Chapter 2

Materials and Methods

Materials

1. Microorganisms

Rhodobacter capsulatus strain SS3 was isolated from Songkhla beach, Songkhla Province by Amornrat Tungprasittiparp (Sattayasamittsathit, 2001). The two mutant strains of *Rhodobacter sphaeroides* ES16 (N20, U7) (Madmarn, 2002) were mutated by using N-methyl-N-nitro-N-nitrosoguanidine (NTG) and ultraviolet (UV), respectively. The cultures were maintained on GM agar slant and after incubation for 48 h under anerobic-light condition at 37 C, they were stored in a refrigerator and subcultured monthly.

2. Media

- Glutamate-malate (GM) medium contained (g/l) L-glutamic acid 3.8, D,L-malate 2.7, yeast extract 2.0, KH_2PO_4 0.5, K_2HPO_4 0.5, $(NH_4)_2SO_4$ 0.8, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.053, $MnSO_4.5H_2O$ 0.0012 and basal medium (Lascelles, 1956). The pH was adjusted to 7.0.

- Basal medium contained (mg/l) nicotinic acid 1.0, thiamine 1.0 and biotin 0.01 (Watanabe *et al*., 1981).

- Glutamate-glucose (GG) medium contained the same components as GM medium but using 9 g/l (50 mM) glucose instead of DL-malate) (Nishikawa *et al*., 1999).

- Modified glutamate glucose (MGG) medium contained the same components as GG medium but using commercial monosodium glutamate instead of glutamate.

- Monosodium glutamate glucose salt (MGS) medium contained monosodium glutamate, commercial glucose and commercial NaCl grade.

- Monosodium glutamate glucose salt yeast extract (MGSY) medium contained MGS with the addition of 2.0 g/l yeast extract.

3. Chemicals

Chemicals used in the analysis were analytical grade:ALA was purchased from Sigma Chemical Co. (St. Louis, MO,USA). Levulinic acid was from Fluka (Buchs, Switzerland). Glycine, succinate, glutamate, malic acid, pyridoxal phosphate were from Fisher Scientific (NJ, US).

4. Instruments

1. Double – beam Spectrophotometer model U-2000 (Hitachi, Ltd.).
2. Refrigerated centifuge model SCR 20B (Hitachi Koki).
3. pH meter model D-12 (Denver Instrument).
4. Hot air oven model ULM. 500 (Memmert)
5. Fermentor model MDL 300 (B.E. Marubishi) and Controller model MDL-6C (B.E. Marubishi).
6. HPLC equipped with a flame ionization detector (FID) model G 1321 (Hewlett Packard).
7. Lux meter Model LX-50 (Digcon).
8. Incubator shaker model KMC-8480SR-L (LMS Co., Ltd).

Analytical Methods

Growth was determined by measuring the optical density (OD 660 nm) with a spectrophotometer (Sasaki *et al*., 1987) and obtained the dry cell weight (DCW) from the standard curve (Noparatnaraporn *et al*., 1986).

Extracellular ALA was measured colorimetrically by the method of Mauzerall and Granick (1956). A 0.01-ml aliquot of culture from each well was transferred to glass tube. Acetate buffer (0.1 ml, 1M, pH 4.7) containing 1 % acetylacetone was added to each well. ALA and acetylacetone condensed to form a pyrrole compound, 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole, in the acetate buffer at 100 °C. After heating for 15 min, the glass tube was quickly cooled on ice. Then 0.1 ml of Ehrlich reagent (50 ml acetic acid solution containing 2 % (w/v) ρ-dimethyl-aminobenzaldehyde and 8 ml of 70% perchloric acid) was added to each well. The pyrrole compound in each well is purple-red by allowing it to react with ρ-dimethylaminobenzaldehyde in the Ehrlich reagent. The purple-red color resulting from the Ehrlich reagent was quantified at 533 nm.

Intracellular ALA was determined using HPLC according to the method of Lin *et al*. (1989). Cell collected by centrifugation (10,000*xg* for20 min) were washed with 0.02 M Tris-HCl buffer (pH 7.4) 2 times and resuspended in 1 ml of 0.04 M Tris-HCl, then freeze at -20 º C, ruptured by Ultra-sonic vibrator for 15 min, followed by removal of the cell debris by centrifugation at 10,000*xg* for 20 min. The supernatant was analyzed by HPLC. Supernatant (10 µl) in a glass tube was added with 3.5 ml of a mixture acetylacetone, ethanol and water (15 : 10 : 75 ; by vol) containing 4 g of sodium chloride per liter and then with 450 µl of aqueous formalin (85 ml of formalin per liter). The solution was heated in boiling water for 30 min and then cooled in a water bath. The HPLC system (pre-column) with a fluorescence detector (Shimadzu, LC-10A, Osaka, Japan) was used by applying 20 µl solution to an injector sample loop. The column used was hyprsil ODS-3 (5 µM, 250 x 4.6 mm i.d., GL Science Inc. Tokyo, Japan) kept at 40 C. Finally the elution was performed with an aqueous solution containing methanol (HPLC grade; Merck) and 2.5% acetic acid (60:40 v/v) at a flow rate of 0.6 ml/min by a constant flow pump. Spectrofluorometer was used to monitor the fluorescence intensity of the eluate at 473 nm (excitation wavelength 363 nm). The correlation of peak area and standard ALA concentration (commercial ALA) were plotted as standard curve of intracellular ALA.

Enzyme assays

1. 5-Aminolevulinic acid synthase (Burnham, 1970)

Stock substrate mixture was prepared by mixing 5 ml of glycine, 5 ml of succinate, 5 ml of 0.1 M $MgCl_2$ and 2.5 ml of Tris-HCl buffer. This may be kept at -15 C.

Stock cofactor mixture was prepared by mixing 2 ml of 0.2 ATP, 1.75 ml of 0.01 M CoA, 1.35 ml of 0.01 M pyridoxal phosphate, and 2.0 ml of distilled water. This solution may be kept at -15°C.

To a test tube (13 x 100 mm) was added 0.15 ml of cofactor mixture and 0.35 ml of substrate mixture. Water and enzyme were added to 1 ml. This assay mixture contained: glycine, 100 µM; succinate, 100 µM; pyridoxal phosphate, 0.27 µ M. The tubes were placed in a water bath at 37°C for 30 minutes. At the termination of incubation, 0.5 ml of 10 % trichloroacetic acid was added and the mixture was

centrifuged for 5 minutes in a bench-top centrifuge. After centrifugation, the contents of the assay tube were decanted into test tubes containing (18x200 mm) 2 ml of 1 M acetate buffer pH 4.7. Two drops (0.05 ml) of acetyl acetone were added and the tubes capped with marbles were heated in a boiling water bath for 15 minutes. Upon cooling, 3.5 ml of modified Ehrlich reagent was added and the optical density was determined at 556 nm after 20 minutes.

One unit of ALA synthase was defined as the amount of enzyme that catalyzed the formation of 1 μM of ALA per hour at 37°C.

Specific activity was expressed as units per mg of protein.

Protein was determined by the colorimetric method of Lowry *et al*. (1951) with crystalline bovine serum albumin as standard.

2. 5-Aminolevulinic acid dehydratase (Nakagawa *et al*, 1999)

One milliliter of reaction mixture, containing 50 mM potassium phosphate buffer (pH 7.5), 6.5 mM ALA, 5 mM $MgCl_2$ and the cell-free extract (0.5 to 1.0 mg protein), was incubated in a test tube at 37°C for 60 min. The reaction was started by transferring the test tube from an ice bath to a water bath at 37°C and terminated by the addition of 2 ml of 10 % tricholoacetic acid. The mixture was centrifuged at 10,000*xg* for 20 min. To a measured aliquot of 1 ml supernatant fluid, an 3 ml of the Ehrlich's reagent was added and after 10 min, the optical density at 555 nm was determined. The molar extinction coefficient used was 62 x 10^3. One ml of supernatant fluid was diluted with 3 ml HCl and incubated in the light (3,000 lux) overnight. The amount of porphyrin was determined at the wavelength of 406 and 430 nm with molar extinction coefficient ($\triangle\varepsilon^{406\text{-}403\ nm}$) of 53x$10^3$. The number of micromoles of porphobilinogen (PBG) converted to porphyrin was calculated by multiplying the micromoles of porphyrin by 4. Therefore, total PBG (nM) = (porphyrin x 4) + PBG (nM).

Methods

1. Mutagenesis and screening on mutant producing the highest ALA concentration

1.1. Strain improvement by mutagenesis

1.1.1. Mutagenesis by UV (Madmarn, 2002)

The cells of wild type strain grown under aerobic-dark condition at 37°C for 24 h were harvested at logarithmic phase by centrifugation (10,000 x g, 20 min) at 4 °C and washed twice with Tris - maleic (TM) buffer (50 mM, pH 6.0). The cells were resuspended in TM buffer at a concentration of 5-8 x 10^8 cell/ml (Tanaka *et al*., 1991). Ten ml of the cell suspension was taken to sterile petri dish with a magnetic stirrer then placed in a laminar flow and used the wave length of 240 nm (about 15 cm. from UV lamp). Samples were taken at 0, 1, 3, 5, 7, 10, 15 and 20 min to measure for 10% survival. The samples were diluted and spreaded on GM medium agar plates and incubated under aerobic-dark condition at 37°C, then the colonies appeared on the plates were counted. From the graph plotted between time and %survival, the time that gave 10% survival was selected for UV treatment. The petri dish was kept in dark condition for 2 h, then the cell was inoculated into GM medium for 2% (v/v) and cultivated for 2 days under aerobic-dark condition at 37 °C). Centrifugation and washing with TM buffer.

1.1.2 Mutagenesis by NTG (Tanaka *et al*., 1991)

N-methyl-N-nitro-N-nitrosoguanidine (NTG), 100 mg/ml, was added into the cell suspension which was prepared as described aboved. After incubation for 1 hr under aerobic-dark condition at 37°C, the cells were centrifuged and washed with Tris-maleic (TM) buffer (50 mM, pH 6.0) and resuspended in the same buffer. This N-methyl-N-nitro-N-nitrosoguanidine (NTG) treated cell suspension was inoculated into GM medium (10% V/V) and cultivated for 2 days. Cells were harvested by centrifugation and washing with TM buffer.

1.2 Screening for the highest ALA-producing mutant strain

The cells, treated by UV or NTG, were resuspended in GM medium containing 100 μg/ml of penicillin, then incubated for 2 h with mild shaking at 37° C. Mutant cells were concentrated by this treatment since non-mutant cells which grew well on GM medium were killed by penicillin whereas some mutants survived. Then cells were centrifuged and washed with TM buffer and resuspended in TM buffer (50 mM, pH 6.0) at the suitable concentration for spreading onto GM medium plates. The colonies were then patched onto fresh media agar plates (17 colonies/plate) to serve as the master plate. After incubation under aerobic-dark condition at 37°C for 2-3 days, the colonies were inoculated into test tubes (21 mm

x 200 mm) containing 10 ml of GM medium and cultivated under aerobic-dark condition at 37 °C. After cultivation for 48 h, the ALA accumulated in the culture medium of each test tube was measured for extracellular ALA.

Mutants that secreted higher amount of extracellular ALA than their parent strain were inoculated into 250 ml flask containing 100 ml of GM medium and incubated under aerobic/dark condition at 37°C. Samples were taken every 24 h up to 3 days cultivation to measure for the extracellular ALA. The mutant producing the highest ALA concentration was selected for optimization studies.

2. Optimization for ALA production by the selected mutant strain

Starter preparation

Starter culture was prepared by inoculating the selected mutant into a 250 ml flask containing GM medium + 3% NaCl and incubated under aerobic-dark condition at 37°C for 24 h. The culture was diluted with the GM medium + 3 % NaCl to obtain OD_{660} of 0.5 before using as the starter culture.

Time course for ALA production from the mutant

Cultivation in GM medium was performed as described above with the addition of 10% starter culture. Samples were taken every 3 h in the first 24 h, then every 12 h up to 3 days cultivation to measure for pH, OD_{660}, dry cell weight, extracellular ALA and intracellular ALA concentrations, as well as activities of ALA synthase and ALA dehydratase.

The influence of the following parameters was investigated and the factor giving the best result was selected for the subsequent studies.

2.1 Effect of using glucose instead of malate

Glucose replaced malate in the GM medium, so called GG medium. Comparison on growth and ALA production from the selected strain of halotelerant photosynthetic bacteria was cultivated in GG medium and GM medium and incubated under aerobic-dark condition at 37 C for 4 days. Samples were taken every 3 h in the first 24 h, then every 12 h up to 3 days cultivation to measure for pH, OD_{660}, dry cell weight and extracellular ALA. The medium giving the highest ALA production was selected for further studies.

2.2 Effect of levulinic acid (LA) concentration and number of repeated addition

Cultivation was carried out as described above in time course study and the concentration of levulinic acid (LA) was varied at 5, 10, 15 and 20 mM. The first addition was at the middle log phase (results from time course study). Repeated additions of LA were conducted when the activity of ALA dehydratase tended to increase. The samples were taken every 12 h and pH was measured before and after each LA addition. The optimum concentration of LA and number of LA addition were selected based on the increase of ALA concentration and its productivity.

2.3 Effect on the addition of C_5 pathway precursors

Glutamate and malic acid are precursors of ALA biosynthesis via C_5 pathway. Cultivation was performed in GM medium + 3% NaCl. Levulinic acid was added at the optimum concentration and number of addition (result from section 2.1) and the two precursors were added at the concentrations of 2, 4, 6, 8 g/l (13.6, 27.2, 40.8 and 54.4 mM, respectively) for glutamate and 1, 2, 3, 4 g/l (7.5, 15, 22.5 and 30.0 mM, respectively) for malic acid. The experiments were conducted on factorial design as shown below.

Number	Glutamate conc. (mM)	Malic acid conc. (mM)
1	13.6	7.5
2	13.6	15.0
3	13.6	22.5
4	13.6	30.0
5	27.2	7.5
6	27.2	15.0
7	27.2	22.5
8	27.2	30.0
9	40.8	7.5
10	40.8	15.0
11	40.8	22.5
12	40.8	30.0

Number	Glutamate conc. (mM)	Malic acid conc. (mM)
13	54.4	7.5
14	54.4	15.0
15	54.4	22.5
16	54.4	30.0

2.4 Effect on the addition of C_4 pathway precursors

Glycine and succinate are precursors of ALA biosynthesis via C_4 pathway. Cultivation was performed in GM medium + 3 % NaCl. Optimum levulinic acid concentration (results from section 2.1) was added and the two precursors were added at the concentrations of 2.5, 5 , 7.5, 10 mM (0.19, 0.38, 0.57, 0.75 g/l, respectively) for glycine and 10, 20, 30, 40 mM (1.18, 2.36, 3.54 and 4.72 g/l, respectively) for succinate. The experiments were conducted on factorial design as shown below.

Number	Glycine conc. (mM)	Succinate conc. (mM)
1	2.5	10
2	2.5	20
3	2.5	30
4	2.5	40
5	5	10
6	5	20
7	5	30
8	5	40
9	7.5	10
10	7.5	20
11	7.5	30
12	7.5	40

Number	Glycine conc. (mM)	Succinate conc. (mM)
13	10	10
14	10	20
15	10	30
16	10	40

2.5 Effect of C_4 and C_5 pathway precursors and their mixture

Biosynthesis of ALA in GM medium + 3 % NaCl with the addition of optimum concentration of C_5 pathway precursors (result from section 2.2) and C_4 pathway precursors (result from section 2.3) as well as the mixture of all precursors were compared and the best condition was selected.

2.6 Effect of initial pH

The initial pH of the optimal medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 using 6 N HCl or 3 N NaOH and used for cultivation of the selected strain in GM medium + 3% NaCl.

2.7 Effect of volatile fatty acids

Acetic acid, propionic acid, butyric acid and mixed volatile fatty acids were studied by adding at the concentration of 0, 0.5, 1, 2 and 3 g/l to GM medium + 3% NaCl with the optimal initial pH (result from section 2.4).

2.8 Effect of $MgCl_2.6H_2O$

The effect of $MgCl_2.6H_2O$ concentration was studied at 5, 10, 15 and 20 mM.

2.9 Effect of pyridoxal phosphate

The effect of pyridoxal phosphate concentration was studied at 10, 20, 30 and 40 μM.

2.10 Effect of controlling pH

The inoculum (5%) was added into a 3 l fermentor containing 1.5 l optimal medium (result from section 2.8). Cultivations were carried out under uncontrolled and controlled pH.

2.11 Effect of NaCl concentration

The effect of NaCl concentration was studied at 0, 1, 2 and 3

3. Comparison between analytical grade medium and commercial grade medium for 5-aminolevulinic acid production

The selected strain of halotelerant photosynthetic bacteria was cultivated in the following media:

1. Glutamate-malate (GM) medium
2. Glutamate-glucose (GG) medium
3. Modified glutamate glucose (MGG) medium
4. Monosodium glutamate glucose salt (MGS) medium
5. Monosodium glutamate glucose salt yeast extract (MGSY) medium
6. Optimized GM medium

The samples were taken every 3 h in the first 24 h, then every 12 h up to 3 days cultivation to measure for pH, OD, dry cell weight, extracellular ALA and intracellular ALA concentrations in each medium. The medium giving the highest ALA production was selected.

Kinetic parameters for batch cultures were as following (Prasertsan, 1993; Doelle, 1997):

1. Specific growth rate (μ) = $(\ln X - \ln X_0)/\Delta t$ (h^{-1})

   When X = final biomass concentration (g/l)

   X_0 = initial biomass concentration (g/l)

   Δt = elapsed time (h)

2. Maximum specific growth rate (μ_m) was calculated by plotted $1/\mu$ vs. $1/S$ obtained a linear slope that intercept value on ordinate was $1/\mu_m$

   or $\mu = \mu_m \, S/K_s+S$

   $(\mu_m) = \mu \, (K_s+S)/S$

   when μ = specific growth rate (h^{-1})

   K_s = saturation constant (g/l)

   S = substrate concentration (g/l)

3. Maximum productivity (R_m) = P_m/t (μM ALA h^{-1})

when P_m = maximum product concentration (μM)