valine, L-phenylalanine, and fatty acids are shown in Figure 1 with key genes targeted by metabolic engineering highlighted.

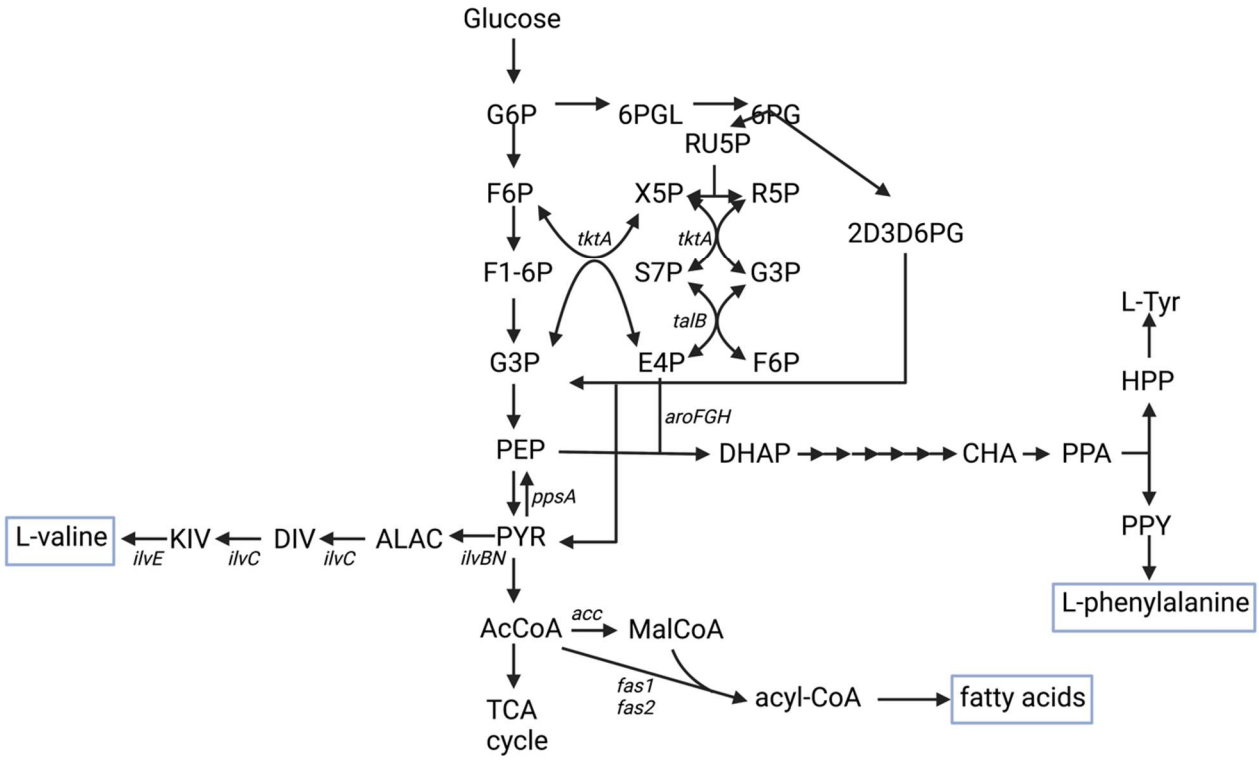


**Figure 1.** Schematic of central metabolism showing key chemical compounds (abbreviated in all capital letters) and gene targets (lowercase italics) for the production of L-valine, L-phenylalanine, and fatty acids. Depicted engineering targets correspond to references [11–16] discussed in the text.

### *3.1. Valine Synthesis*

L-valine is noted as one of the limiting essential amino acids in livestock feed and is naturally present in low quantities in photosynthetic microorganisms. Metabolic engineering approaches have sought to increase the production of L-valine with much of the work occurring in *Corynebacterium glutamicum* and *Escherichia coli*. Typically, the approaches to increasing L-valine production have included combinations of gene deletions to remove competing pathways, overexpression to increase activity through key pathways, increasing precursor and co-factor pools, and manipulating regulatory interactions.

Early efforts to over-produce L-valine in *E. coli* demonstrated the approach of increasing the pyruvate pool, overexpressing genes of the L-valine synthesis pathway, and targeting global regulation. This approach was achieved by deleting *ilvA* to increase the pyruvate pool, overexpressing *ilvBN* and *ilvCED* to increase L-valine production, and overexpression of the global regulator *lrp*. When grown in fed-batch cultures, the engineering strain of *E. coli* was able to produce 60.7 g/L L-valine [11]. Building upon this blueprint for increasing L-valine production, additional work has been done to reduce feedback inhibition of bottleneck synthesis enzymes (acetohydroxy acid synthase) by heterologous expression of homologues while adding a new design component of balancing co-factor regeneration of NADH for use in L-valine synthesis. This combined approach was able to produce up to 84 g/L L-valine [17].

An example approach implemented in *C. glutamicum* created an engineered strain with three gene deletions (*aceE, alaT, ilvA*) to remove competing pathways that would drain the precursor pyruvate (*aceE* and *alaT*) or lead to the production of isoleucine via L-threonine (*ilvA)*, overexpression of genes for production of L-valine (*ilvB, ilvN, ilvC*), and overexpression of the global regulator *lrp* produced up to 51 g/L L-valine in fed-batch cultures [12]. The importance of maintaining or increasing the pyruvate precursor pool has been a particular focus and high L-valine producing strain of *C. glutamicum* was used to analyze additional mechanisms that could be used to maintain the pyruvate pool. Through metabolomic analysis, it was found that a high pyruvate pool was maintained by reducing intracellular levels of methionine, aspartic acid, isoleucine, and glycine along with lower levels of alanine and TCA cycle intermediates [18]. Targeting these pathways might provide additional avenues for maintaining pyruvate levels and could be coupled with the overexpression of genes along the L-valine catabolic pathway (*ilvB, ilvN, ilvC*).

### *3.2. Phenylalanine Synthesis*

L-phenylalanine has been a target for biological production for its variety of uses in the food industry (human and livestock) and as a precursor for a number of high-value chemical products, including pharmaceuticals. As with L-valine production, much of the research has been done in model organisms, especially *E. coli*.

When considering L-phenylalanine synthesis, the first pass approaches typically consider: (1) increasing the precursor pools of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), (2) removing regulatory inhibition to production (*tyrR* and *trpR*), and (3) overexpressing rat-limiting genes (*aroFGH*). Due to L-phenylalanine synthesis requiring precursors from two areas of central metabolism (pentose phosphate and glycolysis), approaches to increasing the precursor pool need to be multi-faceted [19]. E4P availability is typically addressed by increasing the expression of transketolase (*tktA*) and transaldolase (*talB*) necessary for E4P synthesis. Additionally, carbon flux can be diverted into the pentose phosphate pathway by restricting glycolysis (e.g., disrupting phosphoglucose isomerase, *pgi*), but this also would affect the production of PEP.

Intracellular PEP pools are typically manipulated by: (1) inhibiting/removing downstream drains, (2) removing alternative consumption of PEP in the PTS transport system, and (3) addressing the regulation of the PEP pool. Inactivation of pyruvate kinase (*pykF*) or phosphoenolpyruvate carboxylase (*ppc*) has been used to limit the conversion of PEP to pyruvate or oxaloacetate, respectively. While this approach could increase PEP availability, it will also restrict intracellular resources by affecting the TCA cycle, so another approach that has been utilized is to overexpress phosphoenolpyruvate synthetase (*ppsA*) to increase the regeneration of PEP from pyruvate. During glucose uptake in *E. coli*, the PTS transport system utilizes approximately 50% of the intracellular PEP [13] so while the approach of targeting the PTS system is important in *E. coli*, it will not be important to address for a photosynthetic organism. Finally, the intracellular pool of PEP in *E. coli* is regulated by the CsrA regulatory protein. Increases in the intracellular PEP pool have been achieved either by deleting *csrA* [20] or increasing the expression of *csrB* which has a negative regulatory interaction with *csrA* [21].

With the variety of uses of L-phenylalanine, some work has begun to engineer photosynthetic microorganisms for L-phenylalanine overproduction. One notable study used the heterologous expression of three *E. coli* genes in *Synechocystis* PCC6803 to increase the production of L-phenylalanine (and L-tyrosine). The *E. coli* phosphoenolpyruvate synthetase gene (*ppsA)* was used to increase PEP production from pyruvate and the *aroG* and *tyrA* genes were used to increase flux through the amino acid synthesis pathways resulting in an L-phenylalanine titer of 580 mg/L [14].

### *3.3. Fatty Acid Synthesis*

While much of the synthetic design and engineering for the production of L-valine and L-phenylalanine have been done in *E. coli* and *C. glutamicum*, fatty acid/lipid overproduction has largely progressed in yeast and algae.

Much of the early metabolic engineering for fatty acid production was started in *Saccharomyces cerevisiae* largely due to the established knowledge base and availability of genetic engineering tools. One of the advantages of using *S. cerevisiae* is that the conversion of malonyl-CoA and acetyl-CoA to long-chain fatty acids (C16/C18) involves the fatty acid synthase complex that is composed of only two genes (*fas1*,*fas2*) as compared to other organisms that may have a larger number of genes (10 for *Escherichia coli*) making overexpression of the entire complex easier [16]. By using the approach of overexpressing the key catabolic genes (*acc*, *fas1*, *fas2*) a four-fold increase in fatty acid content was achieved in *S. cerevisiae* [15].

Given the recursive nature of the synthesis of fatty acids, one study that applied a variety of synthetic biology techniques to engineering very long-chain fatty acids in *S. cerevisiae* utilized a combination of heterologous expression and dynamic control to selectively produce the fatty alcohol docosanol ($C_{22}H_{46}O$) [22]. While the native *S. cerevisiae* fatty acid synthase complex produces C16/C18 fatty acids, very long chain fatty acids (C22/C24) were produced by using the compatible FasI system from *Mycobacterium vaccae*. The *M. vaccae* gene, *mvfas*, was successfully expressed as a fusion protein with an acyl carrier protein synthase (*acps*) in a fatty acid auxotroph of *S. cerevisiae*. Additionally, the terminal enzyme for production was selected to be the acyl-CoA reductase from *Arabidopsis thaliana* and dynamic control of expression was achieved to segregate biomass production from fatty acid synthesis by testing different carbon-source responsive promoters (high glucose vs. high ethanol). Using these combined approaches allowed the selective production of C22 fatty acids at an amount of 83.5 mg/L [22].

In terms of platform organisms for producing fatty acids, oleaginous yeasts may be one of the most logical choices for development due to their natural high acetyl-CoA flux and ability to sequester fatty acids/oils. In particular, *Yarrowia lipolytica,* has been a targeted oleaginous yeast for the production of oleochemicals. One of the attributes of *Y. lipolytica* that make it attractive as a host for lipid synthesis is that it effectively sequesters oils in lipid inclusion bodies and achieves lipid content up to 40% of its cell dry weight [23]. Some of the foundational work on *Y. lipolytica* began with using a multiplex, combinatorial approach to screen different engineered genotypes finding that a *pex10*, *mfe1*, leucine+, uracil+, DGA1p overexpression genotype produced lipid titer of 6.00 g/L with lipid content of 74% of cell dry weight [24]. A follow-up study focused on improving understanding of the native fatty acid synthesis pathways and rewiring the pathways to decouple fatty acid synthesis from metabolic constraints (i.e. lipid synthesis during nitrogen starvation) achieving a lipid titer of 66.4 g/L [25].

Further development of *Yarrowia lipolytica* for fatty acid synthesis has been enabled through the development of a variety of synthetic biology tools (promoter characterization, CRISPR/Cas9, efficient transformation, marker recycling) [23,26]. Improvements in lipid content have been achieved by utilizing heterologous expression of key genes such as overexpression of *dga1* from *Rhodosporidium toruloides* and *dga2* from *Claviceps purpurea* that produced a strain with 77% lipid content [27]. Other research has been conducted to increase the acetyl-CoA pool by developing alternative mechanisms for acetyl-CoA generation

including expression of the peroxisomal carnitine acetyltransferase (*perCAT2*) from *Saccharomyces cerevisiae* (produced 66.4 g/L lipids) [25] and heterologous expression of phosphoketolase (*xpkA*) and acetate kinase (*ack*) for conversion of xylose to acetate with the acetate being converted to acetyl-CoA by acetyl-CoA synthetase (*acs*), resulting in 67% lipid content [28]. In addition to increasing the acetyl-CoA pool, it was found that NADPH would limit the activity of the fatty acid synthase complex resulting in multiple approaches to convert glycolytic NADH to cytosolic NADPH for lipid synthesis with the best results producing a strain with 99 g/L lipid titer at 67% lipid content [29].

In addition to research using *S. cerevisiae* and *Y. lipolytica*, interest in using microalgae for fatty acid synthesis was spurred in part by the prospect of a sustainable process for biodiesel production. In microalgae, two main mechanisms exist for fatty acid synthesis, fatty acid synthase (FAS) and triacylglycerol (TAG) synthesis. The fatty acid synthase pathway for lipid synthesis is similar to the synthesis pathways in yeast described above with an acetyl-CoA carboxylase (*acc*) gene responsible for the conversion of acetyl CoA to malonyl CoA and downstream elongation to C16/C18 fatty acids. Interestingly, in microalgae, overexpression of *acc* did not increase fatty acid production and was not a limiting step [16]. Instead, the downstream step of transferring malonyl CoA to the acyl carrier protein (ACP) to form malonyl CoA-ACP by the malonyl CoA-ACP transacylase was found to increase lipid production in both *Nannochloropsis* [30] and *Schizochytrium* [31].

Work in microalgae on the triacylglyceride synthesis pathway has made steady progress in improving lipid yields. Systematically, it has been demonstrated that overexpression of genes along the pathway to convert acyl CoA/acyl ACP to triacylglycerides has improved lipid production. Overexpression of glycerol-*sn*-3-phosphate acyl-transferase (GPAT) for conversion of glycerol-3-phosphate to lysophosphatidate improved lipid production in *Chlamydomonas reinhardtii* (50% TAG content per cell dry weight) [32] and *Phaeodactylum triornuum* (43% TAGE content per cell dry weight) [33]. Overexpression of lysophosphatidate acyl-transferas (LPAAT) showed 20% increase lipid content in *Chlamydomonas reinhardtii* [34]. The final step in triacylglyceride synthesis involves diacylglycerol acyltransferase (DGAT) and is thought to be the rate-limiting step. Overexpression of DGAT has been demonstrated to increase TAG content in various microalgae including *Phaeodactylum tricornutum* [35], *Nannochloropsis salina* [36], and *Nannochloropsis oceanica* [37].

## 4. Conclusions and Outlook

Globally there is a growing need to develop additional food sources with high nutritional value. When coupled with climate/environmental concerns, high-density culture of photosynthetic microorganisms may become viable sources of nutritional supplementation. However, it is necessary to consider the amount and distribution of macro- and micro-nutrients that could be provided by photosynthetic microorganisms. In considering *Nostoc*, *Anabaena*, and *Spirulina*, it can be seen that each organism has varied nutritional value (e.g., mineral, carbohydrate, fat content). By cataloging the baseline nutritional content of each species, specific components can be targeted for increased production through targeted genetic engineering approaches; for the species considered here, we focused on L-valine, L-phenylalanine, and fat content.

In considering approaches that have been utilized for increasing the production of L-valine, L-phenylalanine, and fat/fatty acids, there were some similar approaches that were utilized across metabolic engineering work across multiple species. Approaches typically include: overexpressing upstream genes to increase intracellular precursor pools, removing alternative sinks/drains, overexpressing genes for rate-limiting synthesis steps, altering regulatory mechanisms, and regenerating cofactors. More elaborate approaches have rewired all the pathways within a subsystem, and developed novel pathways/cycles for precursor/co-factor generation, and dynamic control strategies. By compiling research associated with increasing production of L-valine, L-phenylalanine, and fatty acid synthesis from multiple organisms it is possible to discern common, successful design strategies that could be implemented in photosynthetic microorganisms, but one caveat is that species/strain specific physiological/metabolic differences could influence the effectiveness of applying a design strategy from one organism to another. A prime example of this is that overexpression of *acc* to produce malonyl CoA in yeasts leads to increases in fatty acid production, but a similar approach was ineffective in *Chlamydomonas reinhardtii*. Overall, we hope that metabolic engineering and synthetic biology approaches will continue to be leveraged to develop new, nutritional food sources to address the growing global food insecurity issues.

## Supplementary Material

The following supporting information can be found at: https://www.sciepublish.com/index/journals/article/sbe/25.html/id/18.

## Author Contributions

NLR and SSF both contributed to the conception of this manuscript, compiling references, and writing of the manuscript.

## Ethics Statement

Not applicable.

## Informed Consent Statement

Not applicable.

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## Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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