

ORIGINAL SCIENTIFIC PAPER

# Characteristics and selection of cultures of photosynthetic purple non-sulphur bacteria as a potential 5-aminolevulinic acid producers

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## Abstract

Wild strains of purple non-sulphur bacteria: *Rhodospirillum rubrum* B-6505, *Rhodopseudomonas palustris* B-6506, *Rhodobacter capsulatus* B-6508 and *Rhodobacter spheroides* B-6509 were studied as 5-ALA (5 aminolevulinic acid) producers. Selected strains were subjected to mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine to obtain a strain with high 5-ALA producing capacity. After mutagenesis 19 stable mutant strains were selected from *Rhodobacter capsulatus* B-6508 and *Rhodobacter sphaeroides* B-6509. On the basis of obtained results, mutant strain of *Rhodobacter capsulatus* B-6508 has shown the highest potential for 5-ALA production. The most favorable conditions for growth and 5-ALA production by mutant strain *R. capsulatus* B-6508 were observed in media composed of glutamate and malate, light at 2000 Lux, microaerophilic conditions and temperature of 28 °C. In these conditions, the highest 5-ALA concentration (179 mg/L) was detected together with the highest bacterial physiological activity. The prolongation of mutant strain *R. capsulatus* B-6508 cultivation time after glycine, succinate and levulinic acid addition is related to the reduction of 5-ALA concentrations (e.g. 124.5 mg/L after 48 h and 89.5 mg/L after 72 hours). In the light/aerobic conditions *R. capsulatus* B-6508 produced only 58.1 mg/L of 5 ALA. Furthermore, in dark conditions even lower biomass and 5-ALA concentrations were observed during *R. capsulatus* B-6508 cultivation.

**Keywords:** purple non-sulphur bacteria, *Rhodobacter capsulatus*, 5-aminolevulinic acid, optimization of cultivation conditions.

## Sažetak

Divlji sojevi ljubičastih ne-sumpornih bakterija *Rhodospirillum rubrum* B-6505, *Rhodopseudomonas palustris* B-6506, *Rhodobacter capsulatus* B-6508 i *Rhodobacter spheroides* B-6509 proučavani su kao potencijalni proizvođači 5-ALA (5-aminolevulininska kiselina). Odabrani sojevi podvrgnuti su postupku mutageneze s *N*-metil-*N*-nitro-*N*-nitrosoguanidinom s ciljem dobivanja sojeva s visokom sposobnošću sinteze 5-ALA. Nakon procesa mutanogeneze selekcionirano je 19 stabilnih sojeva iz vrsta *Rhodobacter capsulatus* B-6508 i *Rhodobacter sphaeroides* B-6509. Na temelju rezultata istraživanja vidljivo je da genetički modificirani soj *Rhodobacter capsulatus* B-6508 ima najveći potencijal za proizvodnju 5-ALA. Najpovoljniji uvjeti za rast *R. capsulatus* B-6508 i proizvodnju 5-ALA zabilježeni su na hranjivoj podlozi koja je sadržavala malat i glutamat, imala intenzitet svjetla od 2000 Lux, mikroaerofilne uvjete i temperaturu od 28°C. U tim uvjetima registrirana je najveća 5-ALA koncentracija (179 mg/L) zajedno s najvećom fiziološkom aktivnošću bakterijske biomase. Produljenje vremena uzgoja *R. capsulatus* B-6508 nakon dodataka glicina, sukcinata i levulinske kiseline povezano je sa smanjenjem 5-ALA proizvodnje (npr. 124.5 mg/L nakon 48 h odnosno 89.5 mg/L nakon 72 h). Uzgoj *R. capsulatus* B-6508 u aerobnim uvjetima uz prisustvo svjetla kroz 48 sati dao je samo 58.1 mg/L 5-ALA. Nadalje, kod uzgoja *R. capsulatus* B-6508 bez prisustva svjetla zabilježene su još manje koncentracije bakterijske biomase i 5-ALA.

**Ključne riječi:** purpurne nesumporne bakterije, *Rhodobacter capsulatus*, 5-aminolevulininska kiselina, optimizacija uvjeta uzgoja.

## Introduction

The photosynthetic bacteria can be found in fresh, salt, acidic and basic waters, and also in various wastewaters. These bacteria play important roles in CO<sub>2</sub> assimilation and nitrogen fixation (Lascelles et al, 1978). One of the major groups are

purple photosynthetic bacteria. The purple bacteria are a small group of Gram-negative eubacteria, consisting of only about 30 species. They are unicellular, reproduce by binary fission, and in most cases are motile by flagella. All purple bacteria are capable of growing anaerobically in the light with CO<sub>2</sub> as the carbon source and reduced inorganic compounds as the



electron donor (Madigan and Jung 2009). The only pathway for carbon fixation by purple bacteria is the Calvin cycle. There are two divisions of photosynthetic purple bacteria, the purple sulphur bacteria and the purple non-sulphur bacteria.

The purple sulphur bacteria use the inorganic sulphur compounds such as hydrogen sulphide as the electron donor. These organisms are predominantly aquatic, found in sulphate-rich environments, and are mostly strictly anaerobes that require vitamin B12 as the only growth factor.

The purple non-sulphur bacteria (PNSB) use various organic compounds as electron donors including fatty acids, other organic acids such as succinate or malate, primary and secondary alcohols, carbohydrates and even aromatic compounds. Under phototrophic (anoxic/light) conditions, typical purple non-sulphur bacteria can grow photoautotrophic with H<sub>2</sub> or low levels of sulphide as electron donors; a few species can use S<sub>2</sub>O<sub>3</sub> or Fe<sup>2+</sup> as photosynthetic electron donors (Ehrenreich and Widdel, 1994; Brune, 1995). However, most purple non-sulphur bacteria grow best as photoheterotrophs in media containing a readily useable organic compound, such as malate or pyruvate, and ammonia as nitrogen source (Sojka, 1978). Most purple non-sulphur bacteria have a predominantly photoheterotrophic mode of metabolism.

The PNSB have been classified as anoxygenic phototrophic bacteria, and are divided into 6 genera. Bacteria from genus *Rhodospirillum* have spiralshaped cells and 0.5 to 1.5 µm width. This genus uses polar flagella for motility. The bacteria have intracytoplasmic photosynthetic membranes arranged in vesicles, lamellae, or stacks, but not as finger-like intrusions of cytoplasmic membrane. *Rsp. rubrum*, *Rsp. fulvum* *Rsp. oxigenis*, *Rsp. photometricum* and *Rsp. meliscchianum* belong to this genus (Madigan and Jung, 2009). The *Rhodopseudomonas* cells are rod-shaped and display polar growth, dividing asymmetrically. They use flagella for motility. These bacteria contain intracytoplasmic photosynthetic membranes. *Rps. palustris*, *Rps. acidophila*, *Rps. rutica*, *Rps. viridis* belong to this genus. The bacteria of the *Rhodomicrobium* genus are ovoid. Their peritrichously arranged flagella are used for motility. Reproduction of these organisms occurs by budding. Members of this genus have staked intracytoplasmic membranes. *Rhodomicrobium vannielii* is a member of the genus (Madigan and Jung 2009). The cells in the *Rhodopila* genus are spherical to ovoid. They use flagella for motility. The photosynthetic membranes of these bacteria are vesicular. The *Rhodopila* can grow at low pH and biotin and p-aminobenzoic acid are required as growth factor. These cells are sensitive to oxygen. *Rhodopila globiformis* is a member of this genus. *Rhodocyclus* bacteria are slender, curved cells and are 0.3-0.7 µm in diameter. The bacteria in this genus are non-motile or motile by polar flagella. They have intracytoplasmic membranes which form small finger-like intrusions of the cytoplasmic membrane (Madigan and Jung 2009). The genus *Rhodobacter* was formerly included in the genus *Rhodopseudomonas*, but is now differentiated by their invaginating intracytoplasmic membranes that can appear vesicular in thin sections. The cells are ovoid to rod-shaped and the bacteria multiply by binary fission. They can be motile or nonmotile. All species require thiamine and most of them require biotin. Additional vitamin requirements are variable among the species. The photosynthetic pigments in purple bacteria are "Bchl a" (bacterial chlorophyll a) and carotenoides

of the spheroidene group. *Rba. sphaeroides*, *Rba. sulfidophilus*, *Rba. capsulatus*, *Rba. blasticus*, and *Rba. adriaticus* are species of this genus (Madigan and Jung, 2009).

5-aminolevulinic acid (5-ALA) is an intermediate in synthesis of different tetrapyrrole molecules in all living organisms, i.e. chlorophyll, hem or vitamin B12 (Kang et al, 2012). There are two different pathways in which 5-ALA can be produced: C4 pathway (Shemin pathway) which is present in purple bacteria, yeasts and mammalian cells and C5 pathway which is present in many plants and some microorganisms (Woodard and Dailey, 1995). Today, 5-ALA is mostly produced using microbial fermentation, namely by photosynthetic bacteria because chemical synthesis of 5-ALA has lower yields and is more complicated in comparison to microbial production (Liu et al, 2014). Production of 5-ALA has been reported using both wild strains of bacteria and their mutants. Application of mutant strains is far more suited for 5-ALA production. So far many different strains of photosynthetic bacteria together with their mutants have been tested for their 5-ALA production capacities (Sasaki et al, 1991; Xiu-yan et al, 2005; Liu et al, 2015; Meng et al, 2016). Both chemically defined and complex media can be used (Heiko et al, 1993). 5-ALA can be used as an effective herbicide, or as a plant stress tolerance enhancer (Nunkaew et al, 2014). It is not harmful to crops, animals or humans and it is biodegradable which makes it interesting from ecological point of view. Much attention was also dedicated to its great potential in the field of medicine, namely tumor-localizing and photodynamic therapy (Seiji et al, 1999).

In this research, strains of purple non-sulphur bacteria belonging to genera *Rhodobacter*, *Rhodopseudomonas* and *Rhodospirillum* were isolated and genetically modified by chemical mutagenesis. Obtained mutant strains were used in further research which the main goal was to define the optimal bioprocess conditions for purple non-sulphur bacteria growth and 5-ALA production.

## Materials and methods

### Microorganisms

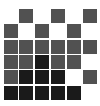
In this study, *Rhodospirillum rubrum* B-6505, *Rhodopseudomonas palustris* B-6506, *Rhodobacter capsulatus* B-6508 and *Rhodobacter sphaeroides* B-6509 from the Culture Collection of the Laboratory of Energy Alternative Sources, Scientific and Production Center "Armbiotechnology" of the National Academy of Sciences of the Republic of Armenia were used as wild strains as well as mutants. Wild strains of microorganisms were isolated from local mineral water sources around the city of Yerevan (Republic of Armenia).

### Cultivation media

In this research, following media were used for cultivation of purple non-sulphur bacteria:

a) Ormerod agar medium (Ormerod et al. 1961):

2.0 g/L Na-malate, 0.1 g/L yeast extract, 0.2 g/L MgSO<sub>4</sub> x 7H<sub>2</sub>O, 0.08 g/L CaCl<sub>2</sub> x H<sub>2</sub>O, 0.01 g/L FeSO<sub>4</sub> x 7H<sub>2</sub>O, 0.9 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.6 g/L KH<sub>2</sub>PO<sub>4</sub>, 1.25 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.02 g/L EDTA, 0.028 g/L H<sub>3</sub>BO<sub>3</sub>, 0.021 g/L MnSO<sub>4</sub> x 4H<sub>2</sub>O, 0.075 g/L Na<sub>2</sub>MoO<sub>4</sub> x 2H<sub>2</sub>O, 0.0024 g/L ZnSO<sub>4</sub> x 2H<sub>2</sub>O, 0.01 g/L Cu(NO<sub>3</sub>)<sub>2</sub> x 3H<sub>2</sub>O 20g/L agar pH = 6.8-7.3.



b) GA (glutamate / acetate) liquid medium (Suwansaard 2010):

1.5 g/L yeast extract, 1.64 g/L  $C_2H_3NaO_2$ , 2 g/L  $C_5H_8NO_4Na$ , 0.8 g/L  $(NH_4)_2SO_4 \times 7H_2O$ , 0.5 g/L  $KH_2PO_4$ , 0.5 g/L  $K_2HPO_4$ , 0.2 g/L  $MgSO_4 \times 7H_2O$ , 0.053 g/L  $CaCl_2 \times 2H_2O$ ,  $1.2 \times 10^{-3}$  g/L  $MnSO_4 \times 7H_2O$ ,  $1.0 \times 10^{-3}$  g/L thiamin-HCl,  $1.0 \times 10^{-3}$  g/L nicotinic acid,  $1.0 \times 10^{-5}$  g/L biotin, pH = 6.8-7.0.

c) GG (glutamate / glucose) liquid medium (Nishikawa et al. 1999):

1.5 g/L yeast extract, 3.6 g/L  $C_6H_{12}O_6$ , 2 g/L  $C_5H_8NO_4Na$ , 0.8 g/L  $(NH_4)_2SO_4 \times 7H_2O$ , 0.5 g/L  $KH_2PO_4$ , 0.5 g/L  $K_2HPO_4$ , 0.2 g/L  $MgSO_4 \times 7H_2O$ , 0.053 g/L  $CaCl_2 \times 2H_2O$ ,  $1.2 \times 10^{-3}$  g/L  $MnSO_4 \times 7H_2O$ ,  $1.0 \times 10^{-3}$  g/L thiamin-HCl,  $1.0 \times 10^{-3}$  g/L nicotinic acid,  $1.0 \times 10^{-5}$  g/L biotin, pH = 6.8-7.0.

d) GM (glutamate / malate) liquid medium (Nishikawa et al. 1999):

1.5 g/L yeast extract, 2.7 g/L  $C_4H_4Na_2O_5$ , 2 g/L  $C_5H_8NO_4Na$ , 0.8 g/L  $(NH_4)_2SO_4 \times 7H_2O$ , 0.5 g/L  $KH_2PO_4$ , 0.5 g/L  $K_2HPO_4$ , 0.2 g/L  $MgSO_4 \times 7H_2O$ , 0.053 g/L  $CaCl_2 \times 2H_2O$ ,  $1.2 \times 10^{-3}$  g/L  $MnSO_4 \times 7H_2O$ ,  $1.0 \times 10^{-3}$  g/L thiamin-HCl,  $1.0 \times 10^{-3}$  g/L nicotinic acid,  $1.0 \times 10^{-5}$  g/L biotin, pH = 6.8-7.0.

#### Selection of media for purple non-sulphur bacteria growth

From Petrie dishes, previously grown bacterial biomass of *Rhodospirillum rubrum* B-6505, *Rhodopseudomonas palustris* B-6506, *Rhodobacter capsulatus* B-6508 and *Rhodobacter sphaeroides* B-6509 on Ormerod agar medium, was taken with microbiological inoculation loop into test tubes with 5 mL of sterile water. This prepared inoculum was homogenized and 2 mL were taken for inoculation of 200 mL flasks containing 25 mL of liquid media (GM, GG and GM) with different carbon sources (acetate, glutamate and malate). Total of 12 flasks were used and they were kept on a rotary shaker in light conditions (1500-1800 lux) at 28°C. After 5 days of cultivation, samples were taken and analyzed. Based on optical density (520 nm) and microscopical evaluation of bacterial activity, appropriate cultivation media was chosen for further research.

#### Chemical mutagenesis

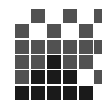
Selected strains of purple non-sulphur bacteria *Rhodopseudomonas palustris*, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* were genetically modified by nitrosoguanidine (N-methyl-N-nitro-N-nitrosoguanidine). The process of mutagenesis was performed according to the Nishikawa et al (1999). Obtained genetically modified strains were cultivated in GM medium (flasks with 20 mL of medium) in light and dark conditions. After 2 days of cultivation, 1 mL of bacterial cultivation medium was taken from light and dark cultivation and used for inoculation of Petri dishes containing GM medium with added 20 g/L of agar. Inoculum contained approximately 1 g/L dry weight of biomass. Inoculated Petri dishes were kept in both dark and light (1800-2000 lux) conditions at 28°C. After 2-3 days of cultivation on the GM-agar, mutant strains that had shown the highest visible amount of grown biomass were selected and used for further research.

#### Comparison of mutants and wild strains of non-sulphur purple bacteria for 5-ALA production

Selected mutant strains of non-sulphur purple bacteria, cultivated on Petri dishes under different cultivation (light and dark) conditions were inoculated on microtiter plates. Each microtiter plate well contained 200  $\mu$ L of GM medium and it was inoculated with single-cell colony isolated from Petri dishes. The mutant strains were evaluated for 5-ALA production using the Ehrlich reaction in a 96-well microtiter plate. Plates were incubated at 30°C until visually detectable biomass quantity was observed. Selection of the most promising 5-ALA producing mutants was carried out by the addition of the mixture of succinate (30mM), glycine (30mM) and levulinic acid (15mM) into each microtiter plate well. Succinate and glycine are precursors for 5-ALA production via 5-ALA synthase and levulinic acid is an inhibitor of 5-ALA dehydratase (Suwansaard 2010). After 24 hours of cultivation, each microtiter plate well was checked for accumulated 5-ALA according to the Nishikawa et al. (1999). An indicator of 5-ALA concentration in media is the intensity of the colorimetric reaction. Selected mutants that have shown the highest 5-ALA production were transferred into new test tubes containing fresh GM media for new cultivation in order to obtain bacterial biomass for further research.

#### Optimization of cultivation conditions for 5-ALA production

In order to define optimal conditions for 5-ALA production, cultivation of non-sulphur purple bacterial mutant strain *Rhodobacter capsulatus* B-6508 was carried out in different conditions (medium composition, light/dark regime, aerobic/microaerophilic conditions). Cultivation of bacterial biomass lasted 24 to 120 h depending on the experimental setup. Inoculum for cultivation was grown in light conditions under 2000 Lux (28 °C) in Erlenmeyer flasks (without mixing) for 48 hours. Erlenmeyer flasks with 50 mL of GM media were inoculated with 10 % vol/vol of inoculum. The impact of the dark and light regime, as well as aerobic and microaerophilic conditions, on the bacterial growth and 5-ALA production was also studied. The light regime was established by cultivation in luminostat with a constant light source intensity of 2000 Lux and cultivation temperature (28 oC). The dark regime was achieved by cultivation in flasks that were enveloped in dark paper and aluminum foil in the thermostat, without light. Aerobic cultivation was carried out by shaker mixing intensity of 150 min<sup>-1</sup> for surface aeration and the free flow of air through the sterile porous filter. Bacterial cultivation in microaerophilic conditions was carried out by low shaker mixing intensity (<50 min<sup>-1</sup>) to prevent surface aeration as well as without any free air flow in the flask. In these conditions, oxygen present in the liquid free volume of flask can be used for bacterial physiological activities. After defined biomass cultivation period (24, 48, 72 h) a mixture of glycine, succinate and levulinic acid (GSLA) was added to stimulate the 5-ALA production. The quantity of added GSLA mixture was defined to obtain the concentration in cultivation medium of 30 mmol/L of glycine, 30 mmol/L of succinate and 15 mmol/L of levulinic acid. After addition of GSLA, cultivation was carried out for another 24 to 72 h, depending on the experimental setup. Samples from each flask (2 mL) were taken for analytical purposes. After centrifugation at 8000 min<sup>-1</sup> for 20 min supernatant was used



for 5-ALA determination by using Suwansaard (2010) protocol. Separated biomass was dried at 105°C (24 hours) for determination of dry biomass concentration. The growth rate of studied bacterial strains was determined by the standard kinetic procedure (Novak 2015).

#### Spectrophotometric determination of 5-ALA (modified Erlich reaction)

Samples taken from cultivation broth were centrifuged and supernatant was used for further analysis. Sample of 0.2 mL was mixed together with 0.65 mL of acetaldehyde-acetone buffer (acetate buffer with 1% of acetylacetone). Sample was well mixed and then put at 100°C for 20 minutes. After defined time (20 min) sample was cooled at 0°C and then 0.65 mL Erlich reagents (acetic acid with 2% w/v polymethylaminobenzaldehyde and perchloric acid; Sato et al. 1981) was added. After 20 minutes, sample absorbance was measured on a spectrophotometer at 553 nm and 5-ALA concentration was determined from the calibration curve.

**Table 1.** Comparison of different chemically defined media for phototrophic cultivation of purple non-sulphur bacteria

Strains	Nutrient media / growth characteristics		
	GM (glutamate-malate)	GG (glutamate-glucose)	GA (glutamate-acetate)
<i>Rhodospirillum rubrum</i> B-6505	++	-	++
<i>Rhodopseudomonas palustris</i> B-6506	++	-	+
<i>Rhodobacter capsulatus</i> B-6508	++	+++	+
<i>Rhodobacter spheroides</i> B-6509	++	+++	+

**Legend:** «-» - no growth, «+» - negligible growth, «++» - good growth, «+++» - excellent growth.

On the basis of these results it is obvious that the GG (glutamate-glucose) media is only suitable for cultivation of *Rhodobacter capsulatus* B-6508 and *Rhodobacter spheroides* B-6509. On the other hand, GM (glutamate-malate) media is suitable for cultivation of all selected strains. Bacterial growth on the GA (glutamate-acetate) medium was on the lowest level compared to other two media. Therefore, for further research GM media was selected. Willison (1988) reported that *Rhodobacter capsulatus* B-6508 prefers organic acids as malate or lactate as a carbon source, whereas slower growth was observed on media with sugars as a carbon source.

#### Mutagenesis and isolation of 5-ALA producing strains

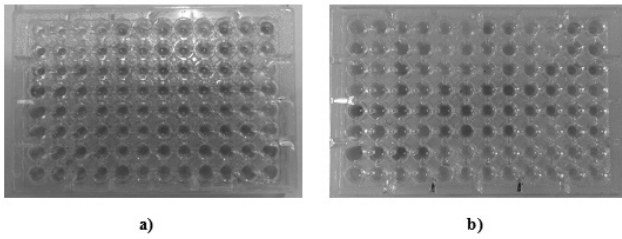
As wild strains of purple non-sulphur bacteria have a very low production rate of 5-ALA (Tangprasittipap and Prasertsan 2002), for example only 11 mg/L in our research, strains of *Rhodopseudomonas palustris*, *Rhodobacter capsulatus* and *Rhodobacter spheroides* were subjected to mutagens with N-methyl-N-nitro-N-nitrosoguanidine in order to obtain mutants that have a higher capacity for 5-ALA production. Total of 19 stable mutant strains were obtained from *Rhodobacter capsulatus* B-6508 (strain marks: D-7, D-8, E-1, E-5, E-6, E-10, F-5, F-6, G-3, G-4) and *Rhodobacter sphaeroides* B-6509 (strain marks: A-12, B-10, C-8, D-7, E-10, F-7, F-10, G-9, G-10). Mutant strains of

## Results and discussion

### Selection of media for cultivation of purple non-sulphur bacteria

Wild strains of purple non-sulphur bacteria: *Rhodospirillum rubrum* B-6505, *Rhodopseudomonas palustris* B-6506, *Rhodobacter capsulatus* B-6508 and *Rhodobacter spheroides* B-6509 were inoculated in different cultivation media in order to define the most adequate carbon source for biomass growth. Three different media were used, GA (glutamate-acetate), GG (glutamate-glucose) and GM media (glutamate-malate), where glutamate serves as a nitrogen source. During purple non-sulphur bacteria cultivations optical density of medium was monitored in order to evaluate the intensity of bacterial growth. Furthermore, the bacterial morphological stage was also examined under a microscope. Results of these experiments are presented in Table 1.

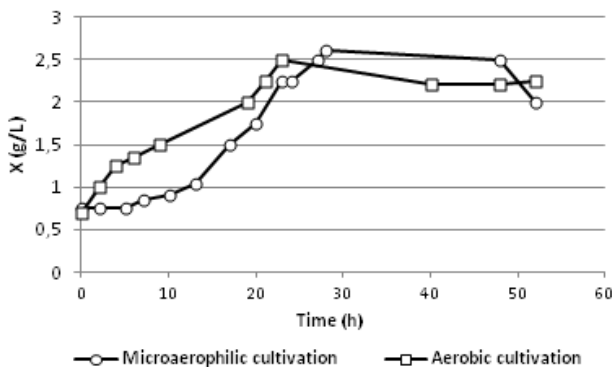
*Rhodopseudomonas palustris* (B-6506) have shown that they can grow under dark conditions, but they were not able to produce 5-ALA. Mutant strains of *Rba. capsulatus* B-6508 and *Rps. sphaeroides* B-6509 did not grow in dark conditions. However, in light conditions (1800 - 2000 Lux) only the growth of selected mutants of *Rba. capsulatus* B-6508 and *Rps. sphaeroides* B-6509 was observed. The photosynthetic bacterium accumulates 5-ALA, which is a precursor in tetrapyrrole biosynthesis, under light illumination and upon addition of levulinic acid as an inhibitor of 5-ALA dehydratase (Suwansaard M., 2010). Based on the colorimetric determination of 5-ALA ten mutants of *Rhodobacter capsulatus* B-6508 have shown the approximately similar intensity of color reaction (Fig. 1b) that was considerably higher compared to the mutants of *Rhodopseudomonas palustris* B-6506 (Fig. 1a). Therefore, for further research, mutant strain *Rhodobacter capsulatus* B-6508 E-10 was selected due to its higher potential for 5-ALA production.



**Figure 1.** Microtiter plate containing mutants of strain *Rhodospirillum rubrum* B-6506 (a) and strain *Rhodospirillum rubrum* B-6508 (b) (quantitative reaction with Ehrlich reagent: purple colour indicates positive reaction)

### Optimization of cultivation conditions for 5-ALA production

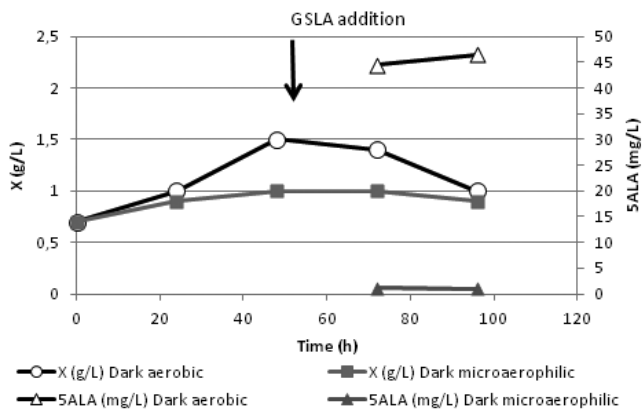
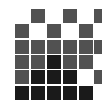
At the beginning of this research, growth in the light regime under aerobic and microaerophilic conditions was studied. As it can be seen in Figure 2, the maximum biomass concentration in microaerophilic cultivation was 2.6 g/L and in aerobic 2.5 g/L. The initial concentration of biomass in both experiments was 0.7 g/L. After 48 h of cultivation, biomass concentration in microaerophilic conditions was 2.5 g/L and in aerobic conditions 2.2 g/L. At this time point, the mixture of glycine, succinate and levulinic acid (GSLA) was added. 5-ALA concentration was determined after further 24 h of cultivation (72 h of total cultivation time). In aerobic conditions 5-ALA concentration reached the value of 58.1 mg/L and in microaerophilic conditions 86.49 mg/L, respectively. Based on the obtained results it is obvious that light combines with microaerophilic conditions are favorable for 5-ALA synthesis. Therefore, the further optimization of mutant strain *Rhodospirillum rubrum* B-6508 E-10 cultivation was performed in these conditions. The activity of 5-ALA synthetase is considerably depending on the oxygen concentration and its higher concentrations can cause the loss of pigmentation due to the impact on the 5-ALA synthetase activator (glycine and succinate). The shift of aerobic cultivation conditions into microaerophilic conditions is related to the slight increase of 5-ALA production (Tangprasittipap and Prasertsan 2002).



**Figure 2.** Changes of mutant strain *Rhodospirillum rubrum* B-6508 E-10 biomass concentration during cultivation in microaerophilic and aerobic conditions at 27-29 °C and light intensity of 2000 Lux.

On the basis of results presented in Figure 2, the growth rate of mutant strain of *Rhodospirillum rubrum* B-6508 E-10 was determined by the standard kinetic procedure. In microaerophilic conditions, *Rhodospirillum rubrum* B-6508 E-10 reached the growth rate of 0.05 h<sup>-1</sup> and in aerobic conditions 0.035 h<sup>-1</sup>, respectively. The maximum biomass concentration was observed in the time period of 22 - 28 h what is important for the addition of glycine, succinate and levulinic acid (GSLA) during 5-ALA production. It is known that the addition of GSLA has to be done in the high exponential bacterial growth phase in order to obtain the highest 5-ALA yields (Liu et al. 2014; Liu et al. 2015). In this research, selected mutant strain of *Rhodospirillum rubrum* B-6508 E-10 shows also the ability to produce relatively small amounts of 5-ALA (8 mg/L) even without GSLA addition. The maximum biomass concentration during *Rhodospirillum rubrum* B-6508 E-10 cultivation in microaerophilic conditions was observed after approximately 24 h and it did not decrease significantly for another 24 hours. This observation confirms that the addition of GSLA has to be performed after 24 hours or in a later cultivation stage.

In another experimental setup, prolonged cultivation after GSLA addition of *Rhodospirillum rubrum* B-6508 E-10 in microaerophilic light conditions was tested. It was determined that bacterial biomass concentrations were at approximately similar levels (2.3 g/L after 24 hours, 2.5 g/L after 48 hours and 2.25 g/L after 72 hours). However, it was observed that 5-ALA concentration was reduced with prolongation of cultivation time due to the negative impact of light, biomass and medium (e.g. pH alteration) on the 5-ALA stability. In these experiments, the most physiologically active biomass and the highest 5-ALA concentration (179 mg/L) were observed after 24 hours of cultivation after addition of GSLA. Prolongation of cultivation time after GSLA addition on the 48 and 72 hours was related to the reduction of 5-ALA concentration on the 124.5 mg/L and 89.5 mg/L, respectively. As a versatile group of microorganisms purple non-sulphur bacteria can grow under aerobic and anaerobic conditions as well as in dark and light conditions (Suwansaard 2010; Nishikawa et al. 1999). Selected strain *Rhodospirillum rubrum* B-6508 E-10 was cultivated under light conditions and it was proven that light conditions are favorable for 5-ALA production. During purple non-sulphur bacterium cultivation light is used as energy source and therefore it is necessary to optimize the light features in order to reduce the operational bioprocess costs. However, the cultivation of *Rhodospirillum rubrum* B-6508 E-10 has to be examined in dark conditions in order to evaluate the potential of these conditions for bacterial growth and 5-ALA synthesis. In these experiments, *Rhodospirillum rubrum* B-6508 E-10 was cultivated in aerobic and microaerophilic conditions without light (dark conditions) for 48 h and then GSLA mixture was added (Figure 3).



**Figure 3** Change of biomass ( $X$ ) and 5-ALA concentrations during mutant strain *Rhodobacter capsulatus* B-6508 E-10 cultivation in microaerophilic and aerobic conditions without light (dark conditions)

As it can be seen in Figure 3, very low 5-ALA concentration (below 2 mg/L) was observed during *Rhodobacter capsulatus* B-6508 E-10 cultivation in dark microaerophilic conditions. On the contrary, during *Rhodobacter capsulatus* B-6508 E-10 cultivation in dark aerobic conditions considerably higher 5-ALA concentration 46.5 mg/L (31 mg/g dry weight biomass) was detected. ALA synthase activity is the same in cultures grown under high and low oxygen concentration (Biel 1992), but ALA dehydratase activity is lower when aerobic conditions are met (Nishikawa et al. 1999) meaning ALA accumulation is possible during aerobic cultivation. Comparison between *Rhodobacter capsulatus* B-6508 E-10 cultivations in light and dark conditions shows considerably lower bacterial biomass yield as well as 5-ALA yield. It is known that light intensity is an important factor for 5-ALA production (Tangprasittipap and Prasertsan 2002) and together with low oxygen concentration 5-aminolevulinic synthase is stabilized (Abu-Farha et al 2005) so that 5-ALA production can be enlarged. On the basis of previous facts, it is obvious that higher 5-ALA production efficiency can only be obtained during *Rhodobacter capsulatus* B-6508 cultivation in light microaerophilic conditions.

#### The impact of $Fe^{2+}$ ion and vitamin B12 on the growth and 5-ALA production by *Rhodobacter capsulatus* B-6508 E-10

Because of fact that  $Fe^{2+}$  ion and vitamin B12 have a great role in the metabolism of purple bacteria (Liu et al 2015) their impact on the growth and 5-ALA production was also examined during *Rhodobacter capsulatus* B-6508 E-10 cultivations. In GM media  $Fe^{2+}$  and vitamin B12 were added so that their concentrations were 30  $\mu$ M and 2.5 mg/L, respectively. After 48 h of *Rhodobacter capsulatus* B-6508 E-10 cultivation in light microaerophilic conditions it was observed that relatively low biomass concentration ( $\approx$  1g/L) was obtained together with the absence of purple pigment synthesis. Prolongation of this cultivation (5 days) did not show any positive impact on bacterial growth. It is known that addition of  $Fe^{2+}$  ion to media can have a positive impact on the growth of purple bacteria (Liu et al. 2005). However, the results of this study did

not support this claim. In this *Rhodobacter capsulatus* B-6508 E-10 cultivation, pH was relatively quickly increased up to 9.50 what considerably slowed down the bacterial growth, due to the fact that purple bacteria prefer neutral to slightly acidic (pH = 6.5 - 7.0) conditions. Furthermore, this relatively high pH value was probably related to the absence of 5-ALA production although GSLA mixture was added to the medium. This observation is in accordance with literature where is pointed out that growth media should not contain iron or cobalt ions if enlarged 5-ALA accumulation would like to happen during purple bacteria cultivation (Tangprasittipap and Prasertsan 2002). Although metal ions are important elements in regulation of tetrapyrrole biosynthesis in *Rhodobacter sphaeroides*, 5-ALA synthetase is obviously regulated by hem compounds through feedback inhibition or repression under iron sufficient conditions (Sasikala and Ramana 1995).

## Conclusions

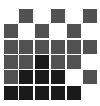
The purple non-sulphur bacteria are able to use various organic compounds for their growth and the medium composed of glutamate and malate (GM) shows the highest potential for their cultivation. The most favorable conditions for *Rhodobacter capsulatus* B-6508 E-10 growth and 5-ALA synthesis are light (2000 Lux), microaerophilic conditions and temperature 28 °C. During *Rhodobacter capsulatus* B-6508 E-10 cultivation in light aerobic conditions lower 5-ALA concentration (58.1 mg/L) was obtained compared to the bacterial cultivation in light microaerophilic conditions (86.59 mg/L). In the dark cultivation of *Rhodobacter capsulatus* B-6508 E-10, aeration is required for 5-ALA production, but considerably lower 5-ALA concentrations (31 mg/L) were detected. Cultivation of *Rhodobacter capsulatus* B-6508 E-10 in light microaerophilic conditions for 24 hours resulted in the most physiologically active bacterial biomass for 5-ALA production (179 mg/L) compared to the bacterial cultivation in the time period of 48 (124.5 mg/L) and 72 (89.5 mg/L) hours.

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