

Full Length Research Paper

# Characterization of a newly isolated *Rubrivivax benzoatilyticus* PS-5 with self-flocculation property and optimization pathway for 5-aminolevulinic acid production

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Six strains of phototrophic purple non-sulfur bacteria were isolated from paddy soil, lake water and pond sediment samples. They showed the ability to produce 5-aminolevulinic acid under anaerobic-light (3,000 lux) condition. A selected strain, designated as PS-5, produced the highest 5-aminolevulinic acid (ALA) concentration (45.10  $\mu\text{M}$ ) and productivity (0.94  $\mu\text{M h}^{-1}$ ) in glutamate-malate (GM) medium as well as possessed self-flocculating ability. Based on physiological and 16S rRNA sequence, the isolate PS-5 was identified to be *Rubrivivax benzoatilyticus*. The optimum concentration of C<sub>4</sub> pathway precursors was 7.5 mM glycine and 10 mM succinate with supplementation of 10 mM levulinic acid. This resulted in 4.2 fold increase of 5-aminolevulinic acid production to 190.74  $\mu\text{M}$  and 2.7-fold increase of its productivity to 2.57  $\mu\text{M h}^{-1}$  compared to the control. In addition, the optimum concentration of C<sub>5</sub> pathway precursor was 50 mM glutamate with the maximum 5-aminolevulinic acid production of 84.12  $\mu\text{M}$  and productivity of 1.56  $\mu\text{M h}^{-1}$ . Therefore, *R. benzoatilyticus* PS-5 could produce 5-aminolevulinic acid using both C<sub>4</sub> and C<sub>5</sub> pathway precursors and preferred C<sub>4</sub> pathway over C<sub>5</sub> pathway as it produced more than 2.3 folds higher 5-aminolevulinic acid concentration.

**Key words:** 5-Aminolevulinic acid, photosynthetic bacteria, self-flocculation, optimization, *Rubrivivax benzoatilyticus*.

## INTRODUCTION

Photosynthetic bacteria (PSB) are gram-negative prokaryotes that convert light energy into chemical energy by the process of anoxygenic photosynthesis. They contain photosynthetic pigments, bacteriochlorophylls and carotenoids, and can grow autotrophically with CO<sub>2</sub> as the sole carbon source

(Pfennig, 1969). PSB has been used for production of 5-aminolevulinic acid commercially with various applications, such as function as a natural photodynamic compound effective as a biodegradable herbicide, growth-promoting factor and a stress tolerance enhancer for plants (Tanaka et al., 1992). Applications of 5-aminolevulinic acid in medical and pharmaceutical field include photodynamic medicine for treating superficial skin cancers, actinic keratoses (Dijkstra et al., 2001), psoriasis (Zeitouni et al., 2003), cutaneous T-cells (Leman et al., 2002), basal cell carcinoma (De Rosa et al., 2000), squamous cell carcinoma (Lee et al., 2010) induce fluorescence diagnosis of pleural malignant tumor (Ali et al., 2011) and the suppression of the inflammatory

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**Abbreviations:** ALA, 5-Aminolevulinic acid; GM, glutamate-malate.

response to coronary and iliac injuries (Sasaki et al., 2005). As a second-generation photodynamic medicine, its outstanding advantages include its minimal side effect, strong curative effect, high penetrability, and low price (Nishikawa et al., 1999). 5-Aminolevulinic acid is nontoxic to humans and animals and it is readily biodegradable, thus causing no adverse effects on the environment (Rebeiz et al., 1988).

Two major pathways for the biosynthesis of 5-aminolevulinic acid have been described (Sasikala et al., 1994). In the Shemin pathway ( $C_4$  pathway), 5-aminolevulinic acid is formed by the condensation of glycine and succinyl-CoA that is catalyzed by aminolevulinic acid synthase (ALAS). This pathway is found in animal cells, yeasts, fungi, and certain bacteria. In the  $C_5$  pathway, 5-aminolevulinic acid is formed in three steps from glutamate (Beale and Weinstein, 1990) and found in plant chloroplasts, cyanobacteria, anaerobic archaeobacteria, *Escherichia coli*, and *Salmonella typhimurium* (Beale, 1999).

In general, the homogeneous characteristic of photosynthetic bacteria in liquid medium make harvesting of biomass relatively high cost. To reduce the production cost, many alternative methods of bacterial cells recovery are proposed such as addition of chemical or biological flocculating agents (Kurane and Matsuyama, 1994) and using flocculating microorganism (Chernyanovskaya, 1990).

Using flocculating strain of photosynthetic bacteria was expected to give three advantages. Firstly, cell mass can be easily harvested by decantation within a few minutes. Secondly, the cells flocs can be used directly in the repeated culture.

Finally, the living flocculating cells can be used for cleaning up the polluted water. Therefore, this work aimed to isolate the flocculating photosynthetic bacteria and optimize the production of 5-aminolevulinic acid from the selected strain.

## MATERIALS AND METHODS

### Medium

The *Rhodospirillum* medium (RM) used for enrichment and isolation contained ( $g\ l^{-1}$ ): 4, D,L malate; 0.1, yeast extract; 0.12,  $MgSO_4 \cdot 7H_2O$ ; 0.076,  $CaCl_2 \cdot 2H_2O$ ; 0.02,  $Na_2EDTA$ ; 0.59,  $KH_2PO_4$ ; 0.39,  $K_2HPO_4$ ; 0.0065,  $FeSO_4 \cdot 7H_2O$ ; 0.0003,  $MnCl_2 \cdot 4H_2O$ ; 0.01,  $ZnSO_4 \cdot 7H_2O$ ; 0.02,  $CoSO_4 \cdot 7H_2O$ ; 0.03,  $H_3BO_3$ ; 0.003,  $NaMoO_4 \cdot 2H_2O$ ; 0.001,  $CuSO_4 \cdot 5H_2O$  (Madukasi et al., 2011).

Modified glutamate-malate medium (GM) without NaCl was used to maintain the bacterial cells and to prepare a starter culture. The medium contained ( $g\ l^{-1}$ ) 2.7, malate; 3.7  $g\ l^{-1}$ , monosodium glutamate; 0.8,  $(NH_4)_2SO_4 \cdot 7H_2O$ ; 0.5, yeast extract; 0.5,  $KH_2PO_4$ ; 0.5,  $K_2HPO_4$ ; 0.2,  $MgSO_4 \cdot 7H_2O$ ;  $1.0 \times 10^{-3}$ , thiamine-HCl;  $1.0 \times 10^{-3}$ , of nicotinic acid and  $1.0 \times 10^{-5}$ , biotin. The initial pH of this medium was adjusted to 7.0 with 5 M NaOH (Tangprasittipap et al., 2007).

Basal medium was used for optimization studies on 5-aminolevulinic acid production. It had the same composition as the modified GM medium but without carbon source.

### Isolation, selection and identification of photosynthetic bacteria

Samples were collected from paddy soil, lake water and pond sediment mud from Songkhla Province, Thailand. The samples (2 g soil or 2 ml) were added into 40 ml *Rhodospirillum* medium (RM) and mixed well. Sterile liquid paraffin (5 ml) was added on top of the medium to create anaerobic condition. Cultivation was conducted at room temperature ( $32 \pm 2^\circ C$ ) under illumination (3,000 lux) with a 100 W incandescent lamp, until the medium turned red. The samples were streaked on modified GM agar plates and incubated under anaerobic-light (3,000 lux) condition. The reddish colonies were purified by subculture several times until pure cultures were obtained. They were kept on the modified GM agar at  $4^\circ C$  until used.

Starter cultures were prepared by transferring a loopfull of cells from the modified GM slant to 100 ml modified GM medium (pH 7.0) and incubated under anaerobic-light (3,000 lux) condition at room temperature ( $32 \pm 2^\circ C$ ) for 24 h. They were harvested by centrifugation ( $8,000 \times g$  for 15 min), and resuspended in the basal medium to a concentration of 0.2 g wet cell weight per 10 ml medium. The cell suspension was transferred to 120 ml serum bottle, each containing 90 ml basal medium and cultivated for 48 h under the same condition as above. Samples were taken for determination of pH and 5-aminolevulinic acid (Mauzeall and Granick, 1956).

Selection was conducted by cultivating all isolates in the modified GM medium plus 10 mM levulinic acid at 24 h (Sasaki et al., 1991) under anaerobic-light (3,000 lux) conditions at room temperature. Preliminary results indicate that without addition of levulinic acid, the isolates could not produce 5-aminolevulinic acid. The highest 5-aminolevulinic acid producing strain was selected and identified according to Bergey's Manual of Systematic Bacteriology (Staley et al., 1989) and the classification was confirmed using 16S rRNA sequencing (Achenbach et al., 2001).

Morphological and physiological characterization of the selected strain was conducted on modified GM medium. Colony pigmentation, margin, and elevation of the colony were recorded while size and shape were determined using Scanning Electron Microscopy (SEM) (JEOL JSM-5800LV) (Harley and Prescott, 2007). Besides, SEM could also illustrate biofilm surface structures at high-resolution by the acquisition of three dimensional images of the surface, revealing details about 1 to 5 nm in size. Cell pigment scans and absorption spectra of the living cells suspension were performed using cell pellets resuspended in 60% sucrose (Truper and Pfennig, 1981). Carbon utilization test was carried out as described by Kantachote et al. (2005).

Identification of the selected strain using molecular technique was also conducted. The primer set 27F-1492R was used in PCR amplification of the 16S rRNA gene fragment of the selected strain. Sequence similarity searches were conducted using the National Center for Biotechnology Information BLAST network service (nucleotide blast). Similar 16S rRNA gene sequences were downloaded from GenBank and manually checked for ambiguous sites using bioedit 7.0.1 software. Alignments were then performed against the 16S rRNA gene sequence of the selected strain, where the pair-wise deletion option for gaps was employed. Phylogenetic tree was constructed from multiple sequence alignment, with related rRNA by the Neighbor Joining analysis with 1000 bootstrap replicates (MEGA version 5.1; Arizona State University, USA), UPGMA, Minimum Evolution or Maximum Parsimony methods (Ramana et al., 2006).

### Effect of precursors on 5-aminolevulinic acid (ALA) production from the selected PSB

Fermentation was carried out in a basal medium (90 ml) containing

(g l<sup>-1</sup>) 0.5, yeast extract; 0.8, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>·7H<sub>2</sub>O; 0.5, KH<sub>2</sub>PO<sub>4</sub>; 0.5, K<sub>2</sub>HPO<sub>4</sub>; 0.2, MgSO<sub>4</sub>·7H<sub>2</sub>O; 1.0×10<sup>-3</sup>, thiamine-HCl, 1.0×10<sup>-3</sup>, nicotinic acid and 1.0×10<sup>-5</sup>, biotin, at pH 7.0, and incubated under anaerobic-light (3,000 lux) condition at room temperature (32±2°C). The effect of C<sub>4</sub> pathway precursors (glycine, succinate) and C<sub>5</sub> pathway precursor (glutamate or malic acid) at various concentrations plus 10 mM levulinic acid at 24 h (Sasaki et al., 1987) were investigated by supplementing each precursor into the medium.

#### Effect of inhibitor on ALA production

Various levulinic acid concentrations (2.5-12.5 mM) were added into the medium at 24 h to study their effect on 5-aminolevulinic acid production. The fermentation was conducted in a basal medium as described above with the supplementation of either C<sub>4</sub> or C<sub>5</sub> pathway precursors.

#### Analytical methods

Samples taken during the fermentation runs were centrifuged (at 8,000×g for 15 min) and the supernatant was used for determination of 5-aminolevulinic acid by a colorimetric method modified from the method of Mauzerall and Granick (1956). Specifically, 0.5 ml of either sample or standard was mixed with 1 ml of 1M sodium acetate buffer (pH 4.7), 25 µl of acetylacetone (2,4-pentanedione) was added, and then the mixtures were boiled in a water bath for 15 min. After cooling, 1.75 ml of freshly prepared modified Ehrlich's reagent was added, and 15 min later, the absorbance at A<sub>553</sub> nm was measured. The acetic acid, propionic acid, butyric acid, succinic acid and levulinic acid concentrations were determined using High Performance Liquid Chromatography (HPLC) (Agilent 1200) equipped with a Bio-Rad HPX-87H (300 mm × 7.8 mm) column (Hercules, CA, USA) and a refractive index detector. The samples were diluted with deionized water, filtered through 0.22 µm, 13 mm Nylon membrane filter (Sartorius, German) and then injected in the chromatograph under the conditions: column temperature at 65°C, 5 mM sulfuric acid as mobile phase at a flow rate of 0.8 ml min<sup>-1</sup>, and an injection volume of 20 µl (Kang et al., 2011). The concentration of these compounds was calculated using calibration curves obtained from the standard solutions.

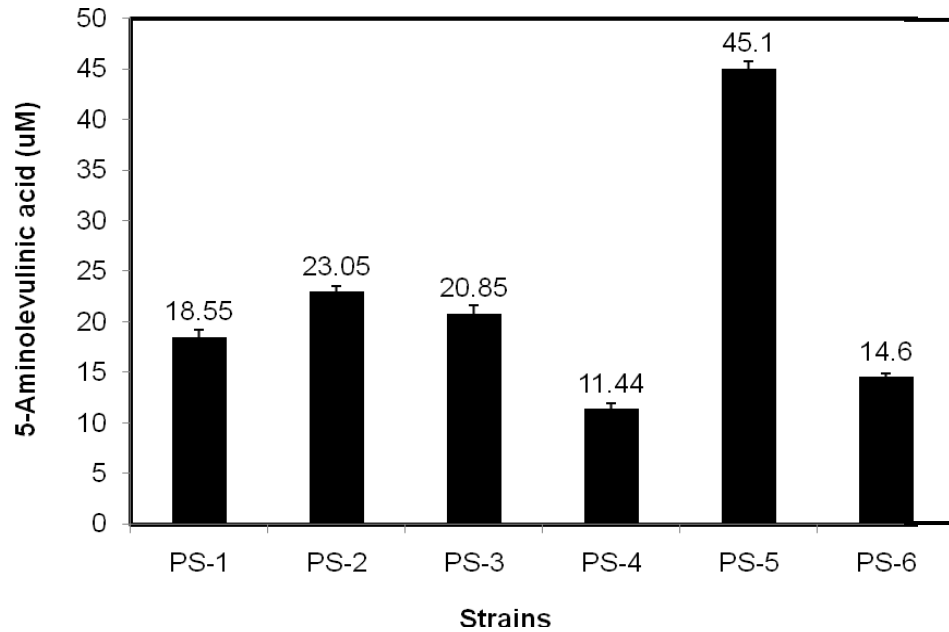
## RESULTS AND DISCUSSION

### Isolation, selection and identification of photosynthetic bacteria

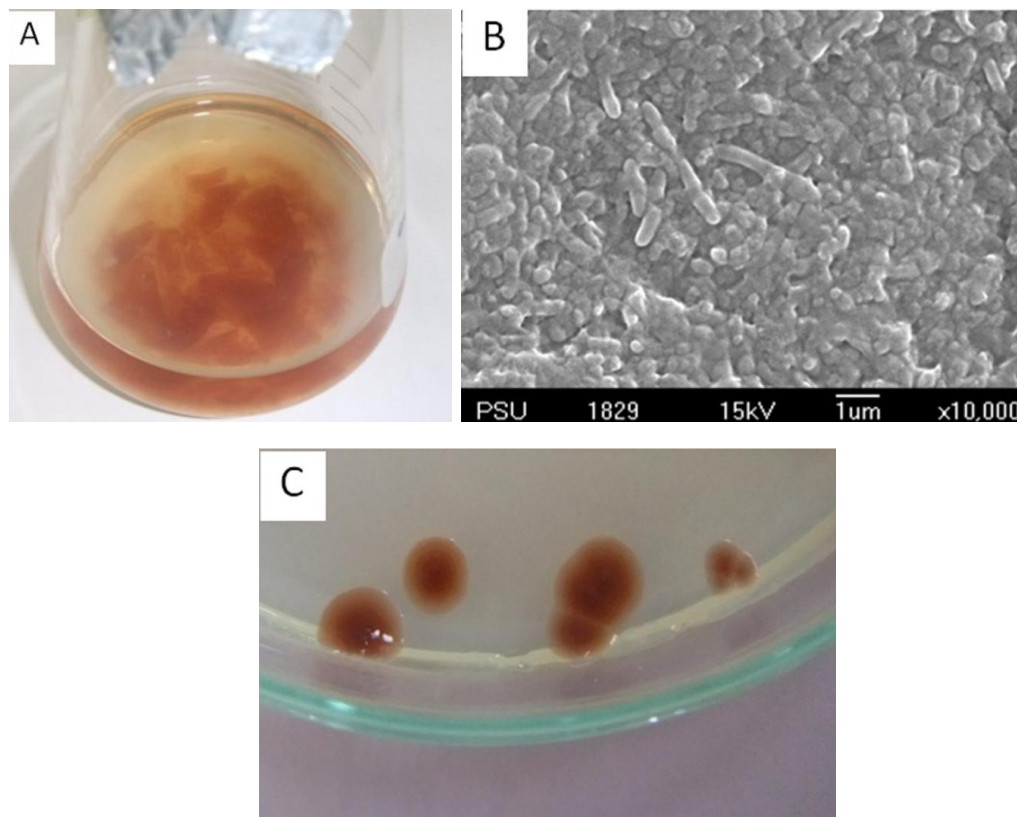
Thirty (30) samples collected from paddy soil, lake, and pond sediment mud were enriched in the RM medium and incubated under anaerobic-light (3,000 lux) condition for growth of photosynthetic bacteria. The RM medium contained high malate that could enhance the growth of photosynthetic bacteria as malate is a photosynthetic reductant that plays a critical role in the noncyclic photophosphorylation of *Rhodospirillum rubrum* (Stenn, 1968). Selective enrichment in liquid media is still an appropriate method for isolation and incubation time of five or more days are required for intense color of purple non-sulfur bacteria. Six colonies, different in forms and colors on the agar plates, were purified by sub-cultured. The six selected isolates producing red, pink, reddish

brown, orange brown and pale pink colonies were designated as PS-1, PS-2, PS-3, PS-4, PS-5 and PS-6, respectively. The occurrence of different colors was dependent on the species of photosynthetic bacteria that contain specific photopigments in their cells particularly carotenoid types (Imhoff, 1988). All six isolates were tested for 5-aminolevulinic acid production in modified GM medium with the addition of 10 mM levulinic acid at 24 h. Levulinic acid is an analogue of 5-aminolevulinic acid and can be used to inhibit ALA dehydratase activity in the biosynthesis of tetrapyrrols by competitive inhibition. Since levulinic acid could retard the growth of photosynthetic bacteria, it should be added in the middle log phase to increase the extracellular 5-aminolevulinic acid concentration (Sasaki et al., 1987). It had been utilized to enhance 5-aminolevulinic acid production effectively in various organisms such as *Rhodobacter sphaeroides* (Sasaki et al., 1991), *Rhodovulum* sp. (Noparatnaraporn et al., 2000), *Chlorella regularis* (Ano et al., 2000), *Methanobacterium thermoautotrophicum* (Lin et al., 1989), *Rhodopseudomonas palustris* (Saikour et al., 2009). Among the six isolates, the strain PS-5 showed the highest 5-aminolevulinic acid production (45.10 µM) at 48 h cultivation with the productivity of 0.94 µM h<sup>-1</sup> (Figure 1). This maximum value was higher than that from *Rhodopseudomonas palustris* KG31 (38.24 µM) after 48 h cultivation in GM medium supplemented with 5 mM levulinic acid at 24 h cultivation under anaerobic-light condition (Saikour et al., 2009).

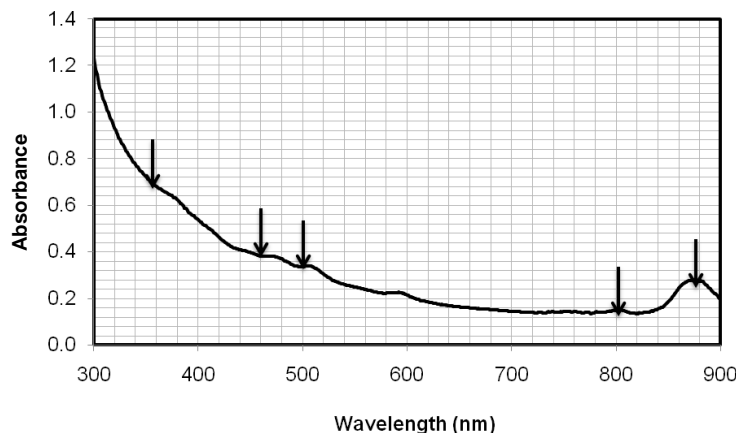
The special property of the isolate PS-5 was self-flocculating ability (Figure 2A) that would benefit the harvesting process using decantation method instead of commonly used energy-intensive centrifugation method. The characteristic of flocculant growth has two forms, compact floc (tightly attachment of cells in flocs) and friable flocs (loosely cells attachment). The SEM of the strain PS-5 illustrated that flocculated cells polarly attached to each other and embedded in gum-like substance, which found covering on the surface of the flocs (Figure 2B). Some flocculating photosynthetic bacteria was reported to produce extracellular polymeric substances (EPS) on the surface of cells (Noparatnaraporn et al., 2000). The arrangement of numerous cells in flocs or pellets of photosynthetic bacteria is relatively uniform in each pattern and seem to be the clusters or layers. In contrast, the cells arranged in multispecies biofilm formed highly complex distribution of microorganisms and structure (Beer et al., 1993). Several flocculating mechanisms were proposed such as bridge formation between the EPS and cells (Urbain et al., 1993), production of extracellular nucleic acids and form structured communities of cells called flocs (as in the genus *Rhodovulum*) (Ando et al., 2006; Watanabe et al., 1998) and production of RNAs (contain mainly fully mature-sized tRNAs and fragments of 16S and 23S rRNAs) (as in the genus *Rhodovulum*) (Ando et al., 2006) and extracellular soluble DNA (*Rhodovulum*



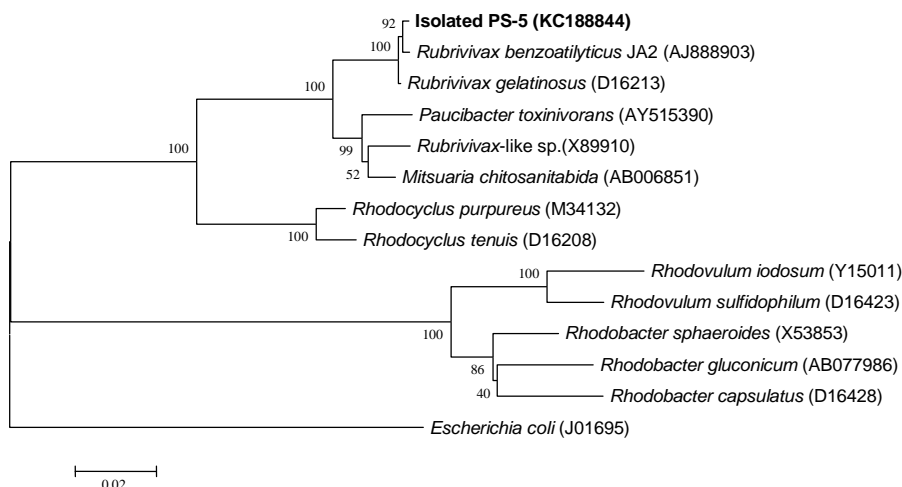
**Figure 1.** 5-Aminolevulinic acid production from cultivation of the isolated strains in modified glutamate-malate (GM) medium with addition of 10 mM levulinic acid at 48 h under anaerobic-light (3,000 lux) condition.



**Figure 2.** Characteristic of photosynthetic bacteria *R. benzoatilyticus* PS-5 growth in modified glutamate-malate (GM) medium (A), Scanning electron micrographs of biofilms formed (B) magnification x 10,000 and colony on GM agar (C).



**Figure 3.** Whole-cell absorption spectrum of the isolate PS-5 under anaerobic-light (3,000 lux) condition. Wavelengths (nm) of absorption maxima are shown at the tops of peaks.



**Figure 4.** Phylogenetic relationship between the isolate PS-5 and other phototrophic purple non-sulfur bacteria, constructed by the Bootstrap percentages that are written on internal branches, neighbor-joining method using the MEGA 5.1 program. Accession numbers for the sequences are shown in parentheses following each strain designation.

*sulfidophilum*) (Suzuki et al., 2009). The isolate PS-5 was chosen for 5-aminolevulinic acid production because it gave the highest 5-aminolevulinic acid and self-flocculation.

Morphology of the isolate PS-5 was rod-shaped, 0.7-1.0  $\mu\text{m}$  wide and 1.5  $\mu\text{m}$  long, and cells multiply by binary fission. Colony appearance on agar plates was round, convex and smooth, with dark orange-brown color (Figure 2C). After growing under anaerobic photoheterotrophic conditions, the living cell suspensions became orange and the absorption spectra showed maxima at 370, 475, 520, 800 and 875 (Figure 3). The main peaks at 800 and 875 nm, which are closely related to bacteriochlorophyll a and b as well as carotenoid, are characteristics of photosynthetic bacteria (Madigan et al., 2000). The isolate PS-5 were tested to determine the

range of suitable electron donors and carbon sources for photosynthetic growth (Table 1). The strain grew photoorganotrophically on a wide variety of organic compounds, including short-chain fatty acids (lactic, formic, acetic, propionic, butyric acid), sugars (glucose, fructose, maltose) and amino acids (glutamic, aspartic) and glycerol. Moreover, this strain showed excellent growth on starch, indicating its production of enzyme amylase. The ability of the isolate PS-5 to assimilate lactic acid but not tartaric acid was opposite to that of *Rubrivivax benzoatilyticus* JA2 previously reported (Ramana et al., 2007).

A typical phylogenetic tree for the relationship of 16S rRNA of the isolate PS-5 to the 16S rRNA of the other phototrophic bacterial strains is shown in Figure 4. It showed that the isolate PS-5 was most closely related to

**Table 1.** Utilization of electron donors and carbon sources used by the isolate PS-5 and *Rubrivivax benzoatilyticus* JA2<sup>a</sup>.

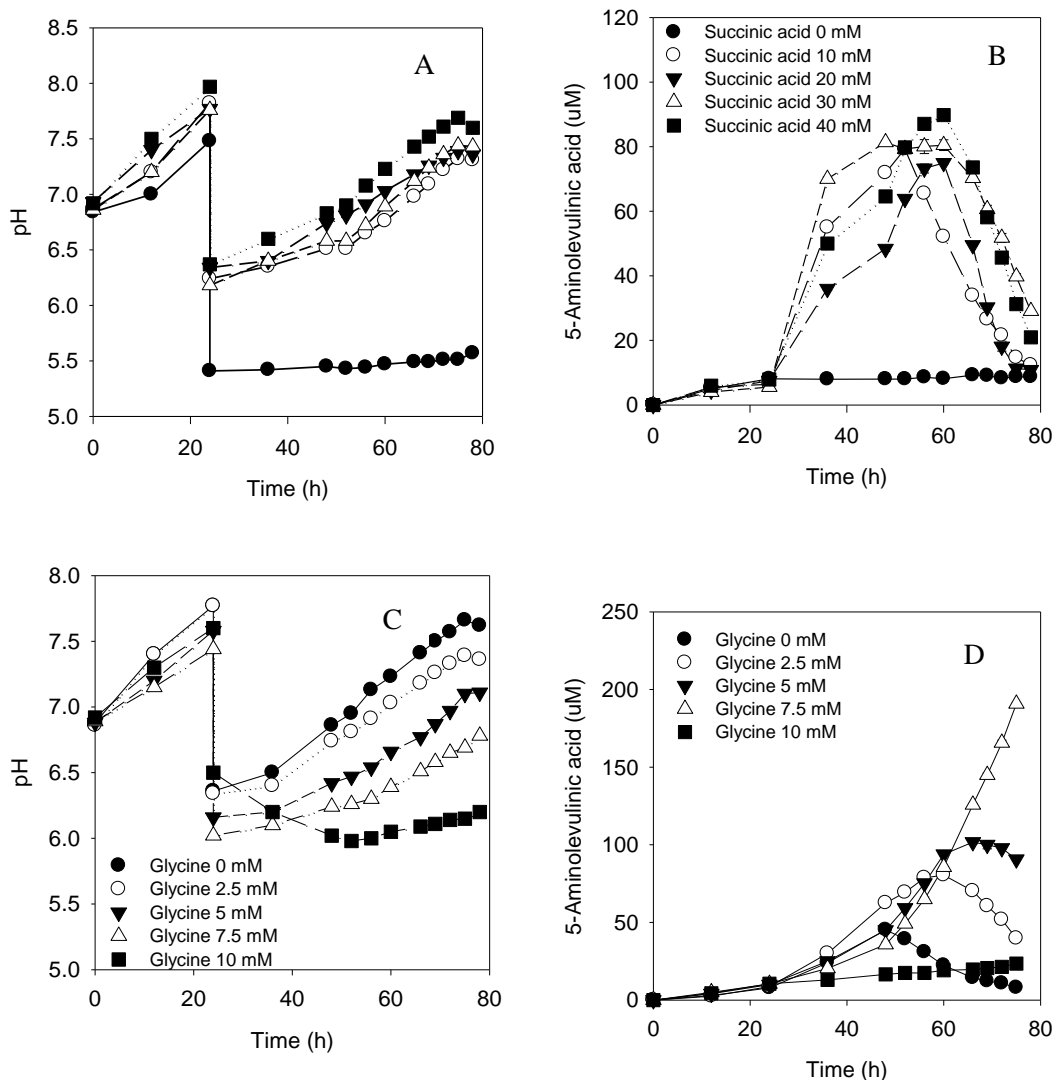
Donor/source	PS-5	<i>R. benzoatilyticus</i> JA2
<b>Organic acids</b>		
Formic acid	+ <sup>b</sup>	+
Acetic acid	+	ND
Propionic acid	+	+
Valeric acid	-	-
Butyric acid	+	ND
Pyruvic acid	++	+
Citric acid	-	-
Lactic acid	+	-
Malic acid	+	+
Succinic acid	+	ND
Tartaric acid	-	+
Fumaric acid	+	+
Benzoic acid	+	+
<b>Sugars and alcohols</b>		
Glucose	++	+
Sorbital	-	ND
Manital	-	-
Sucrose	-	ND
Fructose	++	+
Maltose	++	+
Glycerol	+	+
Ethanol	-	ND
Methanol	-	-
<b>Amino acids</b>		
Glutamic acid	+	+
Aspartic acid	++	ND
Glutamine	-	ND
Glycine	-	ND
<b>Carbohydrate</b>		
Starch	+++	ND
<b>Chemolithoautotrophic substrates</b>		
Sulfide	-	-
Sulfur	-	-
Thiosulfate	-	-

<sup>a</sup>Data for *R. benzoatilyticus* JA2 were obtained from Ramana et al. (2007). All cultures were incubated at room temperature in the presence of light (3,000 lux) for 48 h. <sup>b</sup>+++ , very good growth; ++, good growth; + poor growth; -, no growth; ND, not determined; +++, Excellent growth ( $A_{660nm} > 1.0$ ); ++, good growth ( $A_{660nm} = 0.5-1.0$ ); +, moderate growth ( $A_{660nm} = 0.2-0.5$ ); (+), utilized; ±, some strains only; -, no growth; nt, not determined.

*Rubrivivax benzoatilyticus* (GenBank accession No. AJ888903.1) that was isolated from the rhizosphere of a flooded paddy field near Eluru, Andhra Pradesh, India (Ramana et al., 2007). The accession numbers of the gene sequences submitted to GenBank was KC188844.

#### Effect of C<sub>4</sub> pathway precursor

5-Aminolevulinic acid is formed by the C<sub>4</sub> pathway, called Shemin pathway, in some bacteria including photosynthetic bacteria such as *R. sphaeroides*,



**Figure 5.** Time course of pH change (A, C) and ALA production (B, D) at various succinic acid concentration with 2.5 mM glycine and various glycine concentration with 10 mM succinic acid and adding 10 mM LA at 24h during cultivation of *R. benzoatilyticus* PS-5 under anaerobic-light (3,000 lux) condition at room temperature ( $32\pm^{\circ}\text{C}$ ).

*Rhodopseudomonas* sp.. Biosynthesis of 5-aminolevulinic acid proceeds from  $\text{C}_4$  pathway precursors, glycine and succinyl-CoA through a combined action of the host enzymes, succinyl-CoA synthase and ALA synthase (Sasaki et al., 2002). In addition, substrate inhibition occurred at high initial concentrations of glycine and succinate and could not enhance the production of 5-aminolevulinic acid (Fu et al., 2007). Therefore, the optimum concentration of each precursor in the basal medium is anticipated to be necessary for effective 5-aminolevulinic acid production. In this study, succinic acid was used instead of the expensive succinyl-CoA to reduce the production cost and being another carbon source for cell growth as an indirect precursor. The effect of succinic acid

concentration on growth and 5-aminolevulinic acid production from *Rubrivivax benzoatilyticus*, PS-5 was tested in the range of 0 to 10 mM (0 to  $1.2 \text{ g l}^{-1}$ ) and fix concentration of glycine at 2.5 mM ( $0.19 \text{ g l}^{-1}$ ). Due to self-flocculating property, cell growth was detected indirectly via pH changes during cultivation. The pH increased from 6.8 to 7.3 to 7.8 during growth in the first 24 h cultivation. Addition of levulinic acid resulted in a rapidly drop of pH to 5.4 to 6.3 (Figure 5A), then increased linearly to 7.3 to 7.6 along with a continuous decrease of levulinic acid concentration during 78 h cultivation. It was interesting to find that this strain could not use glycine without succinate, therefore, there was no growth (no pH change) and no consumption of levulinic acid concentration. On the other hand, succinate of 2.5 to 7.5 mM was

**Table 2.** Effect of precursors and levulinic acid concentration.

Parameter	Final pH	Maximum ALA ( $\mu\text{M}$ )	Succinic acid consumption (%)	Levulinic acid consumption (%)
<b>C4 pathway</b>				
<b>Succinic acid (mM) + 2.5 mM glycine</b>				
0	5.57	9.26 $\pm$ 0.07	0	5.34
2.5	7.31	74.95 $\pm$ 1.04	100	100
5	7.43	81.32 $\pm$ 0.49	100	73.39
7.5	7.6	87.06 $\pm$ 1.11	100	76.86
10	7.3	90.59 $\pm$ 0.69	78	66.95
<b>Glycine (mM) + 10 mM succinic acid</b>				
0	7.62	39.26 $\pm$ 1.04	81.24	80
2.5	7.36	80.49 $\pm$ 1.66	75.38	68.8
5	7.11	101.76 $\pm$ 1.11	62.7	47.97
7.5	6.78	190.74 $\pm$ 0.90	48.8	7.32
10	6.2	25.49 $\pm$ 0.97	26.56	2.17
<b>C5 pathway</b>				
<b>Glutamate (mM)</b>				
10	7.17	58.73 $\pm$ 1.04	-	96.92
20	7.32	71.08 $\pm$ 1.66	-	95.65
30	7.44	80.88 $\pm$ 1.46	-	76.98
40	7.4	84.12 $\pm$ 1.39	-	69.37
50	7.39	85.29 $\pm$ 0.21	-	51.22
<b>Malic acid (mM)</b>				
10	7.36	35.29 $\pm$ 1.53	-	87.5
20	7.77	22.06 $\pm$ 0.69	-	89.51
30	7.89	19.12 $\pm$ 0.55	-	93.55
40	7.98	17.35 $\pm$ 0.35	-	90.76
50	7.85	13.53 $\pm$ 0.21	-	91.8
<b>Inhibitor ALA dehydratase</b>				
<b>Levulinic acid (mM)</b>				
2.5	7.63	57.74 $\pm$ 1.73	87.7	90.2
5	7.55	96.18 $\pm$ 1.25	78.87	73.91
7.5	7.33	120.00 $\pm$ 1.39	73.98	61.27
10	7	186.50 $\pm$ 1.11	34.15	25.52
12.5	5.35	73.82 $\pm$ 0.83	27.64	2.27

completely consumed at 70 h cultivation whereas 10 mM succinate was consumed about 79.2% (Table 2). 5-aminolevulinic acid production increased ( $P < 0.05$ ) as succinic acid concentration increased and it was secreted after addition of levulinic acid at 24 h (Figure 5B). 5-aminolevulinic acid production was highest ( $P < 0.05$ ) when succinic acid was supplemented at 10 mM. The

maximum 5-aminolevulinic acid concentration and productivity were 89.8  $\mu\text{M}$  and 1.5  $\mu\text{M h}^{-1}$ , respectively, which was 9.7 times higher than that without succinate. This indicates that appropriate succinate concentration could enhance both cell growth and 5-aminolevulinic acid production. This agreed with the report that succinate could serve as another precursor for 5-aminolevulinic



acid production, as well as a carbon source for cell growth (Qin et al., 2006).

Glycine is another important precursor for 5-aminolevulinic acid production (Choi et al., 1999; Sasaki et al., 2002), therefore, its concentration in the range of 2.5 to 10 mM plus optimum concentration of succinic acid (10 mM) on growth and 5-aminolevulinic acid production was investigated. The pH increased from 6.8 to 7.5 to 7.8 at 24 h and dropped to 6.0 to 6.5 when levulinic acid was added, then increased to 7.3 to 7.6 during 78 h cultivation at low glycine concentration (2.5 to 5.0 mM) (Figure 5C). However, in the presence of high glycine concentration (10 mM) the pH was dropped to 5.98 to 6.2 after levulinic acid addition, and slightly changes thereafter. In addition, only 3.84% of levulinic acid and 26.56% succinate were consumed (Table 2). Therefore, the substrate inhibition was occurred.

Glycine concentration had an effect ( $P > 0.05$ ) on 5-aminolevulinic acid production. The optimum glycine concentration was found to be 7.5 mM, giving the maximum 5-aminolevulinic acid production of 190.74  $\mu\text{M}$  and productivity of 2.54  $\mu\text{M h}^{-1}$  (Figure 5D) which was 2.4 and 1.9 folds higher than those at 2.5 and 5.0 mM, respectively. The negative effect of high concentrations of glycine (10 mM) on 5-aminolevulinic acid production (25.49  $\mu\text{M}$ ) was caused by its inhibitory effect on cell growth from ammonia generated during glycine metabolism (Sasaki et al., 2002). The results agreed with that occurred to *Rhodospseudomonas palustris* KG31 (Saikour et al., 2009). Moreover, high amount of glycine in the medium can inhibit porphyrin formation (Neuberger, 1961). Optimum concentrations of glycine and succinic acid added to the culture medium were different from those in other experiment, that is, 30 mM glycine and 90 mM succinate in a LB complex medium for *Escherichia coli* transformed with ALA synthase gene of *Bradyrhizobium japonicum* (Choi et al., 1999). Besides, the optimum glycine concentration of *Rubrivivax benzoatilyticus* PS-5 (7.5 mM) was lower than that of *Rhodobacter sphaeroides* CR-606 (60 mM) (Nishikawa et al., 1999). Therefore, it depends on bacterial strain, culture condition, and medium used, which may have an effect on the ALA synthase (ALAS) activity levels (Chung et al., 2005).

### Effect of C<sub>5</sub> pathway precursor

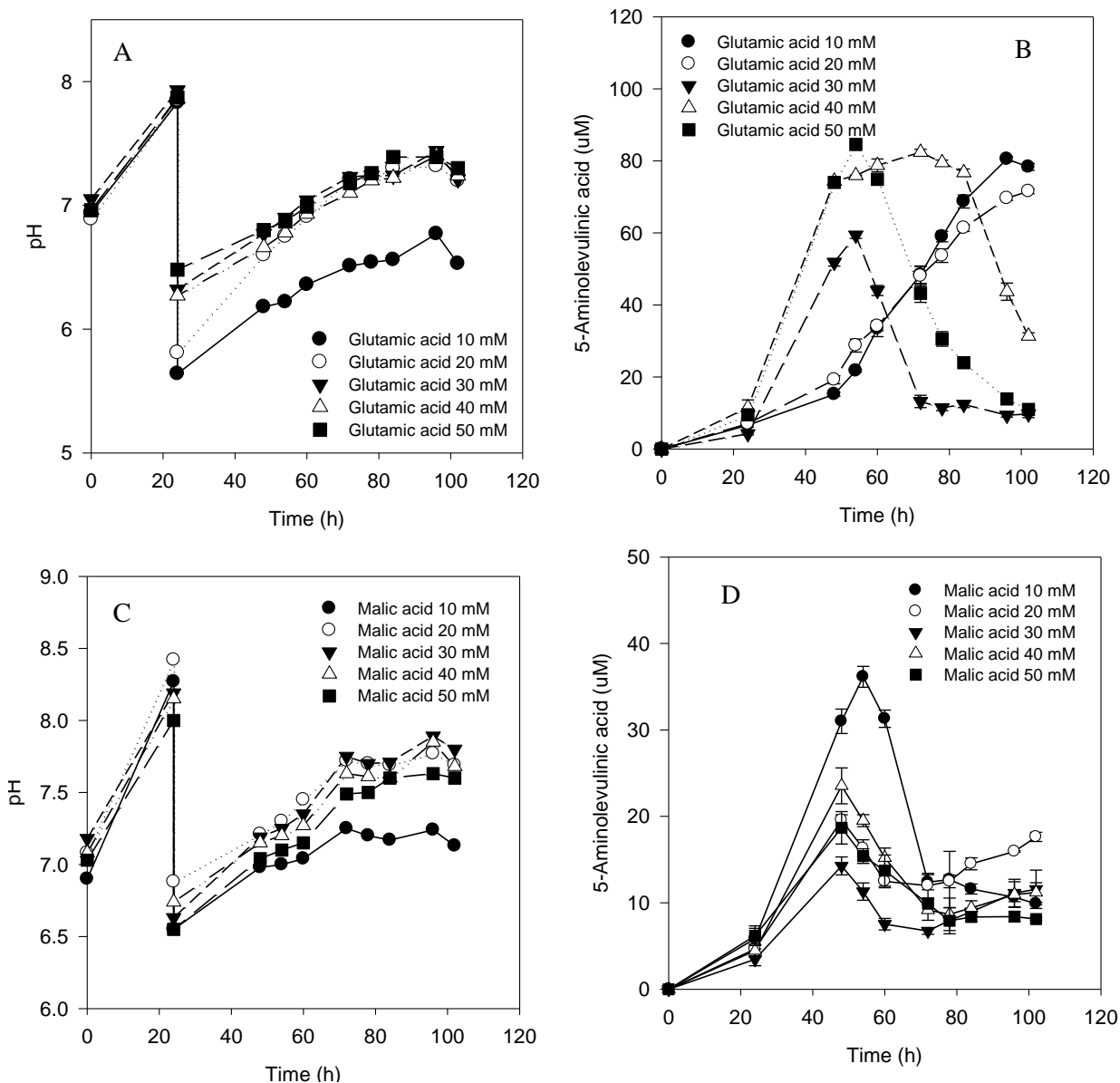
Glutamate is the precursor of 5-aminolevulinic acid biosynthesis in higher plants and most green algae via the light-dependent C<sub>5</sub> pathway in the plastid, called Beale pathway (Reinbothe and Reinbothe, 1996). Phytoflagellate *Euglena gracillis* (Weinstein and Beale, 1983) and the green alga *Scenedesmus obliquus* (Dreschler-Theilmann et al., 1993) have both ALA-forming pathways. Effect of glutamate on growth (pH change) and 5-aminolevulinic acid production from

*Rubrivivax benzoatilyticus* PS-5 was investigated. The change of pH was similar to those of using succinate as a precursor. The pH increased from neutral to alkaline (6.92 to 7.93) at 24 h cultivation and decreased rapidly to acidic pH (pH 5.6) after adding 10 mM levulinic acid, then increased to 7.3 at the end of cultivation (102 h) (Figure 6A). This indicated that glutamate could enhance cell growth.

The optimum glutamate concentration for 5-aminolevulinic acid production was found to be 50 mM, giving the highest 5-aminolevulinic acid production of 84.12  $\mu\text{M}$  at 54 h with the productivity of 1.56  $\mu\text{M h}^{-1}$  (Figure 6B). This may be due to the degradation of 5-aminolevulinic acid into pyrazine compounds and tetrapyrroles (Bunke et al., 2000) or generation of ammonia from assimilation of glutamate that caused the reduction of ALA synthase activity or induced ALA dehydratase activity. Moreover, ALA in aqueous solution was found to be unstable since it is dependent on pH and other factors (Gadmar et al., 2002) such as temperature, and ALA concentration.

It was previously reported that photosynthetic bacteria could not produce 5-aminolevulinic acid via C<sub>5</sub> pathway (glutamate) (Sasikala et al., 1994). However, this work clearly indicated that *R. benzoatilyticus* PS-5 could produce 5-aminolevulinic acid from both C<sub>4</sub> and C<sub>5</sub> pathways precursors in which the product from C<sub>4</sub> pathway was 2-fold higher than that from C<sub>5</sub> pathway. Therefore, it is a special property of *R. benzoatilyticus* PS-5 that it may have the mechanism for changing glutamate to 5-aminolevulinic acid via transfer RNA-dependent five-carbon route and formed by ALA synthase-catalyzed condensation of succinyl coenzyme A and glycine. The biosynthetic source of succinyl coenzyme A of *R. benzoatilyticus* PS-5 may contain  $\alpha$ -ketoglutarate dehydrogenase and use succinyl-coenzyme A as a tricarboxylic acid cycle intermediate (Mayer and Beale, 1991).

Malic acid is a precursor of 5-aminolevulinic acid biosynthesis via C<sub>5</sub> pathway (malate  $\rightarrow$  fumarate  $\rightarrow$  succinate + CO<sub>2</sub>  $\rightarrow$  oxoglutarate  $\rightarrow$  glutarate) (Sasikala et al., 1994). Effect of malic acid concentration (10 to 50 mM) with the addition of 10 mM levulinic acid at 24 h on 5-aminolevulinic acid production was studied. The culture pH increased from neutral to alkaline pH (from 7.0 to 8.42) within 24 h cultivation and decreased rapidly to acidic pH (pH 6.5) after adding 10 mM levulinic acid then increased from 6.92 to about 8.3 at the end of cultivation (102 h) (Figure 6C). Malic acid concentration had no effect on 5-aminolevulinic acid production (Figure 6D) as the values were low ( $P < 0.05$ ) at all malic acid concentrations tested. The 5-aminolevulinic acid production reached the maximum 5-aminolevulinic acid of 35.29  $\mu\text{M}$  obtained at the minimum concentration of malic acid (10 mM) and decreased thereafter ( $P < 0.05$ ). This might be due to the increase of pH resulted from the assimilation of malic acid (Gadmar et al., 2002). Levulinic



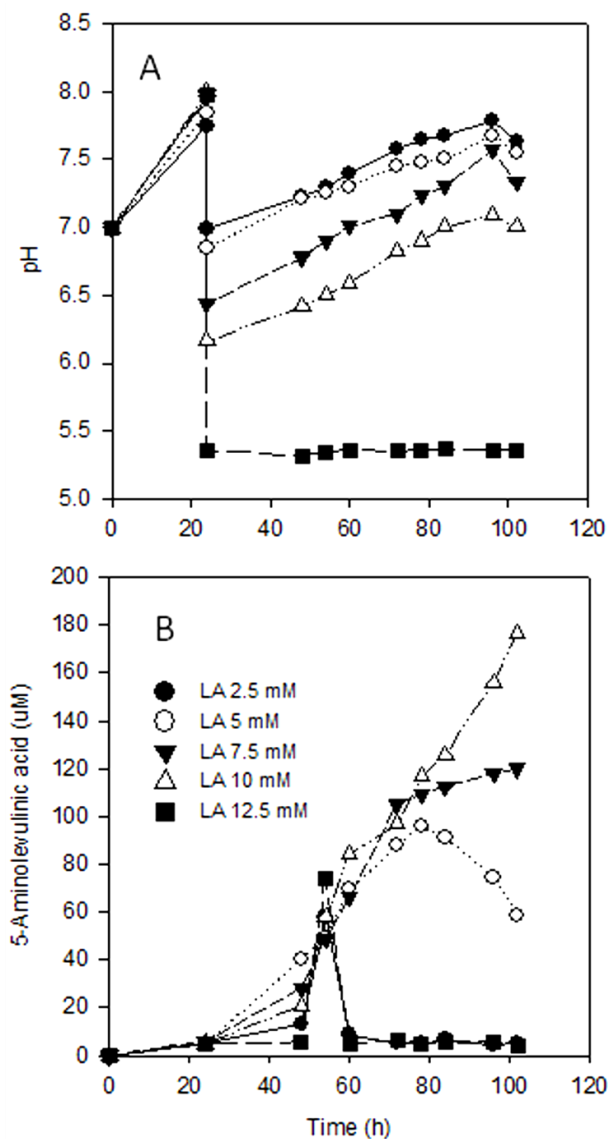
**Figure 6.** Time course of pH change (A, C) and ALA production (B,D) at various glutamic acid concentration and malic acid concentration and adding 10 mM LA at 24h during cultivation of *Rubrivivax benzoatilyticus* PS-5 under anaerobic-light (3,000 lux) condition at room temperature ( $32\pm^{\circ}\text{C}$ ).

acid consumption was in the range of 87.5-91.8% at all malic acid concentrations. Possibly, levulinic acid uptake was stimulated in the presence of malic acid. In addition, malic acid at 30, 40, and 50 mM could be converted to succinic acid at 7.62, 10.75 and 13.04 mM, respectively (data not shown).

The presence of  $\text{C}_4$  precursors could enhance 5-aminolevulinic acid production more effectively than  $\text{C}_5$  pathway precursors. This is the first report that *R. benzoatilyticus* could produce 5-aminolevulinic acid via the consumption of both  $\text{C}_4$  (glycine and succinic acid) and  $\text{C}_5$  (glutamate or malic acid) pathway precursors.

### Effects of LA concentration on ALA production

Levulinic acid is structurally similar to 5-aminolevulinic acid except for the absence of the amino group. The undissociated form of levulinic acid can enter the cell and function as a competitive inhibitor of ALA dehydratase (Sasaki et al., 1997). Since levulinic acid could retard the growth of photosynthetic bacteria, it was recommended to add the middle log phase to increase the extracellular 5-aminolevulinic acid concentration (Sasaki et al., 1987). In this study, levulinic acid was added at 24 h. Effect of levulinic acid concentration (2.5 to 12.5 mM) with



**Figure 7.** Time course of pH change and ALA production at adding various levulinic acid (LA) concentration at 24h during cultivation of *Rubrivivax benzoatilyticus* PS-5 under anaerobic-light (3,000 lux) condition at room temperature ( $32\pm^{\circ}\text{C}$ ).

optimum precursors (7.5 mM glycine plus 10 mM succinate) on 5-aminolevulinic acid production from *R. benzoatilyticus* PS-5 were investigated (Figures 7A and B). Increase levulinic acid concentration from 2.5 to 10 mM caused the decrease of pH from 7.63 to 7.0 at 96 h. At 2.5 mM levulinic acid, the consumption of levulinic acid and succinic acid was 90.20 and 87.70%, respectively (Table 2). This indicates that the lowest concentration of levulinic acid could be uptake more than high concentration (12.5 mM). Moreover, levulinic acid could be metabolized to acetate and propionate and gave the maximum values of 0.23 and  $0.34\text{ g l}^{-1}$ , respectively under low levulinic acid concentration (2.5 mM) (data not

shown). These results agreed with the report that levulinic acid incorporated by a photosynthetic bacterium (*Rhodospseudomonas* sp.) was metabolized to acetate and propionate under anaerobic-light condition (Okayama et al., 1990). Levulinic acid would be incorporated into the main metabolism, which must first pass through glycolysis to form pyruvate and acetyl-CoA (Jaremkó and Yu, 2011).

Levulinic acid supplementation did affect ( $P>0.05$ ) 5-aminolevulinic acid production as it increased from 57.74 to  $176.50\text{ }\mu\text{M}$  with increasing levulinic acid concentration from 2.5 to 10 mM. The decrease at 12.5 mM levulinic acid was due to the drop of pH to 5.3 that strongly suppressed the growth of *R. benzoatilyticus* PS-5 as observed by cells decolorization (data not shown). In addition, levulinic acid also affects the pigment synthesis of the blue-green alga *Agmenellum quadruplicatum* PR-6 chlorophyll, c-phycoyanin, and ALA syntheses (Kipe-nolt and Stevens, 1979). Moreover, the drop of pH that resulted in the decrease of enzyme activities under low pH conditions (Sasaki et al., 1991). For example, at pH 6.0, ALAD activity was relatively inhibited by levulinic acid addition, however, ALAS activity was also low (Noparatnaraporn et al., 2000).

## Conclusion

*R. benzoatilyticus* PS-5 was isolated from pond sediment and found to be a novel 5-aminolevulinic acid producer with self-flocculating property. The optimum precursors of  $\text{C}_4$  pathway for 5-aminolevulinic acid production were 7.5 mM glycine plus 10 mM succinic acid and addition of 10 mM levulinic acid at 24 h cultivation. Moreover, the optimum precursors of  $\text{C}_5$  pathway was  $85.29\text{ }\mu\text{M}$  with the addition of 10 mM levulinic acid at 24 h. The strain preferred  $\text{C}_4$  to  $\text{C}_5$  pathway. Maximum 5-aminolevulinic acid production was  $190.74\text{ }\mu\text{M}$  and the productivity was  $2.54\text{ }\mu\text{M h}^{-1}$ . Further optimization of the fermentation process should improve the 5-aminolevulinic acid production to even higher level.

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