5-aminolevulinic acid from photosynthetic bacteria and its applications

Amornrat Tangprasittipap\textsuperscript{1} and Poonsuk Prasertsan\textsuperscript{2}

Abstract

Tangprasittipap, A. and Prasertsan, P.
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This paper gives an overview on ALA production by photosynthetic bacteria concerning biosynthesis and regulation as well as its application as herbicide, insecticide and growth stimulator. Recent medical applications in the field of photodynamic therapy, cancer treatment, tumor diagnosis and other clinical uses are described.

Key words : 5-aminolevulinic acid, photosynthetic bacteria, ALA production, application

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Received, 28 February 2002 Accepted, 5 June 2002
5-Aminolevulinic acid (ALA) or 5-aminooxypentanoic acid, is an aliphatic precursor of tetrapyrrole biosynthesis present in all living cells. ALA is the natural photodynamic compound effective as a biodegradable herbicide and insecticide harmless for crops, humans and animals (Sasikala et al., 1994) as well as having promotive effect on the growth and photosynthesis of crops and vegetables (Sasaki et al., 1993). Further applications of ALA are now in the area of medicine and pharmacy products (Levy, 1995).

Commercial ALA is produced by chemical synthesis, which involves many complex reactions and causes of high expenditure. Biological production of ALA by algae, anoxygenic photosynthetic bacteria and chemotrophic bacteria (Table 1) is an alternative approach as it is a less expensive method than chemical synthesis. Anoxygenic phototrophic bacteria (APB) can accumulate and excrete high concentration of ALA into the medium, hence it is suitable for commercial exploitation (Sasikala et al., 1994) and it now commercially produces from *Rhodobacter sphaeroides*.

This paper gives an overview on the biosynthesis and regulation of ALA by photosynthe-

### Table 1. Production of ALA by different groups of microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Carbon and nitrogen source</th>
<th>LA (µM)</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Phototrophs</strong></td>
<td></td>
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<td>Algae</td>
<td></td>
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<tr>
<td><em>Agmmennulum quadruplicatum</em></td>
<td>Glutamate</td>
<td>+ 0.225</td>
<td>Kipe-Nolt and Steven, 1980</td>
</tr>
<tr>
<td><em>Cyanidium caldarium</em></td>
<td>Glutamate</td>
<td>+ 0.483</td>
<td>Jugenson et al., 1976</td>
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<tr>
<td><strong>Bacteria, oxygenic phototrophic</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Anacystis nidulans</em></td>
<td>Glutamate</td>
<td>+ 0.38</td>
<td>Anderson et al., 1983</td>
</tr>
<tr>
<td><em>Anabaena variabilis</em></td>
<td>Glutamate</td>
<td>+ 0.019</td>
<td>Avisser et al., 1983</td>
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<tr>
<td><strong>Bacteria, anoxygenic phototrophic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodobacter. sphaeroides</em></td>
<td>Succinate and glycine</td>
<td>+ 0.75</td>
<td>Anderson et al., 1983</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>Succinate and glycine</td>
<td>+ 2-4</td>
<td>Sasaki et al., 1991</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>Succinate and glycine</td>
<td>+ 160.0</td>
<td>Ishii et al., 1990</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>Swine waste (VFA)</td>
<td>+ 4200</td>
<td>Sasaki et al., 1990</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>Mandarin orange peel (modern synthetic waste water)</td>
<td>+ 16000</td>
<td>Sasaki et al., 1993</td>
</tr>
<tr>
<td><em>R. sphaeroides</em></td>
<td>Sewage sludge</td>
<td>+ 9300</td>
<td>Tanaka et al., 1983</td>
</tr>
<tr>
<td><em>Chlorobium limicola</em></td>
<td>Glutamate</td>
<td>+ 3.950</td>
<td>Anderson et al., 1983</td>
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<tr>
<td><strong>Chemotrophic bacteria</strong></td>
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<td><strong>Aerobes</strong></td>
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<tr>
<td><em>Pseudomonas riboflava</em></td>
<td>L-alanine</td>
<td>+ 0.2</td>
<td>Rhee et al., 1987</td>
</tr>
<tr>
<td><em>Propionicbacterium shermanii</em></td>
<td>Succinate and glycine</td>
<td>+ 0.04</td>
<td>Menon and Shemin, 1967</td>
</tr>
<tr>
<td><strong>Anaerobes</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Clostridium thermoaceticum</em></td>
<td>Glucose and L-lysine</td>
<td>+ 155.0</td>
<td>Sjoji et al., 1989</td>
</tr>
<tr>
<td><em>Methanosarcina barkeri</em></td>
<td>Methanol, 2-oxoglutarate</td>
<td>+ 0.4</td>
<td>Lin et al., 1989</td>
</tr>
<tr>
<td><em>Methanobacterium thermoautotrophicum</em></td>
<td>H₂ + CO₂</td>
<td>+ 0.2</td>
<td>Lin et al., 1989</td>
</tr>
</tbody>
</table>

VFA: Volatile Fatty Acid; LA: Levulinic acid; +: Addition

Source: Sasikala et al., 1994
tic bacteria as well as its application as herbi-
cide, insecticide and growth stimulator and in
the medical fields.

Biosynthesis of ALA

The biosynthesis of ALA can be formed via
two distinct metabolic pathways (Figure 1).

1. C₄ pathway (Shermin pathway)

Shermin pathway is observed in mam-
malian cell, yeast, fungi and very common
among the purple non-sulfur photosynthetic group
and a few chemotrophs (Sasaki et al., 1990).
The key enzyme involved in the C₄ pathway of ALA for-
mation is ALA synthetase (EC 2.3.1.37) cata-
lyzing the condensation of succinyl-CoA and
glycine. The ALA synthetase activity was highest
from cells harvested at the logarithmic phase of
growth (Sato et al., 1985).

The culture conditions have high influ-
ence on the synthesis of ALA synthetase and
changing the culture condition from aerobic to
micro-aerobic increased the ALA synthetase
activity by 2-4 fold (Sandy et al., 1985). On the
other hand, the enzyme synthesis in light was
repressed by oxygen and the effect could be
overcome upon the restoration of anaerobic con-
dition (Viale et al., 1983).

2. C₅ pathway

The C₅ pathway is present in higher
plants, algae and several bacteria, (Kajiwara et al., 1994),
indicating the purple and green
sulfur bacteria (Sasikala and Ramana, 1995). In
C₅ pathway, ALA is formed from glutamate or
the α-ketoglutarate via a path that does not
involve the ALA synthetase reaction. The purifi-
cation of the C₅ pathway enzyme indicates that
 glutamate is reduced to ALA in three steps
(Sasikala et al., 1994).

a) Ligation of t-RNA to glutamate catal-
yzed by glutamyl-t-RNA synthetase
b) Reduction of glutamyl-t-RNA to
 generate glutamate-1-semialdehyde
(GSA) catalyzed by glutamyl-t-RNA
reductase (EC 6.1.1.17)
c) Transamination of GSA to generate
ALA catalyzed by GSA aminotrans-
ferase (GSA-AT, EC 5.4.3.8)

Two catalytic mechanisms (Figure 2)
have been proposed for the GSA conversion to
ALA (Grimm et al., 1991). ALA is formed by
accepting and releasing amino group at position
5 and 4 of GSA, respectively (Figure 2a) or two
molecules of GSA oriented head to tail forma-
tion to amino-hemiacetal dimer, which converted
into a double Schiff base and rearranged into the
amino-hemiacetal (exchange amino groups) and
subsequently dissociate into two molecules of
ALA (Figure 2b) (Sasikala et al., 1994).

Regulation of 5-aminolevulinic acid production
in photosynthetic bacteria

1. Carbon and nitrogen sources

Although glutamate is the carbon and
nitrogen source for ALA production by many
microorganisms, ALA production can also be
produced with other carbon sources (Sasikala et al., 1994). Glucose had the advantage of being
are inexpensive source for the industrial produc-
tion of ALA. In the batch fermentation of mutant
strain of R. sphaeroides CR606 accumulated
ALA to level of 20 mM after 18 h with the pro-
duction rate was 1.1 mMh⁻¹ and the yield coef-
ficient for ALA was 40% (mol/mol) of glucose
(Nishikawa et al., 1999).

R. sphaeroides can utilize volatile fatty
acid (VFA) such as acetic, propionic and butyric
acid as carbon and energy sources (Sasaki et al.,
1987). In addition, VFAs produced from the
anaerobic digestion liquor of sewage sludge was
reported to be the carbon source for production
of ALA, up to 9.2 mM, by R. sphaeroides with
repeated addition of the glycine and glutamic
acid as the organic nitrogen source (Tanaka et
al., 1994). The effluent of anaerobic mandarin
orange peel (Tinpi) supplemented with glycine
could be used to produce 5-aminolevulinic acid
from R. sphaeroides (Sasaki et al., 1993).

2. Precursors

The addition of precursors (succinate
and glycine) in the range of 20-60 mM into the
digestion liquor medium resulted in the positive
effect to ALA production of R. sphaeroides IFO
Figure 1. Biosynthesis of 5-aminolevulinic acid (ALA) via $C_4$ and $C_5$ pathway

PALA: pyridoxyl phosphate; TPP: thiamine pyrophosphate; ALAS: ALA synthetase; ALAD: ALA dehydratase; GSA: glutamate 1-semialdehyde

Source: Sasikala et al., 1994
12203, but above 80 mM of precursors resulted in a negative effect on ALA formation. The supply of succinate is sufficient but the glycine supply might limit ALA formation in this culture system (Sasaki et al., 1990). ALA synthetase and ALA dehydratase activities in the cells are not influenced by the simultaneous addition of precursors compared with these activities in the control (no addition of precursor) but the growth is excessively suppressed by the addition of glycine (Sasaki et al., 1991).

The growth and ALA production of Chlorella sp. strain 4S is enhanced by glutamate addition, whereas the addition of succinate and glycine suppresses both (Sasaki et al., 1995). However, the addition of glutamate to the heterotrophic medium culture of Chlorella regularis YA-603 does not enhance ALA production and Shermin pathway is suggested to contribute to ALA production of this strain (Ano et al., 1999).

3. Levulinic acid
Levulinic acid (LA), analog of ALA, is an inhibitor of ALA dehydratase which enhances extracellular ALA formation (Sasaki et al., 1987). In photoheterotrophic culture of R. sphaeroides, repeated addition of LA results in moderate cell growth suppression while extracellular ALA is not produced during the cultivation without the addition of LA. The amount of LA should be as small as possible since LA is expensive compared with glycine and 30 mM LA are recommend for use to save cost (Sasaki et al., 1990). At LA concentration over 50 mM, growth ceased completely and ALA was not excreted (Sasaki et al., 1987).

4. Metal ions
Metal ions particularly Fe$^{2+}$ and Co$^{2+}$ are important elements for regulating tetrapyrrole biosynthesis in R. sphaeroides. ALA synthetase is regulated by heme compound as feedback inhibition or repression under iron-sufficient conditions. Therefore, ALA production medium should contain neither cobalt nor iron to enhance ALA accumulation (Sasikala et al., 1994).

5. Light intensity
Light intensity is an important factor for enhancing ALA formation. Growth of photosynthetic bacteria is found to be independent of light intensity (1-5 klux), while the amount of ALA is reached the maximum value at 3 klux. High illumination (over 5 klux) was not effective for ALA production and low illumination (below 1 klux) produced quite a low growth rate and virtually no formation of ALA (Sasaki et al., 1991).

![Figure 2. Mechanism of the conversion of glutamate 1-semialdehyde to ALA](Source: Grimm et al., 1991)
6. Aeration

Oxygenation is one of the important factors affecting ALA synthetase activity in the loss of pigmentation due to the decrease in the ALA synthetase activator, cysteine trisulfide and glutathione trisulfide (Sandy et al., 1985). Changing the culture conditions from aerobic to microaerobic increased the activity of ALAS by 2-4 folds. The biosynthesis of ALA synthetase under light condition was repressed by oxygen and the effect could be overcome upon the restoration of anaerobic conditions (Viale et al., 1983).

7. pH

The effect of pH (6.0-8.0) of the VFAs culture medium is studied on ALA production by R. sphaeroides. At neutral pH (6.8 and 7.0) extracellular ALA production is up to 16 mM. Under controlled pH 6.8±1, intracellular ALA synthetase activity is significantly enhanced after adding LA, while ALA dehydratase is inhibited to low level. At higher pH (8.0) ALA synthetase activity was low and ALA dehydratase is relatively high (Sasaki et al., 1993). The inhibitory effect of LA for ALA dehydratase activity of R. sphaeroides is strongly dependent on the pH value of the GM medium. At pH 5.5, 5 mM LA inhibited 85% of ALA dehydratase activity (in vitro), while 100 mM LA decreased 45% of ALA dehydratase activity at pH 7.5 (Sasaki et al., 1997).

8. Others factors

Biotin was needed for ALA synthetase activity in the formation of ALA which is the intermediate of bacteriochlorophyll synthesis. Thiamine is the substrate of the coenzyme thiamine pyrophosphate which converts α-oxoglutarate to the C₄-intermediate of Shermin pathway (Lascelles, 1956).

Low molecular weight sulfur compounds of cysteine or glutamine, cysteine trisulfide (CySSSCy), glutathione trisulfide (GSSSG), glutathione and cysteine trisulfide (GSSSCy) and trisulfanedisulfonate (S₅O₆²⁻) regulated the activity of ALA synthetase in vivo. Poly (sulfane) disulfonate (-O-Sn-Sn-SO₄³⁻) and R-Sn-R’ (R and R’ are organic or inorganic group) with n > 3 are exhibited as the activators of ALA synthetase. LA requires the presence of an exogenous thio, such as 2-mercaptoethanol or dithioerythritol to maintain catalytic activity while nitrite in the growth medium inhibited ALA production (Sasaki et al., 1994).

Application of 5-aminolevulinic acid

5-aminolevulinic acid converts molecular oxygen into singlet oxygen when excited by the absorption of light. ALA is potentially useful in agriculture as a herbicide and can be used as an antimicrobial drug. The important reason is that it is nontoxic to mammals, is readily biodegradable, and has no adverse effects on the environment (Tanaka et al., 1992). ALA can also be applied as photodynamic therapy for malignant skin tissue tumors.

1. Herbicide

ALA has photodynamic herbicide properties under appropriate treatment conditions. An immediate dark-incubation period after spraying ALA is an essential step to accumulate tetrpyrrole within the plants. ALA serves as a building block of tetrpyrrole accumulation, while a group of modulators, O-phenanthroline, ethyl nicotinate and 2,2'-depyridyl (DP), have affected to the pattern of tetrpyrrole accumulation and act in concert with ALA. DP is a cheap chemical then it is selected to mix with ALA to enhance the accumulation of tetrpyrrole. During the daylight period, the excess tetrpyrrole produces active oxygen (singlet oxygen) which oxidizes the unsaturated fatty acid on the cell surface (lipoprotein component), thus setting in motion a greatly damaging free-radical chain reaction. The cell membranes become leaky and this in turn results in a rapid and severe dehydration, bleaching and collapse of the leaf and/or hypocotyl tissue (Rebriz et al., 1984). Within 24 h the green plant tissue turns into a brownish desiccated mass of dead tissue (Rebriz et al., 1990). On the other hand, treated plants kept for the same period of time in darkness were unaffected.

The accumulation of tetrpyrrole in plant leaves causes very severe photodynamic
damage and the leave die within a few of hours while the cotyledons, stem and growing point remain unaffected. DICotyledonous weeds such as redroot pigweed, purslane and lambquarter are highly susceptible to the tetrapyrrole induced photodynamic damage. Monocots such as core, wheat, oats and barley were not adversely affected by the spray (Rebriz et al., 1984). The death of plants depended on the ages of plant, ALA concentration, type of modulators, ratio of ALA and modulator, light intensity and kinds of plant treated (Kobayashi and Haque, 1971).

2. Insecticide

Rebriz and his co-worker (1988) developed a novel porphyrin insecticide consisting of modulator of porphyrin, 3.0 mM of ALA plus 30 mM of DP at pH 3.5. When this solution was sprayed on the larvae of Trichoplusia ni (Hubner insects) ALA induced the massive accumulation of protoporphyrin IX causing death in darkness via an unknown mechanism and in the light probably via singlet oxygen formation. Besides the advantages of ALA being nontoxic to non-target organisms, such as other crops, animals and human, it is also difficult for insects to develop resistance against ALA.

3. Growth stimulator

Besides having herbicidal property, ALA is a very good growth simulator when used at low concentrations (Sasaki and Ramana, 1995). ALA has promotive effect on the growth and yield of several crops and vegetables, enhancing photosynthesis, stimulating fixation of CO₂ in light. The ALA also suppresses the respiration and the release of CO₂ under dark (Hotta et al., 1997). The appropriate applications of ALA showed 10-60% promotive effect over the control on radish, kidney beans, barley, potatoes, garlic, rice and corn (Sasaki et al., 1994). In addition, the culture medium from ALA production by R. sphaeroides could be directly used as a fertilizer having herbicide activity (Sasaki et al., 1990).

4. Photodynamic therapy

Photodynamic therapy (PDT) involves the use of photosensitizers (light-sensitive molecules) that are activated by light caused the formation of active forms of oxygen which is resulted in the killing of cell in which the photosensitizers are present, while sparing the normal surrounding tissue (Levy, 1995).

Kennedy et al (1990) proposed the use of tropically ALA based PDT for selected cutaneous disease. The cosmetic results of ALA-PDT treatment are very good and with a minimal effect on the normal skin (Sveanberg et al., 1994). Promising clinical results have been obtained in photosensitizing superficial skin tumors. In contrast, non-superficial and tumors of morpheaform histologic pattern have shown minor response rates only (Martin et al., 1995, Szeimies et al., 1994).

References


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