

Article

Biosynthesis and Application of 2-Amino-3-Methylhexanoic Acid as a Novel Biostimulant for Tea Plants

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Abstract

2-Amino-3-methylhexanoic acid (AMHA), as a naturally occurring α -amino acid, exhibits excellent bioactivity in inducing plant resistance to biotic and abiotic stresses, promoting plant growth and alleviating pesticide damage. However, its low yield in nature has hindered its industrialization. This study aims to address the current challenges in AMHA production, including synthetic difficulties, low yield, and poor production safety. We investigated the production capacity of *Serratia marcescens* NAU002-4 using high-performance liquid chromatography (HPLC), orthogonal experiments, and ion exchange chromatography (IEC), supplemented by precursor feeding assays. Optimization of the fermentation medium revealed that 10 g/L corn steep liquor as the nitrogen source and 10 g/L threonine as the precursor resulted in maximal yield. Further optimization of fermentation parameters—initial pH 7.2, inoculation volume 6%, stirring speed 180 rpm, and aeration rate 350 L/h—led to an AMHA titer of 504 mg/L. In parallel, field trials were conducted to evaluate the effects of pure, biosynthetic AMHA on tea plant growth and photosynthetic performance, using plant physiological data and chlorophyll fluorescence rise kinetics. The results demonstrated that biosynthetic AMHA significantly enhances tea plant growth and photosynthetic efficiency, providing a scientific basis for its development and application as a novel biostimulant.

Keywords: natural product; biostimulant; microbial fermentation; fermentation process; growth promotion

1. Introduction

The global population is projected to reach 9–10 billion by 2050 [1]. As the world's population grows rapidly, so does the demand for food. In addition, frequent occurrence of extreme weather, rise of global temperature, and reduction in available water resources are also huge challenges in agricultural production, which directly or indirectly affect crop yields [2–4]. Pesticides and fertilizers play an important role in boosting grain production and reducing crop diseases and pests. Nearly half of the global population relies on fertilizers to sustain crop growth and thus increase yields. However, the unreasonable and unscientific use of chemical fertilizers will have a negative impact on the quality of agricultural products and the health of the ecological environment. This not only endangers national food security, but also seriously hinders the sustainable development of agriculture. The overuse of traditional chemical synthetic fertilizers can lead to water pollution (such



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as water bloom and red tide) [5], soil fertility destruction, etc., and further damage the ecosystem and biodiversity of the natural environment [6].

To reduce reliance on synthetic agrochemicals such as pesticides and fertilizers and improve the sustainability of agricultural systems, numerous innovative technologies have been introduced over the past few decades. Among them, natural plant biostimulants have emerged as a promising and environmentally friendly alternative [7]. Biostimulants primarily enhance crop quality and yield, as well as improve plant tolerance to various abiotic stresses, by regulating the physiological processes of plants rather than by supplying substantial nutrients. This approach offers significant potential for sustainable agriculture [8–10]. Its application not only alleviates the environmental drawbacks of traditional chemical fertilizers but also increases plant resilience to various stress conditions [11]. In February 2024, the American Association of Plant Food Management Officials (AAPFCO) officially defined “plant biostimulant” as substance that enhance nutrient uptake, stress resistance, or crop quality by stimulating natural physiological processes (<https://cn.agropages.com/News/NewsDetail---36499.htm>, accessed on 8 December 2025). Currently, common biostimulants mainly include seaweed extracts, microbial inoculants, humic substances, protein hydrolysates, and others. In these biostimulants, amino acids and peptides derived from protein hydrolysates are attracting widespread attention in plant science. They are valued for their ability to improve soil health, enhance stress tolerance [12], participate in plant metabolism, promote chlorophyll synthesis, and boost photosynthesis [13,14], thereby supporting overall plant growth.

Currently, amino acids are primarily obtained through chemical synthesis, protein hydrolysis, and microbial fermentation [15–18]. Chemical synthesis is relatively complex, involves significant pollution, and requires expensive catalysts [15]. In addition, chemically synthesized amino acids are mostly achiral, and additional optical resolution steps are required to obtain only *L*-form amino acids from racemic mixtures [16]. Therefore, chemical synthesis is unsuitable for the large-scale industrial production of amino acids. Protein hydrolysis suffers from poor raw material stability [16], generates numerous by-products [15], and has a narrow application scope, being limited to only a few kinds of amino acids [16]. In contrast, microbial fermentation, which has advanced significantly in recent years, converts sugars in the substrate into amino acids. This method offers simplicity, mild operating conditions [17,18], low cost [16–18], and is applicable to the large-scale production of most *L*-amino acids [19]. Moreover, appropriate adjustments and optimizations of the upstream, midstream, and downstream processes can significantly improve the yield of the target product [20,21]. Microbial biotechnology plays a pivotal role in developing a new generation of agricultural inputs that align with the principles of sustainability and circular economy. By utilizing renewable, simple, cheap biomass as raw materials and employing genetically optimized or metabolic modeling, this technology enables the green synthesis of amino acids [15]. These microbial-derived products offer several environmental advantages over conventional agrochemicals, such as lower carbon footprints, reduced soil and water pollution, and enhanced biodiversity in agroecosystems [11]. In summary, microbial fermentation technology is a commonly used method for the biosynthesis of amino acids. By optimizing the fermentation conditions and improving the strains, not only can the production efficiency of the amino acid biostimulant be improved, but the production cost can also be further reduced, thereby increasing market competitiveness of amino acid biostimulants.

2-Amino-3-methylhexanoic acid (AMHA) is a natural α -amino acid found in *Alternaria* Species, and its structure is similar to branched-chain amino acids [22,23]. Our previous research has shown that AMHA possesses a high capacity to induce plant resistance at very low doses, enabling plants to combat a range of biotic and abiotic stresses [24,25].

Exogenous AMHA treatment not only reduced susceptibility to powdery mildew (*Blumeria graminis* f. sp. *tritici*) in wheat, *Pseudomonas syringae* DC3000 in Arabidopsis and Tomato spotted wilt virus in tobacco, but also induced resistance to low temperature in strawberries and tomatoes [26], and to high temperatures in bentgrass, wheat and tomatoes [22,24]. However, the low yield of AMHA in reported fungi has hindered its industrialization process [22]. Previous studies have shown that adding precursor amino acids to the culture medium of some strains can promote AMHA production, with yields reaching up to 500 mg/L, but could not produce AMHA in the conventional medium without precursor amino acids [27,28]. The reaction vessel used in these studies was a conical flask, which has a small production scale, and the cost of the exogenous addition of norvaline was high, making it unsuitable for large-scale production. Tea plant (*Camellia sinensis* (L.) O. Kuntze) is one of the most important economic crops in China for its drinking and medicinal values [29]. Our laboratory has found that AMHA can mitigate the heat damage of tea plants planted in the field by enhancing photosynthetic performance, osmotic regulation, and antioxidant enzyme activity, thereby boosting their thermotolerance [25].

To date, there are no systematic studies investigating the optimized microbial production of AMHA. Although the resistance ability of tea plants to high temperature stress has been explored in previous research [25], the activity of AMHA in promoting the growth of tea plants has not been systematically explored.

The main novelty lies in the scalable production methodology. We reviewed the discovery and production process of biological extract-based biostimulants and proposed a practical step-by-step process [12]. This study aimed to screen and optimize microbial fermentation conditions for efficient AMHA production, purify and structurally confirm the produced compound, and evaluate its plant growth-promoting effects and impact on photosynthetic performance in tea plants. In this study, the yield of AMHA was guaranteed while the production cost was reduced, and a small-scale fermentation process of AMHA was established, which laid a foundation for the development and application of AMHA as a new biostimulant.

2. Materials and Methods

2.1. Material

Strains, Plants and Chemicals

Five strains of *Serratia marcescens*, NAU002-1, NAU002-2, NAU002-3, NAU002-4, and NAU002-5, were distinct isolates from the same soil and isolated by our laboratory. These strains have vigorous metabolism, precise regulatory mechanisms, and are commonly used in industrial production. The tea plants were the cultivar ‘Longjing 43’ (*Camellia sinensis* cv. Longjing 43) and were grown in the tea garden of Jiangsu Bocha Agricultural Technology Development Co., Ltd. (31.95° N, 118.84° E, Nanjing, China).

LB solid medium was used for NAU002 activation. NAU002 was cultured at 37 °C in liquid culture medium containing 10 g/L urea, 7 g/L corn steep liquor powder, 1 g/L K₂HPO₄, 0.5 g/L MgSO₄·7H₂O, and 20 g/L anhydrous glucose, pH 7.0.

AMHA was used in a field experiment and was produced through fermentation by NAU002-4. The solid AMHA was dissolved in sterile water to prepare a 1 mM solution and then diluted to 100 nM. Isabion[®] was obtained from Syngenta Group Corporation Limited (Shanghai, China). The application rate for tea plants was calculated according to the product’s recommended dosage (Isabion stock solution diluted 600-fold).

2.2. Determination of AMHA Content

2.2.1. Preparation of AMHA Standard Curve

The AMHA standard solution was prepared at 25, 50, 100, 200, and 400 mg/L, respectively. The concentration of AMHA was detected by high-performance liquid chromatography (HPLC) (Primade, HITACHI Co., Tokyo, Japan). An Agilent ZORBAX SB-Aq Analytical C₁₈ column (250 mm × 4.6 mm × 5 µm) (Agilent Technologies, Palo Alto, CA, USA) was used for the HPLC analysis. The analytical conditions were as follows: mobile phase, 85% acetonitrile (containing 0.1% formic acid) and 15% water (containing 0.1% formic acid); detection wavelength, 210 nm; injection volume, 5 µL. The standard curve of AMHA was obtained by area normalization.

2.2.2. Detection of AMHA Content in Test Samples

The fermentation broth from each treatment group was centrifuged at 9000 rpm for 6 min and the supernatant filtered through a 0.22 µm hydrophilic polyethersulfone microfiber membrane (Tianjin Branch Billion Lung Experimental Equipment Co., Ltd., Tianjin, China). The supernatant was injected into the chromatographic injection bottle and put into the HPLC instrument, and the analytical conditions were set for detection. The fermentation broth of all treatment groups in the experiment was analyzed and detected by HPLC method. The detection results were calculated based on the standard curve, and the content of AMHA in the fermentation broth of different treatment groups could be obtained.

2.3. Selection of Optimal Fermentation Strains

The five strains of NAU002 were inoculated onto LB solid medium and cultured at 30 °C for 12 h for later use.

To screen the ability of five NAU002 strains to produce AMHA with the addition of different precursor amino acids, 10 g/L threonine (Thr), 10 g/L methionine (Met), 10 g/L norleucine (Nle), and a combination of 10 g/L norvaline (Nva) and 0.5 g/L leucine (Leu) at a 20:1 ratio, were added to the basic liquid medium, respectively. Strains were cultured at 200 rpm and 30 °C for 3 days. HPLC was employed to detect the AMHA content of the fermentation broth.

2.4. Selection of Matrix Components in Culture Medium for NAU002-4

2.4.1. Optimization of Corn Steep Liquor (CSL) Concentration for Strain NAU002-4 Cultivation

CSL was added to the basic medium of NAU002-4 at final concentrations of 0, 3, 7, 14, or 21 g/L, respectively, to identify the optimal content of CSL for NAU002-4 cultivation. After inoculation, strain NAU002-4 was cultured for 3 days at 30 °C. The biomass of NAU002-4 was determined every 24 h by measuring the optical density (OD) of cell suspensions at 540 nm (A₅₄₀) using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Then the addition of CSL was refined to 10, 11, and 12 g/L, through the analysis of each growth kinetics curve. The methods of cultivation and OD measurement were the same as above.

2.4.2. Optimization of the Type and Amount of Precursor Amino Acids in the Medium

Three precursor amino acids including Thr, Met, and Nle were added to the basic liquid medium, at final concentrations of 2.5, 5, 10, or 20 g/L, respectively. At this point, the concentration of CSL in the liquid medium was the optimal level after CSL optimization. The OD value was measured using the methods described above. All experiments were performed with three independent biological replicates.

2.5. AMHA Liquid Fermentation Process Optimization

To obtain the optimal liquid fermentation conditions for NAU002-4 producing AMHA, four technical parameters including initial pH, inoculation volume, stirring speed, and aeration rate of the liquid fermentation process in a 5 L fermenter (BioFlo115, Eppendorf, Hamburg, Germany) were further optimized. A four-factor, three-level orthogonal experiment $L_9(3^4)$ was designed to investigate these parameters (Table 1) and screen out the best fermentation conditions of AMHA.

Table 1. Design of $L_9(3^4)$ orthogonal experiment factors.

Levels	Factors			
	Initial pH	Inoculation Volume (%)	Stirring Speed (rpm)	Aeration Rate (L/h)
1	6.8	2	180	250
2	7.0	4	200	300
3	7.2	6	220	350

2.6. Isolation and Extraction of AMHA

The NAU002-4 fermentation broth was separated by centrifugation at 8000 rpm for 10 min. Activated carbon (200–300 mesh) was added to the supernatant and heated in a boiling water bath to adsorb the pigments. The treated supernatant was adjusted to pH 2 with 2 M HCl. The supernatant was concentrated in a rotary evaporator (EYELA, Tokyo, Japan). The evaporation was stopped when 50–60% of the water had been evaporated. The remaining liquid was then subjected to vacuum filtration to obtain a clear filtrate.

Ion exchange chromatography (IEC) was used to purify AMHA from the fermentation broth. Appropriate amount of 732 cation exchange resin (Na^+ form) was soaked in deionized water for 1–2 h, and then soaked in 3% hydrochloric acid solution for 3–4 h. After that, the resin was rinsed several times with deionized water. The resin was mixed in an appropriate amount of deionized water and poured into the chromatographic column. At least 2 cm of deionized water was retained in the upper layer of the resin. Methods for cation exchange resin activation and column packing were based on those described by Ding, W [30] and Nesterenko, E.P [31]. The fermentation broth was eluted with 0.25%, 0.5%, 0.75%, and 1% ammonia water (*w/w*), respectively. AMHA was identified by filter paper chromatography based on the ninhydrin color reaction principle. The reference standard was a chemically synthesized AMHA that has been structurally confirmed and the purity over 99%. The mobile phase was prepared by mixing *n*-butanol:acetic acid:water (4:1:2, *v/v/v*) with a 0.5% ninhydrin *n*-butanol solution at a ratio of 3:4. Fractions containing AMHA in centrifuge tubes were collected according to the color reaction.

The eluate containing AMHA was evaporated to dryness to obtain the solid. The structure of the compound was analyzed using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, and its purity was determined by HPLC. MS detection was performed according to the method described by Wang et al. [22].

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-400 spectrometer (Bruker, Zuerich, Switzerland). Me_4Si was used for the internal standard. Coupling constants (*J*) are given in Hz. NMR data were analyzed using MestReNova 11.0 software (Mestrelab Research SL, Santiago de Compostela, Spain). The NMR hydrogen spectrum was characterized in the following manner: ^1H NMR (400 MHz, Deuterium Oxide) δ 3.86 (d, *J* = 15 Hz, 1H, CHNH), 2.13–1.94 (m, 1H, CH_3CH), 1.25–1.19 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.12–1.10 (m, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 0.84 (d, *J* = 10 Hz, 3H, CH_3CH), 0.69 (t, *J* = 20 Hz, 3H, CH_3CH_2). The NMR carbon spectrum was characterized in the following

manner: ^{13}C NMR (126 MHz, Deuterium Oxide) δ 172.16 (COOH), 57.67 (CHNH₂), 41.30 (CH₃CH), 33.55 (CH₃CH₂CH₂), 19.61 (CH₃CH₂CH₂), 14.20 (CH₃CH), 13.32 (CH₃CH₂CH₂).

2.7. Treatment of Tea Plant with Reagents

The experiment included three treatments: AMHA (isolated from the fermentation broth of NAU002-4), a blank control (water) and Isabion (a positive control, obtained from Syngenta Group Corporation). Each experimental field plot was 15 m² (30 m × 0.5 m) and included a single row of tea plants, distributed as a randomized block design with three replicates. Each plot contained approximately 100 tea plants. The amount of medicine applied in each plot was calculated according to the amount of 100 mL medicine solution watered per tea plant, and the water consumption was 10 L. A sprinkling kettle was used to evenly water the medicinal solution to each tea plant, and the amount of medicinal solution applied to each tea plant was approximately equal. The first spray was applied on 12 March 2023, and then at 14-day intervals for a total of 3 times. The first application was followed by two additional applications at 14-day intervals, resulting in three applications in total. On the 90th day after the final application, the following parameters were measured: stem diameter, branch number, plant height, and root fresh weight. Plant phenotypes were documented photographically. Additionally, the intact root systems of 2–3 tea plants per treatment were carefully washed, scanned using a root scanner, and their fresh weight was measured. On the 15th and 45th days after the first application, the first leaf below the topmost bud (one bud and one leaf) was sampled from 20 seedlings per treatment per time point. The chlorophyll fluorescence kinetics OJIP curves and chlorophyll fluorescence parameters of the tea plant leaves were measured by Handy-PEA, a plant efficiency meter.

2.8. Determination of Fast Chlorophyll Fluorescence Rise Kinetics and JIP-Test Analysis

On days 15 and 45 after the first treatment with AMHA, Isabion or the control, twenty representative samples of one bud and the first leaf (1B1L) from new shoots were collected from field-grown tea plants in each plot. Before chlorophyll fluorescence measurement, the collected leaves were dark-adapted for at least 30 min at approximately 28 °C. Chl *a* fluorescence rise kinetics OJIP curves of the first fully expanded leaves of all samples were induced by 1 s pulse of red light (650 nm, 3500 μmol (photons) m⁻² s⁻¹) using a Plant Efficiency Analyzer (Handy-PEA fluorometer, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK). The raw fluorescence data were collected by the Handy-PEA v1.30 software and analyzed using Biolyzer 4HP v3.0 software according to the JIP-test [25,32].

The following experimental data from the original measurements were used [32–34]: F_0 , the initial fluorescence at 20 μs when all photosystem II (PSII) reaction centers (RCs) are open (at $t = 0$); F_J , and F_I , the fluorescence intensity at 2 ms (J-step), and 30 ms (I-step); F_M , the maximal fluorescence when all PSII RCs are closed, which is equal to F_P . Absorption (ABS) energy flux per excited leaf cross-section (CS_M), trapped (TR_0) energy flux per CS_M , electron transport (ET_0) energy flux per CS_M and density of PSII Q_A -reducing reaction centers (RCs) per CS_M is defined as ABS/CS_M , TR_0/CS_M , ET_0/CS_M and RC/CS_M , respectively [25,33]. The maximum quantum yield of primary photochemistry (at $t = 0$), TR_0/ABS , is denoted as ϕ_{P_0} . ϕ_{E_0} expresses the quantum yield of electron transport (at $t = 0$), which is given as $\phi_{E_0} = \text{ET}_0/\text{ABS}$. Ψ_{E_0} represents to the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- . PI_{ABS} , a performance index based on the absorption of light energy, is an indicator of the overall photosynthetic activity of PSII, and expressed as: $\text{PI}_{\text{ABS}} = \gamma_{\text{RC}}/(1 - \gamma_{\text{RC}}) \cdot \phi_{P_0}/(1 - \phi_{P_0}) \cdot \Psi_{E_0}/(1 - \Psi_{E_0})$, here γ_{RC} refers to the fraction of reaction center Chl molecules (Chl_{RC}) compared to the total Chl content ($\text{Chl}_{\text{RC}} + \text{Chl}_{\text{antenna}}$). ϕ_{R_0} is used to assess the quantum yield of electron acceptor reduction at the terminal side of PSI acceptor in plants. Performance index for

energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors is defined as $PI_{TOT} = PI_{ABS} \cdot \delta_{Ro} / (1 - \delta_{Ro})$ [24,32–34].

2.9. Data Processing and Statistical Analyses

Statistical analysis of the experimental data was performed using Handy-PEA v. 1.30 software, BiolyzerHP3 v. 5.3 software, and IBM SPSS Statistics 27 software. One-way analysis of variance (ANOVA) and Duncan's multiple comparison test were employed to analyze the significance of differences among different treatment groups ($p < 0.05$). GraphPad Prism 8 software, Microsoft Excel 2016 software, and the Figdraw website were used for the creation of figures and tables.

3. Results

3.1. Strain NAU002-4 Produced AMHA by Fermentation

Previous studies have demonstrated that some bacteria can produce AMHA when cultured in media supplemented with precursor amino acids [23,27,28]. To determine whether NAU002 could produce AMHA when supplemented with the precursor, its fermentation broth and AMHA standard were analyzed by HPLC. The chromatogram for the AMHA standard showed a distinct peak at a retention time of 1.01 min (Figure 1A). Correspondingly, a peak was observed at 1.07 min in the fermentation broth extract, which matched the retention time of the standard, confirming that the fermentation product was AMHA.

Subsequently, a calibration curve of the peak area versus standard concentration was plotted to quantify the AMHA content in different NAU002 strains (Figure 1B). To compare the AMHA production capabilities of different NAU002 strains, five wild-type NAU002 strains were cultured using the streak plate method. However, these strains exhibited considerable variation in colony color under identical medium and conditions, suggesting potentially different metabolic outputs (Figure 1C). HPLC analysis confirmed that all five strains could produce AMHA upon the exogenous addition of precursor amino acids. The AMHA production capacity of the strains from high to low was NAU002-4, NAU002-5, NAU002-1, NAU002-2 and NAU002-3 (Figure 1D). Among the five precursors tested, the AMHA yields for NAU002-4 were 420 mg/L, 422 mg/L, 448 mg/L, and 544 mg/L in media supplemented with Nva and Leu, Thr, Met, and Nle, respectively. Based on these results, NAU002-4 was identified as the optimal strain for fermentation.

3.2. CSL and Threonine Were Identified as the Optimal Nitrogen Source and Precursor Amino Acid in NAU002-4 Medium

In the fermentation industry, CSL serves as a high-quality, cost-effective, and widely available nitrogen source, providing essential nutrients such as amino acids, proteins, vitamins, and minerals for microbial growth. CSL is rich in amino acids, small peptides, and ammonium salts, which are forms of nitrogen sources preferentially utilized by microorganisms without additional hydrolysis or transformation, saving energy. The amino nitrogen in corn pulp provides the direct raw material for protein synthesis and cell proliferation of bacteria [35–38]. To promote the growth of strain NAU002-4 while reducing medium costs, the CSL concentration in the culture medium was optimized. Bacterial biomass of NAU002-4 was monitored by measuring the OD value (Figure 2A). CSL was necessary for growth, as evidenced by an OD value close to zero in its absence. Over three days, OD values increased in a concentration-dependent manner with rising CSL levels. The OD value of the 7 g/L CSL treatment group increased by 100% compared to the 3 g/L treatment group. However, the OD values of the 14 g/L and 21 g/L CSL treatment groups only increased by 4% and 15% respectively compared to the 7 g/L treatment group. Given

that the tested concentration range (0–21 g/L) was broad, we hypothesized that a finer gradient might reveal clearer differences. Therefore, a narrower gradient of 7, 10, 11, 12 and 14 g/L CSL was examined (Figure 2B). The results showed that the OD value increased significantly in the 10 g/L CSL group compared to the 7 g/L CSL group. However, there was no significant difference between 10 and 14 g/L CSL treatments. Thus, 10 g/L was determined to be the most appropriate CSL concentration for the culture medium.

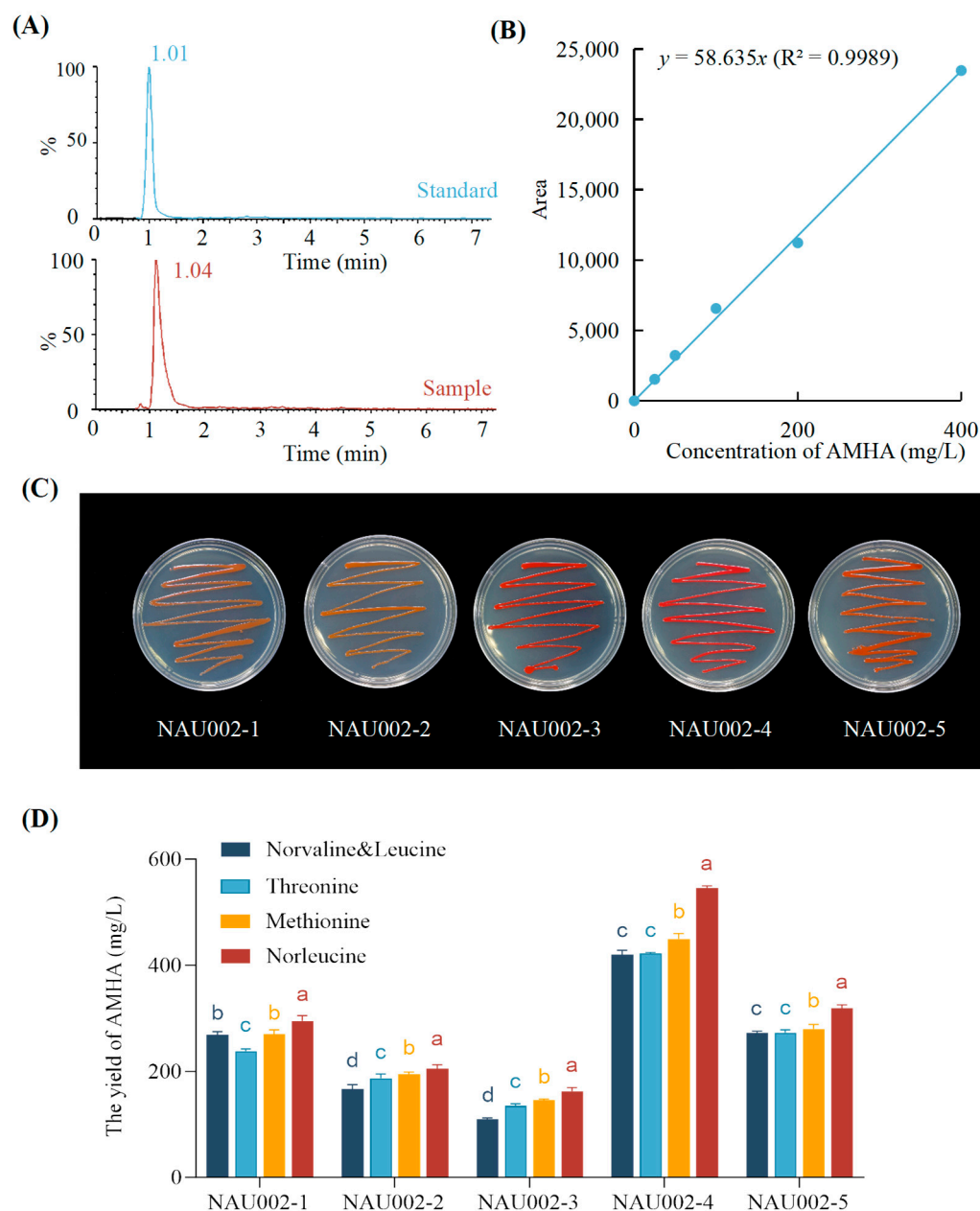


Figure 1. Results of the screening for the optimal fermentation strain: (A) HPLC chromatogram of AMHA standard (above) and AMHA sample (below); (B) Standard curve of AMHA: the abscissa is the AMHA content (25, 50, 100, 200, and 400 mg/L), and measured by HPLC; (C) Agar plate images of five strains of NAU002, from left to right is NAU002-1, NAU002-2, NAU002-3, NAU002-4, and NAU002-5; (D) AMHA production by five strains of NAU002 after fermentation with different precursor amino acids (Norvaline&Leucine, Threonine, Methionine, and Norleucine). The letters a, b, c, and d indicate significant differences ($p < 0.05$).

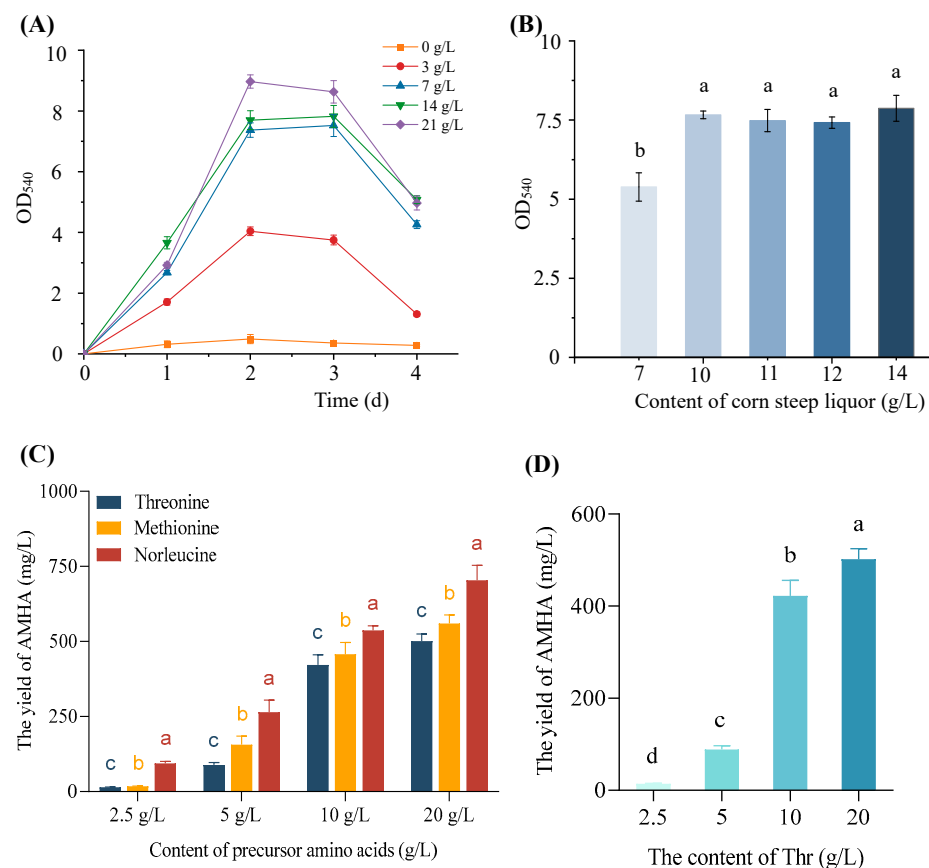


Figure 2. Results of the screening for culture medium components: (A) Effects of different concentrations of CSL in the medium on the growth of NAU002-4; (B) Effects of optimized CSL concentration on the growth of NAU002-4; (C) Optimization results of precursor types and addition levels in the culture medium; (D) Effects of different concentrations of Thr in the medium on the yield of AMHA. Lowercase letters indicate significant differences ($p < 0.05$).

Since the addition of precursor amino acids in the medium is a key factor in the AMHA production by NAU002-4 [27], AMHA content was measured on the third day of fermentation (Figure 2C). Significant differences in AMHA yield were observed among the four precursor treatments (Figure 2C). Specifically, AMHA yield increased with the concentration of Thr, Met, and Nle. Nle resulted in the highest AMHA production, followed by Met and Thr. When the precursor concentration was raised from 5 g/L to 10 g/L, the AMHA yield increased by 376%, 192%, and 103% for Thr, Met, and Nle, respectively. However, with a further increase from 10 g/L to 20 g/L, the AMHA yield only increased by 19%, 22%, and 32%, respectively. So, when the amount of precursor amino acid added was 10 g/L, the yield increase of AMHA was greater and it was the more appropriate concentration. In addition, the yield of AMHA in the Nle treatment group was 27% higher than that in the Thr treatment group, and the yield of AMHA in the Met treatment group was 8% higher than that in the Thr treatment group when the precursor addition was 10 g/L. However, the prices of three amino acids are different that the market prices of Nle and Met are substantially higher than Thr. Although the AMHA yield of 20 g/L Thr treatment was higher than that of 10 g/L treatment, the increase was modest (Figure 2D). Considering that the market prices of Nle and Met and the cost of the Thr addition, a comprehensive cost-benefit analysis identified Thr at 10 g/L as the optimal precursor for AMHA production.

3.3. AMHA Liquid Fermentation Process Optimization

To investigate the scale-up of AMHA liquid fermentation by NAU002-4, an L9(3⁴) orthogonal experimental design was implemented. The design matrix and the corresponding results for various measured indicators are presented in Table 2. As shown, variations in the four tested parameters significantly influenced both the biomass of NAU002-4 and the AMHA yield.

Table 2. Program and results of L9(34) orthogonal experiments.

No.	pH (A)	Inoculation Volume (% B)	Stirring Speed (rpm, C)	Aeration Rate (L/h, D)	OD ₅₄₀	AMHA Content (mg/L)
1	1	1	1	1	24.08	430.63 ± 2.50
2	1	2	3	2	38.08	459.67 ± 2.09
3	1	3	2	3	41.76	467.70 ± 5.34
4	2	1	3	3	34.16	444.40 ± 5.37
5	2	2	2	1	32.88	443.75 ± 4.22
6	2	3	1	2	44.08	480.77 ± 4.22
7	3	1	2	2	33.20	440.73 ± 1.86
8	3	2	1	3	46.40	497.94 ± 3.04
9	3	3	3	1	41.12	461.07 ± 3.41

The effects of initial pH, inoculation volume, stirring speed, and aeration rate on the biomass of NAU002-4 and AMHA concentration on the third day are shown in Table 3. Based on range analysis (R), the factors influencing biomass, in descending order of significance, were: inoculation volume > aeration rate > initial pH > stirring speed. Similarly, for AMHA yield, the order of significance was: inoculation volume > aeration rate > stirring speed > initial pH. These results indicated that among the four factors, inoculation volume and aeration rate were the most critical factors affecting both NAU002-4 biomass and AMHA yield. Accordingly, the optimal conditions for maximum biomass were determined as follows: initial pH of 7.2 (A3), inoculation volume of 6% (B3), stirring speed of 180 rpm (C1), and aeration rate of 350 L/h (D3) (Table 4 and Figure 3).

Table 3. Range analysis of NAU002-4 biomass.

	A	B (%)	C (rpm)	D (L/h)
K1	103.92	91.44	114.56	98.08
K2	111.12	117.36	107.84	115.36
K3	120.72	126.96	113.36	122.32
k1	34.64	30.48	38.19	32.69
k2	37.04	39.12	35.95	38.45
k3	40.24	42.32	37.79	40.77
R	5.60	11.84	2.24	8.08
Optimal level	3	3	1	3
Factors of priority	B > D > A > C			
Optimal technical solution	A ₃ B ₃ C ₁ D ₃			

Table 4. Range analysis of AMHA yield.

	A	B (%)	C (rpm)	D (L/h)
K1	1358.00	1315.76	1409.34	1335.45
K2	1368.92	1401.36	1352.18	1381.17
K3	1399.74	1409.54	1365.14	1410.04

Table 4. Cont.

	A	B (%)	C (rpm)	D (L/h)
k1	452.67	438.59	469.78	445.15
k2	456.31	467.12	450.73	460.39
k3	466.58	469.85	455.05	470.01
R	13.91	31.26	19.05	24.86
Optimal level	3	3	1	3
Factors of priority		B > D > C > A		
Optimal technical solution		A ₃ B ₃ C ₁ D ₃		

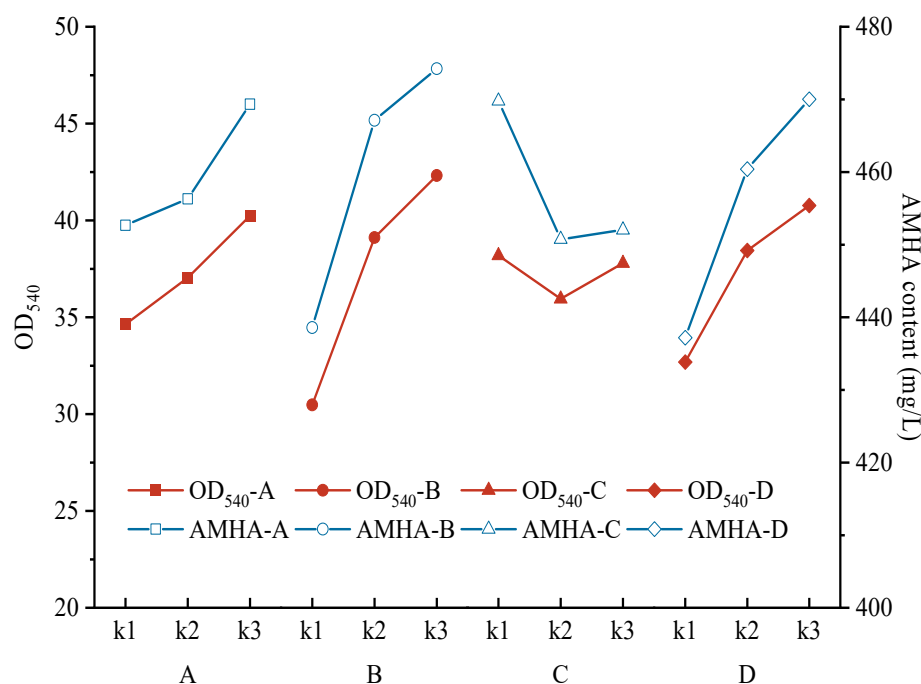


Figure 3. Correspondence between different factor levels with cell concentration as well as AMHA yield on the third day of fermentation: Correspondence between factor A levels and biomass of NAU002-4 (■); Correspondence between factor B levels and biomass of NAU002-4 (●); Correspondence between factor C levels and biomass of NAU002-4 (▲); Correspondence between factor D levels and biomass of NAU002-4 (◆); Correspondence between factor A levels and AMHA yield (□); Correspondence between factor B levels and AMHA yield (○); Correspondence between factor C levels and AMHA yield (△); Correspondence between factor D levels and AMHA yield (◇).

Validation fermentation was carried out using the optimized fermentation conditions in a 5 L fermenter, and the results are shown in Table 5, where the AMHA yield reached 504 mg/L.

Table 5. Results of verification test.

A	B (%)	C (rpm)	D (L/h)	OD ₅₄₀	AMHA Content (mg/L)
7.2	6	180	350	47.76	504.00 ± 1.31

3.4. Purification of AMHA from Fermentation Broth of NAU002-4

During the IEC separation process, ammonia water was used as the eluent to purify AMHA from the fermentation broth (Figure 4A). Specifically, elution was performed with stepwise increases in ammonia concentration from 0.25% to 1%. Initially, no components were eluted at concentrations of 0.25% and 0.5%. When the ammonia concentration was increased to 0.75%, several fractions were collected. The retardation factor (R_f) value of the

eluent was 0.68, which closely matched that of the AMHA standard ($R_f = 0.62$), indicating the presence of AMHA in all three centrifuge tubes eluted with 0.75% ammonia water. Furthermore, a small amount of AMHA was detected in the first centrifuge tube of the 1% ammonia eluent, whereas no AMHA was found in the subsequent second and third tubes, which contained only Thr. Therefore, 0.75% ammonia water was determined to be effective for eluting AMHA with minimal co-elution of other amino acids.

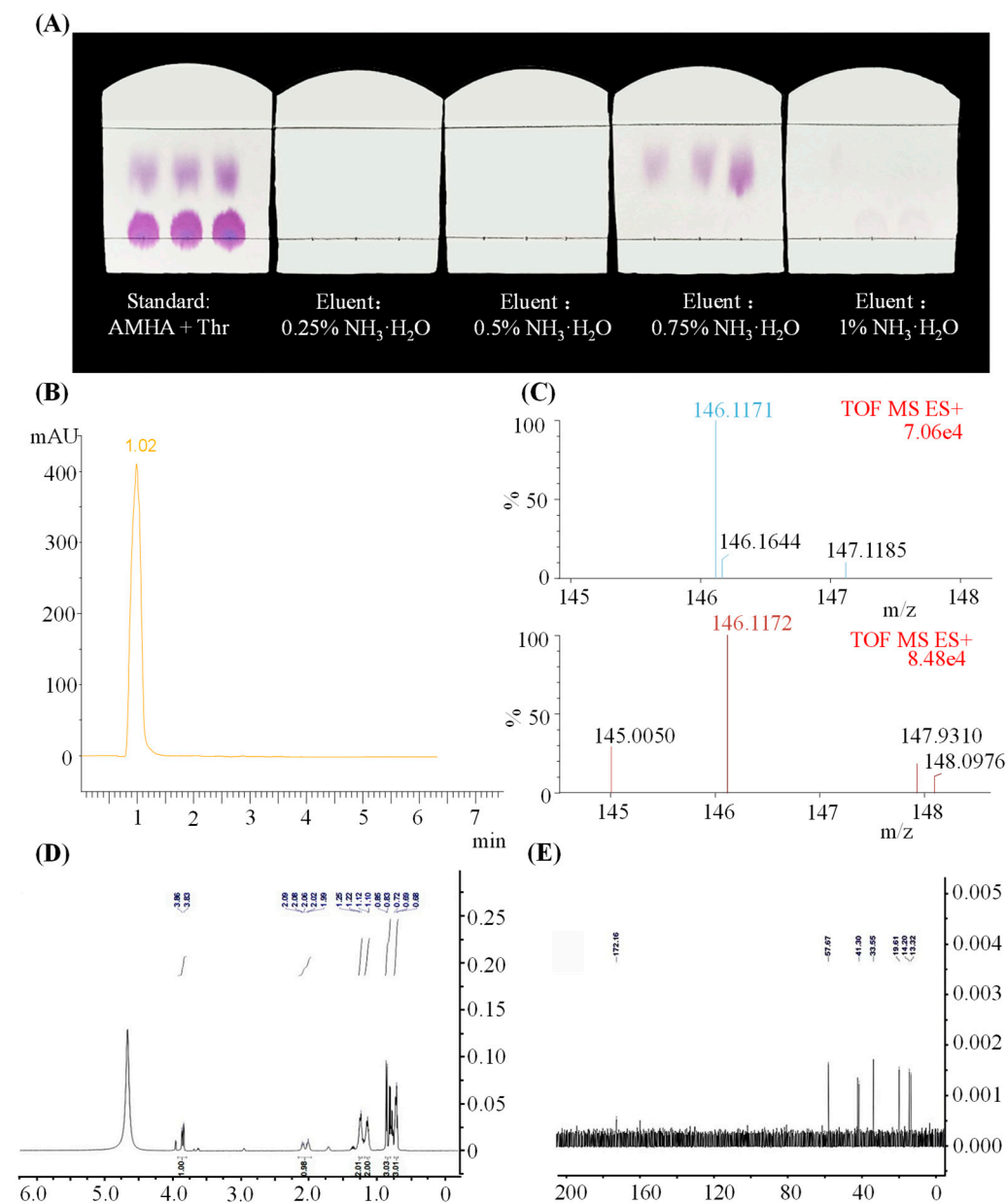


Figure 4. Purification and analysis of AMHA: (A) Filter paper chromatography analysis of the eluate after ion exchange chromatography; (B) HPLC analysis of AMHA samples after filter paper chromatography identification; (C) MS results of AMHA standard (above) and AMHA sample (below); (D) ^1H NMR analysis of the purified AMHA sample (400 MHz, Deuterium Oxide); (E) ^{13}C NMR analysis of the purified AMHA sample (125 MHz, Deuterium Oxide).

The extracted AMHA samples were analyzed by HPLC (Figure 4B). In the chromatogram, a sample peak was observed at a retention time of 0.912 min, closely matching that of the AMHA standard. Notably, the chromatogram exhibited virtually no extraneous peaks, which preliminarily confirmed both the identity and high purity of the eluted compound. To further verify its identity, the sample was subjected to mass spectrometry (MS).

As shown in Figure 4C, the AMHA standard exhibited a precise molecular ion mass of m/z 146.1171 $[M + H]^+$ in positive ion mode, while the sample displayed a corresponding peak at m/z 146.1172 $[M + H]^+$. The nearly identical molecular masses provide further confirmation that the purified compound is AMHA.

Detailed NMR analysis provided further structural confirmation. The ^1H NMR (Figure 4D) spectrum in D_2O displayed characteristic signals corresponding to two methine (CH) protons at δ_{H} 3.86 (1H, d, CHNH_2) and δ_{H} 2.13–1.94 (1H, m, CH_3CH), four methylene (CH_2) protons at δ_{H} 1.25–1.19 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$) and δ_{H} 1.12–1.10 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), six methyl (CH_3) protons at δ_{H} 0.84 (3H, d, CH_3CH) and δ_{H} 0.69 (t, 3H, CH_3CH_2) (Figure 4D). Correspondingly, the ^{13}C NMR (Figure 4E) spectrum of the compound indicated seven absorption peaks, which were assigned to a carboxyl carbon at δ_{C} 172.16 (COOH), two CH carbon atoms at δ_{C} 57.67 (CHNH_2) and δ_{C} 41.30 (CH_3CH), two CH_2 carbon atoms at δ_{C} 33.55 ($\text{CH}_3\text{CH}_2\text{CH}_2$) and δ_{C} 19.61 ($\text{CH}_3\text{CH}_2\text{CH}_2$), and two CH_3 carbon atoms at δ_{C} 14.20 (CH_3CH) and δ_{C} 13.32 ($\text{CH}_3\text{CH}_2\text{CH}_2$) (Figure 4E). The molecular structure of the isolated compound was consistent with that of AMHA standard [22], confirming its identity as AMHA. Furthermore, purity analysis determined that the AMHA sample purified from the fermentation broth had a purity of 97.1%.

3.5. AMHA Promotes the Growth of Tea Plants

Amino acids positively influence plant growth, development, and yield, as they are fundamental to the biosynthesis of proteins, pigments, vitamins, co-enzymes, purine and pyrimidine bases, and other non-protein nitrogenous compounds [39]. Previous studies have indicated that foliar application of the amino acid-based liquid fertilizer Isabion can enhance bud number, bud length, and total bud weight in tea plants, thereby effectively increasing yield [40,41]. To further examine the growth-promoting activity of AMHA, field-grown tea plants were irrigated with water (control), 100 nM AMHA, or commercial plant activator Isabion (positive control). After 90 days, the growth status of the tea plants demonstrated that AMHA significantly promoted growth compared to the control (Figure 5A,B). Treated seedlings exhibited a notable increase in branch and leaf number, along with more vigorous shoot and root development. Statistical analysis of morphological indices revealed that, compared to the control, AMHA and Isabion treatments increased stem diameter by 27.1% and 10.9%, branch number by 39.1% and 54.4%, plant height by 27.7% and 29.2%, and root fresh weight by 127.3% and 31.9%, respectively (Figure 5C–F). Collectively, these results demonstrated that AMHA significantly promotes the growth of tea plants, with its overall effect being slightly superior to that of the positive control Isabion.

3.6. Chlorophyll Fluorescence Rise Kinetics Reveals the Mechanism by AMHA Promotes Photosynthetic II Activity

To explore the mechanism by which AMHA enhances the growth of tea plants, the fast chlorophyll *a* fluorescence transient kinetic technique was employed to analyze the photosynthetic performance of leaves following AMHA treatment. The typical fast chlorophyll fluorescence induction kinetic curve, known as the OJIP curve, comprises the O, J, I, and P phases [32,33,42,43]. The OJIP curves of tea plant leaves at 15 and 45 days after the three treatments are shown in Figure 6B,C. Overall, the fluorescence values in leaves treated with AMHA and Isabion were markedly higher than those in the control. The fluorescence intensity at point P reflects the electron transfer status of PSII [32]. Notably, the point P of tea plant leaves treated with AMHA was significantly elevated, indicating that the pigment content in the leaves treated with AMHA and Isabion was higher than that in the control. After 15 days of treatment, the maximum fluorescence (F_{M}) at point P of tea plant leaves treated with AMHA and Isabion increased by 79.2% and 68.0%, respectively, compared to the control. At 45 days after treatment, the F_{M} of tea plant leaves increased by 36.2%

and 23.7%, respectively. This result indicates that AMHA can enhance the photochemical activity of PSII and promote electron transfer in PSII. Furthermore, the effect of AMHA is superior to that of Isabion.

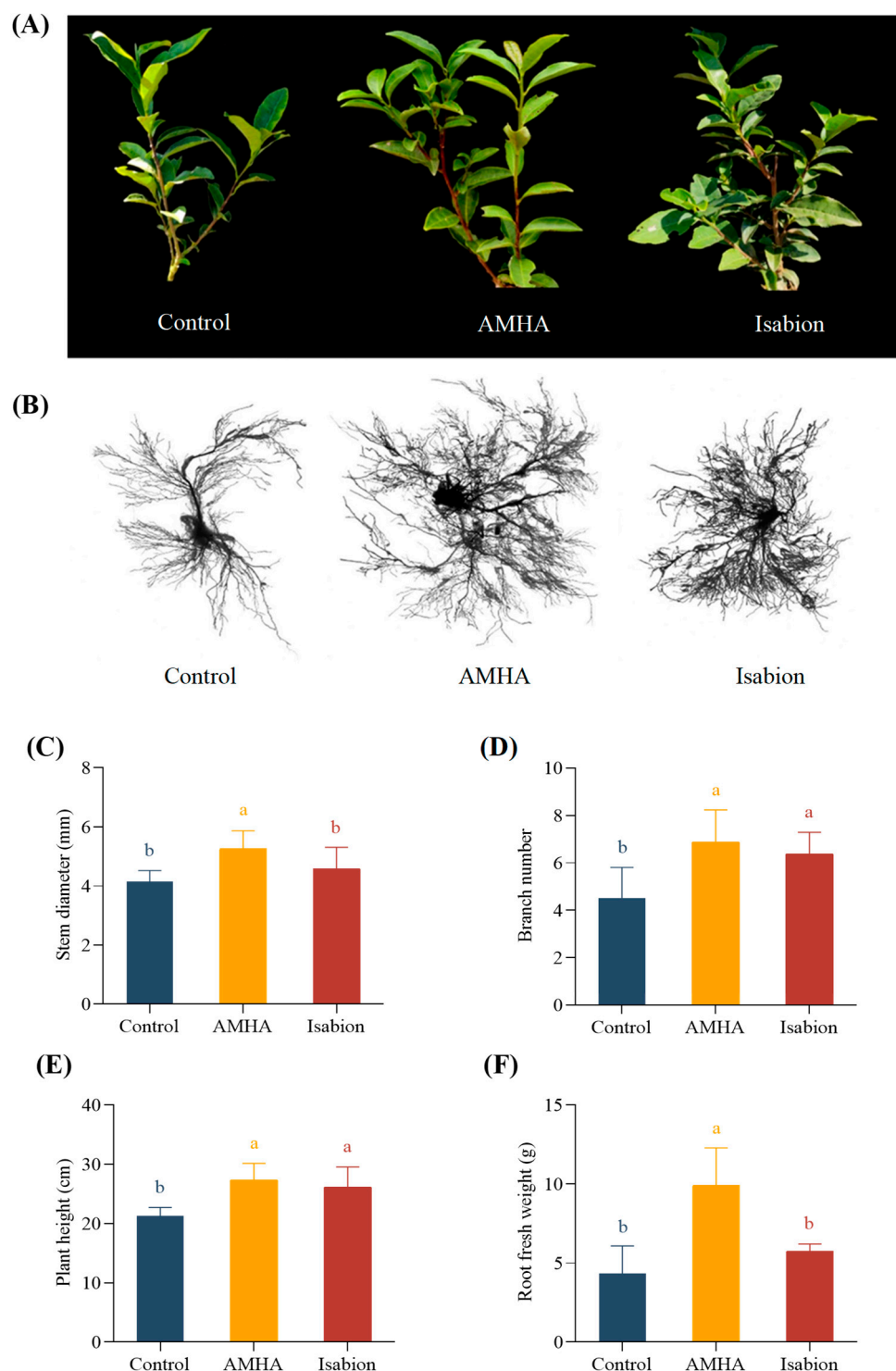


Figure 5. Effects of AMHA on the phenotype of tea plants: (A) Phenotypic photographs of the shoot parts after 90 d of the water application (Control), AMHA application (AMHA), and isabion application (Isabion); (B) Phenotypic photographs of the underground parts after 90 d of the water application (Control), AMHA application (AMHA), and isabion application (Isabion); Effects of AMHA on the physiological indices of tea plants: Stem diameter (C); Branch number (D); Plant height (E); Root fresh weight (F). Lowercase letters indicate significant differences ($p < 0.05$).

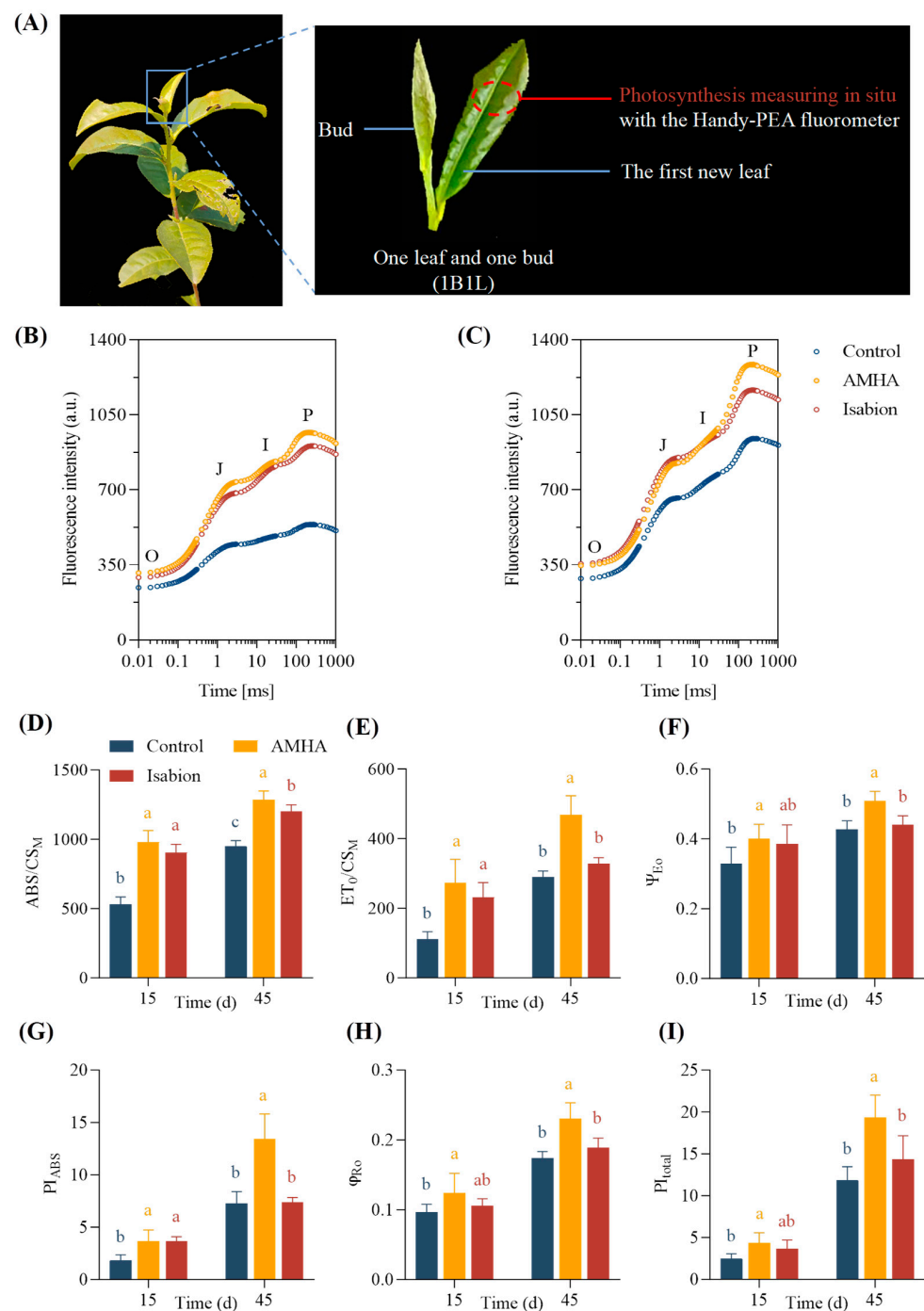


Figure 6. Effects of AMHA on the fast chlorophyll fluorescence transient kinetics in tea plant leaves: (A) The illustration showing a tea sample with one bud and one leaf (1B1L) used for experimental measurements; (B) Effect of different treatment groups on the OJIP curves of tea plant leaves treated with water, AMHA and Isabion 15 days; (C) Effect of different treatment groups on the OJIP curves of tea plant leaves treated with water, AMHA and Isabion 45 days; Fluorescence parameters of tea plant leaves treated with AMHA for 15 and 45 days: (D) The chlorophyll concentration per excited leaf cross-section (ABS/CS_M); (E) The probability that a trapped exciton moves an electron into the electron transport chain beyond QA – (ET_0/CS_M); (F) The probability that a trapped exciton moves an electron into the electron transport chain beyond QA[−] (Ψ_{E0}); (G) Performance index (PI_{ABS}) representing overall photosynthetic efficiency. The values are derived from raw data of the fluorescence rise kinetics shown in (B,C); (H) Quantum yield for reduction of the end electron acceptors at the PSI acceptor side (RE) (ϕ_{R0}); (I) Performance index for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors (PI_{total}). Lowercase letters indicate significant differences ($p < 0.05$).

Among the JIP-test parameters, ABS/CS_M represents the light energy absorbed per unit leaf area and serves as an indicator of the number of antenna pigment molecules or chlorophyll concentration. ET_0/CS_M denotes the electron transport flux per excited leaf cross-section [32,44]. Compared to the control, ABS/CS_M increased by 85.4% and 35.8% (Figure 6D), while ET_0/CS_M increased by 144.1% and 61.5% (Figure 6E), at 15 and 45 days after AMHA treatment, respectively. Ψ_{E_0} represents the probability that an electron moves further than Q_A (Figure 6F). Compared to the control, Ψ_{E_0} increased by 21.2% and 19.0% at 15 and 45 days after AMHA treatment, respectively, indicating enhanced electron transfer capacity in tea plant leaves. The ABS/CS_M , ET_0/CS_M , and Ψ_{E_0} for tea plant leaves treated with AMHA were significantly higher than those of the control, indicating that AMHA treatment enhances light absorption and electron transfer capacity within PSII in tea plant leaves. This results in increased heat dissipation energy, elevated energy flux through the reaction centers, increased number of reaction centers, and improved efficiency of PSII reaction centers in capturing light energy, ultimately promoting the activity of the electron transfer chain.

PI_{ABS} , which combines three independent parameters assessed by the OJIP fluorescence induction curve: γ_{RC} , Ψ_{E_0} , and ϕ_{P_0} , is commonly used to quantify the overall performance of PSII [32]. As shown in Figure 6G, the PI_{ABS} of leaves treated with exogenous AMHA was significantly higher than that of the control (Figure 6G), suggesting that AMHA enhanced the photochemical efficiency of PSII and promoted the activity of the electron transfer chain. In addition, ϕ_{R_0} , a parameter used to evaluate the quantum yield of PSI acceptor-side terminal electron acceptor reduction, represents electron transfer from Q_A -reducing reaction centers to the terminal PSI acceptor. The observed increase in ϕ_{R_0} of tea plant leaves treated with AMHA indicated that AMHA enhanced the quantum yield of PSI acceptor-side terminal electron acceptor reduction (Figure 6H). Furthermore, PI_{total} reflects the performance of the overall ability of electron transfer from the PSII electron donor side to PSI electron acceptor reduction, involving multiple electron transfer processes (Figure 6I). Compared to the control, PI_{total} increased significantly by 79.4% and 64.1% in tea plants treated with AMHA for 15 and 45 days, respectively. Collectively, the significant increases in ϕ_{R_0} and PI_{total} reveal that AMHA promotes electron transfer to PSI in tea seedling leaves, thereby enhancing the electron acceptance capacity of PSI.

This study demonstrated that at 15 and 45 days after treatment, key photosynthetic parameters (ABS/CS_M , ET_0/CS_M , Ψ_{E_0} , PI_{ABS} , ϕ_{R_0} , and PI_{total}) were significantly higher in tea plant leaves treated with either AMHA or Isabion compared to the control (Figure 6D–I). The values of these parameters were better than those of the Isabion treatment group. In addition, combined with the measurement results of the biomass of tea plants, all the indices of the AMHA treatment group were better than those of the control group and Isabion. These results fully proved that the application of AMHA could effectively improve the photosynthetic activity of PSII and enhance the electron transport ability of tea plants, promoted the growth of tea plants, and its activity was better than that of Isabion.

4. Discussion

Current agricultural development faces several challenges, including the high cost, high toxicity, and significant environmental risks associated with traditional chemically synthesized pesticides. Bioactive substances derived from natural products are more environmentally friendly and safer than their conventional counterparts [45–47]. They can significantly reduce the emission of toxic and hazardous substances during both production and waste disposal processes [48]. Many natural products exhibit high bioactivity and have the potential to be developed as biostimulants; however, their extremely low abundance in

natural organisms limits further exploitation. Therefore, the production of these compounds via microbial fermentation represents a direct and effective solution to these challenges.

In our study, shake-flask fermentation experiments showed that adding CSL to the culture medium significantly promoted NAU002-4 growth compared to CSL-free media. This observation is consistent with previous reports on the effectiveness of CSL. For example, Wang et al. [49] used CSL as a carbon source for laccase production by *Trametes versicolor*, achieving a 96.3% increase in yield compared to the control. Similarly, Liu et al. [50] reported that CSL increased the bacterial biomass, stimulated substrate uptake and citric acid (CA) synthesis rates, and ultimately raised the CA yield by 1.24-fold when it replaced yeast extract. CSL is rich in essential nutrients such as vitamins, minerals, amino acids, and growth stimulants, making it a valuable nutritional and functional supplement in fermentation processes [51]. Therefore, this study focused on optimizing the CSL concentration in the fermentation medium to promote bacterial growth while reducing costs.

During the screening of precursor type and concentrations for the fermentation medium, the AMHA yield reached to maximum when the concentration of Nle, Met and Thr was 10 g/L. The AMHA yields under the four precursor amino acid concentrations varied clearly, and there were also significant differences within each group. The research results indicated that when the precursor addition amount was 10 g/L, the AMHA yield of the all three precursor amino acid treatments increased the most compared to the previous concentration treatment. When the precursor addition amount was increased to 20 g/L, the AMHA yield still increased, but the increase was smaller compared to the previous concentration treatment group. This suggests that the optimal addition amount of precursor substances is 10 g/L. If a new type of biostimulant is to be put into commercial application, the production cost is an important factor. The market prices of three amino acids differed substantially. Met was twice as expensive as Thr, while Nle was 4.3 times the price of Thr. Considering both the yield and cost, the addition of 10 g/L Thr was determined to provide the highest cost-effectiveness for large-scale AMHA production.

Precursors play a crucial role in the biosynthesis of target products. The production of AMHA via precursor amino acids is closely tied to their structure and metabolic pathways. Preliminary studies indicate that AMHA is structurally similar to isoleucine (Ile), differing only by one additional carbon atom in its main chain [23]. This similarity suggests a potential biosynthetic pathway for AMHA analogous to that of Ile [23]. Since the synthesis of Ile, a typical branched-chain amino acid, requires Thr as a precursor [52], Thr was selected as one of the precursor amino acids in this study. Furthermore, compared to AMHA, Nle contains only an extra methyl group at the β -position, indicating that Nle could also serve as a precursor in the AMHA synthesis pathway. Met, under the action of adenosyltransferase, can accept an adenosine group from ATP to form S-adenosylmethionine (SAM), which participates in widespread methylation reactions [53]. Therefore, it is hypothesized that Met may donate a methyl group to the β -position of the AMHA precursor backbone via methylation to generate AMHA. Experimental results confirmed that the addition of these precursor amino acids, after optimization of their concentrations, significantly increased the yield of AMHA. The enhanced AMHA production observed upon supplementation with Thr is consistent with the biosynthetic pathway elucidated by Sugiura et al. [23], in which AMHA is derived from Nva via the isoleucine-valine biosynthetic enzymes. However, direct evidence for this pathway in strain NAU002-4, such as enzyme activity assays, gene knockouts, or isotope labeling remain to be confirmed in future work.

During the development of fermentation processes, optimizing fermentation conditions by screening various factor combinations can significantly enhance microbial growth, viability, and the overall yield of the target product [54]. Moreover, microbial metabolic mechanisms are highly complex and sensitive to the culture environment, and subtle envi-

ronment changes can significantly impact the metabolic process and outcomes [55–57]. In this study, we investigated four key parameters, including initial pH, inoculation volume, stirring speed, and aeration rate in the liquid industrial fermentation of NAU002-4. The aim was to optimize the fermentation conditions for AMHA production and thereby identify the optimal combination of technical parameters [58–60]. The results demonstrated that the inoculation volume and aeration rate significantly affected both bacterial concentration and AMHA yield, followed by stirring speed and initial pH. The substantial impact of inoculation volume on NAU002-4 fermentation is likely attributable to its role in establishing the initial microbial population. The larger inoculation volume provided a larger microbial base and faster microbial growth. Aeration rate also had a significant impact on the fermentation process.

Aeration rate also markedly influenced the fermentation process. As a facultative aerobe, strain NAU002-4 required ample oxygen for growth. An increased aeration rate supplied sufficient oxygen and enhanced the mixing and mass transfer rates within the fermentation medium. Zhou et al. [61] found that at a low stirring speed, increasing the aeration rate could promote vigorous microbial growth. Therefore, increasing the aeration rate within a certain range likely stimulated the growth and reproduction of NAU002-4, consequently promoting AMHA production and accumulation in the later fermentation stages [62].

Several studies indicated that impellers in the fermenter generated shear forces during stirring, and excessive shear forces can weaken bacterial growth [63]. Thus, maintaining an appropriate stirring speed was crucial for ensuring the optimal growth of NAU002-4 to achieve higher AMHA yields. Regarding initial pH, its minimal impact on the results could be because the pH gradient tested in this experiment was narrow, and the medium contained phosphate salts that provided strong buffering capacity against pH changes. Furthermore, bacteria typically possess a certain tolerance range for environmental pH, so the initial pH had minimal influence on fermentation.

Regarding the downstream process, when using IEC to purify AMHA from the fermentation broth, the flow rate of the material was required to be low due to the slow diffusion of amino acid ions in the resin, which led to a long filtration time and reduced the overall separation efficiency. Moreover, the complexity of manufacturing the ion exchange resin, the high consumption of regeneration agent, and the overall high cost are constraints in the current production. Therefore, future work could consider optimizing the purification methods for AMHA, such as using extraction technology or membrane filtration technology, to shorten the production cycle and improve downstream separation efficiency.

In plants, over 90% of organic matter is produced through photosynthesis, which is the foundation of plant growth. Enhanced photosynthetic efficiency is key to promoting crop growth and improving yield and quality [64]. In our experiments, the fluorescence curves of tea plant leaves at different time points after AMHA treatment showed a significant increase in overall fluorescence values compared to the control. The pronounced rise in the fluorescence value at point P indicates that the AMHA application promoted electron transfer in the electron transport chain.

Analysis of the JIP-test parameters revealed that both ABS/CS_M and ET_0/CS_M in tea plant leaves treated with AMHA were significantly higher than in the control. This suggested that AMHA optimizes the structure of the antenna complexes and the efficiency of PSII reaction centers in capturing light energy, thereby increasing electron transport chain activity. The elevated values of Ψ_{E_0} and PI_{ABS} suggest that AMHA increased the number of active PSII reaction centers, thereby significantly improving the overall photosynthetic efficiency of PSII. Furthermore, the significant increases in ϕ_{R_0} and PI_{total} indicate that AMHA promoted electron transfer to PSI, thereby enhancing PSI photochemical efficiency.

Taken together, these results demonstrate that AMHA improves the photochemical efficiency of both PSII and PSI, reflected in enhanced electron transport capacity throughout the photosynthetic chain. In addition, as reported in Ji et al. [26], we have conducted field trials and toxicity studies on AMHA application. The results demonstrate that AMHA exhibits low toxicity and poses minimal long-term environmental risk, supporting its safety for agricultural use.

5. Conclusions

In this study, the AMHA high-yielding strain NAU002-4 was screened from five wild-type strains of bacteria NAU002. Through single-factor experiments and orthogonal experiments, the optimal conditions for NAU002-4 to ferment and synthesize AMHA were confirmed to be: initial pH = 7.2, inoculation volume of 6%, stirring speed of 180 rpm, aeration rate of 350 L/h, optimal nitrogen source of 10 g/L corn steep liquor, and optimal precursor amino acid of 10 g/L threonine. Under these conditions, the AMHA concentration in the fermentation broth reached 504 mg/L. Moreover, we found that 0.75% ammonia water could effectively separate AMHA from other impurities, achieving a purity of 97.1%. Additionally, field trials revealed that treatment with AMHA could enhance photosynthesis in tea plant leaves, thereby promoting the growth of tea plants and confirming the growth-promoting activity of AMHA.

In conclusion, this study innovatively established a small-scale process for AMHA production based on the NAU002-4. The key fermentation parameters were optimized, and the plant growth-promoting activity of AMHA was demonstrated. This work provides a theoretical basis and technical support for the future practical application and production of AMHA.

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Conflicts of Interest: The authors declare no competing financial interest.

Abbreviations

The following abbreviations are used in this manuscript:

AMHA	2-Amino-3-methylhexanoic acid
HPLC	High-performance liquid chromatography
IEC	Ion exchange chromatography
CSL	Corn steep liquor
Thr	Threonine
Met	Methionine
Nle	Norleucine
Nva	Norvaline
Leu	Leucine

OD	Optical Density
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
1B1L	One leaf and one bud

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