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Research Article

The Potential Use of Wood Vinegar as an Alternative Inhibitor to Enhance 5-Aminolevulinic Acid Production by *Bacillus paramycoides*

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ABSTRACT

The expensive production of 5-aminolevulinic acid (ALA) as a plant growth stimulator led to an attempt to get an alternative substrate to reduce the production cost. Levulinic acid is the inhibitor of ALA dehydratase in ALA production. Wood vinegar, a liquid gas produced from wood combustion in airtight conditions, is known to contain 12-17 mM of levulinic acid. This study aims to explore the use of wood vinegar as a potential substitute for levulinic acid in the extracellular production of ALA by bacteria identified as *Bacillus paramycoides* through 16S rRNA sequence analysis. Adding precursor and inhibitor glutamate and 1% wood vinegar increased the ALA production to 174.3 µM, while the combination of glutamate and levulinic acid raised the ALA production to 179.9 µM. This study confirmed that wood vinegar can enhance the concentration of ALA and potentially substitute levulinic acid as an inhibitor in ALA production.

1. Introduction

ALA has been used in agriculture as an herbicide, insecticide, growth-promoting factor, and for alleviating plant stress (Sasikala *et al.* 1994; Nishikawa and Murooka 2001; Sasaki *et al.* 2005). However, commercial use of ALA has generally been limited due to the high costs associated with chemical synthesis and concomitant low yields (Nishikawa & Murooka 2001). Therefore, microbial production of ALA has recently been considered an environmentally friendly, sustainable, and renewable methodology (Kang *et al.* 2012).

It is known that ALA is a precursor of tetrapyrrole biosynthesis, including chlorophyll, heme, and vitamin B12, which extensively exists in plants, algae, animals, and bacteria (Rebeiz *et al.* 1984; Beale 1990; Carmichael 1992; Sasikala *et al.* 1994; Sasaki *et al.* 2002). Nishikawa and Murooka (2001) stated that ALA is formed in two different ways: the C5 pathway with glutamate as the precursor and the Shemin (C4) pathway, formed by ALA synthase (ALAS) from succinyl CoA and glycine. However, the synthesis pathway of ALA in most bacteria and animals is similar to that in plants, which is via the C5 pathway and uses glutamate as a precursor (Yonezawa *et al.* 2015).

A precursor is a substance that induces metabolite production in living organisms, including plants and microorganisms (Demain 1998; Nissar *et al.* 2015). Sasaki *et al.* (1991) suggested the need for a precursor in ALA production, and Sasaki *et al.* (1995) demonstrated that the addition of glutamate and levulinic acid (LA) increased the ALA concentrations in *Chlorella regularis* up to 1.9 mM. Four years later, Ano *et al.* (1999) reported

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that *Chlorella regularis* performed better with adding glycine, which enhanced the specific growth rate and ALA production by up to 3.72 mM. In contrast, inhibitors delay metabolic processes (Schonborn 2010). Lee *et al.* (2005) discovered that glucose inhibited ALA degradation by ALA dehydratase (ALAD) and resulted in increased ALA yield. Furthermore, Saikeur *et al.* (2009) showed the positive effect of levulinic acid as an inhibitor of ALAD and enhanced ALA production of *Rhodopseudomonas palustris* KG31.

Many studies have reported the production of ALA by photosynthetic bacteria, rhizobacteria, and recombinant *Escherichia coli*. Nevertheless, a few have observed ALA synthesis from *Bacillus* species. Ahn (2007) reported ALA production (up to 2 mM) in *Bacillus cereus* 1-1 following the addition of glucose, acetic acid, and glutamic acid as precursors. The effect of *Bacillus* species on plant growth was described by Koh and Song (2007); bacteria produce metabolites such as amino acids, organic acids, and growth factors that enhance plant growth.

The production of ALA is considered high due to the high cost of materials. Wood vinegar (WV) was proposed as an economical inhibitor alternative in the liquid fermentation of bacteria-producing ALA. The main aim of this study was to investigate the use of wood vinegar as a potential substitute for levulinic acid in the extracellular production of ALA from *Bacillus paramycoides.* WV is a by-product of wood's distillation process, which is discovered to have 12-17 mM of LA (Matsushita *et al.* 2002). According to Nunkaew *et al.* (2018), the mixed culture of *R. palustris* strains (TN1 14 and PP803) was fed with 1.25% WV at mid-log phase and reached the highest ALA production (130.84 µM). This research provides information about the effect of WV on ALA production by *Bacillus paramycoides*.

2. Materials and Methods

2.1. Bacterial Identification

DNA bacteria was extracted by suspending cells from a 24-hour-old colony into 100 ml of sterile 1 × TE buffer (10 mM Tris-Cl, 1 mM EDTA buffer, pH 7.6) and heating to 100°C for 30 minutes to rupture the cells (Eppendorf Thermomixer Comfort, Germany). After heating, the solution was centrifuged (Heraeus Pico17 centrifuge, UK) at 13,000 g for 15 minutes. The extracted total DNA was transferred into sterile microcentrifuge tubes. For PCR, 2 µL of DNA was used in a 25 µL reaction mixture containing 12.5 µL of DreamTaq™ Green PCR master mix (2 ×) (ThermoFisher Scientific, UK), 8.5 µL of nuclease-free water, and 1 µL (10 µM) of each primer (8F and 805R universal primers). The DreamTaq Green PCR master mix contained DreamTaq DNA polymerase, optimized DreamTaq Green Buffer, 0.4 mM of dNTPs, and 4 mM $MgCl_2$. The quality and concentration of DNA were tested using a nanodrop and a qubit fluorometer, respectively. DNA was sequenced by Eurofins Scientific (UK), and the sequence was compared with the NCBI reference database.

2.2. Bacterial Growth Curve and ALA Production Curve

First, the inoculum was grown on 10 ml of Luria Bertani (LB) broth in a 100 mL flask at room temperature and incubated in the shaker at 150 rpm. After 24 hours, the inoculum was added to 100 ml of LB broth in a 250 ml flask and incubated at 35°C, 150 rpm. Every 2 hours over 24 hours, an aliquot was removed from the flask, and the optical density (absorbance) was determined at 600 nm using an ultraviolet-visible spectrophotometer (Cecil Instruments, UK). The ALA concentration of the medium was quantified at wavelength 553 nm.

2.3. Analytical Determinations

2.3.1. The Effect of Glutamate

10 ml of the inoculum was added into each 250 ml flask containing 40 ml of LB medium (ten replicates). Subsequently, 200 µL of 0.5 M glutamate was added to two flasks at 2, 4, and 8 hours of incubation at room temperature and agitation at 150 rpm. Uninoculated medium and bacteria culture without any addition were used as the control. After 24 hours of incubation, the ALA concentration of samples was analyzed.

2.3.2. The Effect of Levulinic Acid

10 ml of the inoculum was added into each 250 ml flask containing 40 ml of LB medium (ten replicates). Subsequently, 200 µL of 0.5 M levulinic acid was added to two flasks at 4, 8, and 10 hours of incubation at room temperature and agitation at 150 rpm. An uninoculated medium and bacteria culture without any addition was also used as the control. After 24 hours of incubation, the ALA concentration of samples was analyzed.

2.3.3. The Effect of Wood Vinegar

10 ml of the inoculum was added into each 250 ml flask containing 40 ml of LB medium (ten replicates). Subsequently, 200 µL of 0.5 M glutamate was added after 8 hours of incubation and 800 µL of 0.5 M levulinic

acid/WV after 10 hours of incubation according to the following treatments: (i) Medium and bacteria; (ii) glutamate; (iii) Levulinic acid; (iv) WV 1%; (v) WV 2%; (vi) Glutamate + Levulinic acid; (vii) Glutamate + WV 1%; (viii) Glutamate + WV 2%. The three replicates of each treatment were incubated at room temperature and agitation at 150 rpm. After 24 hours of incubation, the ALA concentration of samples was analyzed.

2.4. ALA Measurement

Twenty-four hours of culture was centrifuged. 100 µL of the culture was taken out and dispensed into a 10 ml glass tube and added with 2 ml of acetate buffer containing 1% acetylacetone (76 g sodium acetate, 800 ml ultra-pure water, 70 ml acetic acid, adjusted pH to 4.5-4.6; then 10 ml acetylacetone added and adjusted to a final volume of 1 L with ultra-pure water). The solution was mixed by vortexing for 5 seconds. After mixing, the solution was heated in a water bath at 90°C for 15 minutes, with a set of ALA standards (0-1000 µM ALA (Sigma-Aldrich) in acetate buffer). After 15 minutes, the samples and standards were removed from the water bath and cooled on ice for 10 minutes. A 3.5 ml of Ehrlich's reagent (20 g of p-dimethylaminobenzaldehyde was dissolved in 230 ml sterile water, then added with 600 mL of acetic acid and 170 ml of perchloric acid, stored at 4°C) was added to each tube, mixed, and left at room temperature for 15 minutes. Solutions were vortexed to mix, and the absorbance was measured at 553 nm. The blank used was acetate buffer/acetylacetone solution. The ALA concentrations at each time point were calculated against the standards. The aim was to determine whether a relationship existed between cell number (expressed as OD) and ALA concentration.

2.5. Statistical Analysis

A one-way analysis of variance was conducted on ALA concentrations following the amendment of the incubation medium with the precursor and inhibitor solutions (factors) for the repeated precursor/inhibitor tests. Data were analyzed by GenStat version 19 (VSN International, Hemel Hempstead, UK). Post hoc comparisons between means were based on the Tukey test at the 0.05 probability level.

3. Results

3.1. Bacterial Identification

The 16S rDNA sequence of the bacterium genes was compared to the NCBI (National Centre for Biotechnology Information) reference database and submitted to the NCBI Gene Bank. The bacterium is 100% similar to *Bacillus paramycoides*.

3.2. Bacterial Growth Curve and ALA Production Curve

The bacterial growth curve over the 24-hour incubation period (Figure 1) shows a lag phase between 0-2 hours after inoculation and entering the exponential (log) phase from 3 hours up to 15 hours incubation. Cell numbers (expressed as absorbance) increased 10-fold from the point of inoculation to the start of the stationary phase after 16-18 hours.

The ALA concentration (Figure 1) also increased over the incubation period, with maximum concentrations observed when the cells reached the end of the exponential phase and entered the stationary phase. The final ALA concentration was 102 µM, and the starting concentration at time zero was 56 µM. ALA synthesis thus appeared to increase as cell numbers increased. This is addressed in the following experiments.

A further set of bacterial cells was incubated for 8 hours in order to confirm the optimum time during the cell growth curve to add the precursor (glutamate) and inhibitor (levulinic acid). Ideally, the glutamate should be added during the exponential phase, and LA should be near the end of the phase. This experiment measured the OD every hour after inoculation for 8 hours (Figure 2). The optimum time to introduce the inhibitor was calculated using regression. The exponential phase started 5 hours after inoculation, which is in line with the previous experiment (Figure 1), and the calculated time to add the inhibitor was after 9 hours and 45 minutes of incubation. For the next experiment, the time for adding the inhibitor was rounded to 10 hours after inoculation.

3.3. The Effect of A Precursor and An Inhibitor Addition to The Culture Medium on ALA Production

ALA concentrations within the bacterial culture medium were calculated against the standards using regression. In the first of the two experiments (Figure 3), glutamate addition at 4 hours after inoculation (P4) resulted in the highest ALA concentration (almost 200 µM). Adding glutamate and LA (P3I10 and P4I10) decreased ALA production by the bacteria (123 µM). However, ALA measurement within the uninoculated medium was relatively high (above 150 µM).

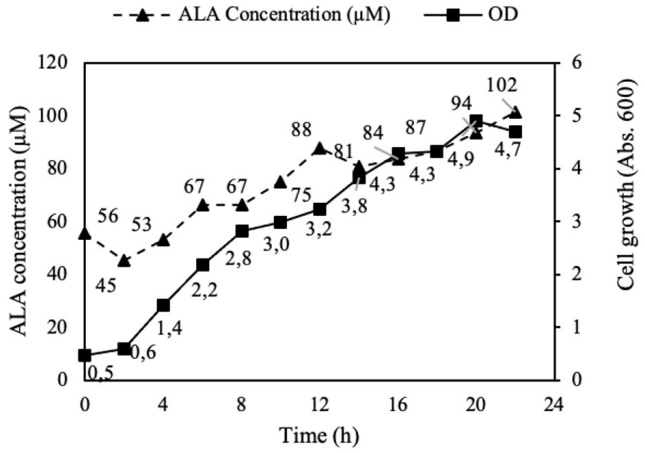

Figure 1. Bacterial growth was expressed as absorbance at 600 nm, and ALA concentrations were measured over a 24-hour incubation period. Bacteria were cultured in LB broth at 35°C

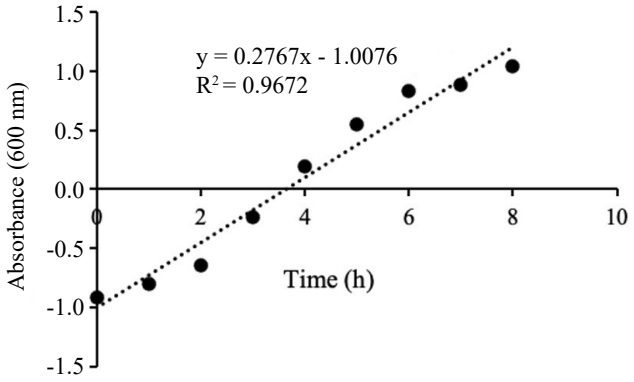

Figure 2. Bacterial growth over an 8 h period in LB broth at 35°C. Data are absorbance values at 660 nm

In the second of the two experiments (where the medium was centrifuged before analysis, a higher concentration of medium was taken for ALA analysis, and amendments were made at more time points), the amendments resulted in ALA concentrations within a lower range as previously measured, but of a similar order of magnitude. However, in this case, the values for the uninoculated medium were much lower (Figure 4). The addition of a single precursor and inhibitor, which were glutamate 8 h and levulinic acid 10 h after inoculation, enhanced the ALA content, respectively 131.7 µM and 179.9 µM.

3.4. The Effect of Wood Vinegar Addition as Levulinic Acid Substitution on ALA Production

As shown in Figure 5, glutamate increased ALA concentration to 151.4 µM. At the same time, adding WV 1% and 2% improved ALA content higher than the glutamate and LA addition, respectively 166.9 µM and 173.6 µM. On the contrary, the combination of precursor and inhibitor, glutamate and LA, enhanced the level of ALA up to 179.9 µM and became the highest value. Followed by adding glutamate and WV 1% with 174.3 µM.

4. Discussion

Based on molecular identification, the bacterial isolate sequence is 100% similar to *Bacillus paramycoides*. According to Osman and Yin (2018), *Bacillus paramycoides* is a plant growth-promoting rhizobacteria

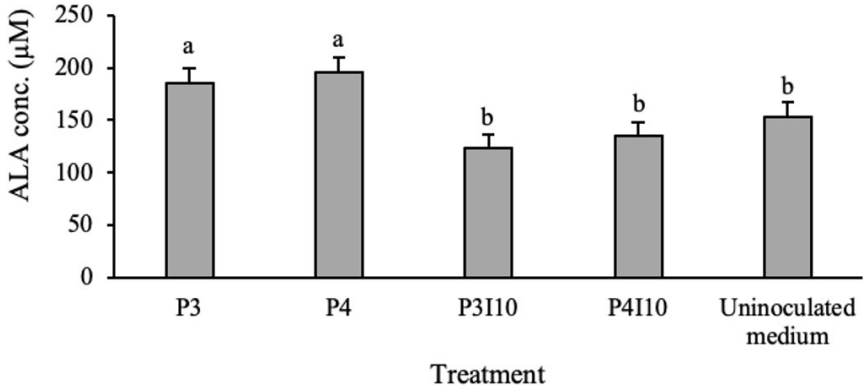

Figure 3. ALA concentrations of the bacterial culture medium following amendments. P3, glutamate addition at 3 h; P4, glutamate addition at 4 h; P3I10, glutamate addition at 3 h and LA amendment at 10 h; P4I10, glutamate addition at 4 h and LA amendment at 10 h. Columns similarly superscripted are not significantly different (Tukey post hoc test). There was a significant treatment effect p = 0.005. Individual error bars are based on the pooled variance estimate from the ANOVA with 10 degrees of freedom

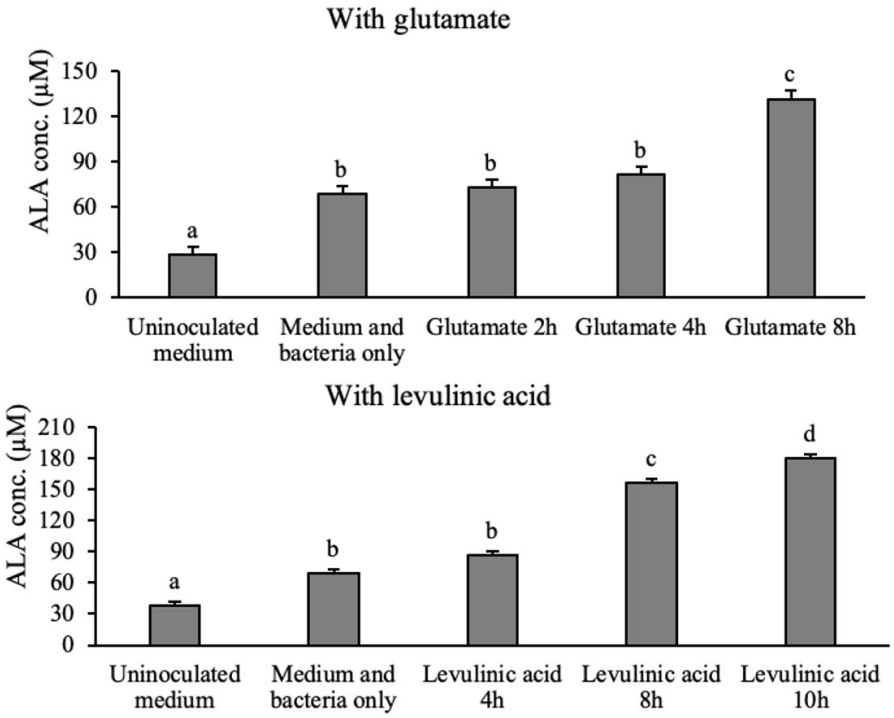

Figure 4. ALA concentrations in the bacterial culture medium following amendments after 2, 4, and 8 h of glutamate (A) and 4, 8, and 10 h of LA (B). Columns similarly superscripted are not significantly different (Tukey post hoc test). There was a significant treatment effect $p<0.001$ for both data sets. Individual error bars are based on the pooled variance estimate from the ANOVA with 10 degrees of freedom

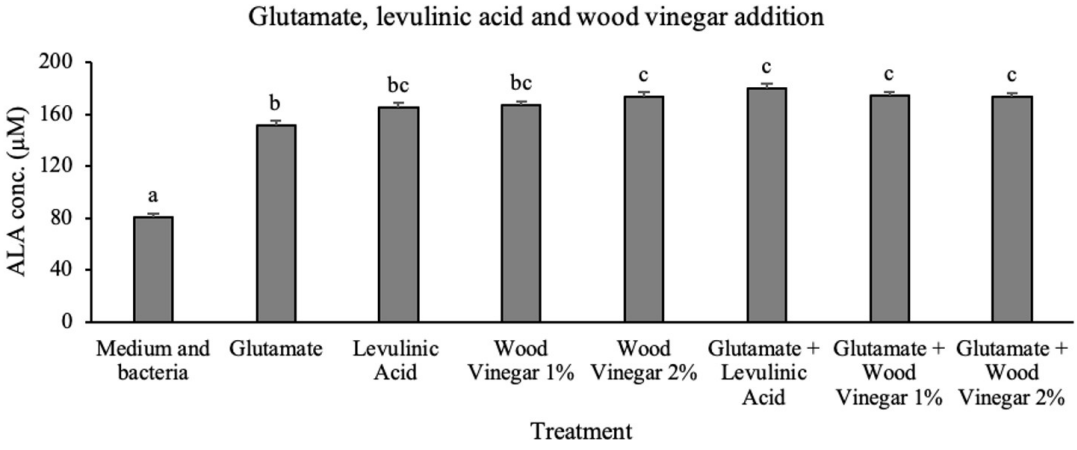

Figure 5. ALA concentrations in the bacterial culture medium following amendments after 8 h of glutamate and 10 h of LA and WV. Columns similarly superscripted are not significantly different (Tukey post hoc test). There was a significant treatment effect $p<0.001$ for both data sets. Individual error bars are based on the pooled variance estimate from the ANOVA with 10 degrees of freedom

that improves the growth of the pea plant (*Pisum sativum* L.). The mechanisms involved in promoting pea growth and biomass were phosphate solubilization, siderophore, and Indole Acetic Acid (IAA) production.

The data for bacterial growth (absorbance) and ALA production (Figure 1) suggest that ALA concentrations within the medium increased with increasing bacterial growth. Bacterial cells excrete ALA into the surrounding medium. Sasaki *et al.* (1987) reported that ALA production in recombinant strains was concomitant with bacterial growth.

The lag phase observed here is normal due to the physiological adaptation of cells to the new culture environment during the early stages (Yates and Smotzer 2007). It was noted that ALA (56 µM) was detected at the first point, suggesting that ALA was produced in the preculture stage. The exponential (log) phase was initiated when the bacteria adapted to the environment; at this point, cells increase in number as well as size, and metabolite production (in this case, ALA) is enhanced (Lee *et al.* 2004; Maier *et al.* 2015). Moving to the middle of the exponential stage, there was a rapid increase in ALA concentration. This contradicts the previous study, which demonstrated that maximum ALA production occurred at the end of the exponential phase (Lee *et al.* 2004). As the cells reached the stationary phase, ALA synthesis decreased, corroborating observations in an earlier study that showed that ALA synthase activity slowed at the onset of the stationary stage (Foley and Beale 1982). The maximum ALA production reached 102 µM, consistent with the work of Foley and Beale (1982), who measured values in the range of 100 µM. Ishii *et al.* (1990) and Saikeur *et al.* (2009) found that *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* KG31 produced ALA up to 160-180 µM. The apparent increase in ALA concentration after 22 h suggests that ALA was a component of secondary metabolites since, according to Singh *et al.* (2017), secondary metabolites are organic compounds that are indirectly connected with the growth, development, and reproduction of microorganisms and are usually formed towards the end of the stationary stage. These contradicts the comment by Ruiz *et al.* (2010) that the formation of microbial secondary metabolites usually occurs during the late log stage.

The optimum time for adding the precursor (glutamate) to the bacterial culture was determined from the first growth curve (Figure 1) by observing the point where the exponential phase started (3-4 hours after inoculation). Theoretically, adding a precursor at the early exponential phase should support ALA synthesis by the bacteria (Saikeur *et al.* 2009). Furthermore, Cihak *et al.* (2017) found that secondary metabolites are usually synthesized at the early phase of microbial growth, contradicting other authors' earlier statements. Weber and Kim (2016) pointed out that metabolic engineering should be performed at the early stage of secondary metabolite production to optimize the production of secondary metabolites.

The second curve (Figure 2) became the base for determining the optimum time for adding the inhibitor (LA). From the regression formulae calculation, the best time for adding the inhibitor was after 9 hours and 45 minutes, or rounded to 10 hours after inoculation. The utilization of an inhibitor was an attempt to enhance the ALA production from the bacteria. Fu *et al.* (2008) stated that the function of an inhibitor was to prevent the degradation of ALA during fermentation, which is caused by the activity of ALAD.

In the present study, the addition of glutamate as a precursor after 4 hours of incubation enhanced the ALA content in the bacterial growth medium. This finding suggests that the precursor supported the bacteria in producing/excreting ALA. Photosynthetic bacteria, such as *Anabaena variabilis, Anacystis nidulans, Chlorobium limicola, Chloroflexus auranticus*, and algae, such as *Cyanidium caldarium* and *Agmenellum quadruplicatum* utilize glutamate as a sole precursor to increase the ALA production (Jugenson *et al.* 1976; Avissar 1980; Kipe-Nolt, 1980; Andersen *et al.* 1983).

The findings in the current investigation using Bacillus paramycoides confirmed the work of Ahn (2007), who synthesized 2.0 mM of ALA from *Bacillus cereus* using glutamic acid, glucose, and acetic acid. The second experiment yielded lower concentrations of ALA from the growth medium than in the first experiment. This might be caused by removing cells during the centrifugation, and any ALA that the lysis of cells might have generated in the first experiment would not be present. Glutamate addition at 8 hours was more effective than the earlier experiment. Nevertheless, both experiments demonstrate that glutamate is an effective precursor for enhancing bacterial ALA production.

Lee *et al.* (2003) proposed the use of an inhibitor in order to limit the formation of ALAD during fermentation as a means of improving ALA yield. However, in the first experiment here, LA reduced ALA production. The reason for this is unknown, but it is worth noting that LA was added to the media, which had also previously been amended with glutamate. It is possible that a form of metabolic feedback occurred, preventing ALA production or excretion, or perhaps an excess of ALA

itself acted as a precursor for other metabolites since the pathways involved are complex. In contrast, in the second experiment, when LA was added after 8 and 10 hours of incubation (in the absence of glutamate), ALA concentrations exceeded those observed in the cultures that had been amended with glutamate. These data support the proposition by Lee *et al.* (2003).

The highest ALA content after the addition of glutamate 8 hours after inoculation and LA addition 10 hours after inoculation corroborate the earlier studies related to the ideal time for adding precursor (glutamate) and inhibitor (LA) in the production of ALA by *Bacillus paramycoides*. This study set out to assess the use of WV as the substitution of LA in improving the extracellular production of ALA. WV is a by-product of the pyrolysis process from biomass charcoal production (Wang *et al.* 2020). This environmentally friendly product was considered a low-cost inhibitor in the liquid fermentation of bacteria-producing ALA, which generally uses levulinic acid.

Levulinic acid (4-oxo-pentanoic acid), containing ketone and carboxylic acid groups, is considered a useful intermediate chemical compound for producing fuels and chemicals (Kumar *et al.* 2019; Pyo 2020). Levulinic acid is also known for its inhibitory properties in glucose fermentation (Li *et al.* 2017). Sasaki *et al.* (1987) found the effect of LA as the inhibitor of ALAD in the extracellular production of ALA. LA addition (10-25 mmol/L) at the mid-log phase was proven to delay the growth and enhance the ALA formation, whereas more than 50 mmol/L compressed both growth and production.

Matsushita *et al.* (2002) and Nunkaew *et al.* (2018) identified LA as one of the compounds of WV, with amounts of 12-17 mM and 9.47±0.34 mM. In this study, the ALA content with the addition of WV was increased, and the result was almost the same as that of LA. This finding confirms that WV could have substituted LA for the ALA production. This is also in accordance with the study of Nunkaew *et al.* (2018), who showed that the optimal condition for enhancing the production of ALA was by adding WV during the middle log phase. With the utilization of WV, it was expected that the ALA production would be higher with lower costs.

The study showed that the addition of glutamate and 1% WV increased the ALA production by *Bacillus paramycoides* to 174.3 µM, almost reaching the same value as the addition of glutamate and levulinic acid, which produced an ALA content of 179.9 µM. Moreover, this research confirmed that WV was potentially used as an alternative economical substitution for LA in ALA production.

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