



รายงานวิจัยฉบับสมบูรณ์

โครงการ

การผลิตแบคทีเรียสังเคราะห์แสงจากทะเลที่ผลิตกรดอะมิโนดีวูลินิก
และการนำไปใช้ประโยชน์ในการเพาะเลี้ยงสัตว์น้ำ

โดย

อมรรัตน์ ตั้งประสิทธิภาพ

พูนสุข ประเสริฐสรรพ

กิจการ สุขุมาศัย

กัลยาณี ศรีชัยญลักษณ์

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คณะผู้วิจัย

สังกัด

- | | |
|-----------------------------------|---|
| 1. นางสาวอมรรัตน์ ตั้งประสิทธิภาพ | ภาควิชาเทคโนโลยีชีวภาพอุตสาหกรรม
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คณะทรัพยากรธรรมชาติ
มหาวิทยาลัยสงขลานครินทร์ |
| 4. ดร.กัลยาณ์ ศรีธัญญลักษณ์ | หน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพ
กุ้ง (CENTEX SHRIMP) คณะวิทยาศาสตร์
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บทคัดย่อ

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ชื่อโครงการ : การผลิตแบคทีเรียสังเคราะห์แสงจากทะเลที่ผลิตกรดอะมิโนลิวูลินิก และการนำไปใช้ประโยชน์ในการเพาะเลี้ยงสัตว์น้ำ

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หน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพกุ้ง (CENTEX SHRIMP)

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ระยะเวลาโครงการ 2 ปี

บทคัดย่อ จากการปรับปรุงสายพันธุ์ของแบคทีเรียสังเคราะห์แสงทนเค็ม *Rhodobacter sphaeroides* SH5 เพื่อเพิ่มการสะสมกรดอะมิโนลิวูลินิก (5-aminolevulinic acid; ALA) ภายในเซลล์ด้วยวิธีการต่างๆ 4 วิธี คือ การใช้แสงอัลตราไวโอเล็ต (UV) การใช้สาร N-methyl-N'-nitrosoguanidine (NTG) การใช้แสง UV ร่วมกับ NTG และการใช้สาร NTG ร่วมกับการใช้แสง UV พบว่า สามารถแยกเชื้อสายพันธุ์กลายได้ จำนวน 50 สายพันธุ์ ซึ่งมีเพียง 5 สายพันธุ์ที่ผลิตกรดอะมิโนลิวูลินิกได้สูง แต่มีเพียง 2 สายพันธุ์ที่มีความคงตัวในการผลิตกรดอะมิโนลิวูลินิก คือ สายพันธุ์กลาย U10 และ N20 อย่างไรก็ตาม ผลผลิตของกรดอะมิโนลิวูลินิกภายในเซลล์ที่ได้ไม่แตกต่างอย่างมีนัยสำคัญกับผลผลิตของสายพันธุ์ดั้งเดิม ดังนั้นจึงใช้สายพันธุ์ดั้งเดิมในการทดลองขั้นต่อไป

จากการศึกษาปัจจัยที่มีผลต่อการเพิ่มปริมาณกรดอะมิโนลิวูลินิกภายในเซลล์ของ *Rhodobacter sphaeroides* SH5 ที่เลี้ยงภายใต้สภาวะให้อากาศไร้แสง พบว่าอาหารเลี้ยงเชื้อสูตร MGG ที่มีการเติมเกลือ (3% NaCl) กรดลิวูลินิกและธาตุเหล็ก เป็นสูตรอาหารที่เหมาะสมโดยให้กรดอะมิโนลิวูลินิกภายในเซลล์สูงสุด (226 $\mu\text{g/gDCW}$) ที่ชั่วโมงที่ 30 และจากการศึกษาเบื้องต้น พบว่า ค่า LD_{50} ของ ALA ต่อกุ้งกุลาดำ (น้ำหนักเฉลี่ย 15 ± 2 กรัม) มีค่า 113 ส่วนในล้านส่วน (ppm) ในระยะเวลา 7 วัน ดังนั้น ALA จึงมีความเป็นพิษต่ำในกุ้งกุลาดำ เมื่อนำเซลล์ที่ผลิต

ได้ไปผสมในอาหารกึ่งกลาดำ จำนวน 5 สูตร เพื่อศึกษาระบบภูมิคุ้มกันของกึ่งกลาดำ พบว่า ทุกสูตรที่ให้กึ่งกินไม่มีผลต่อการเจริญเติบโต อัตราการรอดตาย อัตราการแลกเนื้อ รวมทั้งบางส่วนของระบบภูมิคุ้มกันของกึ่งได้แก่ เอนไซม์โปรตีนออกซิเดส ความต้านทานต่อการติดเชื้อไวรัสตัวแดงดวงขาว ระหว่างกึ่งชุดที่เลี้ยงด้วยอาหารที่ผสมแบคทีเรียและกึ่งชุดควบคุม อย่างไรก็ตาม มีการเพิ่มจำนวนของเม็ดเลือดรวมทั้งเม็ดเลือดที่มีแกรนูโล และเอนไซม์ซูเปอร์ออกไซด์ดิสมิวเตส (SOD) ของกึ่งที่ได้รับอาหารที่มีกรดอะมิโนลิวลินิกบริสุทธิ และกรดอะมิโนลิวลินิกที่อยู่ในเซลล์แบคทีเรียสังเคราะห์แสงมากกว่าที่พบในกึ่งชุดควบคุม ($P>0.05$) และ SOD activity มีค่าสูงสุดในชุดการทดลองที่ใช้เซลล์ของ *Rhodobacter sphaeroides* SH5 ที่ผลิตได้ แสดงว่ากรดอะมิโนลิวลินิกที่อยู่ภายใน ไม่มีผลต่ออัตราการเจริญ แต่มีผลต่อเม็ดเลือดของกึ่งและกิจกรรมของเอนไซม์ ดังนั้น กรดอะมิโนลิวลินิกจึงมีคุณสมบัติเป็นสารกระตุ้นภูมิคุ้มกันให้กับกึ่ง

เมื่อศึกษากลไกการทำงานของกรดอะมิโนลิวลินิกในการเป็นสารกระตุ้นภูมิคุ้มกัน โดยศึกษาจากการแสดงออกของ cytosol haemocyte Mn-SOD ในระดับการสังเคราะห์ mRNA (transcriptional level) โดยวิธี RT-PCR พบว่า ไม่มีความแตกต่างในการทดลองทั้ง 5 ชุด แสดงว่ากรดอะมิโนลิวลินิกไม่มีผลในระดับการสังเคราะห์ mRNA (transcriptional level) โดย Mn-SOD และ SOD activity ที่เพิ่มขึ้นน่าจะเป็นผลจากกลไกการกระตุ้นในระดับการแปลรหัสเป็นโปรตีน (translational level) ในการสังเคราะห์เอนไซม์ซูเปอร์ออกไซด์ดิสมิวเตส

Abstract

Project Code : BGJ4580014

Project Title : Production of Marine Photosynthetic Bacteria Producing 5-Aminolevulinic Acid and Its Application to Aquaculture

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Project Period : 2 years

Abstract Strain improvement of halotolerant photosynthetic bacteria *Rhodobacter sphaeroides* SH5 to increase the accumulation of intracellular 5-aminolevulinic acid (ALA) using 4 mutagenesis treatments; UV, N-methyl-N'-nitro-N-Nitrosoguanidine (NTG), UV+NTG (UVN) and NTG+UV (NUV) mutation was investigated. Among 50 mutants obtained, only 5 strains produced high quantity of ALA in which 2 of them (mutant strain U10 and U20) were stable for ALA production. However, their intracellular ALA were not significantly different from that of the wild type strain. Therefore, the wild type strain was used for further studies.

Factors affecting the accumulation of intracellular ALA of *Rhodobacter sphaeroides* SH5 cultivated under aerobic-light condition were investigated. MGG medium with the addition of 3% NaCl, levulinic acid and iron was the optimum medium giving addition the highest accumulation of intracellular ALA (226 µg/gDCW) after 30 h cultivation. Preliminary test revealed that the LD₅₀ of ALA to black tiger shrimp *Penaeus monodon* (average weight of 15 ± 2 g) was estimated to be 113 ppm for 7 days. Therefore, ALA is considered to be low toxicity substance for shrimp. The bacterial cells were used for preparation of 5 formulae of shrimp feed to test on shrimp immune response. It was found that there was no significantly different

($P < 0.05$) in the growth rate, survival rate, feed conversion rate (FCR), phenoloxidase (PO) activity and disease (WSSV) resistance between both bacterial-fed shrimp and control group. However, the total haemocyte, granular cell number, and superoxide dismutase (SOD) activity in shrimp fed with pure ALA and ALA accumulated in the cells were higher than those of control in all feed formulae tested ($P > 0.05$). The highest SOD activity was found in shrimp fed with the formula containing ALA intact bacterial cells. These results suggested that ALA in the cells of *R. sphaeroides* SH5 had no effect on growth rate but on shrimp haemocytes and its enzymatic activity. Therefore, ALA was considered to be an immunostimulant for shrimp.

Mechanism of ALA acted as an immunostimulant was studied from the expression profile of shrimp cytosol haemocyte Mn-SOD in the synthesis of mRNA (transcriptional level) by RT-PCR. Elongation factor was used as constitutively expressed control gene. The results showed that no significant difference was observed for all 5 treatments suggesting that ALA has no effect at transcriptional level by Mn-SOD. The increase of total SOD activity in circulating haemocytes in shrimp fed immunostimulant, would be resulted from ALA affected to translation level of SOD synthesis

Introduction

5-Aminolevulinic acid (ALA) is the universal biosynthetic precursor of the tetrapyrroles, such as porphyrin, heme, chlorophyll, and vitamins B₁₂. It has several physiological activities. Such as a photodynamic herbicides effect on several plants (Rebeiz *et al.*, 1984), a photodynamic insecticidal effect on several insects such as *Trichoplusia ni* (Rebeiz *et al.*, 1988), plant-growth-regulating properties and may enhance agricultural productivity (Hotta *et al.*, 1997). Moreover, further applications of ALA in medicinal and pharmacological products were also reported (Sasaki *et al.*, 2002).

Research and development to reduce the cost of ALA production has been carried out in various aspects. Relatively large amounts of extracellular ALA production from *Rhodobacter sphaeroides* was achieved by adding levulinic acid (LA), a competitive inhibitor of ALA dehydratase (ALAD), intermittently (Sasaki *et al.*, 1987, 1990). In this condition, supplementing the precursors for synthesis (glycine and succinate) enhanced ALA accumulation (Sasaki *et al.*, 1991). In addition, ALA could be produced extracellularly using volatile fatty acids (VFAs) medium containing acetic, propionic and butyric acids as the carbon substrate (Sasaki *et al.*, 1993). Following this approach, ALA production using mutants of photosynthetic bacteria has been established (Nishikawa *et al.*, 1999; Sasaki *et al.*, 2002).

ALA was reported to have promotive effect on the growth and photosynthesis of crops and vegetable such as rice, barley, potato and garlic by applying small amount of ALA on the leave and roots of the plants (Sasaki *et al.*, 1993). ALA could not only be applied to agricultural sector but also to medical and pharmaceutical fields (Sasaki *et al.*, 2002).

To increase the value of the isolated strain of halotolerant photosynthetic bacteria and its product, 5-aminolevulinic acid (ALA), this investigation aims to increase the intracellular ALA production from *Rhodobacter sphaeroides* SH5 by strain improvement, optimization studies and apply to test its effect on shrimp.

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Objectives

1. To increase the intracellular 5-aminolevulinic acid (ALA) production of the halotolerant photosynthetic bacteria *Rhodobacter sphaeroides* SH5 by mutagenesis using ultraviolet (UV) and N-methyl-N'-nitro-N-Nitrosoguanidine (NTG).
2. To study on factors affecting the increase of intracellular ALA concentration including the effects of substrate concentration, medium composition, sodium chloride concentration, levulinic concentration, iron concentration.
3. To produce large quantity of bacterial cells for preparation of shrimp feed.
4. To investigate on the effect of ALA on the immunity of shrimp.

Materials and Methods

1. Organism and culture media

Halotolerant (0-12% NaCl) photosynthetic bacteria *Rhodobacter sphaeroides* SH5 used in this study was isolated from coastal area in Songkhla Province. The strain was maintained on modified glutamate-malate (GM) medium (Sasaki *et al.*, 1987) containing 3% NaCl, pH 8.0. The medium consisted of 3.8 g/l sodium glutamate, 2.7 g/l DL-malate, 2 g/l yeast extract, 0.5 g/l each of KH_2PO_4 and K_2HPO_4 , 0.8 g/l NH_4SO_4 , 0.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.053 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.012 g/l $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ and basal medium containing 1 mg/l each of nicotinic acid, thiamine, *p*-aminobenzoic acid and 0.01 g/l Biotin. Glutamate-glucose (GG) medium (Nishikawa *et al.*, 1999), MGG medium, MGGY medium and MGY medium containing 3% NaCl, pH 7.0 were used for selection of the optimal medium for intracellular ALA production from *R. sphaeroides* SH5.

2. Analytical methods

2.1 Growth

Growth of the culture was measured by turbidometric method at the wavelength of 660 nm (OD_{660}) and dry cell weight (DCW) (Noparatnaraporn *et al.*, 1986). Light intensity was measured by lux meter.

2.2 ALA determination

Extracellular ALA was determined by colorimetric method (Mauzerall and Granick, 1956), whereas intracellular ALA was determined by fluorometric method (modified from Okayama *et al.*, 1990). The concentration of intracellular ALA was calculated from the peak area with standard sample (Skoog *et al.*, 1996).

Intracellular ALA was determined by fluorometric method (Okayama *et al.*, 1990). For fluorometric method, bacterial cells were washed twice with 20 mM of Tris-HCl buffer (pH 7.4), then the cell suspension (40 mM of Tris-HCl, pH 7.4) and ruptured by ultrasonic homogenizer in ice-bath. The cell debris was removed by centrifugation (10,000 $\times g$, 15 min). Samples amount 50 μ l was pretreated by mixing with 3.5 ml of the mixture of acetylacetone, ethanol and water (15:10:75, v/v), 0.4% NaCl and 450 μ l of aqueous formalin (8.5 % of formalin). The solution was heated in boiling water bath for 30 min and cooled in ice bath. The HPLC system (pre-column) with a fluorescence detector (Shimadzu, LC-10A, Osaka, Japan) was used by applying 20 μ l solution to an injector sample loop. The column used was Inertsil ODS-3 (5 μ M, 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 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).

2.3 ALA synthetase

ALA synthetase was assayed according to the method of Mauzerall and Granick (1956) and Burnham and Lascelles (1963). The reaction mixture contained glycine 100 μ mol, succinate 100 μ mol, Tris-HCl buffer (pH 7.5) 50 μ mol, $MgCl_2$ 10 μ mol, PLAP (pyridoxal-5-phosphate) 0.5 μ mol, CoA 0.5 μ mol, ATP (dissolved in 0.1 ml of 0.7% KOH) 10 μ mol and cell free extract (protein 0.4 mg) in the total volume of 1.0 ml. The mixtures were incubated for 60 min at 37°C in the dark. The

reaction was terminated by adding 1.0 ml of 5% trichloroacetic acid. The mixture was centrifuged at 1,500 $\times g$ for 10 min, 1.0 ml of the supernatant was transferred to the test tube containing 2.0 ml of 1 M acetate buffer (pH 4.7) and 0.05 ml of acetylacetone. The mixture was heated in a boiling water bath for 15 min. After the mixtures had been cooled, 3.5 ml of modified Ehrlich's reagent was added. After 20 minute of incubation at RT, the optical density at 556 nm was measured. The amount of ALA was determined using a molar extinction coefficient of 66×10^3 at 556 nm.

2.4 ALA dehydratase

ALA dehydratase was assayed by a modified method of Shemin *et al.* (1970). The reaction mixture contained ALA 5 μmol , KCl 2 μmol , MgCl_2 5 μmol , Tris-HCl buffer (pH 8.1) 100 μmol and cell free extract (protein 1.2 mg) in a total volume of 0.6 ml. After incubation at 37°C for 60 min, the reaction was terminated by adding 2.4 ml of 5% trichloroacetic acid. The resulting precipitate was removed by centrifugation at 1,500 $\times g$ for 20 min. An aliquot (1 ml) was mixed with 3 ml of modified Ehrlich's reagent and the absorption was read after 10 min. The molar extinction coefficient used was 62×10^3 at 555 nm. The cell free extract used also contained the enzymes which catalyzed the conversion of porphobilinogen (PBG) to porphyrin. To correct for this loss of PBG, 3 ml of 3 N HCl was added to 1 ml of aliquot of trichloroacetic acid-treated supernatant fluid, the solution exposed to a fluorescent lamp overnight and the amount of porphyrin determined using a difference molar extinction coefficient ($\Delta\epsilon^{406-430}$) of 53×10^3 . The total amount of PBG (nmol) was then calculated by multiplying the amount of porphyrin (nmol) by 4 and adding this to the amount of PBG (nmol) obtained above.

2.5 Haemocyte count

Total haemocyte count (THC)

Haemolymph of black tiger shrimp was withdrawn from each apparently healthy shrimp using a 1 ml plastic syringe and diluted with 0.15% trypan blue solution. Haemocytes were counted using a haemocytometer and calculated as number of total haemocytes per cubic millimeter (Supamattaya *et al.*, 2000).

Differentiated hemocyte count (DHC)

Haemolymph samples were collected by using a 1 ml syringe with 25G needle from the base of walking leg. After withdrawal, 25 μl of haemolymph was

mixed with 225 μ l of the mixture of fixative (10% formalin in 0.45 M NaCl) and Bengal rose solution (1.2% Bengal rose in 50% ethanol) in ratio 1:1 (modified from Sritunyalucksana *et al.*, 2004). Granular and agranular cells were determined by haemocytometer and calculated as number of granular, agranular cells and total haemocytes per milliliter.

2.6 Phenoloxidase (PO) activity

Haemocyte lysate supernatant (HLS) was prepared in Na-CAC buffer (sodium cacodylate, 5 mM CaCl_2 , 50 mM MgCl_2 pH 7.0) using a sonicator (Vibra CellTM Sonicator and Materials, USA) at 30 amplitude for 10 second. PO activities from HLS were measured following the modified method of Söderhäll (1988). L-3,4-dihydroxy phenylalanine (L-DOPA) was used as a substrate, and enzyme activity was measured at 490 nm. The protein concentration was determined by the method of Lowry *et al* (1951) using bovine serum albumin as the standard.

Briefly, PO activity was determined by incubation of 200 μ l HLS with the same volume of the serine proteinase, trypsin (1 mg/ml, in cacodylate buffer pH 7.0) for 2 min. Then, each sample was incubated with 200 μ l of substrate, L-3,4-dihydroxy phenylalanine (L-DOPA 3 mg/ml, Sigma) for 2 min, the reaction was then dilute by adding 1,800 μ l of cacodylate buffer, pH 7.0. The development of the red pigment Dopachrome was monitored by its absorbance at 490 nm every 2 min until 40 min. Protein content in HLS was measured using Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as the standard. One unit of specific activity was equivalent to the increase of 0.001 in absorbance/min/mg protein (unit/min/mg protein).

2.7 Superoxidase dismutase activity

Haemolymph was taken from individual shrimp using a needle inserted into the base of walking leg and withdrawn into the syringe containing precooled (4°C) 1% EDTA pH 7.4. The haemolymph suspension was then centrifuged at 3,590 $\times g$ for 2 min at 4°C and washed three times with 1.5% NaCl. The pellet was resuspended in 100 μ l sterilized deionized water.

To measure superoxidase dismutase (SOD) activity, 25 μ l of haemolymph sample were used spectrophotometrically at 505 nm at 37°C. This assay was

performed using Randox Laboratories kits (Crumlin, Antrim, UK) according to manufacturer's instructions. Protein content in HLS was measured using Lowry's method (Lowry *et al.*, 1951) Activities were expressed as international specific activity (unit/mg protein) (Chien *et al.*, 2003).

2.8 Disease resistance

Hemolymph was collected from WSSV-infected *P. monodon*. WSSV-infected hemolymph was withdrawn, diluted ten-fold with PBS buffer (pH 7.4) and distributed in a 1 ml aliquot stored at -80°C until used. Aliquot of WSSV-infected hemolymph was centrifuged 12,000xg for 10 min at 4°C for separation of the cell debris. The supernatant was ten-fold diluted with PBS buffer and filtered with nitrocellulose membrane (0.45 µm) to eliminate bacterial contamination. Then 100 µl of diluent was injected to healthy shrimp (free from WSSV-infection) for determination of ED₅₀ for 10 days. The challenge test with WSSV was performed by injection 100 µl LD₅₀ dose of WSSV to 10 healthy shrimp. Mortality was recorded daily for 10 days after infection (Supamattaya *et al.*, 2000).

2.9 Clearance ability

Vibrio harveyi had been isolated from infected shrimp and was kept in stock culture at Aquatic Animal Health Research Center, Prince of Songkhla University, Thailand. The log phase bacterial suspension was prepared and adjusted to approximately 1×10^7 cell/ml in sterile 1.5% NaCl solution. A bacterial suspension of volume 100 µl was injected into each shrimp. After 3 h, hemolymph from each shrimp was withdrawn and the number of bacteria in the hemolymph was enumerated by total plate count techniques using TCBS (plus 1.5% NaCl). Condition of incubating plates was for 12-18 h at 35°C (Supamattaya *et al.*, 2000).

2.10 Stress test

Shrimp was reared in closed system with reduced dissolved oxygen, decreased 80% volume of seawater in aquarium and cover water surface with plastic 10 h a day for 10 day. During the experiment, shrimp were fed once in the morning and their mortality was daily recorded.

Methods

1. Mutagenesis to increase the intracellular ALA production of *R. sphaeroides* SH5

Mutagenesis treatments of *R. sphaeroides* SH5 strain was divided into 4 treatments, UV, N-methyl-N'-nitro-N-Nitrosoguanidine (NTG), UV+NTG (UVN) and NTG+UV (NUV) mutation. The strain grown under agitation in the dark at 37°C were harvested during the logarithmic phase by centrifugation at 9000 xg for 10 min at 4°C and washed twice with Tris-maleic (TM) buffer (50 mM, pH 6.0). For UV mutation, the cells suspension (5×10^8 cell/ml) were treated with UV lamp (30 cm distances) for 1 min (10% survival) and kept in the dark for 2 h. For NTG mutation, the cell suspension at a concentration 5×10^8 cell/ml were incubated in TM buffer containing 100 mg/l of NTG for 1 h at room temperature then the cells were centrifuged, washed and resuspended in TM buffer. For UVN mutation, the cells were treated with UV lamp then incubated with NTG for 1 h. For NUV mutation, the cells were treated with NTG, then resuspended in fresh TM buffer, treated under UV lamp and kept in the dark for 2 h. All of the cell suspension were added to fresh GM medium (10%, v/v) and cultivated under agitation in the dark at 37°C for 48 h. The mutagenesis cells were harvested, washed twice and incubated in GM media containing 1000 ppm of penG under mild shaking for 2 h according to the standard method of penicillin treatment (modified from Tanaka *et al.*, 1991).

The mutant cells were spread on GM agar plate and incubated at 37°C for 2 d in the dark. The colonies were pitched onto new media plates (50 colonies/plate) and incubated at 37°C for 2 d in the dark. Then the colonies were blotted onto new GM and GM plus prophobilinogen (PBG) plates. Colonies showing better growth on PBG plates than GM plates were selected as the low ALA dehydratase activity mutants (Tanaka *et al.*, 1991, 1994). The better growth colonies were cultured in GM medium under aerobic-dark condition (200 rpm, 37°C) and determined for ALA production. Mutants showed higher ALA production than parent wild type strain were selected and tested for the stability of ALA production by cultivating in 100 ml of GM and GG medium (20 mM glucose) under aerobic-dark condition with agitation (200 rpm, 37°C). The stability of mutant strains was cultivated in both media for determination of extracellular ALA and intracellular ALA production.

2. Factors affecting the increase of intracellular ALA concentration

The selected strain with the highest intracellular ALA content was used to study the effect of various parameters on the ALA production, ALA synthetase and ALA dehydratase activities.

2.1 Effect of substrate concentration and medium composition

The effect of substrate concentration was first determined by cultivating the strain *Rhodobacter sphaeroides* SH5 in GG, MGY, GY and MGG media containing 3% NaCl with the addition of 20 and 50 mM glucose under aerobic-dark condition with agitation (200 rpm) at 37°C.

2.2 Effect of NaCl

The selected medium (from 2.1) was used to determine the effect of NaCl (3%) source using analytical grade NaCl and commercial grade NaCl compare with NaCl-free.

2.3 Effect of levulinic acid (LA) addition

The strain was cultivated in the selected medium (from 2.2) under aerobic-dark condition (200 rpm) at 37°C. After inoculation with 10% starter ($OD_{660} = 0.5$) and cultivation for 24 h, LA was then added at various concentrations (0-15 mM).

2.4 Effect of Fe supplementation

The strain was cultivated in the selected medium (from 2.3) supplemented with iron in the range of 1.5-120 μ M under aerobic-dark condition (200 rpm) at 37°C. The optimal concentration of LA was added at 24 h cultivation.

3. Shrimp feeds preparation

3.1 Biomass production of ALA containing *Rhodobacter sphaeroides* SH5

Three kinds of biomass of halotolerant photosynthetic bacteria were produced as following:

3.1.1 Broken-biomass containing normal level of ALA

3.1.2 Broken-biomass containing high level of ALA

3.1.3 Non-broken-biomass containing high level of ALA

The wild type SH5 was cultivated in the selected medium (from section 2.1) for biomass containing normal level of ALA, the selected medium (from section 2.4) of biomass containing high level of ALA. The 10% inoculum was transferred to a 1-L flask containing 0.48-L of culture medium under aerobic-dark condition (200 rpm) and the temperature was controlled at 37°C. Cells of marine photosynthetic bacteria were harvested at 30 h cultivation by centrifugation (8000 xg, 15 min) at 4°C, washed twice with normal saline (0.85% NaCl) and kept in a deep freezer (-70°C.). The cells were disrupted by freeze-thaw (5 times; -70°C, 60°C 10 min each) and sonication (5 times, 50 Hz, 15 min each). The cells were dried by freeze-dryer and storage in a deep freezer (-70°C) until used. The intracellular ALA content dried halotolerant *R. sphaeroides* SH5 were determined

3.2 Feed preparation

Shrimp feed formulation (Table 2) was used to make the experimental feeds. Feed no.1 was as the control, feed no.2 contained commercial ALA (2 mg/kg), feed no.3 contained 3% broken-biomass with normal level of ALA, feed no.4 contained 3% non-broken-biomass with high level of ALA and feed no.5 contained 3% broken-biomass with high level of ALA. All of components were blended with 30% water and cold extruded through a 2 mm die. The pellets were dried in a forced air-circulation oven at 50°C for 6 hours, and then stored in sealed polyethylene bags in a freezer (-20°C) until used. Proximate analyses following standard AOAC (1984) methods were performed for crude protein (Kjeldahl), crude lipid (Soxhlet), crude ash and moisture content in the feeds.

4. Effect of ALA on the immunity of shrimp

Preparation of shrimp

Black tiger shrimp (*Penaeus monodon* Fabricius) with the average fresh weight of 15±2.0 g each collected from farm in Songkhla province were acclimatized in aerated tanks containing sea water with salinity of about 15 ppt. Shrimp were fed with a commercial diet for at least a week and randomly sampled for detection of bacteria and virus to ensure that they were free of *Vibrio harveyi* and white spot syndrome virus (WSSV).

Table 1 Formulation of feeds used to assess efficacy of ALA on immune response of black tiger shrimp

Ingredient (g)	Feed 1 Control	Feed 2	Feed 3	Feed 4	Feed 5
Fish meal	280	280	265	265	265
Shrimp head meal	100	100	100	100	100
Squid meal	55	55	55	55	55
Soy bean meal	100	100	100	100	100
Gluten	60	60	60	60	60
Wheat	200	200	200	200	200
BHT	0.2	0.2	0.2	0.2	0.2
Mineral mix	20	20	20	20	20
Vitamin mix	5	5	5	5	5
Chloine	3	3	3	3	3
Zeolite	15	15	15	15	15
Rice flour	100	100	100	100	100
lecithin	20	20	20	20	20
Fish oil	20	20	20	20	20
SCP of PSB	-	-	105	105	105
ALA	-	0.007	-	-	-
Total	1000	1000	1000	1000	1000

Lethal dose (LD₅₀) was determined in order to use in the study of the acute-toxicity of ALA on black tiger shrimp. Serial dilution of ALA (0-5 x 10⁵ ppm) which analytical ALA was dissolved in normal saline, one hundred microliter was injected into the last abdominal segment of black tiger shrimp (average weight 15±2.0 g) with the batches of 10 shrimps/dose. The mortality was observed for 7 days and calculated the LD₅₀ by using Probit analysis (Luangthuwapraneet, 1988).

The effect of ALA on immune response of black tiger shrimp was determined both *in vitro* and *in vivo*. Studies degranulation and activator of prophenoloxidase activating enzyme (ppA) were determined *in vitro* in haemocytes (Smith and Söderhäll, 1983). For *in vivo* studies, total haemocytes count (THC), PO activity and disease resistance (WSSV) were determined in shrimp after 3-21 days injection of ALA.

4.1 Immune response in haemolymph of black tiger shrimp (*in vitro*)

In degranulation determination, hemolymph of shrimp was withdrawn from each healthy shrimp using a 1 ml plastic syringe containing precooled (4°C) L-cysteine (30 mg/ml) dissolved in K-199 medium (modified Medium-199 Invitrogen Life Technology; pH 7.4, Itami *et al.*, 1993) as an anticoagulant. The haemocytes were separated from hemolymph by refrigerated centrifugation (800 $\times g$) for 10 min and then washed twice. Haemocytes pellet was resuspended with K-199 medium) and cultured on the cell disk (1 cm diameter; Sumilon, Tokyo) with K-199 containing ALA (0.1×10^3 ppm) and 10% of fetal bovine serum. After incubation at 28°C for 30 min, the cell disk were fixed in 0.125% glutaldehyde in K-199 medium for 10 min and washed with Na-CAC buffer (10 mM sodium cacodylate, 5 mM CaCl_2 , 50 mM MgCl_2 pH 7.0). The cells were stained with Diff Quick (Baxter, Swiss) for determination of the haemocyte number (400 cells per sample) and observation of haemocytes degranulation (400 $\times$ magnification) under phase contrast optics.

For determination of activator of proPO activating enzyme (ppA), hemolymph was withdrawn from individual healthy shrimp using a 1 ml plastic syringe containing precooled AC-1 anticoagulant (Smith and Söderhäll, 1983). Haemolymph obtained from three different individuals pooled in 15 ml conical tube and centrifuged at 8,000 $\times g$, 10 min, at 4°C. The pellet was resuspended in Na-CAC buffer and homogenized with sonicator (Vibra cell, Sonics & Materials INC., USA) at 30 amplitudes for 10 seconds. The supernatant was separated by centrifugation (10,000 $\times g$, 10 min) and referred to as haemocyte lysate supernatant (HLS). PO activities were determined by incubation of 50 μl HLS (triplicate) with 50 μl ALA ($0.1-1 \times 10^4$ ppm in Na-CAC buffer) for 2 min, then 50 μl of L-DOPA (3 mg/ml in Na-CAC buffer) was added. The absorbance at 490 nm was measured every 2 min until 40 min. Blank was 100 μl Na-CAC buffer incubated with 50 μl L-DOPA. A positive control contained 50 μl of HLS, 50 μl trypsin (1 mg/ml from beef pancreases; BDH in Na-CAC buffer, positive control) and 50 μl L-DOPA and a negative control with 50 μl of HLS, 50 μl CAC and 50 ml of L-DOPA. The concentrations of total plasma protein were determined by Bradford (1976) method using bovine serum albumin (Bio-rad Protein assay kit II) as the standard. One unit of PO activity was defined as an increase in absorbance of 0.001/min/mg protein.

4.2 Immune response in black tiger shrimp (*in vivo*)

Black tiger shrimp (average weight 15 ± 2.0 g) were stocked in 3 tons concrete pond at a density of 120 shrimps per 1.5 tons sea water. One hundred microlites of diluted ALA solution (0–1,000 ppm) in normal saline was injected into the last abdominal segment of healthy shrimp. The injected shrimp were fed commercial diet four times a day. After 3–21 days of injection, shrimps were collected to determine the immune response parameters in shrimp such as THC, PO activity and disease resistance (WSSV) for 10 day.

4.3 Assess the potential use of marine photosynthetic bacteria containing ALA to enhance growth, immunology system and/or disease resistance in cultured shrimp

Preparation of shrimp

Healthy black tiger shrimp, *P. monodon* with mean fresh weight of 2–3 g were obtained from Charoen Pokphand Farm (C.P. Group) in Nakhon Si Thammarath province, Thailand. They were stocked in a 3000-L concrete pond with an aerated recirculation system (15‰ salinity). Shrimp were fed with commercial feed for one week.

Experimental design

Shrimp with average fresh weight of 2.5 g were stocked and acclimatized in fiber aquarium (200-L) at a density of twenty shrimp per aquarium. Five replicates were performed for each dietary group. Five experimental diets formulae were conducted to determine the effect of ALA on growth, feed conversion, survival, immunological functions, disease resistance and stress test.

4.3.1 Effect of ALA on growth, feed performance, survival and immunological functions

Shrimp were fed using the experimental feeds five times a day for 8 weeks. The feeding rate was fine adjusted according to water temperature and molting cycle. The experimental data on growth, survival and feed conversion were recorded every 2 weeks. Data from the trials were recorded every 2 weeks and analyzed using analysis of variance (ANOVA) and Duncan's new multiple range tests to determine if differences between population means existed.

After eight weeks of feeding, shrimps from each treatment was collected to determine total haemocyte count (THC), differential haemocytes count (DHC), phenoloxidase (PO) activity, superoxide dismutase (SOD) production, the ability to clear bacterial cells from blood circulate (clearance ability).

4.3.2 Effect of ALA on disease resistance and stress test

Shrimp with average fresh weight of 2.5 g were stocked and acclimatized in fiber aquarium (200-L) at a density of fifteen shrimp per aquarium. Three replicates were performed for each dietary group. Shrimp were fed using the experimental diets five times a day for 8 weeks. The feeding rate was fine adjusted to satiation according to water temperature and molting cycle. After eight weeks of feeding, shrimps from each treatment was collected for determine disease resistance (WSSV) and stress test (hypoxia) (duplication; n = 10) for 10 days post challenge. Mortality was daily recorded for determination of disease resistance and tolerance for hypoxia.

4.4 Mechanism of ALA act as immune response in black tiger shrimp

The *Penaeus monodon* hemocyte cDNA library was constructed using a UNI-ZAP XR cDNA synthesis kit (Stratagene). The primers derived from shrimp haematopoietic tissue Mn-SOD (accession no. BI784454) were synthesized and used to amplify Mn-SOD from haemocyte cDNA library. The amplified PCR product was analyzed on agarose gel electrophoresis and cloned into TA cloning vector (In Vitrogen). The clone with the correct insert size was amplified and subjected to nucleotide sequencing (BSU, Thailand).

Total RNA was prepared from haemocyte by Trizol extraction according to the manufacturer's instruction (Gibco BRL). Hemocyte RNA was extracted, precipitated and resolved in 10 µl distilled water. The protocol for RT-PCR used in this study with total RNA of 150 ng with 25 PCR cycles. To synthesize first strand cDNA by reverse transcription 10 µl reaction volume containing 150 ng total RNA, 50 µM oligo (dT)₁₈, 2 µl first strand buffer (250 mM Tris, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 1 µl of dNTP (10 mM of each dNTP), 0.5 µl of 0.1 M dithiothreitol, 0.5 µl of 20 unit RNase inhibitor (Amersham Bioscience) and 200 units of Superscript™ III (In vitrogen) were reacted for 55°C, 60 min. The synthesis was stopped by heating at 70°C for 15 min.

To study the effect of ALA on expression of Mn-SOD and proPO in shrimp fed with ALA, the first strand cDNA was further amplified by specific primers for shrimp Mn-SOD (accession no. BI784454) and proPO (accession no. AF099741), the forward primer; SF1 (coding sequence), 5'-GAGAAGAAGTTAGCTGAGCTGACT-3' and the reverse primer; SR1 (antisense sequence), 5'-CCACCTCCATTGAACTTGATAGC-3'. PPO16 (coding sequence), 5'-GGCTGGAACACGAGGGAGTT-3' and PPO-5 (antisense sequence) 5'-TCACAGTAACTTCCTTCGG-3'. The primers for proPO were kindly provided by Centex Shrimp, Mahidol University. The PCR reaction composed of 1 μ l first strand cDNA (amplified from 150 ng total RNA), 10 pmol of each primers, PCR buffer (16 mM $(\text{NH}_4)_2\text{SO}_4$, 100 mM Tris, pH 8.3, 0.01% Tween-20), 10 mM dNTP, 1.5 mM MgCl_2 , and 0.625 unit of ImmolaseTM Taq polymerase (Bioline). The thermal profile of PCR was 30 seconds each for denaturing, annealing (55°C), extension step, 25 cycles and a final 5 min post-amplification at 72°C. Elongation factor transcript was analyzed as the internal control. The primers for elongation factor were provided using *Schizophyllum commune* (accession no. X94913). Elongation factor forward primer; EF1 (coding sequence) 5'-GGCTTCAACGTGAAGAACGT-3', and the reverse primer; ER1 (antisense sequence) 5'-TGGTAGTCTTGCCAGATTGT-3' were used. The PCR amplified products were analyzed by agarose gel electrophoresis.

Results and Discussion

1. Mutagenesis to increase the intracellular ALA production of *R. sphaeroides* SH5

Among 50 mutants obtained, five mutant strains; U10, N20, NU42, NU 43 and NU51 produced higher amount of extracellular ALA than the others after 48 h cultivation in GM medium under aerobic-dark condition. Their amounts of ALA were 1.5 fold and 2-3 fold higher than that of the wild strain (SH5) at 24 h and 48 h, respectively. ALA production from these mutant strains was highest at 48 h cultivation (Table 3). However, it was found that ALA production from the three mutant strains; NU42, NU43 and NU51, were not stable when repeated the production in GM medium. Therefore, the mutants U10 and U20 were selected for further study.

Photosynthetic bacteria are able to utilize various kinds of organic substrates, such as sugars, organic acids and volatile fatty acids (VFA) (Sasaki *et al.*, 1998). The effect of carbon source on growth rate of the mutant strain CR-450 was investigated. The growth rate of CR-450 was higher when glucose was used as a carbon source than with DL-malate under agitation in the dark. It appeared that glucose is a satisfactory source of succinyl Co-A under aerobic-dark condition (Nishikawa *et al.* 1999). Comparison on ALA production between wild type and the two selected mutants (U10 and U20) was conducted in GM and GG media. Both mutants accumulated high amount of intracellular ALA in both media, especially GG medium. Glucose in GG medium may replace DL-malate in GM medium for ALA production. However, ALA production from mutant strains was not significantly different from the wild type (Fig. 1). At the same concentration of carbon sources (20 mM) both intracellular and extracellular ALA production in GG medium was higher than in GM medium. The intracellular ALA accumulation in GG medium (0.65 $\mu\text{g/g}$ DCW) was higher than GM medium (0.45 $\mu\text{g/g}$ DCW) at 24 h cultivation. Therefore the wild type SH5 was selected for mass ALA production in GG medium because of inexpensive carbon source.

Table 3 ALA production from four treatments of mutagenesis of *Rhodobacter sphaeroides* SH5 in GG and GM medium under aerobic-dark condition

Strain	ALA production (μ M) in GM medium				
	Batch 1			Batch 2	
	24 h	48 h	72 h	24 h	48 h
UV4	12.92	22.14	17.14	-	-
9	14.93	22.25	17.29	-	-
10	13.19	24.43	17.34	13.18	22.87
18	13.39	23.62	16.80	-	-
20	13.39	24.45	17.13	13.78	25.42
30	13.83	23.45	17.25	-	-
N34	13.05	21.09	16.83	-	-
36	13.24	21.80	17.56	-	-
37	13.14	19.38	16.91	-	-
42	16.21	26.10	14.42	10.88	11.48
43	11.74	23.62	18.52	12.98	11.23
44	13.12	23.12	16.67	-	-
46	14.00	22.78	16.87	-	-
48	13.43	23.13	16.41	-	-
UVN50	12.19	15.98	17.06	-	-
51	18.10	23.77	12.20	10.68	11.88
52	13.79	22.85	16.82	-	-
58	12.82	23.32	15.96	-	-
59	11.53	21.85	16.33	-	-
NUV60	9.36	23.52	9.55	-	-
63	12.53	18.17	17.27	-	-
64	11.99	22.08	17.04	-	-
66	12.20	23.30	16.88	-	-
67	12.13	23.05	16.55	-	-
68	13.27	23.49	17.30	-	-
SH5	16.38	10.68	11.39	15.98	10.84

Strain	ALA production (μ M) in GG medium					
	Batch 1		Batch 2		Batch 3	
	24 h	48 h	24 h	48 h	24 h	48 h
UV 10	25.28	30.98	20.58	27.93	18.28	27.38
UV 20	26.98	23.58	25.32	24.75	22.28	29.48
N 42	22.28	20.98	15.90	12.68	12.78	10.78
N 43	21.68	17.78	15.78	13.48	14.95	12.43
UVN 51	19.98	20.68	14.38	14.98	15.88	19.48
SH5	20.38	27.48	22.92	28.68	21.88	33.88

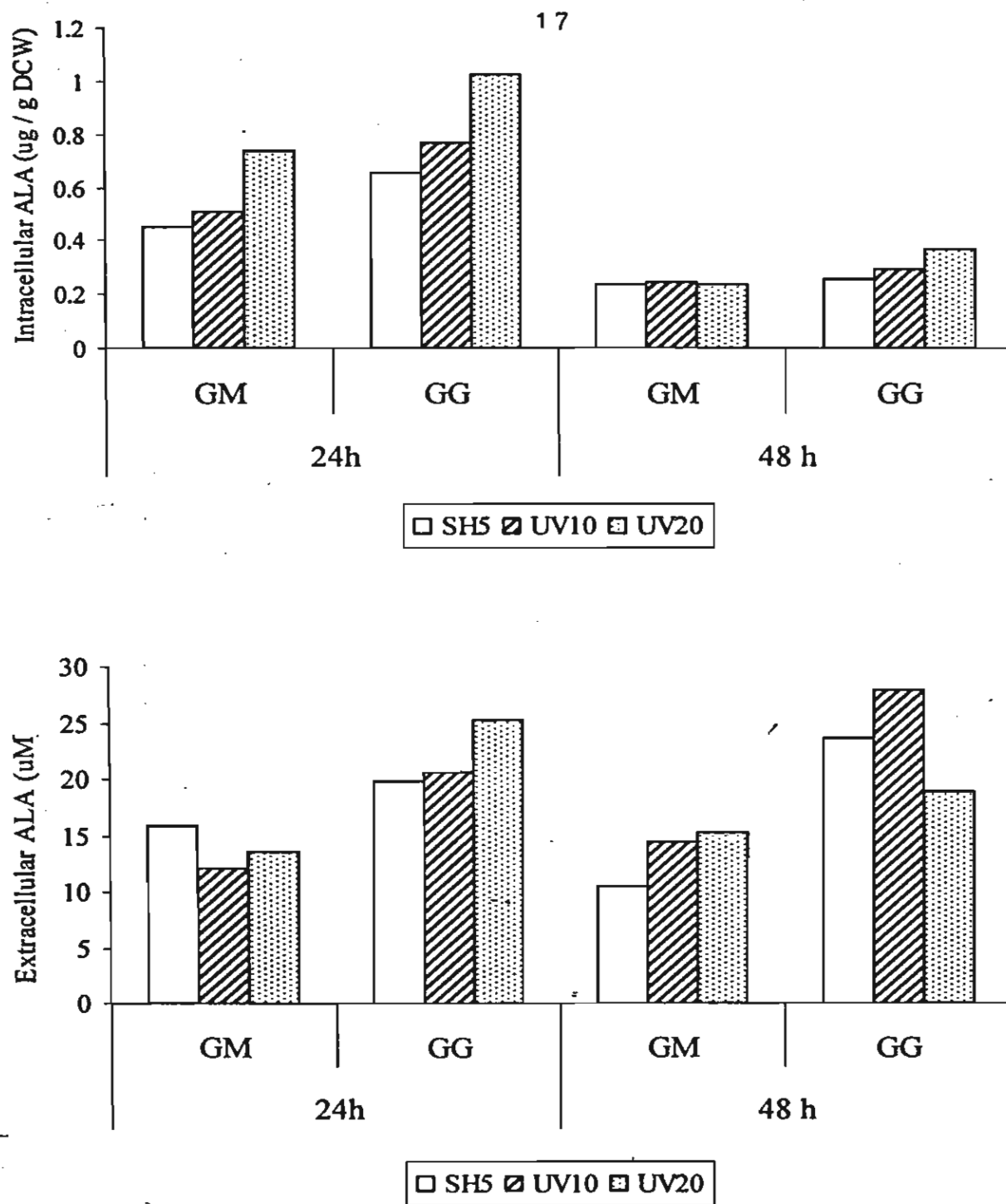


Fig.1 Extracellular ALA production from *Rhodobacter sphaeroides* SH5 and their mutant strains (UV10 and UV20) in GM and GG medium (20 mM glucose) under microaerobic-dark condition (200 rpm) at 37°C

2. Factors affecting the increase of intracellular ALA concentration of

R. sphaeroides SH5

2.1 Effect of substrate concentration and medium composition

ALA is synthesized via the Shemin pathway (Lascelles, 1978) from glycine and succinate. Glucose is a satisfactory source of succinyl Co-A via TCA cycle (Nishikawa *et al.*, 1999). *R. sphaeroides* SH5 was cultivated in GG medium and modified media, MGY, GY and MGG containing 20, 50 mM glucose under aerobic-dark condition. These modified medium contained commercial grade glucose and monosodium glutamate (MSG) to replace analytical grade chemicals in order to reduce the cost of medium. ALA production increased in media containing 50 mM glucose. MGG medium containing 50 mM glucose gave the highest ALA production both intracellular ALA (0.67 $\mu\text{g/g}$ DCW) and extracellular ALA (20.6 μM). Inorganic salts, KH_2PO_4 , K_2HPO_4 and organic cation compound in yeast extract were functioned as buffering mineral for controlling pH changes during cultivation (Ullman *et al.*, 2000). Since *R. sphaeroides* cultivated in the four media (GG, MGY GY and MGG) gave no significant difference in the accumulation of intracellular ALA at 24 h cultivation (Fig. 2), therefore MGG medium containing 20 mM glucose was selected for ALA production.

2.2 Effect of NaCl

R. sphaeroides SH5 was a halotolerant (0-12% NaCl) photosynthetic bacteria (isolated from shrimp farm) and preferred to grow in culture medium containing 3% NaCl (30 ppt salinity). Effect of NaCl both type (analytical and commercial NaCl) and concentration (0 and 3%) on ALA production from *R. sphaeroides* SH5 was investigated. Results indicated that the strain could grow in MGG medium containing commercial grade NaCl and without NaCl, however, the strain could not accumulate intracellular ALA (Fig. 3). Commercial grade NaCl may contain some minerals and salts which affected on the secretion of ALA from the cell. NaCl was found to be necessary for accumulation of intracellular ALA as no accumulation of intracellular ALA in the cells cultivated in MGG medium without NaCl. Therefore, MGG (20 mM) containing 3% NaCl was used for further study.

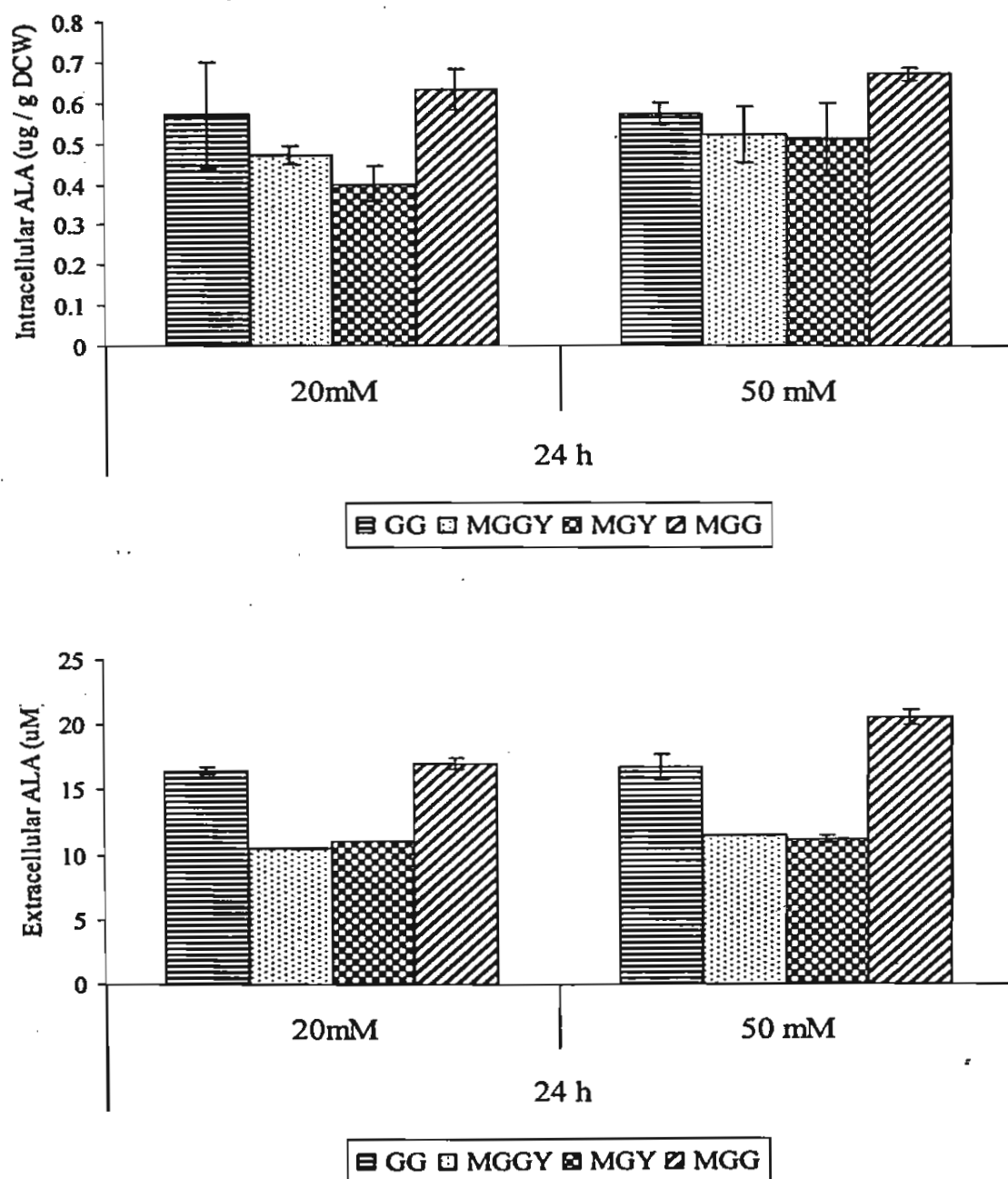


Fig.2 ALA production from *Rhodobacter sphaeroides* SH5 in four different media; GG, MGGY, MGY and MGG media containing 20 and 50 mM glucose under microaerobic-dark condition (200 rpm) at 37°C

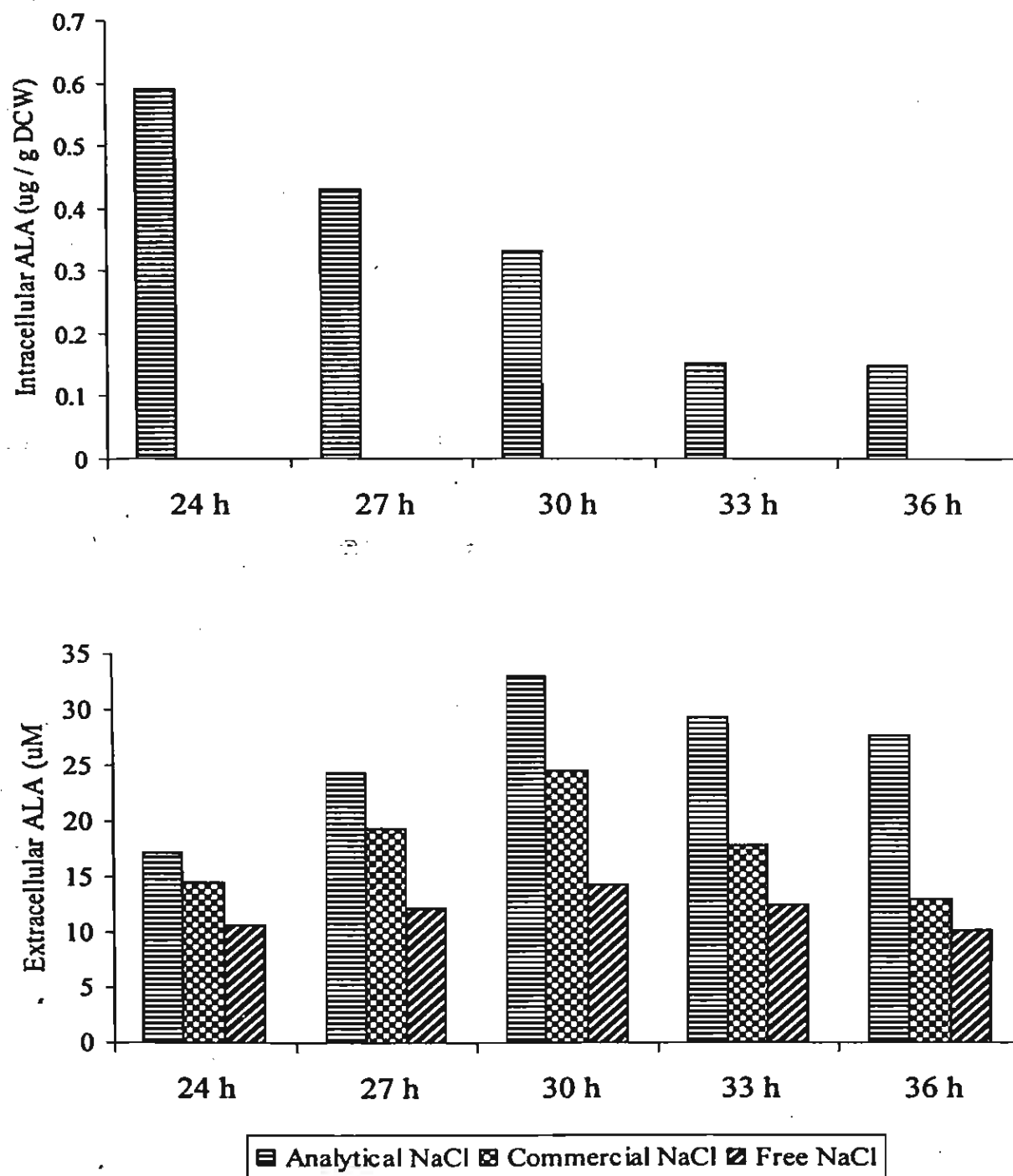


Fig.3 ALA production from *Rhodobacter sphaeroides* SH5 cultivated in MGG medium (20 mM glucose) containing analytical grade, commercial grade, and free NaCl under microaerobic-dark condition (200 rpm) at 37°C

2.3 Effect of LA addition

As levulinic acid (LA) could retard the growth of photosynthetic bacteria, Sasaki *et al.* (1987) recommended that LA should be added in the middle log phase to increase the extracellular ALA concentration. Effect of LA addition on intracellular ALA accumulation in *R. sphaeroides* SH5 was investigated. Preliminary studies indicated that addition of 15 mM LA into MGG medium at 24 h immediately stopped the growth of this strain. Therefore, LA concentration lower than 15 mM was added. Results (Fig. 4) indicated that when 5 mM LA was added into MGG medium containing 20 mM glucose at 24 h cultivation, *R. sphaeroides* SH5 accumulated the highest intracellular ALA concentration (6.66 $\mu\text{g/gDCW}$). Further increase of intracellular ALA accumulation to 14.04 $\mu\text{g/gDCW}$ was achieved in MGG medium containing 50 mM glucose with the addition of 10 mM LA at 24 h cultivation. This was about 20 folds higher than the normal level of intracellular ALA at 24 h cultivation in MGG medium. Higher glucose concentration (50 mM) in culture medium could increase the accumulation of precursor, succinyl Co-A via TCA cycle, for ALA accumulation via Shemin pathway. Therefore, MGG medium containing 50 mM glucose and 10 mM LA addition at 24 h cultivation were the optimal concentration of carbon source and ALA dehydratase inhibitor (LA) for accumulation of intracellular ALA, respectively.