

Article

Nitrogen Availability Influences Biomass Composition in *Yarrowia lipolytica* Grown on Acetate

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Abstract

Microbial protein production from acetate represents a promising route for sustainable protein supply, yet its efficiency is constrained by limited understanding of carbon–nitrogen metabolic coordination. In this study, nitrogen availability was systematically varied to investigate its role in regulating biomass composition and protein biosynthesis in *Yarrowia lipolytica*. Nitrogen limitation markedly reduced cell growth and protein accumulation (19.56% of dry cell weight) while increasing lipid content (up to 34.16%), indicating a altered protein and lipid accumulation under different nitrogen conditions. Transcriptomic analysis revealed a global downregulation of anabolic pathways under nitrogen limitation, accompanied by a shift in nitrogen assimilation from the glutamate dehydrogenase (GDH) pathway to the glutamine synthetase/glutamate synthase (GS–GOGAT) pathway, as well as significant upregulation of genes related to ammonium and amino acid transport. Guided by these findings, metabolic engineering of key nitrogen assimilation pathways was performed. Strains harboring additional copies of *GDH* and *GS* expression cassettes showed increased protein content from 48.52% to 55.77% and improved amino acid composition, whereas strains with an additional copy of the *GOGAT* gene exhibited reduced growth and protein accumulation. These results demonstrate that nitrogen availability regulates biomass composition through coordinated control of nitrogen transport and assimilation, and that balanced upregulation of *GDH* and *GS* genes is an effective strategy to improve microbial protein production from acetate, supporting the development of efficient fermentation processes using low-cost carbon sources.

Keywords: *Yarrowia lipolytica*; acetate utilization; single cell protein; nitrogen assimilation



Academic Editor: Ronnie G. Willaert

Received: 21 April 2026

Revised: 27 May 2026

Accepted: 2 June 2026

Published: 30 June 2026

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1. Introduction

The increasing global demand for sustainable protein sources has stimulated growing interest in microbial protein production as an alternative to conventional agriculture [1].

Compared with plant- and animal-derived proteins, microbial protein offers advantages such as rapid growth, high productivity, and reduced dependence on arable land and climate [2]. Among various carbon substrates, acetate has emerged as a promising feedstock due to its availability from CO₂-derived processes and industrial waste streams, enabling environmentally friendly and carbon-efficient biomanufacturing [3,4]. However, compared with sugar-based substrates, acetate metabolism provides limited energy and reducing power, which constrains cell growth and biosynthesis. Under such conditions, cellular metabolism becomes highly sensitive to nutrient availability, particularly nitrogen [5,6]. Therefore, a deeper understanding of how nitrogen effects biomass composition is essential to enable precise regulation of carbon flux distribution between protein and other biomass components under acetate-based cultivation.

The oleaginous yeast *Yarrowia lipolytica* represents an attractive microbial chassis owing to its Generally Recognized As Safe (GRAS) status, metabolic versatility, and capacity to efficiently utilize acetate [7,8]. To date, most studies on *Y. lipolytica* have focused on lipid production, since nitrogen limitation typically promotes storage lipid accumulation [9,10]. In contrast, its potential for microbial protein production remains underexplored. From a metabolic perspective, nitrogen availability regulates biomass composition and metabolic network activity [11,12]. Microorganisms assimilate ammonium primarily via the glutamate dehydrogenase (GDH) pathway or the glutamine synthetase/glutamate synthase (GS-GOGAT) pathway, which differ in energy requirements and substrate affinity [13,14]. In oleaginous yeasts, nitrogen limitation is known to induce lipid accumulation while repressing protein synthesis, whereas sufficient nitrogen availability promotes protein biosynthesis [15]. However, under acetate-based cultivation—where metabolic resources are inherently constrained—how nitrogen availability affects protein and lipid accumulation, and how distinct nitrogen assimilation pathways are coordinately regulated, remain poorly understood. This knowledge gap hinders the rational design of strategies to enhance protein production in *Y. lipolytica*.

In this study, we systematically investigated the effects of nitrogen availability on protein accumulation in *Y. lipolytica* cultivated on acetate. By constructing a series of nitrogen concentration gradients, we first evaluated changes in biomass formation, protein content, lipid content, and substrate utilization efficiency. Transcriptomic analysis was subsequently performed to examine global gene expression changes and to identify pathways potentially involved in nitrogen assimilation and amino acid biosynthesis. Particular attention was paid to the differential expression of genes associated with the GDH pathway and the GS-GOGAT pathway under nitrogen-limited and nitrogen-replete conditions. Furthermore, metabolic engineering strategies were applied to alter the expression of nitrogen assimilation-related genes and to assess their effects on intracellular protein accumulation and amino acid composition. This work improves the understanding of nitrogen-regulated biomass composition and protein biosynthesis in *Y. lipolytica* and provides a potential strategy for enhancing microbial protein production from acetate.

2. Materials and Methods

2.1. Genetic Manipulation for the Construction of Plasmids and Strains

All strains used here are listed in Supplementary Table S1. All vectors described in this paper were constructed via Gibson assembly and are listed in Supplementary Table S2. All the fragments used for overexpression of genes were amplified by PCR using primers as described in Supplementary Table S3. Nucleotide sequences of functional genes used to construct engineering strains are listed in Supplementary Table S4. Cloning was performed with chemically competent *Escherichia coli* strain DMT or DH5 α .

In this study, the wild-type strain W29, which carries Cas9 and nourseothricin resistance genes integrated at the KU70 locus and is maintained in our laboratory stock, was used for preliminary fermentation under different C/N ratios and for transcriptomic analysis. The engineered strain HR9, featuring knockout of the lipid biosynthesis pathway and enhanced acetate utilization, was employed for functional validation of GDH, GS, and GOGAT. The chemically competent cells DMT and DH5 α were purchased from TransGen Biotech (Beijing, China). Genome integrations were carried out using a CRISPR/Cas9-based strategy that employed a single gRNA plasmid and an integrative donor plasmid. Verification of integration results was performed by Sanger sequencing. All genes used in this study are endogenous to *Yarrowia lipolytica* strain W29: *GDH* (YALI1_F23664g), *GS1* (YALI1_F00821g), *GS2* (YALI1_D16151g), and *GOGAT* (YALI1_B26112g).

2.2. Media and Growth Conditions

YPD medium was used for strain activation and routine cultivation. It contained (per liter): 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone and 20 g L⁻¹ glucose as the sole carbon source. For solid medium preparation, 15 g L⁻¹ agar was supplemented. If needed, nourseothricin was added after autoclaving to achieve a final concentration of 0.25 g L⁻¹. Acetate-based minimal medium (MM) was formulated as follows. Each liter of deionized water contained 7.5 g (NH₄)₂SO₄ (unless otherwise specified for nitrogen gradient experiments), 14.4 g KH₂PO₄, 0.5 g MgSO₄·7H₂O and 16.3 g sodium acetate, together with 1 mL vitamin solution and 2 mL trace metal solution [16]. The initial pH of the medium was adjusted to 6.0 $\pm$ 0.1 using sodium hydroxide prior to autoclaving. In nitrogen gradient cultivation trials, molar carbon-to-nitrogen (C/N) ratios were calculated according to total carbon supplied by 200 mM sodium acetate and total nitrogen derived from ammonium sulfate. Four concentrations of (NH₄)₂SO₄ were established: 0.1, 1.0, 7.5 and 15 g L⁻¹, yielding corresponding C/N ratios of 263:1, 26.4:1, 3.5:1 and 1.7:1, respectively. All other medium ingredients remained unchanged throughout the experiment. All chemical reagents used in this study were purchased from Solarbio (Beijing, China). Kanamycin and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Shake Flask Culture and Determination of Cell Content

Y. lipolytica cells pre-cultured in activation medium (YPD) were inoculated into 250 mL shake flasks containing 50 mL MM supplemented with 200 mM sodium acetate and 7.5 g/L (NH₄)₂SO₄, and cultivated at 30 °C and 220 rpm until OD₆₀₀ reached 6–8 to prepare a common seed culture. For each nitrogen condition, an appropriate volume of this seed culture was transferred into fresh 250 mL flasks containing 50 mL MM with 200 mM sodium acetate and the designated (NH₄)₂SO₄ concentration (0.1, 1.0, 7.5, or 15 g/L) to achieve an initial OD₆₀₀ of 0.05. After 39 h of shake flask cultivation, cells were harvested by centrifugation at 2000 $\times$ g for 30 min, washed three times with deionized water, and lyophilized to constant weight using a freeze-dryer for further analysis. Three biological replicates were performed per condition.

Crude lipid content determination [17,18]: The lyophilized cell biomass was ground in a mortar for 15 min, and the weight of the cell powder was recorded (m_1). The powder was resuspended in 3 mL of 4 mol L⁻¹ hydrochloric acid in a glass tube, followed by gentle shaking for 1.5 h. The mixture was then boiled in a water bath for 8 min and frozen at -20 °C for 30 min. Subsequently, 6 mL of chloroform: methanol (1:1, *v/v*) was added, and the mixture was centrifuged at 2000 $\times$ g for 10 min. The supernatant was transferred to a clean test tube, mixed with 3 mL of 0.15% NaCl solution, and centrifuged again. Finally, the supernatant was transferred to a pre-weighed clean test tube and evaporated to dryness

using a nitrogen blow-down concentrator. The mass of crude lipid was determined using an analytical balance (m_2). The crude lipid content was calculated as:

$$\text{Crude lipid content (\%)} = m_2/m_1 \times 100\%$$

Crude protein content determination: The crude protein content was quantified by elemental analysis using a FlashSmart CHNS/O analyzer (Thermo Fisher Scientific, Milan, Italy). Approximately 3.0–4.0 mg of freeze-dried biomass was combusted in pure oxygen at 950 °C. The resulting total nitrogen was detected by a thermal conductivity detector and subsequently converted to crude protein using a nitrogen-to-protein conversion factor of 6.25.

$$\text{Protein (\%)} = \text{Nitrogen (\%)} \times 6.25$$

Amino acid content analysis: Accurately weighed solid samples (20–50 mg, with a protein content of at least 10 mg g^{−1}) were placed into hydrolysis tubes, and 10 mL of 6 M analytical-grade HCl was added. The tubes were purged with nitrogen for 30 s, sealed, and hydrolyzed at 110 °C for 22–24 h in an oil bath. After cooling to room temperature, the hydrolysates were filtered through a 0.45 µm membrane and diluted to 50 mL with deionized water in a volumetric flask. A 2 mL aliquot of the filtrate was transferred to a 50 mM evaporation vial and evaporated to near dryness twice at 85 °C to remove residual hydrochloric acid. The residue was then dissolved in 1 mL of sodium citrate buffer (pH 2.2), filtered through a 0.22 µm membrane, and analyzed for amino acid composition. Under these conventional acid hydrolysis conditions, tryptophan is known to be unstable and is largely destroyed; therefore, tryptophan could not be reliably quantified.

Amino acid analysis was performed using a Biochrom 30+ automatic amino acid analyzer (Biochrom, Cambridge, UK) equipped with a Na-type cation-exchange column (200 × 4.6 mm, 8 µm). Separation was achieved using gradient elution with three sodium citrate buffers (pH 3.20, 4.25, and 6.45) at a flow rate of 25 mL h^{−1}. The post-column ninhydrin reagent was delivered at 10 mL/h, and the reaction coil temperature was maintained at 138 °C. The column temperature was programmed as follows: hold at 44 °C for 10 min, then increase to 47 °C and hold for 13 min, then increase to 98 °C and hold for 26 min. Amino acids were detected at 570 nm, except proline, which was monitored at 440 nm. The injection volume was 20 µL. Quantification was carried out using the Sigma AAS18 amino acid standard mixture diluted fivefold (Sigma-Aldrich, St. Louis, USA) with sodium citrate buffer to final concentrations of 0.5 µmol mL^{−1} for each amino acid (0.25 µmol mL^{−1} for cystine). Instrument performance specifications included a detection limit of 2.5 pmol for aspartic acid (S/N = 2), a retention time reproducibility with CV ≤ 0.3%, and a peak area reproducibility with CV ≤ 1.0%.

2.4. Determination of Acetate Consumption by HPLC (Simplified Version)

Before harvesting, 1 mL of the culture was taken and centrifuged at 10,000 rpm for 10 min. The supernatant was then used for HPLC analysis. Acetate concentration in culture supernatant was determined by HPLC (Agilent 1260 Infinity, Santa Clara, CA, USA) equipped with an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) and a UV detector at 210 nm. The column temperature was 40 °C. The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.6 mL min^{−1}, and the injection volume was 20 µL. A calibration curve was prepared using sodium acetate standards (20–200 mM, R² > 0.999). Samples were centrifuged (12,000 × g, 10 min) and filtered (0.22 µm) before analysis. Acetate consumption was calculated as the difference between initial and residual concentration. All measurements were performed in triplicate.

2.5. Analytical Methods

Cell density, as the optical density at 600 nm, was determined using a spectrophotometer (UNICO, Santa Clara, CA, USA). Dry cell weight (DCW) was determined by taking 1 mL of the yeast culture, centrifuging to collect the cells, freeze drying to constant weight, and weighing the dried material on a precision balance to obtain the DCW (g L^{-1}).

The specific OD-based growth rate (μ) was calculated during the exponential phase using the following equation:

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

where X_1 and X_2 are the biomass (OD_{600} value in this work) at the time points t_1 and t_2 , respectively.

2.6. Transcriptome Sequencing and Analysis

For transcriptomic analysis, the strain W29 was cultivated in MM supplemented with different concentrations of $(\text{NH}_4)_2\text{SO}_4$ (0.1, 1.0, 7.5, and 15 g/L). Cells grown under nitrogen-limited conditions (0.1 g/L) were harvested at 48 h; cells cultured at the other nitrogen concentrations were harvested at 24 h. Cell pellets from 50 mL cultures were obtained by centrifugation at $2000 \times g$ and washed three times with PBS. Three biological replicates were performed per condition.

For transcriptomic analysis, total RNA was extracted using an RNA extraction kit. RNA integrity and purity were strictly evaluated using agarose gel electrophoresis, NanoDrop spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA), Qubit fluorometric quantification, and capillary electrophoresis to ensure that the samples met the requirements for library construction. Sequencing was performed on the Illumina NovaSeq 6000 platform using a paired-end strategy with a read length of PE150 (Illumina, San Diego, CA, USA).

Each sample generated approximately 46.42 million raw reads. After quality filtering, 46.05 million clean reads were retained for downstream analysis. The average Q30 value was 97%, and the GC content ranged from 53% to 54%. Clean reads were mapped to the reference genome of *Y. lipolytica* (ASM176148v1) using HISAT2 V2.2.1). The average mapping rate was >85%. Gene expression levels were quantified using HTSeq (V0.6.1) based on raw read counts. Raw count data were normalized using DESeq2 (V1.26.0). Differentially expressed genes (DEGs) were identified using DESeq2 with thresholds of $|\log_2\text{FoldChange}| \geq 1$ and adjusted p -value < 0.05. The raw RNA-seq datasets generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1468311.

3. Results

3.1. Effects of Nitrogen Availability on Growth and Protein Accumulation

To investigate the effects of nitrogen availability on cell growth and protein synthesis, four culture conditions with distinct nitrogen source concentrations (0.1, 1.0, 7.5, and 15 g L^{-1}) were established based on a modified minimal medium. Due to significant differences in growth kinetics among the tested conditions, the exponential growth phase for each group was individually determined according to the linear relationship between $\ln(\text{OD}_{600})$ and cultivation time. Under nitrogen-limited conditions (0.1 g L^{-1}), a pronounced delay in the onset of exponential growth was observed, with the exponential phase occurring approximately between 20 and 50 h (Figure 1a). Under the condition with 0.1 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, the specific OD-based growth rate was $0.066 \pm 0.008 \text{ h}^{-1}$, which was lower than those under the other three nitrogen concentrations: 1.0 g L^{-1} ($0.162 \pm 0.010 \text{ h}^{-1}$), 7.5 g L^{-1} ($0.203 \pm 0.003 \text{ h}^{-1}$), and 15 g L^{-1} ($0.177 \pm 0.016 \text{ h}^{-1}$). In contrast, cells culti-

vated under the three nitrogen-replete conditions exhibited comparable growth kinetics, with similar specific OD-based growth rate of approximately 0.17 h^{-1} (Figure 1a and Supplementary Table S5). Consistent with the reduced growth rate, biomass accumulation under nitrogen limitation (0.1 g L^{-1} , $1.57 \pm 0.04 \text{ g} \cdot \text{L}^{-1}$ DCW), reaching only about one-third of that obtained under nitrogen-replete conditions ($4.61\text{--}4.71 \text{ g L}^{-1}$, Figure 1b). In parallel, both the protein content and protein fraction were substantially reduced, with protein accounting for only $19.56 \pm 0.45\%$ of total biomass under nitrogen limitation, while the other three nitrogen-replete conditions maintained a protein content of approximately 50% (Figure 1c). These results indicate that nitrogen deficiency severely restricts not only cell growth but also protein biosynthesis. Further analysis of acetate consumption revealed that substrate utilization was markedly suppressed under nitrogen-limited conditions ($4.52 \pm 0.10 \text{ g L}^{-1}$) compared with nitrogen-replete conditions ($10.91\text{--}12.33 \text{ g L}^{-1}$, Figure 1d). The reduced acetate uptake was accompanied by decreased biomass formation and protein synthesis under nitrogen deficiency, suggesting impaired cellular growth and biosynthetic activity. In contrast, no significant differences in growth or protein accumulation were observed among the three nitrogen conditions (1.0 , 7.5 , and 15 g L^{-1} , Figure 1c,d). Notably, increasing nitrogen concentration beyond a certain threshold did not further enhance protein synthesis, implying that intracellular nitrogen metabolism is subject to more complex regulatory mechanisms rather than simple substrate-driven effects. The decrease in protein fraction was accompanied by a reduction in absolute protein content, indicating that the observed trend was not merely due to changes in biomass composition but reflected a genuine limitation in protein biosynthesis.

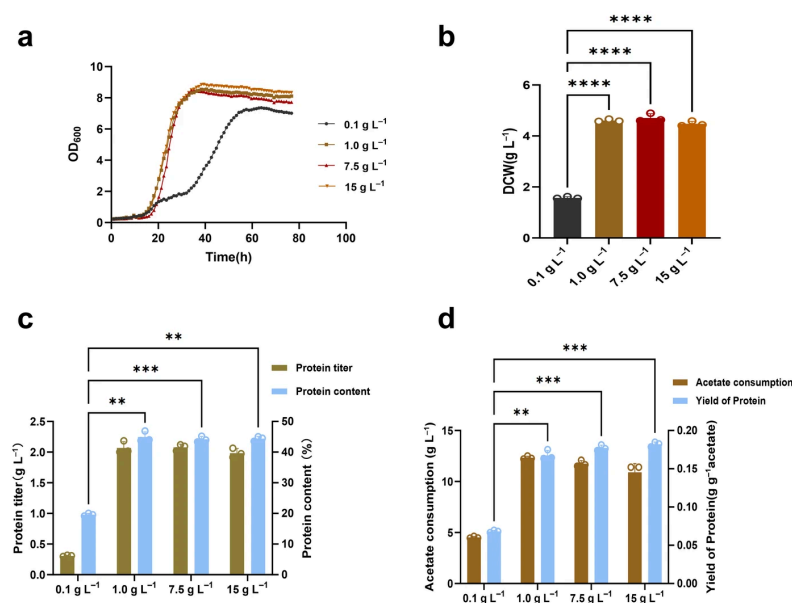


Figure 1. Effects of different nitrogen levels on cell growth and protein accumulation in *Y. lipolytica*. (a) Growth curves under different nitrogen conditions. (b) Biomass accumulation under different nitrogen conditions. Asterisks denote significant differences relative to the $0.1 \text{ g} \cdot \text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$ group: **** $p < 0.0001$. (c) Protein accumulation under different nitrogen conditions (protein content: ** $p = 0.001$ for $15 \text{ g} \cdot \text{L}^{-1}$ vs $0.1 \text{ g} \cdot \text{L}^{-1}$; *** $p < 0.001$ for $7.5 \text{ g} \cdot \text{L}^{-1}$ vs $0.1 \text{ g} \cdot \text{L}^{-1}$, ** $p = 0.0031$ for $1.0 \text{ g} \cdot \text{L}^{-1}$ vs $0.1 \text{ g} \cdot \text{L}^{-1}$) (d) Relationship between acetate consumption and protein accumulation under different nitrogen conditions (yield of protein: *** $p < 0.001$ for $15 \text{ g} \cdot \text{L}^{-1}$ vs $0.1 \text{ g} \cdot \text{L}^{-1}$; *** $p < 0.001$ for $7.5 \text{ g} \cdot \text{L}^{-1}$ vs $0.1 \text{ g} \cdot \text{L}^{-1}$, ** $p = 0.0028$ for $1.0 \text{ g} \cdot \text{L}^{-1}$ vs $0.1 \text{ g} \cdot \text{L}^{-1}$). All strains were cultivated in 250 mL shake flasks containing 50 mL of MM with 200 mM sodium acetate. All data are presented as mean value and standard deviations of three independent biological replicates and error bars show standard deviations. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Source data are provided as Supplementary Materials Data S1.

3.2. Nitrogen Availability Regulates Biomass Composition Between Lipid and Protein Biosynthesis

To further elucidate how nitrogen availability influences intracellular biomass composition, lipid accumulation was quantified under different C/N conditions in terms of lipid content (%), lipid mass per dry cell weight) and lipid titer ($\text{g}\cdot\text{L}^{-1}$, absolute lipid concentration). As shown in Figure 2a, under nitrogen-limited conditions ($0.1\text{ g}\cdot\text{L}^{-1}\text{ (NH}_4\text{)}_2\text{SO}_4$), lipid content reached $34.16 \pm 1.51\%$ of DCW, which was higher than that observed under nitrogen-replete conditions (for $1.0\text{ g}\cdot\text{L}^{-1}$: $17.52 \pm 1.22\%$; $7.5\text{ g}\cdot\text{L}^{-1}$: $11.82 \pm 1.01\%$; $15\text{ g}\cdot\text{L}^{-1}$: $11.73 \pm 1.49\%$). However, this increase in lipid content did not translate into a corresponding increase in lipid titer. Due to the significantly reduced biomass accumulation under nitrogen limitation (Figure 1), the overall lipid titer remained relatively low ($0.55 \pm 0.02\text{ g}\cdot\text{L}^{-1}$), indicating that enhanced lipid accumulation at the cellular level was offset by limited cell growth. In contrast, the highest lipid titer was observed at a moderately low nitrogen concentration ($1.0\text{ g}\cdot\text{L}^{-1}\text{ (NH}_4\text{)}_2\text{SO}_4$, Figure 2a), where a balanced metabolic state was achieved. Under this condition, cells maintained relatively high biomass while still allocating a substantial fraction of acetate toward lipid synthesis, resulting in maximal lipid production ($0.82 \pm 0.01\text{ g}\cdot\text{L}^{-1}$). Further increases in nitrogen availability (7.5 and $15\text{ g}\cdot\text{L}^{-1}\text{ (NH}_4\text{)}_2\text{SO}_4$) led to no significant differences in either lipid content or lipid titer (lipid content: $11.82 \pm 1.01\%$ and $11.73 \pm 1.49\%$; lipid titer: 0.55 ± 0.02 and $0.52 \pm 0.01\text{ g}\cdot\text{L}^{-1}$), suggesting that lipid biosynthesis was suppressed under nitrogen-replete conditions and that excess nitrogen did not further affect biomass composition. To better visualize the relationship between these two major biomass components, the relative proportions of protein and lipid content were plotted across different nitrogen conditions (Figure 2b). An opposite variation trend was observed between protein yield and lipid yield, which may reflect a potential competitive distribution of cellular resources under different nitrogen conditions. As nitrogen availability increased from limiting to sufficient levels, cellular biosynthesis gradually shifted from lipid accumulation toward protein synthesis, resulting in an increase in protein yield from $0.068 \pm 0.002\text{ g}\cdot\text{g}^{-1}$ acetate to $0.177 \pm 0.004\text{ g}\cdot\text{g}^{-1}$ acetate, accompanied by a corresponding decrease in lipid yield from $0.119 \pm 0.006\text{ g}\cdot\text{g}^{-1}$ acetate to $0.047 \pm 0.007\text{ g}\cdot\text{g}^{-1}$ acetate. To further assess substrate utilization efficiency, the relative contributions of protein and lipid to total biomass were analyzed (Figure 2c). Under nitrogen-limited conditions, a larger fraction of acetate was diverted toward lipid biosynthesis, while a smaller proportion was incorporated into protein. In contrast, under nitrogen-replete conditions ($7.5\text{ g}\cdot\text{L}^{-1}$), cells were more inclined toward protein synthesis. These results suggest that nitrogen availability plays an important role in regulating biomass composition and protein/lipid accumulation.

Importantly, the observed increase in protein proportion under higher nitrogen conditions was accompanied by a corresponding increase in absolute protein fraction, rather than being solely attributable to reduced lipid accumulation (Figure 1c). This finding confirms that nitrogen availability actively promotes protein biosynthesis rather than passively altering biomass composition. Collectively, these results demonstrate that nitrogen availability plays an important role in regulating protein and lipid accumulation, thereby determining the overall biomass composition in acetate-grown cells.

3.3. Transcriptomic Analysis Reveals Nitrogen-Dependent Changes in Biomass-Related Metabolic Pathways

To elucidate the transcriptional responses associated with nitrogen-regulated protein accumulation and changes in biomass composition, transcriptomic analysis was performed to compare gene expression profiles under nitrogen-limited ($0.1\text{ g}\cdot\text{L}^{-1}$) and nitrogen-replete ($7.5\text{ g}\cdot\text{L}^{-1}$) conditions. A total of 2702 differentially expressed genes (DEGs) were identified, including 1399 upregulated and 1303 downregulated genes in the nitrogen-limited group

versus the nitrogen-replete group (Figure 3a), indicating extensive transcriptional reprogramming in response to nitrogen availability. To further characterize transcriptional responses associated with nitrogen availability, amino acid-related and lipid-related pathways were manually curated from KEGG annotations and comparatively analyzed (Figure 3b and Supplementary Figure S1). The numbers of upregulated and downregulated genes within each pathway were quantified to evaluate pathway-level transcriptional changes. Overall, most pathways exhibited a predominance of downregulated genes under nitrogen-limited conditions, indicating a general suppression of cellular anabolic metabolism.

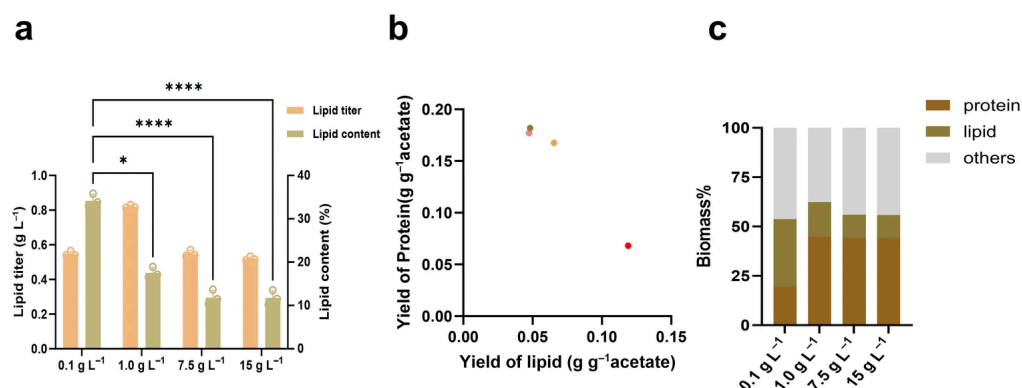


Figure 2. Effects of nitrogen availability on biomass composition between lipid and protein biosynthesis in *Y. lipolytica*. (a) Lipid production under different nitrogen conditions (lipid content: **** $p < 0.0001$ for 15 g·L⁻¹ vs 0.1 g·L⁻¹; **** $p < 0.0001$ for 7.5 g·L⁻¹ vs 0.1 g·L⁻¹, * $p = 0.0165$ for 1.0 g·L⁻¹ vs 0.1 g·L⁻¹). (b) Relationship between lipid yield and protein yield from acetate (i.e., the amount of lipid/protein produced per gram of acetate). The three close points (1.0, 7.5, 15 g·L⁻¹) showed similar lipid and protein yields, whereas the condition at 0.1 g·L⁻¹ (red point) exhibited significant differences in cellular components compared to the other control groups. (c) Distribution of major biomass components (%) under different nitrogen conditions. All strains were cultivated in 250 mL shake flasks containing 50 mL of MM with 200 mM sodium acetate. All data are presented as mean value and standard deviations of three independent biological replicates and error bars show standard deviations. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Source data are provided as a Supplementary Materials Data S1.

Specifically, several amino acid-related pathways, including β -alanine metabolism, alanine, aspartate and glutamate metabolism, tyrosine metabolism, valine, leucine and isoleucine degradation, and tryptophan metabolism, showed reduced transcriptional activity, with downregulated genes generally exceeding upregulated genes within each pathway. Similarly, lipid-related pathways, including fatty acid metabolism, fatty acid degradation, and biosynthesis of unsaturated fatty acids, also displayed an overall downregulation trend. These results suggest that nitrogen limitation broadly suppresses biosynthetic and metabolic activities associated with both protein- and lipid-related metabolism.

To further identify key responsive genes under nitrogen limitation beyond pathway-level analysis, the top 10 most strongly upregulated genes were extracted based on log2foldchange and manually annotated using NCBI and UniProt databases (Figure 3c). Notably, these genes were not enriched in KEGG or GO functional categories but were predominantly predicted to encode membrane-associated transport proteins or transport-related regulatory factors. Functional annotation revealed that the majority of these genes are involved in ammonium transport, amino acid permeases, and transport regulation, indicating a pronounced enhancement of transmembrane nitrogen uptake and intracellular redistribution capacity under nitrogen-limited conditions. The consistent upregulation of these transport-related genes suggests that cells respond to nitrogen scarcity not only by adjusting intracellular metabolic pathways but also by actively strengthening nutrient

acquisition systems at the membrane level. This observation highlights a multi-layered adaptive response to nitrogen limitation, in which transport processes play a critical role in maintaining intracellular nitrogen availability when external supply is restricted. Such reinforcement of nitrogen uptake capacity may represent a primary compensatory mechanism that precedes or complements downstream metabolic reprogramming.

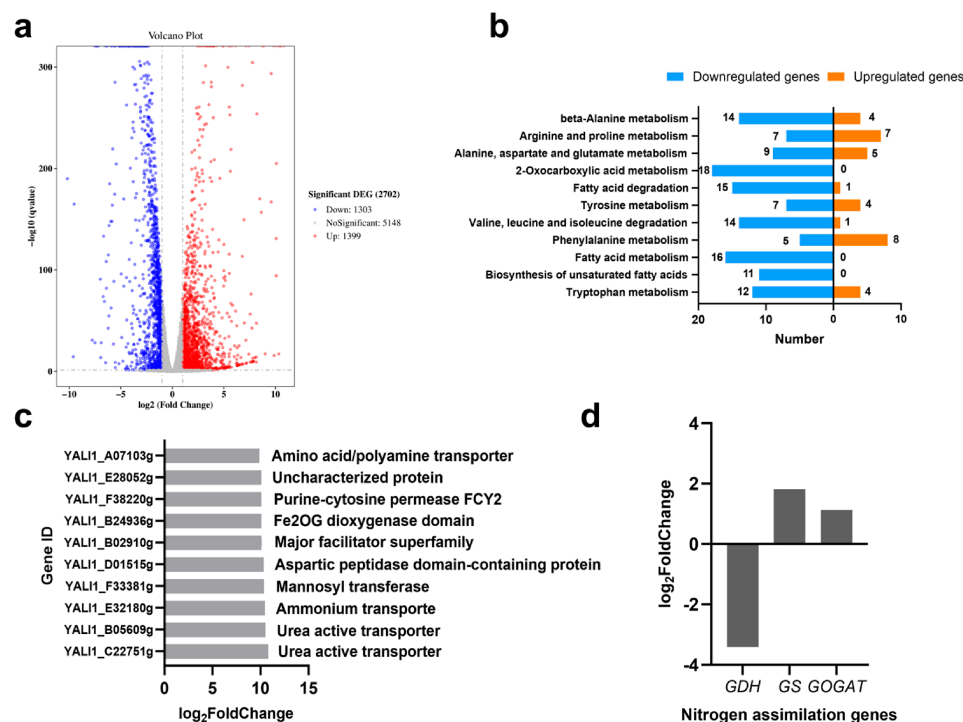


Figure 3. Transcriptomic analysis reveals nitrogen metabolism regulation in *Y. lipolytica* under nitrogen-limited conditions. Transcriptome comparison was performed between cells grown under nitrogen-limited (0.1 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, C/N = 263:1) and nitrogen-replete (7.5 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, C/N = 3.5:1) conditions. Three biological replicates were analyzed per condition. (a) Volcano plot showing differentially expressed genes (DEGs). Vertical dashed lines indicate the significance threshold of $|\log_2\text{FC}| = 1$, and the horizontal dashed line indicates $q = 0.05$ ($-\log_{10}(q\text{-value}) = 1.301$). Red dots represent significantly upregulated genes ($\log_2\text{FC} > 1$, $q < 0.05$), blue dots represent significantly downregulated genes ($\log_2\text{FC} < -1$, $q < 0.05$), and gray dots represent non-significant genes. (b) Distribution of upregulated and downregulated genes in selected KEGG pathways related to amino acid metabolism and fatty acid metabolism. (c) Top 10 most significantly upregulated genes under nitrogen-limited conditions, annotated based on NCBI and UniProt databases. (d) Expression changes ($\log_2$ fold change) of key nitrogen assimilation genes, including GDH, GS, and GOGAT, under nitrogen-limited conditions. Positive values indicate upregulation under nitrogen limitation; negative values indicate downregulation. Significance thresholds are as in (a).

In addition to enhanced transport capacity, differential regulation was also observed in key intracellular nitrogen assimilation pathways. To further elucidate how assimilated nitrogen is incorporated into central metabolism, the expression patterns of core ammonium assimilation enzymes were examined. The expression of *GDH* was significantly downregulated under nitrogen limitation, whereas genes encoding *GS* and *GOGAT* were markedly upregulated (Figure 3d). This contrasting expression pattern could be interpreted as a possible shift in nitrogen assimilation strategy from the *GDH*-dependent pathway toward the high-affinity *GS*-*GOGAT* pathway under nitrogen-limited conditions. Given that both pathways converge on glutamate production, a central nitrogen donor for amino acid biosynthesis, this transcriptional reprogramming likely reflects an adaptive mechanism to optimize nitrogen utilization under nutrient-limited conditions. When integrated with the

biomass composition results (Figure 2), these findings suggest that nitrogen availability is associated with coordinated regulation of nitrogen assimilation and biomass composition. Under nitrogen limitation, although high-affinity nitrogen assimilation pathways are activated, insufficient nitrogen supply and restricted carbon metabolism ultimately limit protein biosynthesis and favor lipid accumulation. In contrast, under nitrogen-replete conditions, efficient nitrogen assimilation supports amino acid and protein synthesis, leading to higher protein accumulation and reduced lipid content.

3.4. Functional Validation of Nitrogen Assimilation Pathways Through Targeted Gene Overexpression

To further validate the role of nitrogen assimilation in regulating protein biosynthesis, a stepwise metabolic engineering strategy was implemented based on an acetate-utilization-enhanced strain (HR9). Strains harboring additional copies of expression cassettes of key genes involved in nitrogen assimilation, including *GDH*, *GS*, and *GOGAT*, were sequentially constructed to evaluate their cumulative effects on protein accumulation and cellular metabolism (Figure 4a). Introduction of an additional *GDH* expression cassette into the HR9 strain increased the protein content from $48.52 \pm 1.95\%$ in the control strain to $52.44 \pm 3.02\%$. Subsequent introduction of an additional *GS* expression cassette further raised the protein accumulation to $55.77 \pm 0.95\%$. During this process, the biomass yield remained unchanged (approximately $3.6 \text{ g} \cdot \text{g}^{-1}$). Meanwhile, the protein content of the strain harboring both *GDH* and *GS* additional copies increased by 7.25% relative to that of the parental strain HR9. The sequential introduction of *GDH* and *GS* additional copies led to a gradual increase in the protein content of the strains, suggesting a potential synergistic effect of *GDH* and *GS* in promoting nitrogen incorporation into biomass. This stepwise improvement suggests that reinforcing both direct ammonium assimilation (via *GDH*) and high-affinity nitrogen assimilation (via *GS*) effectively increases intracellular nitrogen flux toward amino acid and protein biosynthesis. However, upon further introduction of an additional *GOGAT* expression cassette into the strain already harboring *GDH* and *GS* additional copies, the protein content decreased to $50.02 \pm 0.37\%$, accompanied by reduced biomass (from $0.36 \pm 0.0042 \text{ g} \cdot \text{g}^{-1}$ to $0.304 \pm 0.0012 \text{ g} \cdot \text{g}^{-1}$ acetate). This result suggests that, unlike *GDH* and *GS*, introduction of additional *GOGAT* copies does not contribute to enhanced protein accumulation and may instead impose a detrimental effect on cellular metabolism. To further characterize the protein quality of the engineered strain, the strain harboring both *GDH* and *GS* additional copies was selected for amino acid composition analysis (Figure 4b). Compared with the control strain, the relative abundance of nearly all amino acids was significantly increased, with the exception of cysteine and tryptophan (which could not be quantified). Notably, glutamate exhibited the most pronounced increase, consistent with its central role as a key nitrogen donor in amino acid biosynthesis. In addition, eight quantified amino acids showed varying degrees of enhancement, including histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine, highlighting the improved nutritional quality of the microbial protein produced by the engineered strain (Figure 4c). The observed enhancement in amino acid content can be attributed to improved nitrogen assimilation and redistribution toward biosynthetic pathways. *GDH* directly converts ammonium into glutamate using reducing equivalents, while *GS* enhances ammonium assimilation efficiency through ATP-dependent glutamine formation. The combined action of these two pathways likely increases the intracellular pools of glutamate and glutamine, thereby supporting the synthesis of a broad range of amino acids. In contrast, we hypothesize that the negative effect observed upon additional *GOGAT* copy introduction may be attributable to metabolic and energetic imbalance. The *GS*-*GOGAT* cycle is highly demanding in terms of both ATP (*GS*) and reducing equivalents (*GOGAT*). A potential increase in *GOGAT* activity might lead to overconsumption of

NAD(P)H and accelerated depletion of α -ketoglutarate, a key intermediate linking carbon and nitrogen metabolism. This could constrain flux through the tricarboxylic acid (TCA) cycle, thereby limiting energy generation and precursor supply required for biomass and protein synthesis. Furthermore, disproportionate expression of GOGAT relative to GS may disrupt the balance of the nitrogen assimilation cycle, resulting in inefficient flux distribution and reduced overall metabolic efficiency.

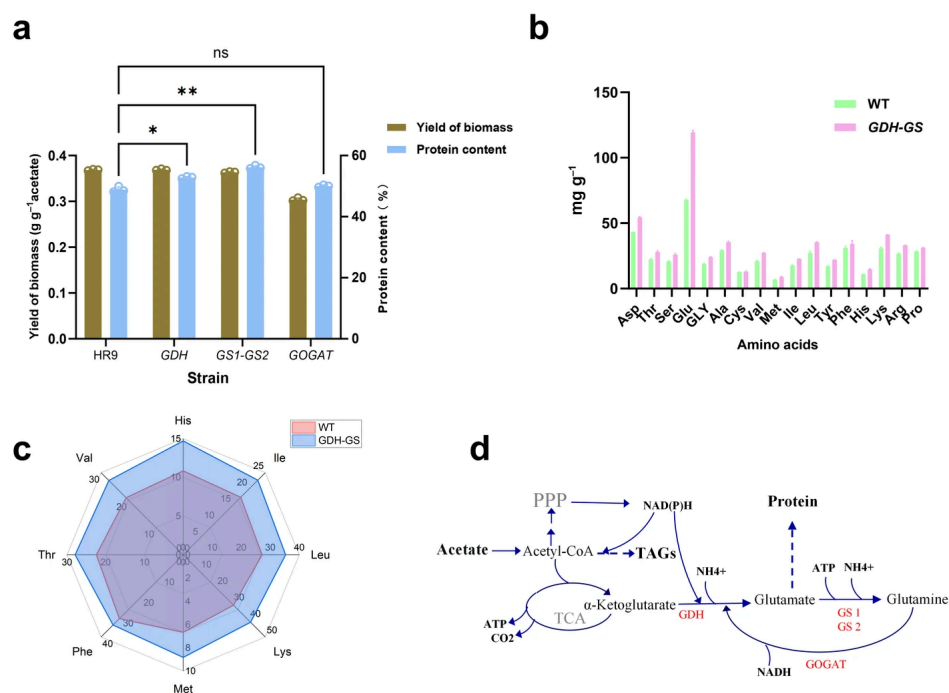


Figure 4. Functional validation of key nitrogen assimilation genes and metabolic analysis in *Y. lipolytica*. (a) Biomass formation and protein accumulation in strains harboring additional copies of key nitrogen assimilation genes. The strains used were: Control (parental strain, HR9), GDH (strain overexpressing *GDH*), GS-GDH (strain overexpressing *GDH* and *GS*), and GOGAT (strain overexpressing *GDH*, *GS*, and *GOGAT*). (protein content: ns = 0.4432 for 15 g·L⁻¹ vs 0.1 g·L⁻¹; ** $p = 0.0023$ for 7.5 g·L⁻¹ vs 0.1 g·L⁻¹, * $p = 0.0242$ for 1.0 g·L⁻¹ vs 0.1 g·L⁻¹). (b) Changes in amino acid composition in engineered strains. (c) Radar plot comparing the relative abundance of eight quantified amino acids between engineered strains and the wild-type strain. (d) Schematic representation of metabolic pathways for protein biosynthesis from acetate. TAGs, triacylglycerols; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle. Red labels indicate key enzymes involved in nitrogen assimilation. All data are presented as mean value and standard deviations of three independent biological replicates and error bars show standard deviations. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Source data are provided as a Supplementary Materials Data S1.

Taken together, these results suggest that coordinated introduction of additional copies of *GDH* and *GS* may promote nitrogen assimilation and protein biosynthesis, while further introduction of an additional copy of *GOGAT* without balanced regulation leads to metabolic burden and diminished performance. Importantly, the improved amino acid profile, particularly the increased levels of quantified amino acids, highlights the potential of the engineered strain as a microbial protein source for feed applications.

Based on the combined results of biomass composition analysis, transcriptomic profiling, and metabolic engineering experiments, an integrated model is proposed to illustrate how nitrogen assimilation may influence protein biosynthesis and lipid accumulation in the acetate-utilizing system (Figure 4d). As shown in Figure 4d, acetate is assimilated into central metabolism via acetyl-CoA and subsequently enters the tricarboxylic acid (TCA) cy-

cle, where key intermediates such as α -ketoglutarate serve as critical nodes linking carbon and nitrogen metabolism. At this metabolic branch point, cellular metabolism may shift between lipid accumulation and amino acid/protein biosynthesis depending on nitrogen availability and assimilation capacity.

4. Discussion

In this study, we systematically investigated how nitrogen assimilation influences biomass composition and protein biosynthesis in an acetate-utilizing *Y. lipolytica*. By combining transcriptomic analysis with metabolic engineering, we observed that nitrogen availability strongly affects the balance between protein and lipid accumulation through coordinated regulation of nitrogen assimilation pathways. In particular, strains harboring additional copies of *GDH* and *GS* showed increased protein accumulation and improved amino acid composition, whereas further introduction of an additional *GOGAT* copy was associated with impaired cell growth and protein synthesis, suggesting that excessive nitrogen assimilation flux may disrupt intracellular metabolic and energetic balance. These findings suggest the importance of balanced nitrogen assimilation for efficient microbial protein production from acetate.

Beyond increasing total protein content, the observed improvement in amino acid composition may further enhance the biological value of the produced microbial protein. Quantified amino acids are critical indicators of protein nutritional quality for food and feed applications [19,20]. In this study, simultaneous introduction of additional copies of *GDH* and *GS* increased the abundance of eight quantified amino acids, with glutamate showing the most pronounced increase. As glutamate serves as a central nitrogen donor for amino acid biosynthesis, the observed increase in glutamate may have promoted overall amino acid synthesis and nitrogen incorporation efficiency [21]. In addition, improved nitrogen assimilation might contribute to more efficient acetate utilization toward biomass formation rather than carbon loss to storage compounds such as lipids, thereby potentially improving protein yield from acetate. These results suggest that optimization of nitrogen metabolism could simultaneously improve both protein quantity and nutritional quality.

Nitrogen assimilation through the *GDH* and *GS*–*GOGAT* pathways has been widely studied in bacteria and model yeasts, where these pathways are known to exhibit different affinities and energetic requirements under varying nitrogen conditions [22,23]. In *Saccharomyces cerevisiae* and several filamentous fungi, the *GS*–*GOGAT* pathway is generally activated under nitrogen limitation due to its higher ammonium affinity, whereas *GDH* is typically associated with nitrogen-rich conditions and rapid biomass synthesis [14,24,25]. Our transcriptomic results showed a similar transition from *GDH* to *GS*–*GOGAT* under nitrogen limitation in *Y. lipolytica*, indicating a conserved adaptive response among microorganisms. However, although cells initiated this adaptive mechanism and shifted nitrogen assimilation to a higher-affinity mode, growth remained severely inhibited. In addition to insufficient nitrogen supply, acetate itself may impose constraints in terms of energy consumption and pH, further aggravating the growth inhibition [26,27]. Moreover, unlike previous studies mainly focused on nitrogen stress adaptation or amino acid metabolism, our work further suggests that balanced introduction of additional copies of these pathway genes may be associated with improved microbial protein production from acetate. Furthermore, the negative effect associated with additional *GOGAT* copy introduction suggests that excessive nitrogen assimilation may impose additional NADPH and ATP burdens, highlighting the importance of balanced nitrogen assimilation and energy metabolism during metabolic engineering.

Overall, this study provides mechanistic insights and practical strategies for improving nitrogen utilization and protein biosynthesis in acetate-based fermentation systems.

These findings may facilitate the development of efficient microbial protein production processes using low-cost and sustainable carbon substrates, including acetate derived from renewable resources.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12070315/s1>. Figure S1: Bar chart of significantly enriched KEGG pathways; Table S1: All strains used in this study; Table S2: The plasmids used in this study; Table S3: The primers used in this study; Table S4: The nucleotide sequences used in this study; Table S5: Specific growth rates under different nitrogen conditions. Supplementary Data S1: Raw numerical data; Supplementary Data S2: Differentially expressed genes; Supplementary Data S3: KEGG enrichment outputs.

Author Contributions: H.J. and D.L. provided overall guidance for the project. D.L. proposed a series of experimental ideas based on cultivation experiments of strains under different nitrogen levels, transcriptomic analysis, and validation. R.H. and D.L. participated in the design and implementation of cultivation and validation under different nitrogen levels, as well as the statistical integration and analysis of transcriptomic data, and strain modification and validation experiments. X.S., Z.Z., and K.S. participated in strain modification and fermentation experiments, while W.L. and Y.L. provided technical guidance for the experiments. D.L., H.J., and R.H. co-wrote the paper. All authors participated in discussions of the results and provided assistance during the preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Key R&D Program of China (2024YFA0918100 to Y.W.L.), Tianjin Science and Technology Planning Project (Grant number 25ZXZSS00100 to H.F.J.), CAS Project for Young Scientists in Basic Research (Grant number YSBR-072-4 to H.F.J.), Cosychem Technology (Tianjin) Co., Ltd., Tianjin 300450, PR China and Tianjin Science and Technology Plan Key R&D Program (24YFYSHZ00050 to H.F.J.). The funder, Cosychem Technology (Tianjin) Co., Ltd., had no involvement in the study design, data analysis, or manuscript preparation. The authors declare no conflicts of interest.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors. BioProject accession number PRJNA1468311.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

GDH	glutamate dehydrogenase
GS	glutamine synthetase
GOGAT	glutamate synthase
DCW	Dry cell weight

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