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RESEARCH ARTICLE

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Production of 5-aminolevulinic acid from hydrolysates of cassava residue and fish waste by engineered *Bacillus cereus* PT1

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Abstract

The economical production of 5-aminolevulinic acid (ALA) has recently received increasing attention for its extensive use in agriculture. In this study, a strain of *Bacillus cereus* PT1 could initially produce ALA at a titre of 251.72 mg/L by using a hydrolysate mixture of low-cost cassava residue and fish waste. The integration of endogenous *hemA* encoding glutamyl-tRNA reductase led to a 39.30% increase in ALA production. Moreover, improving cell permeability by deletion of the LytR-CpsA-Psr (LCP) family gene *tagU* led to a further increase of 59.73% in ALA production. Finally, the engineered strain *B. cereus* PT1-*hemA*-Δ*tagU* produced 2.62 g/L of ALA from the previously mentioned hydrolysate mixture in a 7-L bioreactor. In a pot experiment, foliar spray of the ALA produced by *B. cereus* PT1-*hemA*-Δ*tagU* from the hydrolysates increased salt tolerance of cucumber by improving chlorophyll content and catalase activity, while decreasing malondialdehyde content. Overall, this study demonstrated an economic way to produce ALA using a microbial platform and evidenced the potential of ALA in agricultural application.

INTRODUCTION

As a non-proteinogenic five-carbon amino acid and an essential intermediate in the synthesis of various tetrapyrroles, including heme, chlorophylls and vitamin B_{12}, 5-aminolevulinic acid (ALA) has a great application potential in agriculture and medicine (Kang et al., 2012). In agriculture, ALA has been proved as a novel plant growth regulator in enhancing plant resistance against numerous abiotic stresses (Phour et al., 2018) by improving photosynthesis activities (Liu et al., 2013) and stimulating antioxidant systems to scavenge malondialdehyde (MDA) in plants (Rhaman et al., 2021). ALA could also improve the growth, yield and quality of plants (Wu et al., 2019). The global market size of ALA might be expanded from US$ 96 million in 2020 to US$ 118 million by 2026 (https://www.mrinsights.biz/report/global-5-aminolevulinic-acid-hydrochloride-market-growth-2021-2026).

Currently, the commercial production of ALA relies on chemical synthesis and the complex synthesis reactions and low production efficiency led to the high price of ALA (Kang et al., 2017; Su et al., 2021). Microbial production of ALA is an alternative to chemical synthesis

and might have advantages in economical practicability and sustainability (Kang et al., 2012). In microorganisms, ALA can be synthesized via two natural pathways, including the C4 pathway in purple non-sulphur photosynthetic bacteria (*Rhodobacter sphaeroides*) (Kang et al., 2017), and the C5 pathway in many bacteria, such as *Escherichia coli* (Li et al., 1989) and *Salmonella typhimurium* (Wang et al., 1999). Previous studies had mainly focused on increasing ALA production efficiency by engineering *E. coli* and *Corynebacterium glutamicum* strains (Su et al., 2021). One bioengineering strategy was the heterologous expression of *alaS* gene (encoding ALA synthase) from *R. sphaeroides* into *E. coli* (Zhu et al., 2019) or *C. glutamicum* (Feng et al., 2016; Yang et al., 2016) to produce ALA via C4 pathway. Another strategy was to co-express *hemA* (from *S. typhimurium*, encoding glutamyl-tRNA reductase) and *hemL* (from *E. coli*, encoding glutamate-1-semialdehyde aminotransferase) genes in *E. coli* (Cui et al., 2019; Zhang et al., 2019) or *C. glutamicum* (Zhang et al., 2020) to produce ALA via C5 pathway.

To date, glucose as a carbon source (Su et al., 2021), and tryptone, yeast extract (Yu et al., 2015) or ammonium sulphate (Zhang et al., 2019) as nitrogen sources had been used in almost all the reported ALA production procedures, in which addition of inducers and antibiotics were also required for the engineering of strains. The high cost of these procedures limited ALA microbial production and application (Chen et al., 2020). Large amounts of waste biomass, such as cassava residue (CR) (Wang et al., 2012) and fish waste (FW) (Huo et al., 2010), are produced annually in China, which could be cheap resources for microbial synthesis of value-added products such as ALA (Yu et al., 2018), since the acid hydrolysates of CR and FW are rich in glucose (Cai et al., 2012), amino acids and growth factors (Samat et al., 2018).

Characterized by fast growth and good environmental compatibility (Xiang et al., 2020), *Bacillus* strains had been used as chassis cells for the production of poly-3-hydroxybutyrate (Rami Reddy Tadi et al., 2021), lactic acid (Wan et al., 2014) and 2,3-butanediol (Białkowska et al., 2015), but not yet for ALA. The thick peptidoglycan layer in the cell wall of Gram-positive bacteria, like *Bacillus* might have limited the release of metabolites from cells. Therefore, the weakening of peptidoglycan synthesis in *Bacillus subtilis* could enhance cell permeability and improve yield of some products (Laneelle et al., 2013), such as recombinant enzymes (Yadav et al., 2020) and poly-γ-glutamic acid (GABA) (Li et al., 2021). The LytR-CpsA-Psr (LCP) protein family is a key factor influencing transmembrane transport in Gram-positive bacteria (Li et al., 2020b). In *Bacillus* species, TagT, TagU and TagV proteins belonging to the LCP family are wall teichoic acid (WTA) transferases (Kawai et al., 2011). It was reported that a triple disruption of the corresponding genes *tagTUV* arrested cell growth, while a single mutation of *tagT*, *tagU* or *tagV* only changed the structure of cell walls but had indiscernible effects on cell growth (Kawai et al., 2011). However, it is not clear whether the cell wall structure changes caused by these single mutants could affect the efflux of metabolites.

Previously, a strain *Bacillus cereus* PT1 isolated from the rhizosphere of *Portulaca oleracea* L. generated 50 mg/L of ALA via C5 pathway using glucose as substrate (our unpublished data). To achieve economical production of ALA, this study was performed using *Bacillus cereus* PT1 and with biomass-based substrates as carbon and nitrogen sources. Firstly, ALA production from hydrolysates of cassava residue (CR) and fish waste (FW) was tested and the medium components were optimized. Secondly, an increased ALA production from the biomass hydrolysates was achieved by endogenous *hemA*-integrated overexpression. Thirdly, the LCP family gene *tagU* was deleted in the *hemA*-integrated mutant to further increase ALA production by improving cell wall permeability. Finally, the plant growth promoting (PGP) potential of ALA produced from the mixture of CR and FW acid hydrolysates was verified by a pot experiment. The results evidenced the possibility of using low-cost agricultural waste to produce ALA with PGP function.

EXPERIMENTAL PROCEDURES

Bacterial strains, biomass materials and bacterial cultivation

The parent strain *B. cereus* PT1 originated from rhizosphere of *Portulaca oleracea* had been stocked in the China General Microbiological Collection Center in Beijing under the accession number of CGMCC 16179. Its mutants of PT1-*hemA*, PT1-*hemA*-*hemL*, PT1-Δ*tagU* and PT1-*hemA*-Δ*tagU* were constructed. Cassava residue (CR) was donated by the Beijing Starch Factory, China, while the fresh fish waste (FW) was bought from a local aquaculture farm, minced using a blender and stored at –20°C until further use.

Escherichia coli DH5α was cultivated in Luria-Bertani (LB) medium. *B. cereus* PT1 and its derives were cultivated in Tryptic Soy Broth (TSB) medium. Antibiotics ampicillin and erythromycin for marker selection were added at a respective concentration of 100 μg/ml and 10 μg/ml. *B. cereus* strains were pre-cultured overnight in TSB to obtain seed cultures from a single colony. Aliquots of 0.3 ml seed cultures were inoculated into 300 ml shake-flasks containing 30 ml of basal medium (Glucose 5.0 g; Tryptone 5.0 g; Yeast extract 2.5 g; 50 mM of acetic acid–sodium acetate buffer, in 1 L of distilled water; pH 7.3 ± 0.2) with cultivation at 37°C with shaking (200 rpm).

Preparation of CR and FW hydrolysates

CR was treated with the acid hydrolysis method under the described protocol (Liu et al., 2016) and freeze-dried for subsequent use as the carbon source (CRH) to produce ALA. Minced FW was thawed overnight at 4°C, diluted with distilled water (1:3 v/v), heated at 121°C for 60 min and centrifuged (6000 × *g*) for 20 min. The supernatant was collected to obtain the soluble peptones A. The residual solid fraction collected after centrifugation was hydrolysed again with diluted sulphuric acid (pH 4.0) at 121°C for 20 min (solid–liquid ratio, 1:3), and the supernatant was collected by using the above-mentioned method to obtain the soluble peptones B. Finally, soluble peptones A and B were mixed and freeze-dried as FWH for further experiments.

Optimization of the medium for ALA production

Effects of carbon sources on ALA production of *B. cereus* PT1 were studied in 30 ml of modified basal medium, in which glucose, maltose, glycerol, sucrose or lactose were added separately with a final concentration of 10 g/L. The effects of nitrogen sources were also evaluated in the basal medium and the modified basal medium, in which tryptone, beef extract, ammonium sulphate, and urea with a final concentration of 10 g/L were used separately as nitrogen source; meanwhile, 10 g/L of glucose were added as the optimized carbon source and yeast extract was increased to 5 g/L. The inoculation and incubation conditions were the same as mentioned above. Aliquots (0.5 ml) of the culture were sampled at 6, 12, 18 and 24 h to evaluate the ALA production with the modified Ehrlich's reagent (Yu et al., 2020). To lower the cost of feedstock, CRH and FWH were used to replace carbon and nitrogen source in the optimized medium (10 g/L glucose, 10 g/L of tryptone, 5 g/L of yeast extract, 50 mM acetic acid–sodium acetate buffer), respectively. Glucose and xylose contents were 84 and 10 g/L in CRH as determined by a HPLC analysis (Zhu et al., 2016). The protein content was 10.14 g/L in FWH as measured using the Quick Start™ Bradford 1× Dye Reagent (BIO-RAD). CRH with an equivalent 10 g/L of glucose and FWH with an equivalent 0.66 g/L of proteins were added separately into the optimum media to replace glucose and tryptone, respectively. To test the effects of carbon/nitrogen (C/N) ratio, CRH and FWH concentrations in the medium were adjusted to the glucose/protein (g/L) ratios: G5P0.5, G10P0.8, G10P1.2, G20P1.5, G25P1.2 and G25P1.5, respectively. The cell growth and ALA production in the CRH-FWH media were determined after 18 h incubation, using the methods mentioned above.

Construction of mutants for *B. cereus* PT1

Gene modification of strain PT1 was performed to improve its ALA production. According to the genome sequence of strain PT1 (accession number CP089518 in NCBI database), genes *hemA*, *hemL* and *tagU* or their promoters were separately cloned into the plasmids according to the reported protocols (Miller, 1992) and then transformed into strain PT1 in this study, as listed in Table 1. *E. coli* DH5α was used as the host for plasmid construction. Restriction endonucleases and PrimeSTAR HS DNA polymerase were purchased from Takara Bio Inc. (Beijing China). Genstar Plasmid Extraction kit (Shenzhen, China) and Tiangen Gel

TABLE 1 Bacterial strains, mutants and plasmids used or constructed in the study

Strains or plasmid	Relevant characteristics	Source or references
Strains		
E. coli DH5α	*F-*, *deoR*, *endA1*, *gyrA96*, *hsdR17*, *supE44*, *thi1* *recA1*, *λ-*, *ΔlacU169*	Lab stock
B. cereus PT1	Wild-type strain	This study
PT1-*hemA*	PT1 with integration of *hemA* under HpaII promoter	This study
PT1-*hemA*-*hemL*	PT1 with integration of *hemA* and *hemL* under HpaII promoter	This study
PT1-Δ*tagU*	*B. cereus* PT1 Δ*tagU*	This study
PT1-*hemA*-Δ*tagU*	PT1Δ*tagU* with integration of *hemA* under HpaII promoter	This study
Plasmids		
pRN5101	*Bacillus*-*E. coli* shuttle, temperature-sensitive plasmid, Amp^r, Ery^r	Villafane et al. (1987)
pR-*hemA*	Integrative transformation vector for overexpression of *hemA*	This study
pR-*hemL*	Integrative transformation vector for overexpression of *hemL*	This study
pR-*tagU*	Integrative transformation vector for deletion of the *tagU* gene	This study

Abbreviations: Amp, ampicillin; Ery, erythromycin; r, resistance.

Purification kit (Beijing, China) were utilized for DNA extraction and gel purification, respectively.

As a key gene in ALA synthesis (Zhang et al., 2020), endogenous *hemA*-cloned plasmid pMA5-*hemA* was transformed by the way of electroporation (Lu et al., 2012) into *B. cereus* PT1 to check if its overexpression could enhance ALA production. To overcome the potential cell stress caused by plasmid duplication and resistance to antibiotics (Yi & Ng, 2021), *hemA* under the constitutive strong promoter P_{HapII} was integrated into the *amyA* gene to construct the engineering strain PT1-*hemA*. To test the effects of *hemL* on ALA production, *hemL* under promoter P_{HapII} was integrated into the *mel* site of strain PT1-*hemA* by the way of specific recombination, using temperature-sensitive plasmid pRN5101.

In *Bacillus* species, LCP enzymes played major roles in peptidoglycan synthesis, among which TagU had a higher catalytic activity than TagT and TagV (Gale et al., 2017). To explore the effects of LCP enzyme defect on ALA production, *tagU* was deleted through a double crossover within homologous sequences. *tagU* gene in *B. cereus* PT1 was deleted by amplifying the upstream homologous arm and the downstream homologous arm in chromosomal sequences, generating pRN5101 derivative, pR-*tagU*. Recombination was performed as described (Bravo et al., 1996) to generate strain PT1-*hemA*-Δ*tagU*. Finally, the mutants PT1-*hemA*, PT1-*hemA*-*hemL*, PT1-Δ*tagU* and PT1-*hemA*-Δ*tagU* were obtained (Table 1) and used for the subsequent study.

ALA production and growth of PT1 mutants

The ALA production of *B. cereus* PT1-*hemA*-Δ*tagU* cultured with a mixture of CRH and FWH was investigated in batch process. For batch culture of the engineered strain, 100 ml of seed cultured in TSB medium for 10 h were transferred into a 7-L fermenter filled with 3 L of modified basal medium containing CRH at a glucose concentration of 40 g/L and FWH at a protein concentration of 2.5 g/L. During the fermentation, the saturation percentage of dissolved oxygen was maintained between 30% and 40% by controlling the stirring at a speed of less than 600 rpm and the injecting at a maximum flow rate of 1 vvm (air volume/culture volume/min). The culture pH was maintained at 6.5 by adding 2.0 M of NaOH. Samples of 5 ml were taken every 2 h up to 20 h of incubation. The samples were centrifuged (6000 × *g*, 20 min) and the supernatant was collected for ALA quantification with the modified Ehrlich's reagent assay. Next, 0.4 ml of supernatant was mixed with 0.2 ml of 0.2 M acetate buffer (pH 4.6) and 0.1 ml of acetylacetone. Subsequently, the mixture was heated at 100°C for 15 min, then cooled to room temperature, and mixed with 0.7 ml of freshly prepared Ehrlich's reagent (Shemin & Russell, 1953). After 10 min of incubation at room temperature, the absorbance at 554 nm was measured.

In addition, intracellular ALA concentration, cell density and cell morphology of the cell permeability of mutants (Δ*tagU*) were determined. For intracellular ALA quantification, cells were harvested by centrifugation, washed twice with distilled water and lyophilized. Then the dry cells were sonicated three times for 10 min with 40% of power in 50 mM of phosphate sodium buffer (pH 7.0). After centrifugation, the supernatant of the sonication lysate was used to determine intracellular ALA contents. For cell morphology observation, the cells were cultured in liquid basal medium to the exponential growth phase and were stained with SYTO 9 dye (Invitrogen Inc., Carlsbad, Calif.). After incubation in dark for 15 min, the bacteria were imaged using a confocal microscope with a 488 nm laser. Lengths of one hundred cells on their long axis were measured by ImageJ software (available at http://rsbweb.nih.gov/ij/) (Li et al., 2020a).

ALA purification by iron-exchange column

To best remove other metabolites in the culture, purification of ALA from the fermentation broth of recombinant PT1 was performed using strong cation (Seplite® LX-010) exchangers. Resin was pre-conditioned according to the manufacturer's instructions prior to use and then placed in a column (2 cm × 40 cm) containing distilled water and filled up to 100 cm^3. Prior to the adsorption process, the culture broth pH was adjusted to 3.0–3.5 with sulphuric acid. The broth was recycled two times at a flow rate of 10 ml/min. In the elution process, solution of 0.3 M ammonium hydroxide was used to release ALA bound to the resin. After adsorption and elution, the eluent was freeze dried and the obtained biological ALA powder presented a purity of 80%.

Plant material and treatments

Cucumber seeds were surface sterilized with 3% (w/v) sodium hypochlorite for 10 min, and rinsed with distilled water. The seeds were germinated at 28°C in dark for 48 h in Petri dishes lined with double-layer moistened filter paper. The germinated seeds were sown in 200-ml opaque plastic containers containing nutrient soil and vermiculite (1:3, v/v). The seedlings were grown in a climate chamber with a temperature of 25–26°C, a humidity of 50–60% and a cycle of 16-h light/8-h darkness. After 7 days of germination, uniform plants were subjected to treatment of 150 mM of NaCl in the 1/2 MS (Murashige & Skoog Basal Salts) medium. At the same time, 20 mg/L of biological ALA obtained in this study and 20 mg/L of commercial ALA (purchased from Tokyo Chemical Industry [TCI, Tokyo, Japan], purity

>98%) were sprayed to completely wet sides of the leaves every 3 days for 2 weeks, and distilled water was sprayed as the blank control. Plant fresh weight of the cucumber seedlings were recorded after 21 days of growth. The roots were dried at 60°C for 72 h, and the root dry weight was recorded.

Pigment contents, MDA level and CAT activity in cucumber plants treated with ALA

In this analysis, fresh leaf (0.2 g) of cucumber seedlings was cut into pieces and immerged in 10 ml of acetone (80% v/v) overnight at 4°C in dark. Then the supernatant was used for measuring the absorbance using a spectrophotometer at 663, 645 and 470 nm. The concentrations of chlorophylls *a* and *b* were calculated using the equation of Arnon (1949), and carotenoids with the equation of Wellburn and Lichtenthaler (1984). Another aliquot of fresh leaf (0.5 g) was triturated in 10 ml of phosphate buffer (50 mM; pH 7.0) with a pestle and mortar. After centrifugation (12,000 × *g*, 15 min, 4°C), the supernatant was collected to analyse its MDA content and activity of catalase (CAT) with commercial kits (Jiancheng Biotech Co. Ltd., Nanjing, China) according to the manufacturer's instructions.

Statistical analysis

In this study, all experiments were performed in triplicate (shake flask studies) or duplicate (bioreactor broth). Statistical significance was determined through the two-tailed Student's t-test, using $p < 0.05$ as cut-off for significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

RESULTS AND DISCUSSION

Effect of carbon and nitrogen sources on ALA production by *B. cereus* PT1

Carbon and nitrogen sources play important roles in ALA production and cell growth (Lee et al., 2005; Sattayasamitsathit & Prasertsan, 2014). In this study, glucose was verified to be the most effective carbon source among the five tested compounds for the growth and ALA production of strain PT1, which generated 79.24 mg/L of ALA after an 18 h incubation (Figure 1A). The efficiency of glucose utilization was better than those of the other substrates (Figure S1). Although glycerol, sucrose and lactose were beneficial to the growth of strain PT1, ALA thus produced was only a quarter of that with glucose as substrate. These results were consistent with a previous report that higher ALA accumulation was obtained from the culture of recombinant *E. coli* strain with glucose as carbon source than from that with other substrates (Liu et al., 2010). With glucose as carbon source, tryptone was identified as the optimum nitrogen source for strain PT1 growth and contributed to significantly higher ALA production than other nitrogen sources (146.31 mg/L) (Figure 1B). Among the tested nitrogen sources, urea and ammonium sulphate resulted in poor ALA production and cell growth.

ALA production from acid hydrolysates of biomass residues

To reduce cost in carbon/nitrogen sources and improve market competitiveness, the acid hydrolysates of renewable biomass CR (cassava residue) and FW (fish waste) were used to replace glucose and tryptone, respectively.

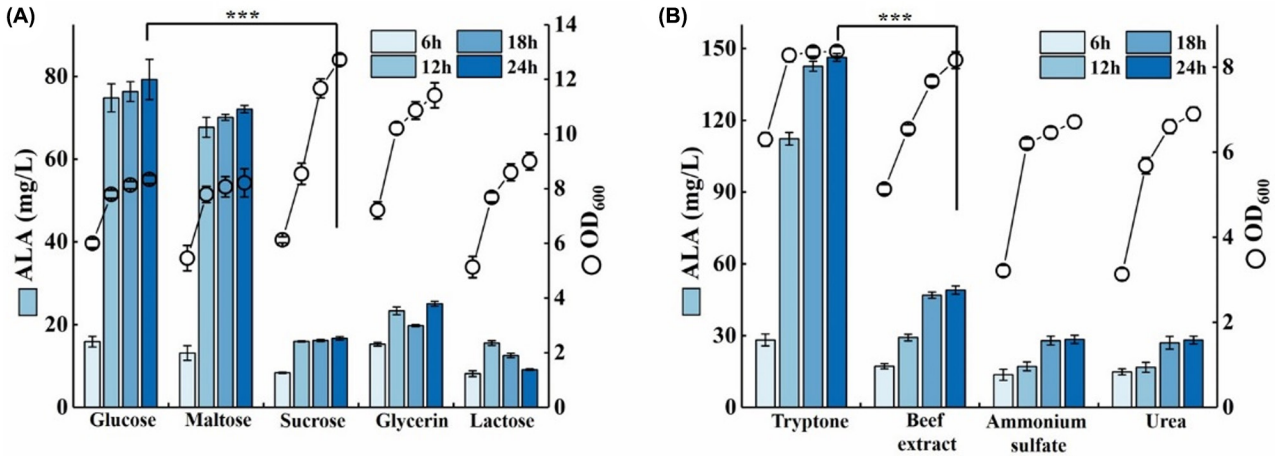

FIGURE 1 Effects of different carbon sources (A), nitrogen sources (B) on ALA production (bars) and biomass (dots) by strain *B. cereus* PT1. Cultivation was performed in 300-ml Erlenmeyer flasks with 30 ml of modified medium at 37°C and 200 rpm for 48 h. Samples were taken and measured with an interval of 6 h. The data presented were the means of three replicates along with standard deviation. "***" represented significant difference ($p < 0.001$) in the two-tailed Student's *t*-test. ALA: 5-aminolevulinic acid; OD: Optical density.

Using CR hydrolysate (CRH) as carbon source and tryptone as nitrogen source, the ALA production of *B. cereus* PT1 reached 139.12 mg/L after 18 h, similar to that using glucose (142.63 mg/L) (Figure 2A,B). In addition, strain PT1 showed unimpaired growth with acid hydrolysate (Figure 2A). These results proved CRH an economical and efficient carbon source for ALA production by *B. cereus* PT1. With glucose as carbon source, FW hydrolysate (FWH) contributed to an ALA production of 195.51 mg/L by strain PT1 after 18 h, which was 37.68% higher than that using tryptone and yeast extract of the same protein content as nitrogen sources (Figure 2C). As reported previously, fish waste could provide beneficial peptides for microbial synthesis (Zainuddin et al., 2020), which might result in the increase in ALA production by strain PT1.

Besides the types of carbon/nitrogen sources, the carbon/nitrogen (glucose/protein) ratio is also critical for ALA production. As shown in Figure 2D, the highest biomass and ALA yield (251.72 mg/L) were achieved in the medium G20P1.5, which was composed of CRH containing 20.0 g/L of glucose, and FWH containing 1.5 g/L of protein. The cell growth and ALA production were decreased at lower and higher CRH/FWH ratios. Previously, Chen et al. (2020) tested ALA production by engineered *C. glutamicum* using enzymatic hydrolysate of CR, but the cost of enzymatic hydrolysis was relatively high. In another case, *R. sphaeroides* was able to produce ALA by utilizing wastewater (Liu et al., 2018), but its growth was slow and the ALA production was relatively low, requiring illumination. This study for the first time described the effective utilization of CRH/FWH mixture for ALA production, which might provide an alternative economic strategy for ALA production by microbial fermentation, and promote the resourceful utilization of CR and FW.

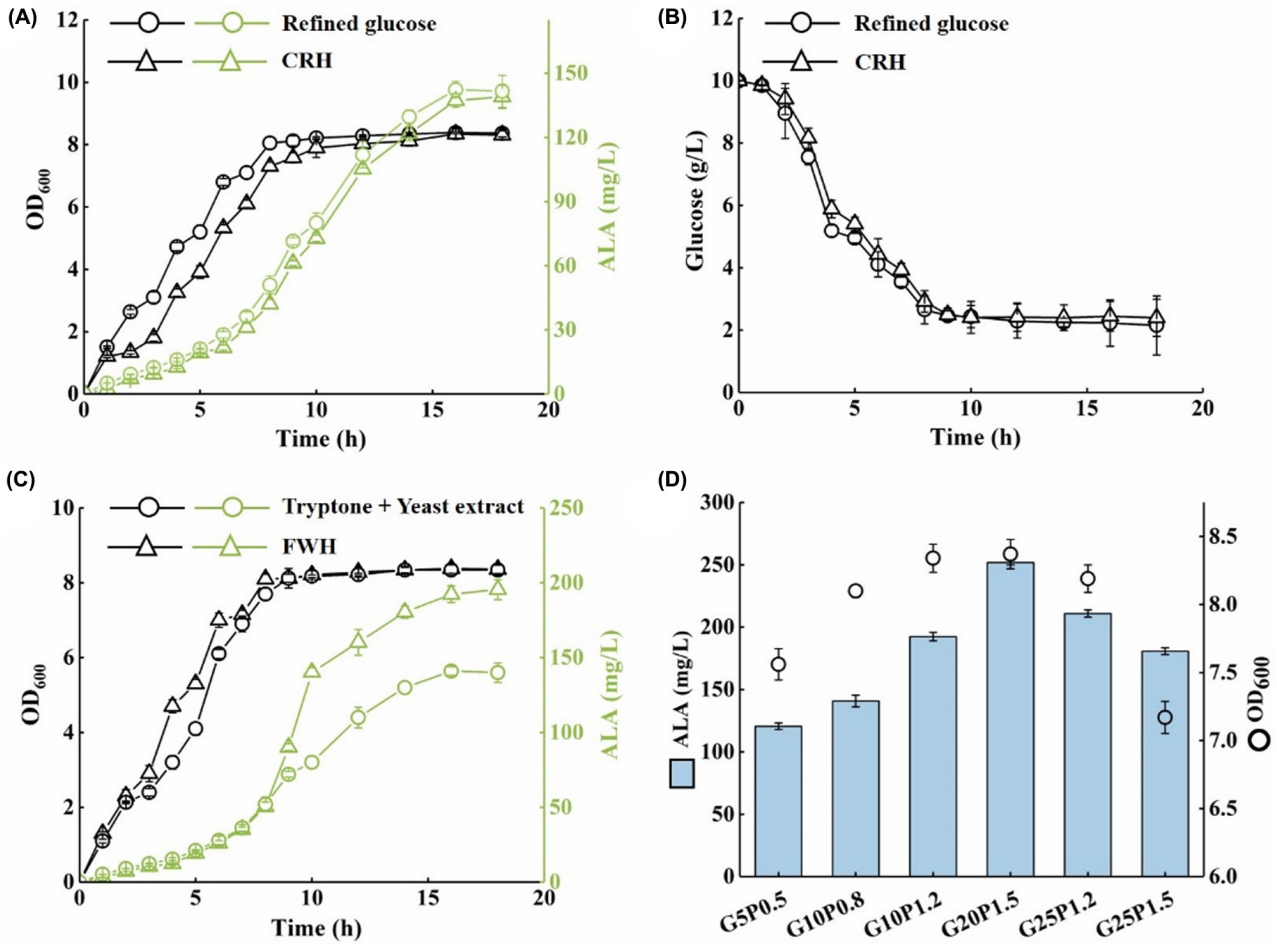

FIGURE 2 Production performance of *B. cereus* PT1 using acid hydrolysates. The ALA production, biomass (A) and glucose consumption (B) of strain *B. cereus* PT1 using refined glucose or cassava residue hydrolysate (containing 20 g/L of glucose) as carbon source (tryptone and yeast extract as nitrogen source). (C) The ALA production and biomass of *B. cereus* PT1 using tryptone and yeast extract or fish waste hydrolysate (containing 0.66 g/L of protein) as nitrogen source (with refined glucose as carbon source). (D) Effects of glucose to protein concentration (G/P) ratio in cultivation medium on ALA production (bars) and OD_{600} (dots) by *B. cereus* PT1 strain. Glucose (g/L) from cassava residue hydrolysate and protein (g/L) from fish waste hydrolysate. Cultivation was performed in 300-ml Erlenmeyer flasks with 30 ml of modified medium at 37°C and 200 rpm for 18 h. The data presented were the means of three replicates along with standard deviation. ALA, 5-aminolevulinic acid; OD, Optical density.

ALA production by overexpression of *hemA* and improved cell wall permeability

HemA and HemL are key enzymes for ALA biosynthesis (Zhang et al., 2020). In this study, overexpression of *hemA* was first conducted by introducing the plasmid pMA5-*hemA* into strain PT1, but the engineered strain *B. cereus* PT1-p*hemA* (harbouring plasmid pMA5-*hemA*) exhibited decreased growth and ALA production compared to those of the wild-type strain (Figure S2), which might be owing to possible plasmid duplication and resistance to antibiotics caused cell stress. To avoid these disadvantages, the engineered strain PT1-*hemA* was generated by integrating *hemA* under HpaII promoter into the chromosome of PT1 strain with the homologous region of the *amyA* gene. The mutant PT1-*hemA* increased ALA production (350.64 mg/L), biomass and glucose utilization by 39.30%, 15.23% and 12.49% over the parent strain after 24 h of incubation, respectively (Figure 3A,B). Furthermore, *hemL* was integrated into the chromosome of strain PT1-*hemA* and a recombinant strain PT1-*hemA*-*hemL* was obtained, but this recombinant strain generated similar ALA (356.36 mg/L) and biomass (OD_{600}: 10.33) compared to those of the strain PT1-*hemA* (Figure S3). These results suggested that *hemA* was a more critical gene than *hemL* in ALA synthesis by *B. cereus* PT1.

To explore the effects of LCP enzyme defect on ALA production, *tagU*-deleted mutants PT1-Δ*tagU* and PT1-*hemA*-Δ*tagU* were constructed. As shown in Figure 4A, PT1-Δ*tagU* and PT1-*hemA*-Δ*tagU* with *tagU* deleted notably increased ALA production to 410.28 mg/L and 560.08 mg/L, 61.97% and 59.73% higher than those by the strains PT1 and PT1-*hemA* (Figure 3A), respectively. Meanwhile, the mutant PT1-Δ*tagU* exhibited 21.42% and 16.58% increases in cell biomass (Figure 4B) and glucose utilization (Figure 4A) compared to those of the strain PT1 (Figure 3A,B), respectively. Although the OD_{600} and glucose consumption of strain PT1-*hemA*-Δ*tagU* (Figure 4A,B) were comparable to those of strain PT1-*hemA* (Figure 3A,B), PT1-*hemA*-Δ*tagU* achieved an approximately 6.6-folds increase in viable bacteria count after 12 h of cultivation, compared to those of strain PT1-*hemA* (Figure 4C). LCP proteins (TagT, TagU and TagV) displayed functional redundancy in *Bacillus subtilis* (Kawai et al., 2011) and regulated bacterial cell autolysis (Chatfield et al., 2005) Hence the deletion of *tagU* might retard autolysis and enhance cell viability.

Moreover, the intracellular level of ALA in mutant PT1-*hemA*-Δ*tagU* was dramatically decreased by 41.69% (Figure 4C) compared to that in PT1-*hemA* after 12 h of cultivation. The released intracellular ALA not only enhanced the accumulation of extracellular ALA, but also reduced the feedback inhibition of ALA synthesis. Additionally, to investigate the effects of *tagU* deletion on cell morphology, strain PT1-Δ*tagU* cells were labelled with fluorescent dye SYTO 9 and observed by exciting with a 488 nm argon ion laser. As shown in Figure 4D, the length of *tagU* mutant cells became shorter (decreased by 18.47%) than that of the wild-type cells, which led to higher specific surface area, contributing to ALA efflux improvement. The above results indicated that deleting *tagU* might weaken the synthesis of peptidoglycan layer and facilitate its nutrient absorption and ALA export, thus promoting cell growth and ALA production.

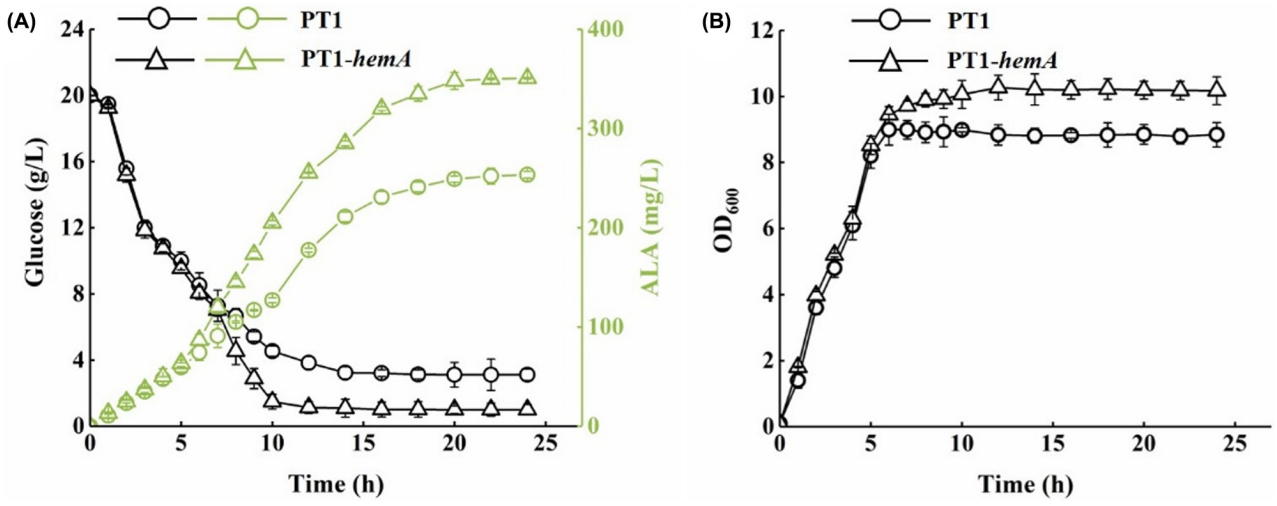

FIGURE 3 The ALA production, glucose consumption (A) and OD_{600} (B) of overexpression of endogenous *hemA* in strain *B. cereus* PT1. PT1: Wild-type strain *B. cereus* PT1. PT1-*hemA*: Strain PT1 was integrated with one copy of *hemA* under HpaII promoter on the *amyA* site. Cultivation was performed in 300-ml Erlenmeyer flasks with 30 ml of acid hydrolysates CRH-FWH medium (containing 20 g/L glucose and 1.5 g/L protein) at 37°C and 200 rpm for 24 h. The data presented were the means of three replicates along with standard deviation. ALA, 5-aminolevulinic acid; CRH, Cassava residue hydrolysate; FWH, Fish waste hydrolysate; OD, Optical density.

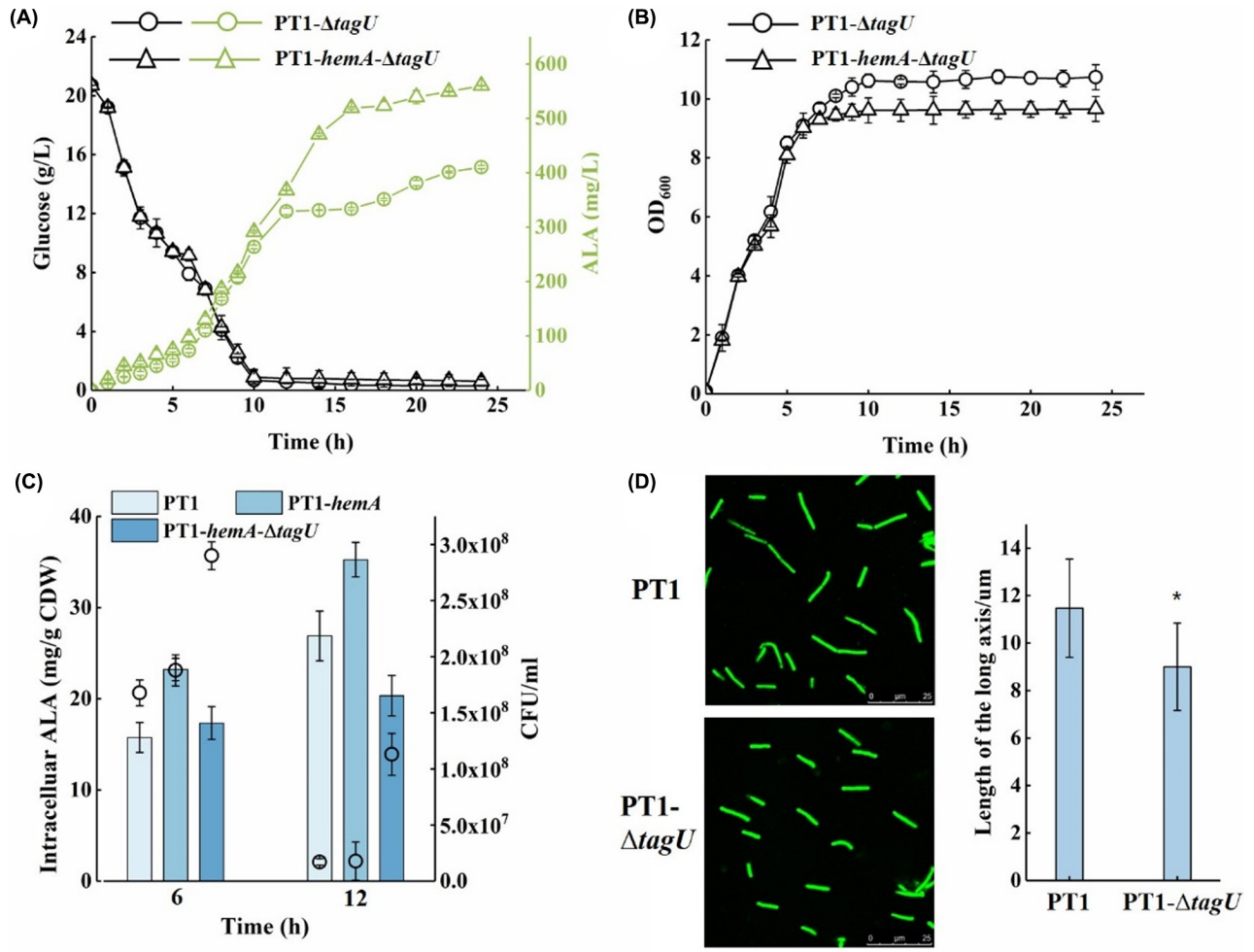

FIGURE 4 The ALA production, glucose consumption (A) and biomass (B) of PT1 and PT1-*hemA* strains with *tagU* gene deleted, respectively. (C) Intracellular ALA (bars) and CFU (dots) were enumerated at 6 h and 12 h culture of PT1, PT1-*hemA* and PT1-*hemA*-Δ*tagU* strains, respectively. (D) Fluorescence image and bacterial long-axis length of the PT1 wild type and the *tagU* defect mutant by STYO 9 green. The cells were cultured in liquid medium to the exponential growth phase. PT1-Δ*tagU*: *B. cereus* PT1 Δ*tagU*; PT1-*hemA*-Δ*tagU*: *B. cereus* PT1 with *hemA* integrated and *tagU* deleted. Cultivation was performed in 300-ml Erlenmeyer flasks with 30 ml of acid hydrolysates CRH-FWH medium (containing 20 g/L of glucose and 1.5 g/L of protein) at 37°C and 200 rpm for 24 h. The data presented were the means of three replicates along with standard deviation. ALA, 5-aminolevulinic acid; CDW, Cell dry weight; CFU, Colony forming unit; CRH, Cassava residue hydrolysate; FWH, Fish waste hydrolysate; OD: Optical density; . "*" represented significant difference ($p < 0.05$) in the two-tailed Student's *t*-test.

ALA production from biomass hydrolysates

In bath fermentation, with the mixture of CRH and FWH (containing 40 g/L glucose and 2.5 g/L protein) as substrates, *B. cereus* PT1-*hemA*-Δ*tagU* achieved an ALA production of 2.62 g/L, a highest OD_{600} value of 32.37, and a glucose utilization of 95% after 20 h of cultivation (Figure 5A). These results demonstrated the potential to use CRH and FWH as substrates for ALA microbial production in industrial scale, which might greatly decrease the production cost (Mudaliar, 2015), compared to that with refined glucose and nitrogen sources (Chen et al., 2020; Kang et al., 2017). It was reported that the highest titre of ALA production via C5 pathway was 5.25 g/L (1.5 L medium/3-L fermenter) by engineered *C. glutamicum* with total glucose and ammonium sulphate consumption of 52.50 g and 24.00 g, respectively (Yu et al., 2015). Since the prices of CR (~20 USD/tonne) (Chen et al., 2020) and FW (~129 USD/tonne) (Kratky & Zamazal, 2020) are much lower than that of glucose (~521 USD/tonne) (Chen et al., 2020) and ammonium sulphate (~362 USD/tonne) (Errico et al., 2018), using CR and FW to produce an equal titre of ALA by engineered strain PT1-*hemA*-Δ*tagU* would reduce the cost of substrates by 72.70%. Furthermore, inducers and antibiotics were not required for ALA production by engineered strain PT1-*hemA*-Δ*tagU*. High yield of ALA in fed-batch fermentation might be achieved by further optimizing the culture conditions (pH, temperature, aeration, feeding profile).

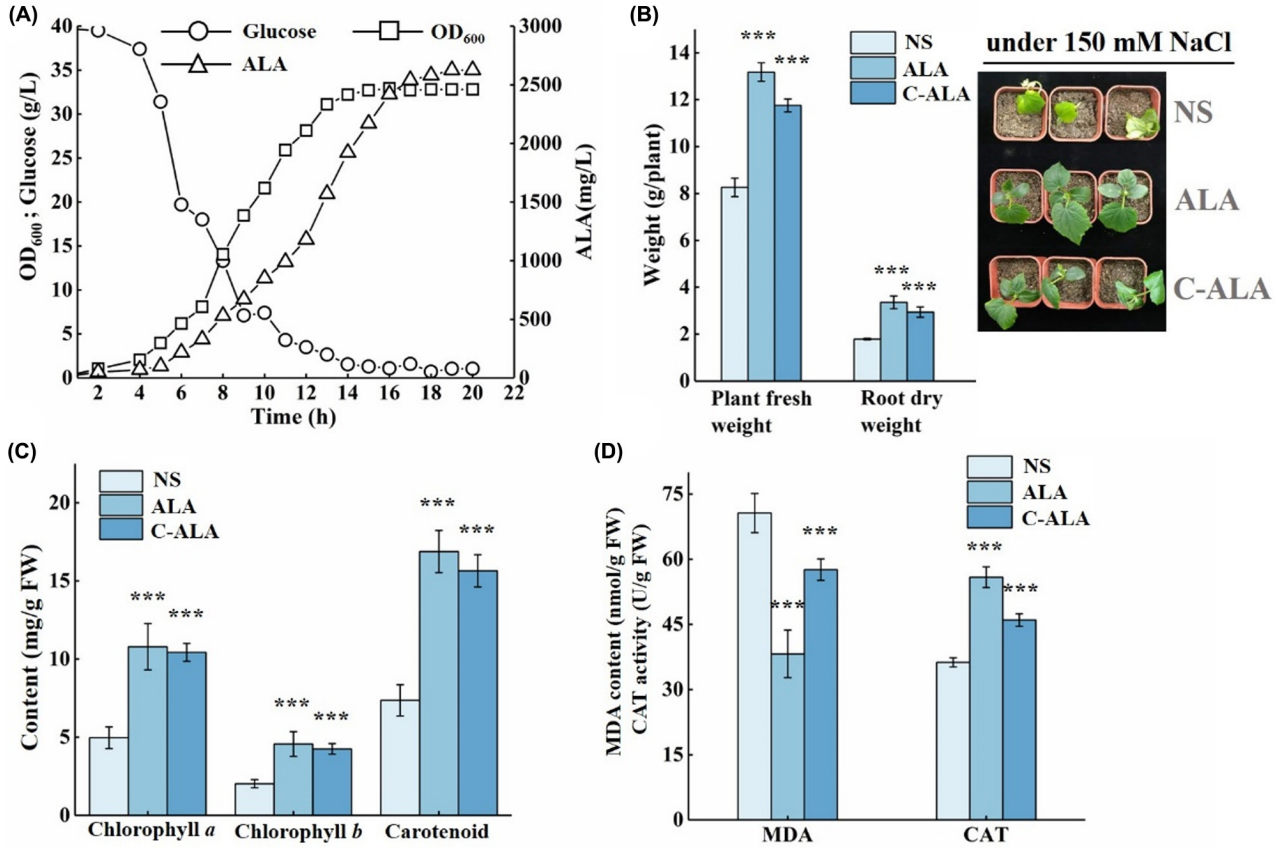

FIGURE 5 (A) Batch fermentation for 5-ALA production from acid hydrolysates of cassava residue and fish by-product using recombinant strain PT1-*hemA*-Δ*tagU*. Square, OD_{600}nm; circle, glucose; triangle, ALA. PT1-*hemA*-Δ*tagU*: *B. cereus* PT1 with *hemA* integrated and *tagU* deleted. Cultivation was performed in 7-L fermenters with 3 L of acid hydrolysates CRH-FWH medium (containing 40 g/L of glucose and 2.5 g/L of protein) at 37°C for 20 h. ALA, 5-aminolevulinic acid; CRH, Cassava residue hydrolysate; FWH, Fish waste hydrolysate; OD, Optical density. Effects of biological ALA (containing ALA 20 mg/L) and commercial ALA (20 mg/L, chemical synthesis) on cucumber seedlings under salt stress (150 mM NaCl). Plant fresh and root dry weight of cucumber seedlings (B). Chlorophyll *a*, chlorophyll *b* and carotenoids contents (C), MDA contents and CAT activity (D). NS: No ALA spray; distilled water; ALA: Biological ALA purified from the fermentation broth of recombinant strain PT1. C-ALA: Commercial ALA from chemical synthesis and purity >98%. FW: Fresh weight; MDA: Malondialdehyde CAT: Catalase. The data presented were the means of three replicates along with standard deviation. "***" represented significant difference ($p < 0.001$) in the two-tailed Student's *t*-test.

Application of ALA in cucumber culture

By passing the fermentation broth through a cation exchange column, ALA powder with a purity of 80% was obtained. The effects of biological ALA (containing 20 mg/L ALA) or commercial ALA (20 mg/L, chemical synthesis) on cucumber growth under NaCl stress were investigated by foliar spray. The results showed that both the ALA products could significantly increase cucumber fresh weight by 59.56% and 42.34%, root dry weight by 87.44% and 64.20%, respectively (Figure 5B). The application of biological ALA significantly enhanced the contents of chlorophylls *a*, *b* and carotenoids by 117.20%, 125.43% and 129.35%, respectively, similar with those of commercial ALA (Figure 5C).

The oxidative stress and antioxidant enzymatic activity were determined in terms of malondialdehyde (MDA) content and catalase (CAT) activity in leaves of cucumber seedlings. Although, significant changes of MDA content and CAT activity were observed in both the biological ALA treatment and commercial ALA treatment (Figure 5D), MDA content and CAT activity in biological ALA treatment were 27.43% lower and 27.21% higher than those in commercial ALA treatment (Figure 5D), respectively. These results implied that the biological ALA had better effects on improving plant salt tolerance and additional benefits to plants, in comparison with chemically synthesized ALA.

In summary, *Bacillus cereus* PT1 was an efficient native ALA producer with a yield of 251.72 mg/L using CRH and FWH as carbon and nitrogen sources. A respective increase of 39.30% and 59.73% in ALA production were observed by integrating endogenous *hemA* to the chromosome or deleting LCP family gene (*tagU*) in this strain. Finally, the recombinant strain PT1-*hemA*-Δ*tagU* reached an ALA production of 2.62 g/L in a 7-L fermenter using CRH and FWH without any addition of inducers and antibiotics, with a splendid expectation for microbial production of ALA. For the first time, this study reported that ALA

produced from the acid hydrolysates of agricultural biomass by recombinant PT1 could enhance chlorophyll contents in cucumber seedling, as well as the resistance to NaCl stress. This study laid a foundation for low-cost fermentative production of ALA and its potential application in agriculture.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in the article and supporting information.

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SUPPORTING INFORMATION

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