

# **Influence of Glycine, Succinate, Levulinic Acid and Glutamate Concentrations on Growth of Purple Non-Sulfur Photosynthetic Bacteria and 5-Aminolevulinic Acid Production**

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**ABSTRACT:** Purple non-sulfur photosynthetic bacteria (PNSB) and their product 5-aminolevulinic acid (5-ALA) have broad usage in different fields of medicine, agriculture, bioremediation, etc. As demand for aminolevulinic acid is increasing continuously, new bio-processes and new producers of 5-ALA are researched and optimized. Strain of purple non-sulfur bacteria *Rhodobacter azotoformans* isolated from mineral waters of Armenia is potential producer of 5-aminolevulinic acid. For better optimization of 5-ALA production, set of experiments was conducted in order to determine influence of glycine, succinate, levulinic acid and glutamate on the growth and production of 5-ALA. As addition of mentioned chemical compounds in nutrient media is needed for promotion of 5-ALA production, it was tested what is the optimal concentration of glycine, succinate, levulinic acid and glutamate during cultivation to obtain highest yields. From the results it can be concluded that maximal 5-ALA concentration was achieved in batch cultivation when concentration of glycine was 40 mM, succinate 30 mM, levulinic acid 15 mM and concentration of glutamate 4g/l. Concentration of 5-ALA was in this case 385 mg/L. Addition of glycine only yielded lower concentrations (183.75 mg/L). Concentrations of added glycine and succinate above 40 mM didn't yield higher concentrations of 5-ALA. Increase of glutamate concentration above 4 g/L also doesn't increase 5-ALA yields.

**KEYWORDS:** purple non-sulfur photosynthetic bacteria, nutrient media, 5-aminolevulinic acid, synthesis precursors.

## **I. INTRODUCTION**

Purple non-sulfur photosynthetic bacteria (PNSB) are versatile microorganisms that can grow under photoautotrophic, photoheterotrophic and chemoheterotrophic conditions [6]. For their growth they are able to assimilate various organic acids, vitamins, aminoacids, carbohydrates, alcohols, etc. however, use of organic substances differs significantly from strain to strain [9,13]. Capability of usage of organic substances in the presence of light is a characteristic for all purple bacteria, but the metabolic pathways in which organic substrates are utilized depend on the characteristics of the organisms, the nature of the compounds and on growth conditions. The best substrates for the growth of photosynthetic bacteria (PB) are most often dicarboxylic acids, pyruvate, lactate and acetate [12]. The organic substrates have two functions in bacterial photosynthesis and metabolism, that is, as a donor of hydrogen and at the same time as a source of carbon [12]. With presence of organic compounds in growth media photosynthetic bacteria do not require the presence of inorganic hydrogen, and in some cases do not need exogenous carbon dioxide. This is the case when

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microorganisms grow in the presence of acetate, pyruvate, malate, fumarate, i.e. compounds that can be incorporated into the constructive processes of cells [12, 13]. Also other carbon sources can be used for growth of PNSB. As an example of unusual carbon source consumption for growth and synthesis, *Rhodospseudomonas (Rps.) palustris*, is able to assimilate formate, as well as benzoate and other aromatic compounds. *Rhodomicrobium (Rbi.) vannielii* and *Rmi. fuloum* grow well on nutrient media with ethanol, and some representatives of *Rhodospseudomonas (Rps.)* in the presence of carbon monoxide and methane. Those strains have a potential in bioremediation of polluted soil.

In this study, we have examined 5-ALA production by purple non-sulfur photosynthetic bacteria, taking into consideration the effects of succinate, glutamate, acetate, levulinic acid and glycine addition as a precursor under constant light regime, temperature and pH [16, 17]. Microbial production of 5-ALA has many advantages over chemical production, such as a simpler process control, high efficiency, without production of toxic compounds and process sustainability. Application of 5-ALA in medicine for example in photodynamic therapy for cancer patients is reported [10]. 5-ALA is also used in the detection of glioma cells during surgery [18]. 5-ALA as a versatile chemical can be also used in agricultural applications as an herbicide, insecticide and growth promoting factor and also can increase salt and cold temperature tolerance in plants [23]. 5-ALA is an intermediate of tetrapyrrol biosynthesis. There are two biochemical pathway by which ALA can be synthesized, C4 and C5 pathway. In the C4 pathway of ALA formation, 5-ALA is produced in animals, fungi and alphaproteobacteria. In this pathway, 5-ALA is synthesized from the condensation of glycine and succinate, which is catalyzed by 5-ALA synthase enzyme. The C5 pathway of ALA formation is present in plants, algae, cyanobacteria, most eubacteria and some anoxygenic photosynthetic bacteria [11,24,25]. Also it has been found that a bacteria *Methanobacterium thermoautotrophicum* [7] and algae such as *Agmenellum quadruplicum*, *Anacystis marina* [6] and *Chlorella vulgaris* [2] can excrete 5-ALA extracellularly when levulinic acid as a competitive inhibitor of ALA-dehydratase in tetrapyrrol biosynthesis [5] was added to the medium. Production of ALA from glutamate by anoxygenic photosynthetic bacteria such as *Chlorobium limicola*, *Chloroflexus aurantiacus* and *Rps. palustris*, as well as oxygenic photosynthetic bacteria, such as *Anacystis nidulans* are also reported [1].

It is well known that photosynthetic bacteria have the ability to synthesize tetrapyrrol via 5-ALA intracellularly from glycine and succinate by ALA-synthetase (Shemin pathway) [14, 15, 26]. Wild strains have very low yield of 5-ALA, so optimization of the production is needed. One of the solutions is usage of genetically modified strains of PNSB that have higher 5-ALA yield [20]. Another solution is application of microorganisms that are commonly used in biotechnological production of different compounds as genetically engineered *E. coli* and *Corynebacterium glutamicum*[4]. *Escherichia coli* strains have been successfully genetically modified by hem A gene transfer from *Bradyrhizobium japonicum* [3] and *Rba. capsulatus* [7].

Mineral waters in the Republic of Armenia have abundance of new and useful microorganisms that have great potential for production of biofuels [8] and specific biochemicals [20]. Isolated strains of purple non-sulfur bacteria were tested for their 5-ALA production capability in previous work and one potential strain was selected as an ALA producer [20]. This strain was characterized as *Rba. azotoformans*.

In this work optimization of nutrient media for 5-ALA production by *Rba. azotoformans* was performed in order to obtain higher yields of 5-ALA. Influence of glutamate as a nitrogen source was tested as well as influence of addition of glycine, succinate and levulinic acid during growth on 5-ALA synthesis.

## II. RELATED WORK

**Microorganisms.** Working microorganism used in this research was mutant strain *Rba. azotoformans* E10 from the culture collection of photosynthetic bacteria of the Laboratory of Energy Alternative Sources. Working microorganism was firstly classified as *Rba. capsulatus*, but de novo classification by 16S rRNA sequences with high level reliability form a single cluster (85%) and obtained phylogenetic tree was classified as *Rba. Azotoformans*. This tested strain used in this research has 99% sequence similarity with the corresponding nucleotide sequence of the strain *Rba. azotoformans* strain JCM 9340 (NR\_113300.1).

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Methods of selection and mutagenesis were described previously in paper by [20].

**Nutrient media.** Glutamate malate (GM) media (g/L) was proven to be the most suited for growth of bacterial biomass (dark / light and aerobic / microaerophilic conditions) [20]: 1.5 g/L of yeast extract, 2.7 g/L of  $C_4H_4Na_2O_5$  (malate, 178.1 g/mol), 2 g/L of  $C_5H_8NO_4Na$  (monosodium glutamate, 169.1 g/mol), 0.8 g/L of  $(NH_4)_2SO_4 \times 7H_2O$  (ammonium sulfate, 132.1 g/mol), 0.5 g/l of  $KH_2PO_4$  (monopotassium phosphate, 136.1 g/mol), 0.5 g/L of  $K_2HPO_4$  (dipotassium phosphate, 142 g/mol), 0.2 g/L of  $MgSO_4 \times 7H_2O$  (magnesium sulfate 120.4 g/mol), 0.053 g/L of  $CaCl_2 \times 2H_2O$  (calcium chloride, 110.0 g/mol),  $1.2 \times 10^{-3}$  g/L of  $MnSO_4 \times 7H_2O$  (manganese sulfate),  $1.0 \times 10^{-3}$  g/L of thiamin-hydrochloride,  $1.0 \times 10^{-3}$  g/L of nicotinic acid,  $1.0 \times 10^{-5}$  g/L of biotin, pH 6.8-7.0 [19, 20].

## Analytical methods

**Determination of biomass concentration.** Samples from cultivation were taken in predetermined time, centrifugation at 8000rpm and cell dry weight was determined by drying in oven at 105°C for 24 h to constant mass [19, 20].

**Determination of 5 ALA concentrations.** Supernatant after centrifugation was used for 5 ALA determinations. Sample of 0.2 mL was mixed together with 0.65 mL of acetaldehyde-acetone buffer (acetate buffer with 1% of acetyl acetone). Sample was well mixed and then put at 100 C for 20 minutes. After 20 minutes sample was cooled to 0°C and then 0.65 mL Ehrlich reagents (acetic acid with 2% w/v polimethylaminobenz-aldehyde and perchloric acid) was added. After 20 minutes samples were analyzed on spectrophotometer at 553 nm wave length [19, 20].

## Cultivation of *Rba. azotoformans* in different nutrient media composition for optimization of 5 ALA production

**a) cultivation of *Rba. azotoformans* in different nutrient media composition for optimization of 5 ALA production with different levulinic acid concentrations.** In first set of experiments influence of addition of different LA concentration on production of 5-ALA was tested. Batch cultivation of bacterial biomass lasted 72 h. Inoculum for cultivation was grown in light conditions under 2000 Lux and at temperature of 29°C in Erlenmeyer flasks without mixing for 48 h. The flasks with 25ml of GM media were inoculated with 10%, v/v of inoculum. After set period of cultivation time for biomass growth (24 h) a mixture of glycine, succinate and levulinic acid was added to stimulate the production of 5-ALA. Concentration of levulinic acid, glycine and succinate added to GM media is in Table 1. Samples were taken in set time intervals and analyzed. 2 ml of suspension from each flask was taken and centrifuged at 8000 rpm for 20 min. Centrifuged biomass was used for determination of dry biomass concentration by drying in oven at 105°C for 24 h. Supernatant was used for determination of 5-ALA.

Table 1. Initial concentrations of succinate, glycine and levulinic acid for cultivation of mutant strain *Rba. azotoformans* E10 during experimental determination of influence of added levulinic acid on 5 ALA production

Precursors	Concentrations, mM								
Succinate	30	30	30	40	40	40	50	50	50
Glycine	30	30	30	40	40	40	50	50	50
Levulinic acid	10	15	20	10	15	20	10	15	20

**b) cultivation of *Rba. azotoformans* in different nutrient media composition for optimization of 5 ALA production with different glycine and succinate concentrations.** In second set of experiments influence of addition of different glycine and succinate concentration on production of 5-ALA was tested. In this experimental setup concentration of levulinic acid was always 15 mM, and concentrations of added glycine and succinate were changed. Concentrations of added glycine and succinate are in Table 2. Cultivation was carried out as described in first part (a)

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Table 2. Initial concentrations of succinate, glycine and levulinic acid for cultivation of mutant strain *Rba. azotoformans* E10 during experimental determination of influence of added glycine and succinate on 5 ALA production

Precursors	Concentrations, mM															
Succinate	30	40	50	60	30	40	50	60	30	40	50	60	30	40	50	60
Glycine	30	30	30	30	40	40	40	40	50	50	50	50	60	60	60	60
Levulinic acid	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15

c) *cultivation of Rba. azotoformans in different nutrient media composition for optimization of 5 ALA production with different initial glycine, succinate and glutamate concentrations.* In third set of experiments influence of addition of different glycine, succinate concentration and glutamate on production of 5-ALA was tested. GM media with different concentrations of glutamate was prepared (2-6 g/L). In first part only addition of glycine was tested (Table 3).

Table 3. Initial concentrations of glutamate, glycine and levulinic acid during cultivation of mutant strain *Rba. azotoformans* E10

Precursors	Concentrations													
Glutamate, g/L	2	2	2	2	4	4	4	4	4	6	6	6	6	6
Glycine, mM	20	30	40	50	20	30	40	50	20	30	40	50	20	30
Levulinic acid, mM	15													

In the second part the addition of glycine and succinate (Table 4) on production of 5-ALA was tested. Concentration of levulinic acid was 15 mM. Cultivation was carried out as described in first part (a).

Table 4. Initial concentrations of glutamate, glycine, succinate and levulinic acid during cultivation of mutant strain *Rba. azotoformans* E10

Precursors	Concentrations														
Glutamate, g/L	2	2	2	2	4	4	4	4	4	4	6	6	6	6	6
Glycine, mM	30	40	50	40	30	40	50	40	50	30	40	50	40	50	
Succinate, mM	30	30	30	40	30	30	30	40	50	30	30	30	40	50	
Levulinic acid, mM	15														

### III. EXPERIMENTAL RESULTS

#### **Influence of succinate, glycine and levulinic acid concentrations on ALA production during cultivation of mutant strain *Rba. azotoformans* E10.**

Various concentrations of glycine, succinate and levulinic acid were added to the culture broth in order to evaluate the influence of glycine, succinate, levulinic acid on the growth of purple non-sulfur photosynthetic bacteria and the synthesis of 5-ALA. Glycine and succinate are precursors of 5-ALA biosynthesis, and levulinic acid is an inhibitor of 5-ALA dehydratase enzyme [16], therefore, glycine, succinate, levulinic acid were used during this research to achieve higher extracellular 5-ALA yield.

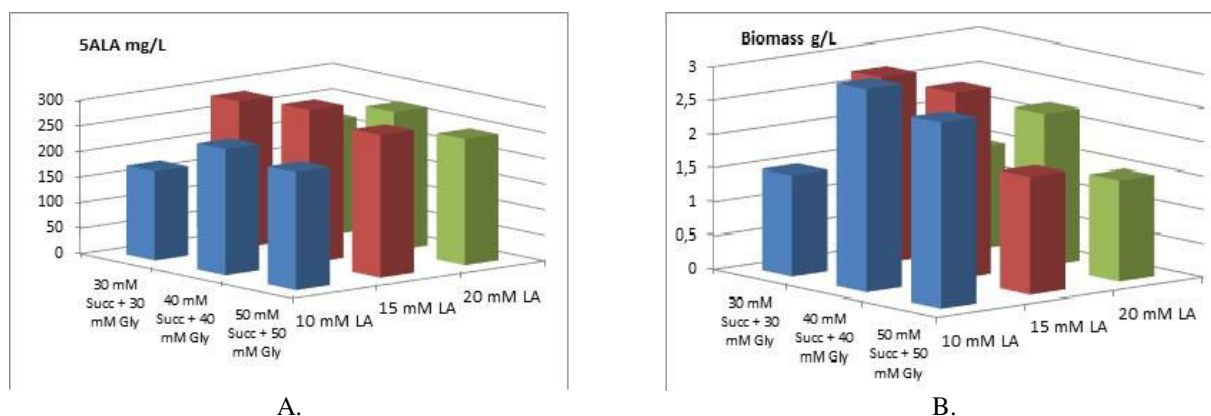


Fig. 1. Influence of different initial concentrations of succinate, glycine and levulinic acid on ALA formation during the cultivation of mutant strain *Rba. azotoformans* E10 (A) and biomass concentration (B). The concentration of Levulinic acid is 10, 15 and 20 mM respectively and the concentrations of succinate + glycine are 30+30mM, 40 + 40mM and 50 + 50mM respectively

From the results on Figure 1 it can be seen that addition of 15 mM of levulinic acid has most positive influence on 5-ALA production. Also, addition of 20 mM of levulinic acid yields higher concentration of 5-ALA than 10 mM of LA when initial concentration of succinate and glycine is higher than 30 mM. Maximal concentration of 5-ALA was 298 mg/L and it was produced in cultivation with 40 mM of succinate, 40 mM of glycine and 15 mM of levulinic acid. In this experiment the biomass concentration was 2.7 g/L.

### Influence of different succinate and glycine concentrations on ALA production during cultivation of mutant strain *Rba. azotoformans* E10.

From the above described experiments, it was determined that when concentration of Levulinic acid (LA) in cultivation media was 15 mM, we obtained best results regarding 5-ALA yield. For the next experimental setup, a detailed study of the effects of succinate and glycine on ALA production was examined. Concentration of levulinic acid was 15 mM for all experiments. Results obtained are shown in figure 2.

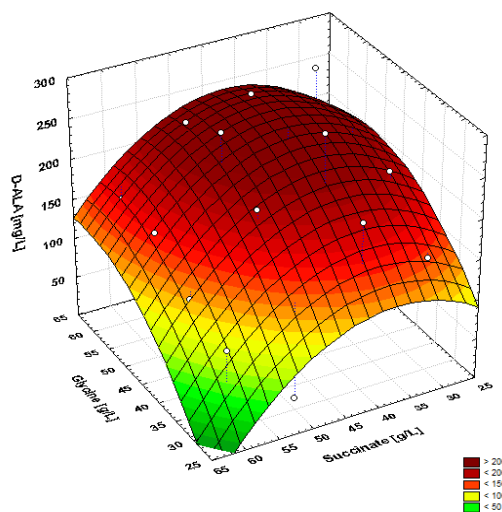


Fig. 2. Influence of different succinate and glycine concentrations on ALA production during cultivation of mutant strain *Rba. azotoformans* E10



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As shown in Figure 2, the highest concentrations of 5-ALA were obtained when the initial concentrations of succinate and glycine were 40/40 and 50/50 mM, respectively. At the same time, it has been shown that the use of low concentrations of glycine and succinate is not effective for 5-ALA production, since, presumably, a bacterial biomass is sufficiently active, and low concentrations of precursors are not sufficient for high 5-ALA yield. On the other hand, high concentrations of succinate and glycine (60/60 mM) have an inhibitory influence on extracellular ALA production.

Simultaneously, we have established that high concentrations of glycine in the media do not inhibit significantly the growth and production in our case. In literature it is stated that concentrations of glycine above 80 mM have negative influence on 5-ALA production [18]. In our experiments those high concentrations were not tested. From the biochemistry of 5-ALA synthesis it can be seen that succinyl-CoA and glycine are substrates for ALAS enzyme and therefore for synthesis of 5-ALA, and succinyl-CoA is synthesized from  $\alpha$ -ketoglutarate via  $\alpha$ -ketoglutarate dehydrogenase. Alpha-ketoglutarate is synthesized from glutamate via transaminase. As we have glutamate in our media as a nitrogen source (among others) this means that it is possible that glutamate is converted to  $\alpha$ -ketoglutarate, then to succinyl-CoA and with addition of succinate as a precursor for ALA synthesis, we have enough of succinyl in the media for ALA synthesis and therefore supply of glycine might limit the 5-ALA formation. Glutamate can enhance growth of biomass and 5-ALA production [21, 22, 25].

### **Influence of initial glutamate concentration on biomass growth and 5-ALA production during cultivation of mutant strain *Rba. azotoformans* E10.**

Glutamate is an important carbon and nitrogen source for growth of photosynthetic bacteria. Succinyl-CoA and glycine are precursors for 5-ALA, which is synthesized via ALAS enzyme. Succinyl-CoA is synthesized from alpha-ketoglutarate via alpha ketoglutarate dehydrogenase and Alpha ketoglutarate is synthesized from glutamate via transaminase.

Table 5. Influence of different initial glutamate and added glycine and levulinic acid concentrations on biomass and ALA formation by mutant strain *Rba. azotoformans* E10

Glutamate, g/L	Glycine, mM	Levulinic acid, mM	ALA, mg/L	Bacteria biomass, g/L
2	20	15	42.5	1.2
2	30	15	93.75	2.25
2	40	15	121.25	1.25
2	50	15	11.25	1.25
4	20	15	92.5	1.5
4	30	15	183.75	2.25
4	40	15	171.25	1.5
4	50	15	71.25	1.5
6	20	15	57.5	1.2
6	30	15	146.25	2.5
6	40	15	146.25	1.5
6	50	15	10	1.5

With this experiment it was proven that glutamate from the media is partially used for biomass growth and after addition of glycine and levulinic acid, ALA-dehydrogenase is inhibited and extracellular production of ALA occurs. After 24 h, enough glutamate is in the media for ALA production. Maximal ALA concentration was 183.75 mg/L when 30 mM of glycine was added and glutamate concentration was 4 g/L (Table 5.).

At the same time it is shown that if glutamate concentration is increased in nutrient medium and additional succinate added with glycine, the output of 5-ALA increases even more.

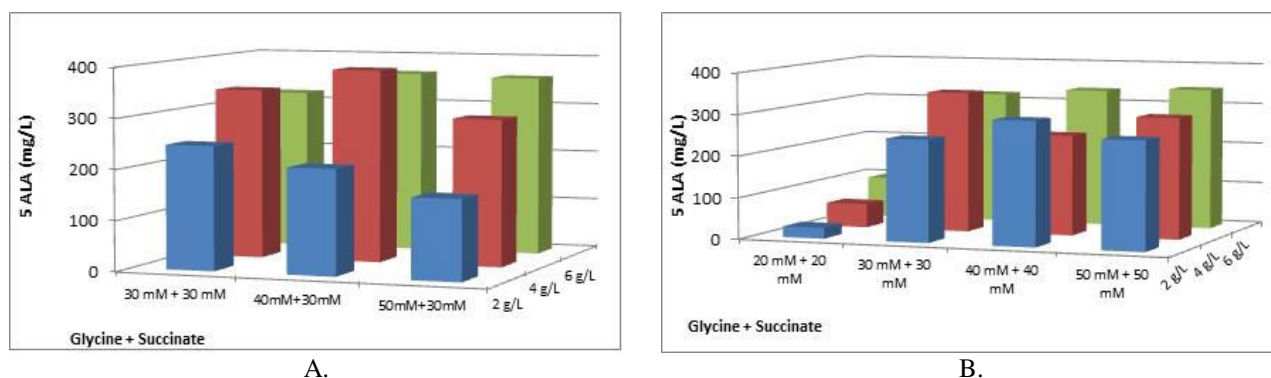


Fig. 3. Influence of different initial glutamate concentration and added glycine / succinate / LA (15 mM) on ALA production during cultivation of mutant strain *Rba. azotoformans* E10 (A - comparison when only glycine concentration is increased, B – comparison when glycine and succinate are increased equimolar)

As presented on the Figure 3, only addition of 30 mM of glycine with 4 g/L of glutamate in medium, ALA yield is 183.75 mg/L. In case if additional 30 mM of succinate is added, ALA synthesis increases by 85% and is 340 mg/L. In the case when 40 mM glycine and 30 mM succinate are added to the nutrient medium, the yield of ALA increases by 123% and is 385 mg/L.

#### IV. CONCLUSION

Thus, it was found that the mutant E10 of *Rhodobacter azotoformans* strain B-6508 has a large potential as a producer of 5-aminolevulinic acid. It was shown that the formation of ALA was closely related to intracellular ALA-synthetase activity produces by the mentioned microorganism. Light intensity, temperature, as well as supplementation with glycine, succinate, levulinic acid and glutamate as precursors were also an important factors for enhancing ALA formation. It was detected that upon addition of 30 mM glycine to the nutrient medium, the concentration of 5-ALA reaches 183.75 mg/L, with an initial glutamate concentration of 4 g/L in the medium. At the same time, it was found that when 30 mM succinate was added to the nutrient medium, the concentration of 5-ALA reached 340 mg/L, and when 40 mM glycine and 30 mM succinate were added to the medium, the concentration of 5-ALA increased to 385 mg/L.

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