Chapter 3

Results and Discussion

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

Mutagenesis on *Rhodobacter capsulatus* SS3 and *R sphaeroides* N20 and U7 using UV and NTG was conducted. From these methods, 323 strains, 145 colonies and 178 colonies by using UV and NTG, respectively were obtained and tested for ALA production. The ALA production from these 323 mutant strains cultivated in 10 ml glutamate-malate (GM) medium under aerobic-dark condition at 37 C for 48 h were given in Table 1-6. Among them, sixteen mutant strains in which four strains mutated by UV (NU13, SU5, SU14 and SU47) and twelve strains mutated by NTG (DN22, DN44, DN61, UN7, UN37, UN39, SN8, SN21, SN22, SN28, SN33 and SN45), gave ALA productivity at higher levels than their parent strains significantly ($p< 0.05$) at 0.376, 0.338, 0.305, 0.349; and 0.323, 0.294, 0.315, 0.367, 0.311, 0.337, 0.294, 0.275, 0.326, 0.467, 0.311, 0.343 μM/g cell h^{-1}, respectively. The extracellular ALA production and the maximum values of 25.57, 37.75, 33.45, 35.21, 38.96, 34.78, 42.39, 41.07, 34.37, 45.52, 36.15, 34.13, 41.91, 43.65, 36.13 and 32.15 μM, respectively, were achieved after 48 h cultivation while their wildtype, *Rhodobacter capsulatus* SS3, *sphaeroides* N20 and U7, were 26.59, 24.83 and 30.97 μM, respectively. Almost all mutants mutated by NTG accumulated extracellular ALA higher than those mutated by UV.

The selected sixteen mutant strains were cultivated in 100 ml of GM medium in 250 ml flask under aerobic-dark condition and the kinetic parameters are shown in Table 7. The specific growth rate (μ_{max}) of these sixteen mutant strains were almost the same (appoximately 0.08 h^{-1}) except the strains DN22, SN28, SN33 rate (appoximately 0.07 h^{-1}) and the strain SU14 (0.06 h^{-1}). The maximum productivity for ALA production from the strains SN28, SU47, NU13, DN22, SN33, SU14, SN22, UN7, SN45, SN8, UN37, SU5, UN39, SU21, DN61

Table 1 Comparison on ALA production from UV mutant strain of *Rhodobacter sphaeroides* U7 after 48 h cultivation in GM medium under aerobic-dark condition at 37 C

Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})	Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})
DU1	15.47	0.166	DU33	3.36	0.084
DU2	17.60	0.169	DU34	15.27	0.152
DU3	8.64	0.093	DU35	7.85	0.105
DU4	9.97	0.216	DU36	8.96	0.107
DU5	15.69	0.168	DU37	14.64	0.134
DU6	15.36	0.152	DU38	6.73	0.102
DU7	16.51	0.185	DU39	8.64	0.142
DU8	6.36	0.621	DU40	12.40	0.113
DU9	8.64	0.107	DU41	5.78	0.099
DU10	11.85	0.113	DU42	15.52	0.144
DU11	9.53	0.109	DU43	12.35	0.122
DU12	15.28	0.171	DU44	19.15	0.186
DU13	9.55	0.095	DU45	4.16	0.090
DU14	16.14	0.161	DU46	13.25	0.153
DU15	6.88	0.077	DU47	9.98	0.126
DU16	15.22	0.148	DU48	4.13	0.054
DU17	11.64	0.112	DU49	6.33	0.085
DU18	6.89	0.068	DU50	9.03	0.108
DU19	7.15	0.078	DU51	6.15	0.076
DU20	11.35	0.117	DU52	9.55	0.108
DU21	7.82	0.089	DU53	16.14	0.175
DU22	12.25	0.119	DU54	6.17	0.088
DU23	8.47	0.103	DU55	8.15	0.122
DU24	9.15	0.101	DU56	8.26	0.106
DU25	12.15	0.133	DU57	7.25	0.103
DU26	13.85	0.165	DU58	11.17	0.144
DU27	8.15	0.081	DU59	8.95	0.142
DU28	7.79	0.099	DU60	7.64	0.105
DU29	6.56	0.053	DU61	15.25	0.113
DU30	8.12	0.096	DU62	12.21	0.112
DU31	15.50	0.115	DU63	8.15	0.097
DU32	14.13	0.207	DU64	11.13	0.137
			Wild type (U7)	30.97	0.325

Table 2 Comparison on ALA production from UV mutant strain of *Rhodobacter sphaeroides* N20 after 48 h cultivation in GM medium under aerobic-dark condition at 37 C

Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})	Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})
NU1	8.51	0.117	NU16	9.47	0.108
NU2	12.28	0.112	NU17	12.24	0.112
NU3	4.80	0.070	NU18	11.21	0.111
NU4	9.78	0.212	NU19	10.00	0.109
NU5	7.36	0.688	NU20	8.23	0.557
NU6	16.47	0.168	NU21	9.56	0.109
NU7	8.98	0.107	NU22	9.71	0.160
NU8	9.60	0.109	NU23	10.19	0.110
NU9	14.30	0.132	NU24	9.14	0.108
NU10	13.57	0.147	NU25	9.70	0.109
NU11	8.25	0.110	NU26	7.13	1.416
NU12	8.85	0.099	NU27	10.81	0.104
NU13	25.57	0.376	NU28	9.60	0.109
NU14	10.08	0.109	NU29	9.96	0.109
NU15	9.50	0.108	NU30	8.05	0.105
			NU31	8.67	0.155
			Wild type (N20)	24.83	0.280

Table 3 Comparison on ALA production from UV mutant strain of *Rhodobacter capsulatus* SS3 after 48 h cultivation in GM medium under aerobic-dark condition at 37 C

Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})	Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})
SU1	11.35	0.156	SU26	11.44	0.111
SU2	11.87	0.121	SU27	17.63	0.169
SU3	12.35	0.181	SU28	16.94	0.167
SU4	11.59	0.127	SU29	13.18	0.113
SU5	37.75	0.338	SU30	12.33	0.112
SU6	10.50	0.107	SU31	6.01	0.129
SU7	11.57	0.112	SU32	11.22	0.122
SU8	16.82	0.154	SU33	13.62	0.130
SU9	15.35	0.141	SU34	11.96	0.137
SU10	10.87	0.117	SU35	18.54	0.177
SU11	9.47	0.126	SU36	16.59	0.164
SU12	12.34	0.138	SU37	9.12	0.084
SU13	14.46	0.160	SU38	14.85	0.121
SU14	33.15	0.305	SU39	6.45	0.113
SU15	16.82	0.167	SU40	19.24	0.199
SU16	9.45	0.087	SU41	18.98	0.182
SU17	12.20	0.112	SU42	21.54	0.228
SU18	13.56	0.138	SU43	16.54	0.171
SU19	10.69	0.110	SU44	15.84	0.120
SU20	17.60	0.174	SU45	8.22	0.078
SU21	6.78	0.055	SU46	6.13	0.086
SU22	9.40	0.204	SU47	35.21	0.349
SU23	15.472	0.114	SU48	12.22	0.174
SU24	12.33	0.112	SU49	16.52	0.153
SU25	11.75	0.112	SU50	11.30	0.122
			Wild type (SS3)	26.59	0.260

Table 4 Comparison on ALA production from NTG mutant strain of *Rhodobacter sphaeroides* U7 after 48 h cultivation in GM medium under aerobic-dark condition at 37 C

Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})	Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})
UN1	9.38	0.096	UN33	11.62	0.112
UN2	9.75	0.097	UN34	10.28	0.110
UN3	9.55	0.096	UN35	10.34	0.110
UN4	9.64	0.097	UN36	11.79	0.112
UN5	14.11	0.147	UN37	34.37	0.311
UN6	12.92	0.129	UN38	10.07	0.109
UN7	41.07	0.367	UN39	45.52	0.337
UN8	10.66	0.137	UN40	11.79	0.112
UN9	13.45	0.133	UN41	9.14	0.108
UN10	16.42	0.169	UN42	10.10	0.109
UN11	9.78	0.097	UN43	10.24	0.110
UN12	9.55	0.096	UN44	6.24	0.100
UN13	8.83	0.095	UN45	21.90	0.178
UN14	15.33	0.168	UN46	10.09	0.109
UN15	10.90	0.098	UN47	8.88	0.107
UN16	9.09	0.096	UN48	10.88	0.111
UN17	11.53	0.116	UN49	11.74	0.112
UN18	10.36	0.103	UN50	11.36	0.111
UN19	18.09	0.166	UN51	9.78	0.109
UN20	14.73	0.137	UN52	9.42	0.108
UN21	7.71	0.137	UN53	13.87	0.159
UN22	10.20	0.097	UN54	12.06	0.112
UN23	7.18	0.159	UN55	13.21	0.160
UN24	11.27	0.125	UN56	11.63	0.119
UN25	16.84	0.169	UN57	15.15	0.164
UN26	11.60	0.119	UN58	11.23	0.113
UN27	8.60	0.095	UN59	14.57	0.167
UN28	9.54	0.096	UN60	11.78	0.112
UN29	7.71	0.093	UN61	13.01	0.126
UN30	20.55	0.184	UN62	15.38	0.166
UN31	11.76	0.135	UN63	11.09	0.111
UN32	10.80	0.105	UN64	13.25	0.157
			Wild type (U7)	30.97	0.325

Table 5 Comparison on ALA production from NTG mutant strain of *Rhodobacter sphaeroides* N20 after 48 h cultivation in GM medium under aerobic-dark condition at 37 C

Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})	Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})
DN1	9.65	0.109	DN33	10.81	0.111
DN2	10.96	0.111	DN34	8.70	0.107
DN3	9.10	0.133	DN35	6.67	0.102
DN4	8.61	0.186	DN36	10.27	0.110
DN5	9.98	0.109	DN37	8.49	0.106
DN6	11.11	0.111	DN38	8.30	0.106
DN7	38.96	0.110	DN39	6.98	0.115
DN8	11.32	0.111	DN40	8.44	0.106
DN9	9.52	0.108	DN41	9.00	0.108
DN10	8.53	0.107	DN42	8.06	0.106
DN11	12.03	0.112	DN43	8.63	0.107
DN12	14.19	0.159	DN44	65.74	0.241
DN13	9.90	0.192	DN45	8.42	0.106
DN14	11.15	0.111	DN46	9.39	0.108
DN15	9.76	0.109	DN47	8.39	0.106
DN16	11.42	0.111	DN48	8.06	0.175
DN17	11.64	0.059	DN49	7.80	0.137
DN18	11.36	0.111	DN50	9.03	0.179
DN19	10.10	0.109	DN51	8.67	0.107
DN20	10.70	0.110	DN52	9.62	0.109
DN21	9.49	0.108	DN53	7.64	0.105
DN22	10.60	0.323	DN54	7.26	0.103
DN23	12.70	0.118	DN55	6.85	0.102
DN24	14.87	0.154	DN56	8.26	0.106
DN25	9.96	0.109	DN57	7.26	0.103
DN26	9.05	0.108	DN58	8.19	0.106
DN27	13.85	0.137	DN59	6.35	0.101
DN28	8.36	0.106	DN60	7.64	0.105
DN29	14.15	0.136	DN61	42.39	0.272
DN30	9.09	0.108	DN62	12.21	0.112
DN31	15.49	0.144	DN63	9.02	0.108
DN32	11.90	0.112	DN64	8.66	0.107
			Wild type (N20)	24.83	0.280

Table 6 Comparison on ALA production from NTG mutant strain of *Rhodobacter capsulatus* SS3 after 48 h cultivation in GM medium under aerobic-dark condition at 37 C

Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})	Mutant	ALA_{max} (μM)	R_m (μM/g cell h^{-1})
SN1	11.29	0.111	SN26	14.78	0.141
SN2	19.83	0.118	SN27	15.90	0.146
SN3	14.46	0.110	SN28	43.65	0.467
SN4	11.91	0.112	SN29	20.00	0.184
SN5	11.54	0.112	SN30	19.39	0.179
SN6	8.96	0.107	SN31	16.75	0.157
SN7	13.46	0.114	SN32	15.58	0.149
SN8	36.15	0.294	SN33	36.13	0.331
SN9	20.33	0.182	SN34	21.24	0.199
SN10	12.35	0.165	SN35	17.96	0.162
SN11	10.78	0.110	SN36	17.39	0.179
SN12	14.53	0.155	SN37	14.79	0.142
SN13	4.57	0.515	SN38	18.79	0.208
SN14	12.36	0.113	SN39	19.65	0.162
SN15	13.28	0.113	SN40	16.20	0.156
SN16	12.32	0.112	SN41	16.91	0.155
SN17	15.38	0.153	SN42	9.13	0.103
SN18	13.21	0.113	SN43	16.21	0.174
SN19	14.81	0.187	SN44	18.26	0.174
SN20	12.73	0.113	SN45	32.15	0.343
SN21	34.13	0.275	SN46	21.13	0.229
SN22	41.91	0.326	SN47	14.12	0.138
SN23	13.10	0.125	SN48	16.26	0.150
SN24	10.67	0.110	SN49	19.82	0.194
SN25	23.79	0.181	SN50	23.56	0.210
			Wild type (SS3)	26.59	0.260

Table 7 Kinetic parameters from each mutant strain in GM medium under aerobic-dark condition

Strains	Kinetic parameters				
	X_{max} (g/l)	μ_{max} (h^{-1})	ALA_{max} (μM)	R_m (μM ALA h^{-1})	R_m (μM /g cell h^{-1})
By NTG					
DN22	1.455	0.071	26.51	0.30	0.227
DN44	3.814	0.085	24.22	0.11	0.030
DN61	4.006	0.085	34.33	0.14	0.037
UN7	2.664	0.082	38.12	0.24	0.090
UN37	3.546	0.083	41.09	0.19	0.059
UN39	4.142	0.088	44.10	0.18	0.044
SN8	3.456	0.083	45.65	0.22	0.073
SN21	4.361	0.087	46.80	0.18	0.043
SN22	3.321	0.083	54.16	0.27	0.082
SN28	2.121	0.074	61.54	0.48	0.546
SN33	2.426	0.076	41.15	0.28	0.127
SN45	3.146	0.084	44.35	0.23	0.077
By UV					
NU13	1.614	0.071	30.31	0.31	0.218
SU5	2.984	0.083	31.95	0.18	0.060
SU14	0.954	0.064	15.33	0.27	0.307
SU47	1.895	0.078	37.08	0.33	0.181

and DN44 were 0.48, 0.33, 0.31, 0.30, 0.28, 0.27, 0.27, 0.24, 0.23, 0.22, 0.19, 0.18, 0.18, 0.18, 0.14, 0.11 µM/g cell h^{-1}, respectively. *R. capsulatus* SN28 gave the highest extracellular ALA productivity (0.48 µM/g cell h^{-1}) than the others (0.11-0.33 µM/g cell h^{-1}) significantly.

Comparison on the cultivation between *R. capsulatus* SS3 and its wild type *R. capsulatus* SN28 in GM medium are given in Figure 6 and Figure 7 and the kinetic parameters is shown in Table 8. The pH of the culture in GM medium under aerobic-dark condition had the same trends towards alkaline values but pH changes of *R. capsulatus* SN28 (from pH 7.00 to 9.11 at 96 h) increased more rapidly than the wild type strain (from pH 7 to 8.93 at 96 h). Although these two strains had rather similar dry cell weights (1.87 and 1.79 g/l, respectively) at the end of the cultivation, their ALA productivity of the mutant strain SN28 was 1.18 µM ALA /h while that of the wild type strain SS3 was only 0.37 µM ALA /h. Therefore, the mutant produced ALA nearly 3.2 times higher than the wild type strain.

R. capsulatus SS3 and the mutant strains *R. capsulatus* SN28 were examined by transmission electron microscopy (TEM) and scanning electron microscope (SEM). The pictures (Figure 8-10) showed that the cells of wild type strain SS3 and mutant strain SN28 were about 1.0 µm in diameter and the latter growing by binary fission. TEM images showed that *R. capsulatus* SN28 had thinner cell wall than the wild type. It is possible that the mutant can secrete ALA more than the wild type.

The mutant SN28 was finally selected since this strain had higher cell yield (2.23 g/l) with lower ALAD activity (74.64 units) than the wild type (2.07 g/l and 163.73 units, respectively) after 36 h cultivation. The ALA synthase (ALAS) activity of the mutant SN28 (14.21 units) was higher than that of the wild type strain (8.19 units) after 60 h cultivation while its ALA dehydratase (ALAD) activity (200.01 units) was lower than the wild type strain (287.15 units).

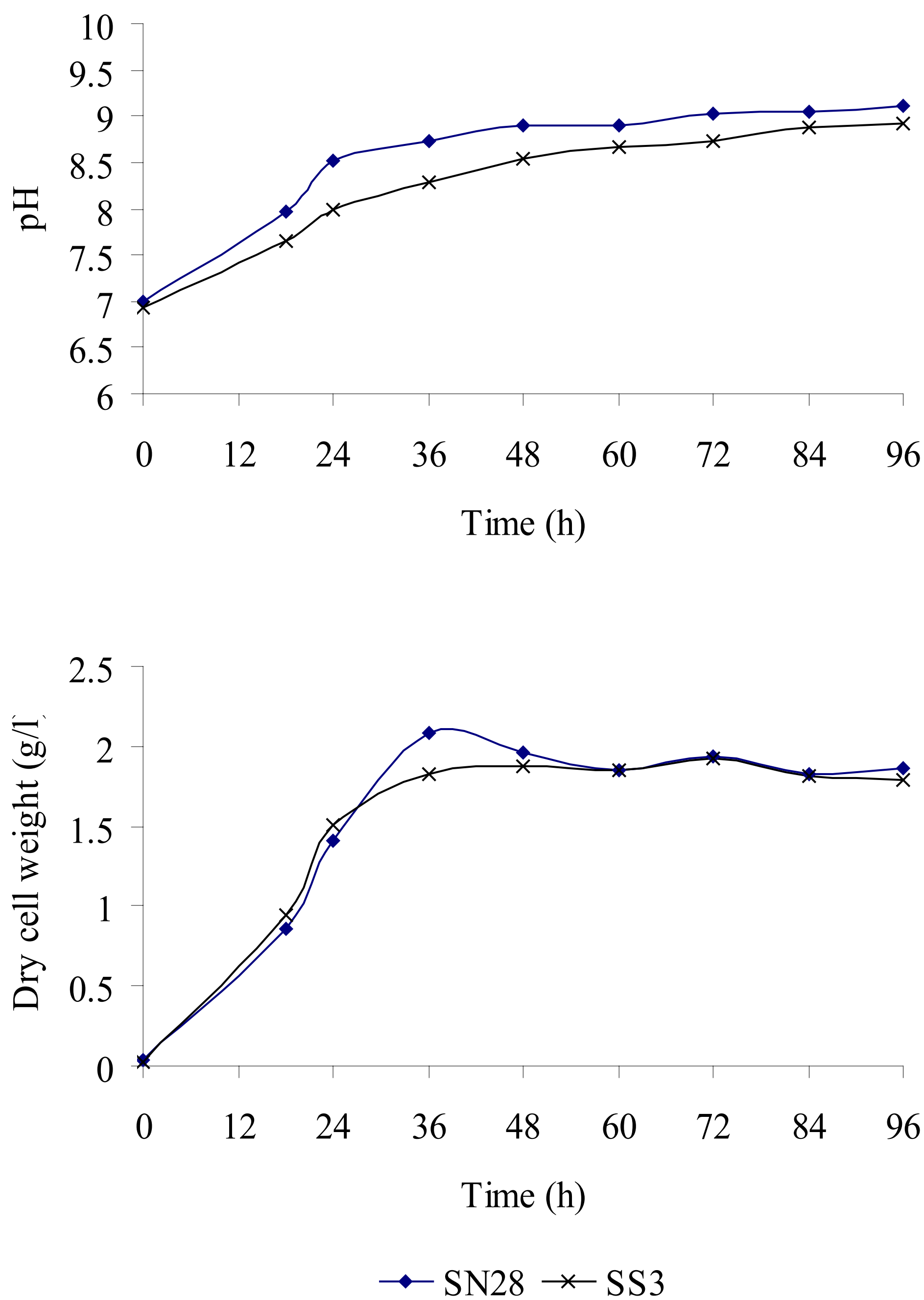


Figure 6 Growth of the NTG mutant *Rhodobacter capsulatus* SN28 and the wild-type *Rhodobacter capsulatus* SS3 in GM medium under aerobic-dark condition at 37°C

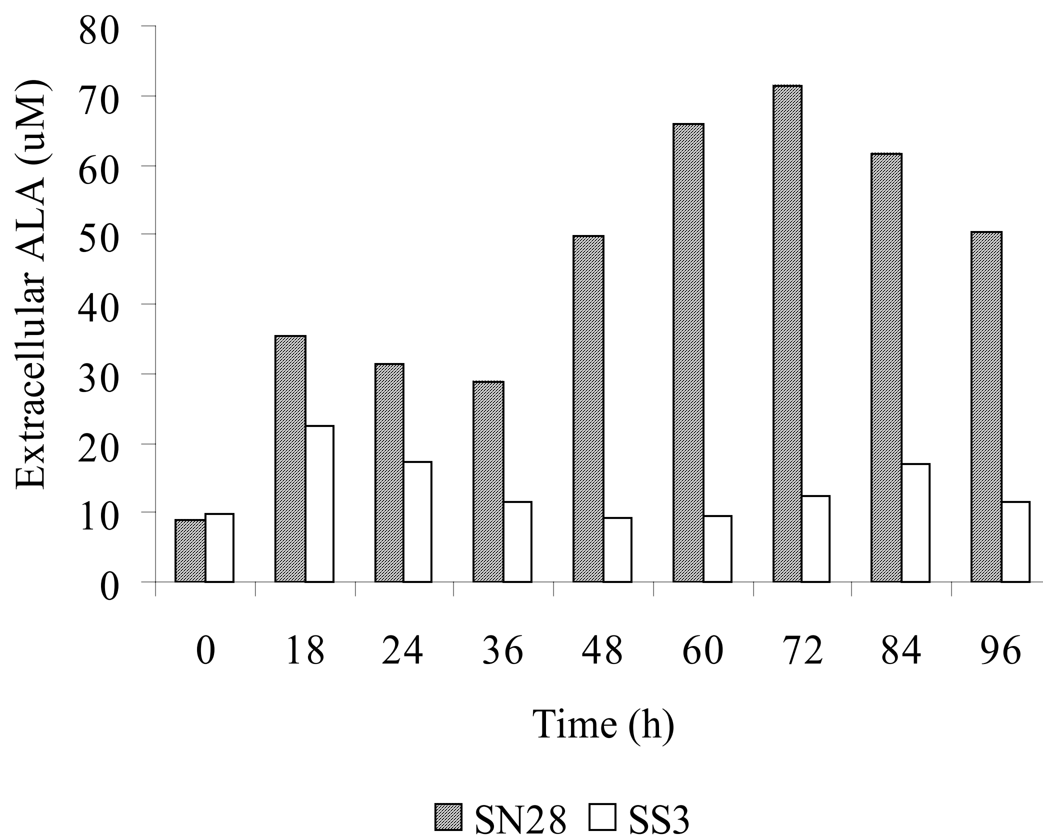
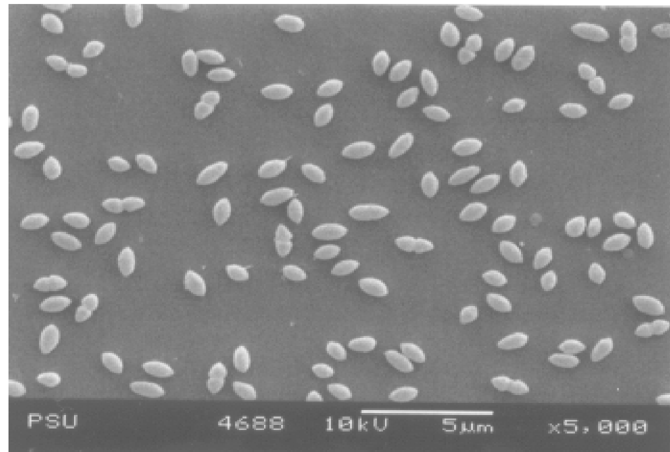


Figure 7 Extracellular ALA production of the NTG mutant *Rhodobacter capsulatus* SN28 and the wild-type *R. capsulatus* SS3 in GM medium under aerobic-dark condition at 37°C

Table 8 Kinetic parameters of growth and ALA production of the selected mutant (*Rhodobacter capsulatus* SN28) compared to the wild type strain (*Rhodobacter capsulatus* SS3) in GM medium under aerobic–dark condition at 37 C

Parameter	Unit	Wild type strain	Mutant strain
x_{max}	g/l	1.92	2.11
μ_{max}	h^{-1}	0.07	0.07
Extracellular ALA			
ALA_{max}	μM	22.34	71.25
R_m	μM ALA/h	0.37	1.18
Enzyme activity			
ALAS (60h)	mol ALA/h/mg protein	8.19	14.21
ALAD (36h)	nmol PBG/h/mg protein	163.73	74.64

(A)



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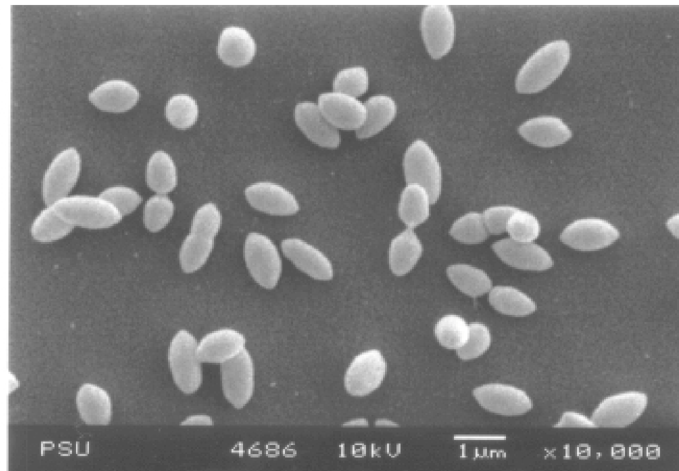

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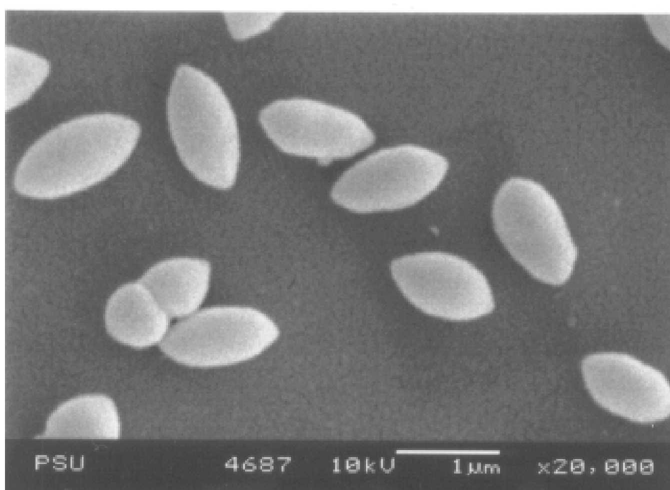
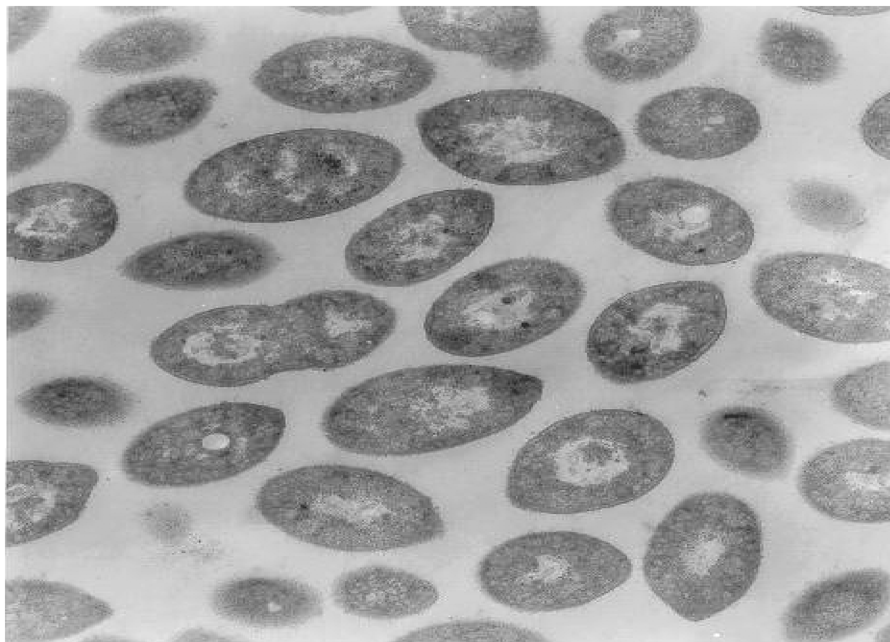


Figure 8 SEM images of the mutant strain *Rhodobacter capsulatus* SN28

(A) magnification x 5,000, (B) magnification x 10,000, (C) magnification x 20,000

(A)



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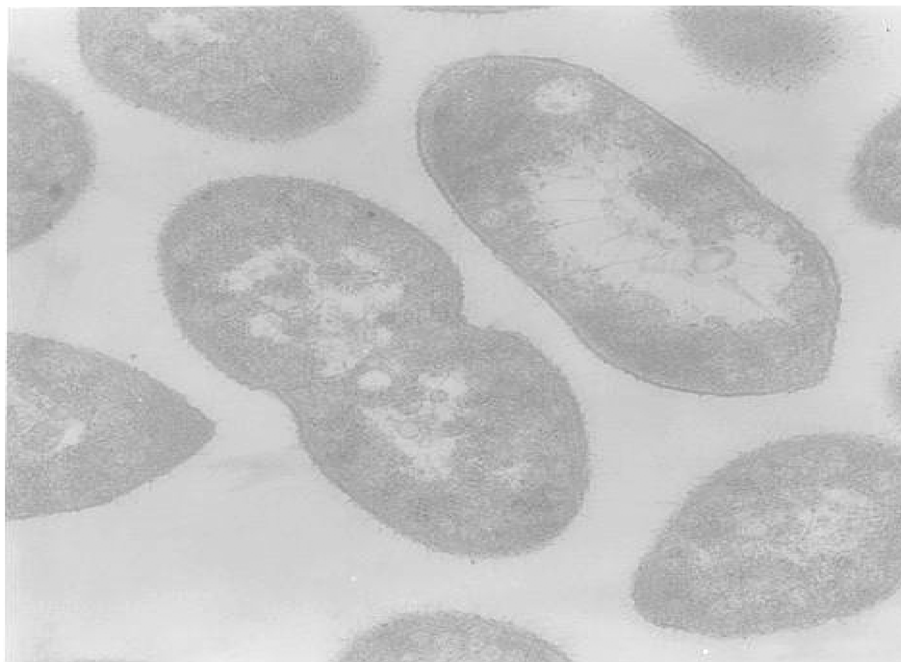
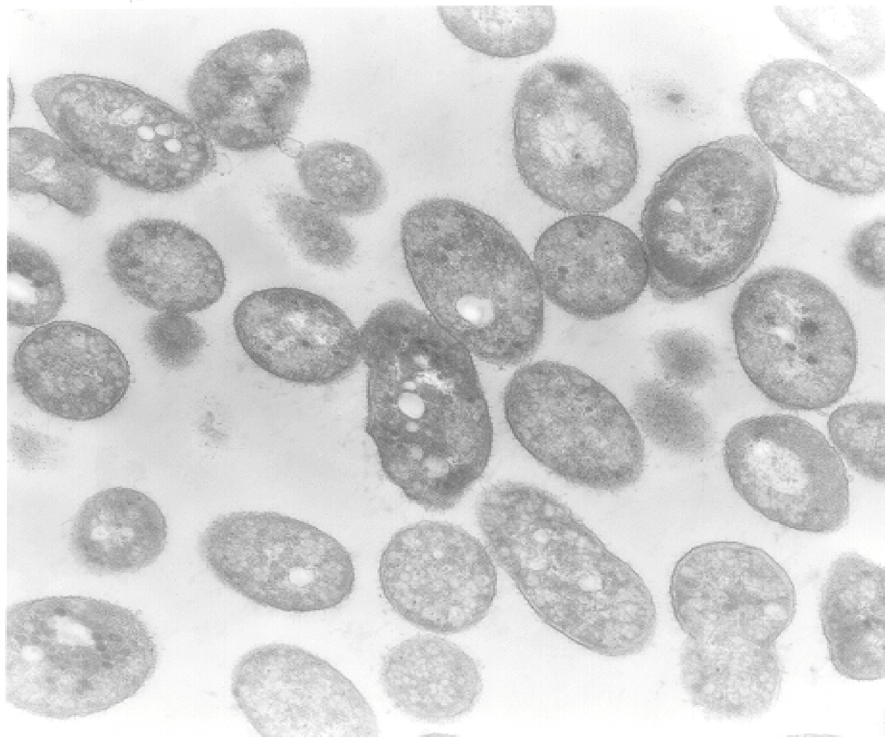


Figure 9 TEM images of the mutant strain *Rhodobacter capsulatus* SN28
(A) magnification x 10,000, (B) magnification x 20,000

(A)



(B)

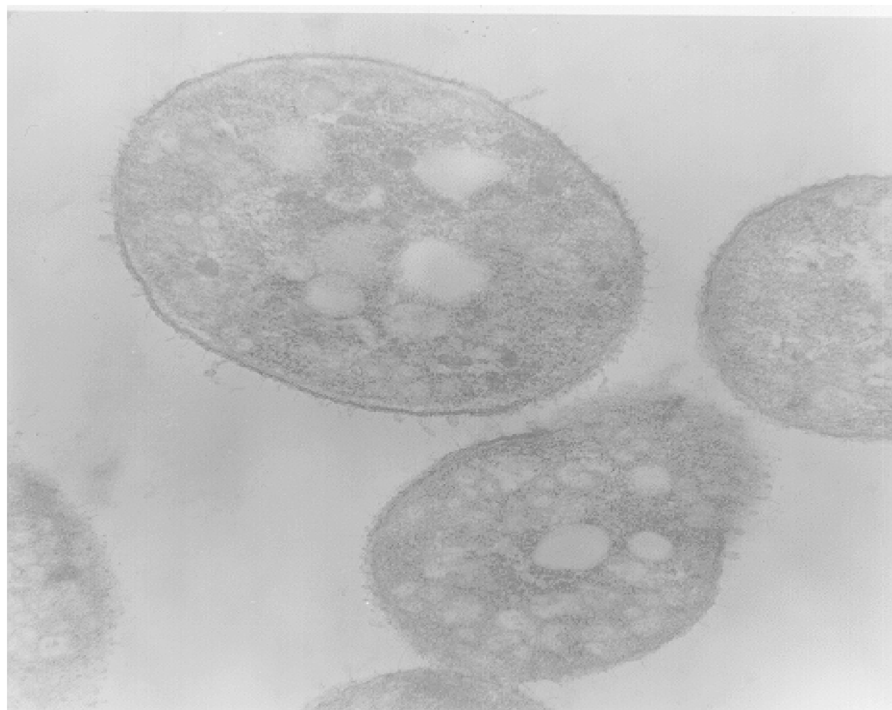


Figure 10 TEM images of the wild type *Rhodobacter capsulatus* SS3

(A) magnification x 10,000, (B) magnification x 30,000

2. Optimization for ALA production by the selected mutant strain

2.1 Effect of using glucose instead of malate

Comparison on growth and extracellular ALA production of *R. capsulatus* SN28 cultivating in GM medium and GG medium (glucose replaced malate) under aerobic-dark condition were conducted (Figure 11 and 12). The maximum dry cell weights in these two media were 2.09 and 3.92 g/l, respectively or about two times higher in GG medium. The maximum extracellular ALA, increased to 97.13 µM in GG medium or 1.5 folds higher than incubated in GM medium after 60 h cultivation under aerobic-dark condition. During cultivation in GM medium and GG medium, pH of the culture moved towards alkaline values (pH 6.86.to 7.74 and 8.73 within the first 36 h) and pH 9.04 and 8.38 at 96 h cultivation. Although, these two media had different cell growth, GG medium gave the highest cell growth (3.02 g/l dry cell weight) while GM medium gave low growth values (2.13 g/l dry cell weight). Rapid increase of pH changes to alkaline could affect the stability properties of ALA (Gadmar *et al*., 2002) which was relatively low extracellular ALA in GM medium.

Kinetic parameters from cultivation in GM and GG media are shown in Table 9. The specific growth rate in GM medium was 0.12 h^{-1} while it was 0.13 h^{-1} when grew on GG medium. The maximum productivity increased from 1.10 to 1.62 µM ALA h^{-1}. This result showed that the GG medium was more suitable than GM medium for growth and ALA production from the mutant strain SN28. However, the intracellular ALA from cultivation in GM medium was slightly higher than in GG medium (1.02 and 0.98 µM/g mol, respectively). Glucose was a better carbon source than malate as it is a satisfactory source of succinyl-CoA via TCA cycle under aerobic-dark condition (Nishikawa *et al.,* 1999). It was reported that the growth rate of the mutant strain CR-450 was higher with glucose than DL-malate as the carbon source (Sasaki *et al*., 1991). From this result, GG medium was selected for further studies.

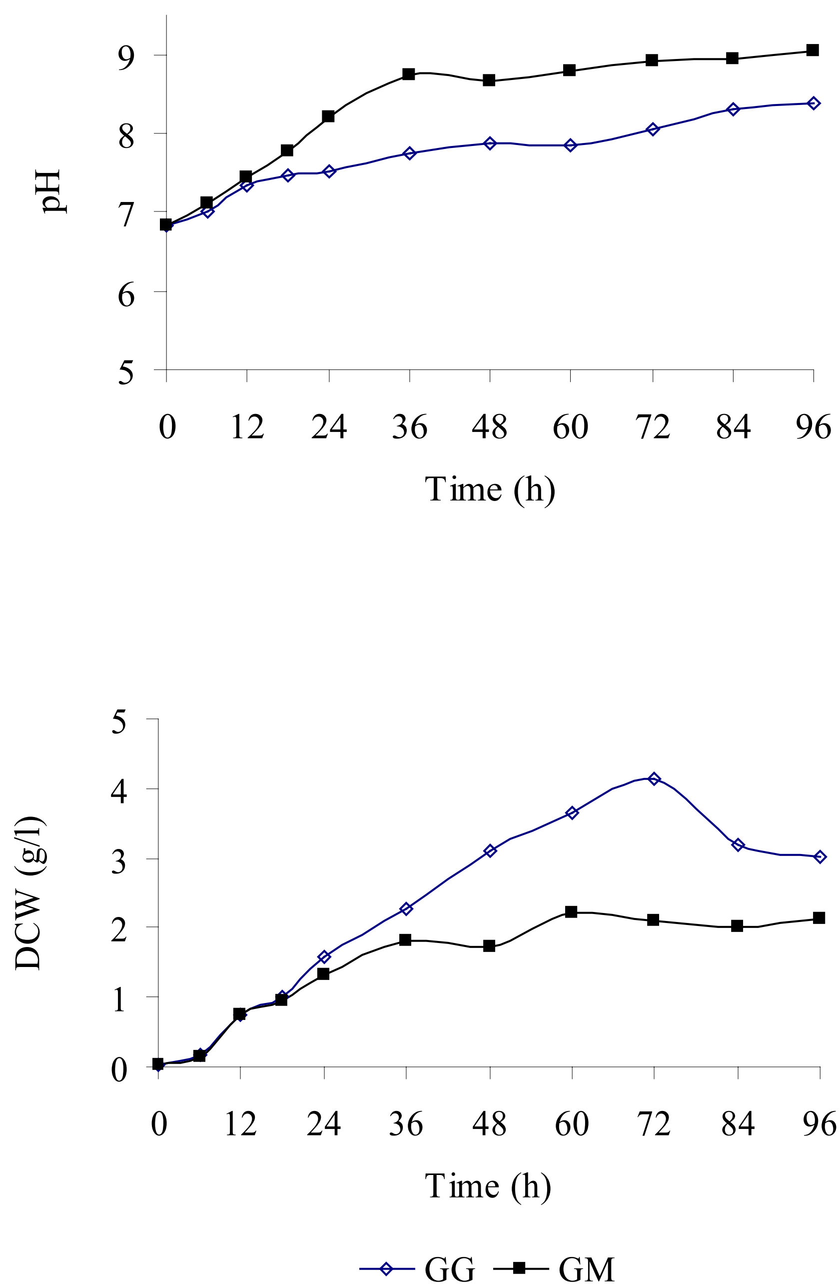


Figure 11 pH and growth during cultivation of the *Rhodobacter capsulatus* SN28 in glutamate-malate (GM) medium and glutamate-glucose (GG) medium under aerobic-dark condition at 37°C

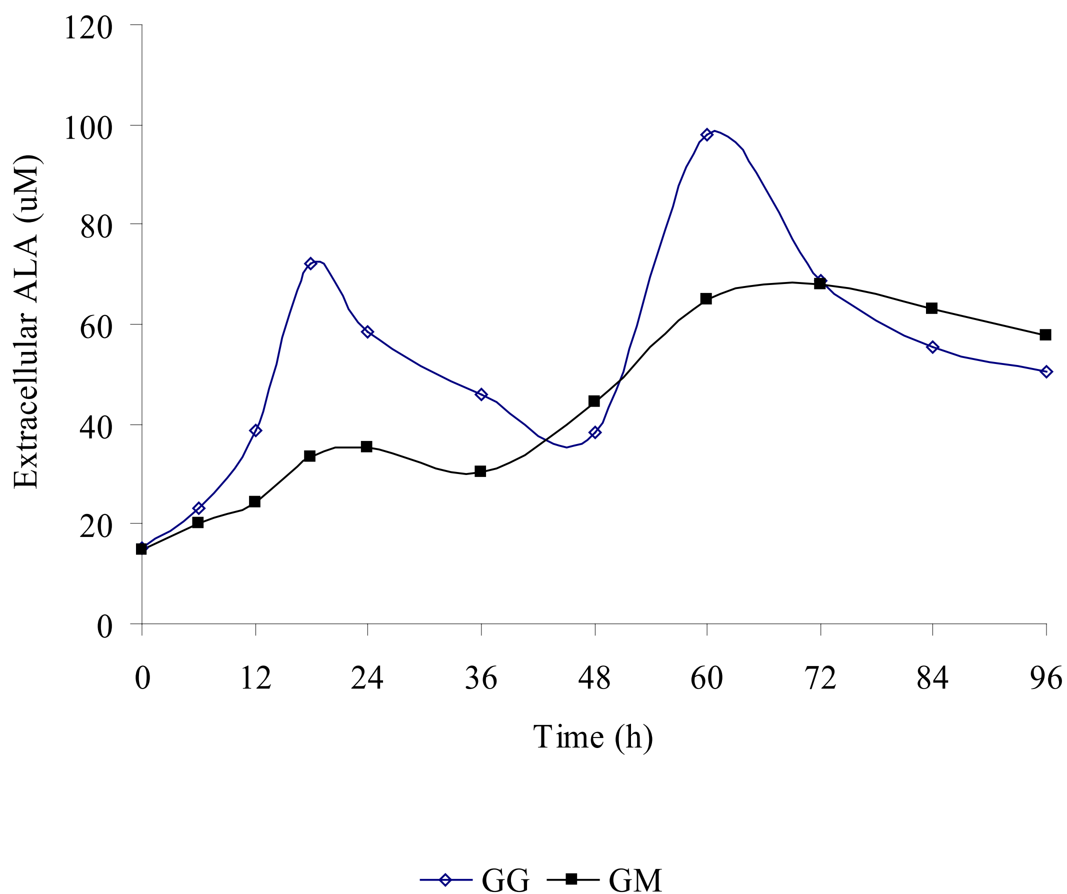


Figure 12 Extracellular ALA production during cultivation of the *Rhodobacter capsulatus* SN28 in glutamate-malate (GM) medium and glutamate-glucose (GG) medium under aerobic-dark condition at 37°C

Table 9 Effect of medium formular (GM and GG medium) on growth and ALA production of *Rhodobacter capsulatus* SN28

Parameter	GM medium	GG medium
Maximum dry cell weight (g/l)	2.09	3.92
Specific growth rate (h^{-1})	0.12	0.13
Extracellular ALA (µM)	64.77	97.13
Production rate (µM h^{-1})	1.10	1.62
Intracellular ALA (µM/g mol) at 24 h	1.02	0.98
Production rate (µM/g cell h^{-1})	0.59	0.42

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

ALA was synthesized mainly by ALA synthase and then converted to tetrapyrrols by ALA dehydratase. The latter enzyme could be inhibited in this presence of LA, a competitive inhibitor. The result of the time course (Fig 11). on growth and ALA production by SN28 cultivated in GG medium + 3% NaCl under aerobic-dark condition at 37°C, the middle of the exponential growth phase was found to be 36 h and the activity of ALA dehydratase tended to increase at this time (Figure 13 and Table 10). Thus, LA was added into the medium at 36 h cultivation.

The inhibitory effects of LA on ALAD in *Rhodobacter capsulatus* SN28 were investigated by varying the LA concentration (5-20 mM LA) and number of repeated addition of LA (1-3 times) during the cultivation in 100 ml GG medium under aerobic-dark condition at 37 C. Results indicated that when no LA was added into the medium, 97.13 µM of ALA was produced (Figure 13). The maximum dry cell weight was 3.54 g/l and the pH of the culture increased from 6.90 to 8.28 at 96 h cultivation. When 5, 10 and 15 mM of LA was added, ALA production increased and the maximum extracellular ALA concentrations were 102.55, 103.26 and 129.28 µM, respectively. Cell growth was almost retarded by LA addition and completely suppressed at 20 mM LA. The maximum dry cell weights were 3.21, 3.39, 3.42 and 2.66 g/l, respectively. Extracellular ALA formation seemed to be accelerated when growth was retarted by LA addition. The results were in agreement with previous report (Sasaki *et al*., 1987) that LA addition in middle log phase (active growth phase) affectively blocked ALA metabolism, resulting in the retardation of cellular growth and excretion of the ALA formed. However, higher concentration of LA (20 mM) could not further enhance ALA accumulation (48.92 µM after 72 h cultivation) and the pH of the culture was dropped suddenly to 5.0. The very low pH (5.0) caused the drop of ALA production which might due to the decrease of other enzyme systems such as energy-harvesting enzymes under low pH conditions (Noparatnaraporn *et al*., 2000).

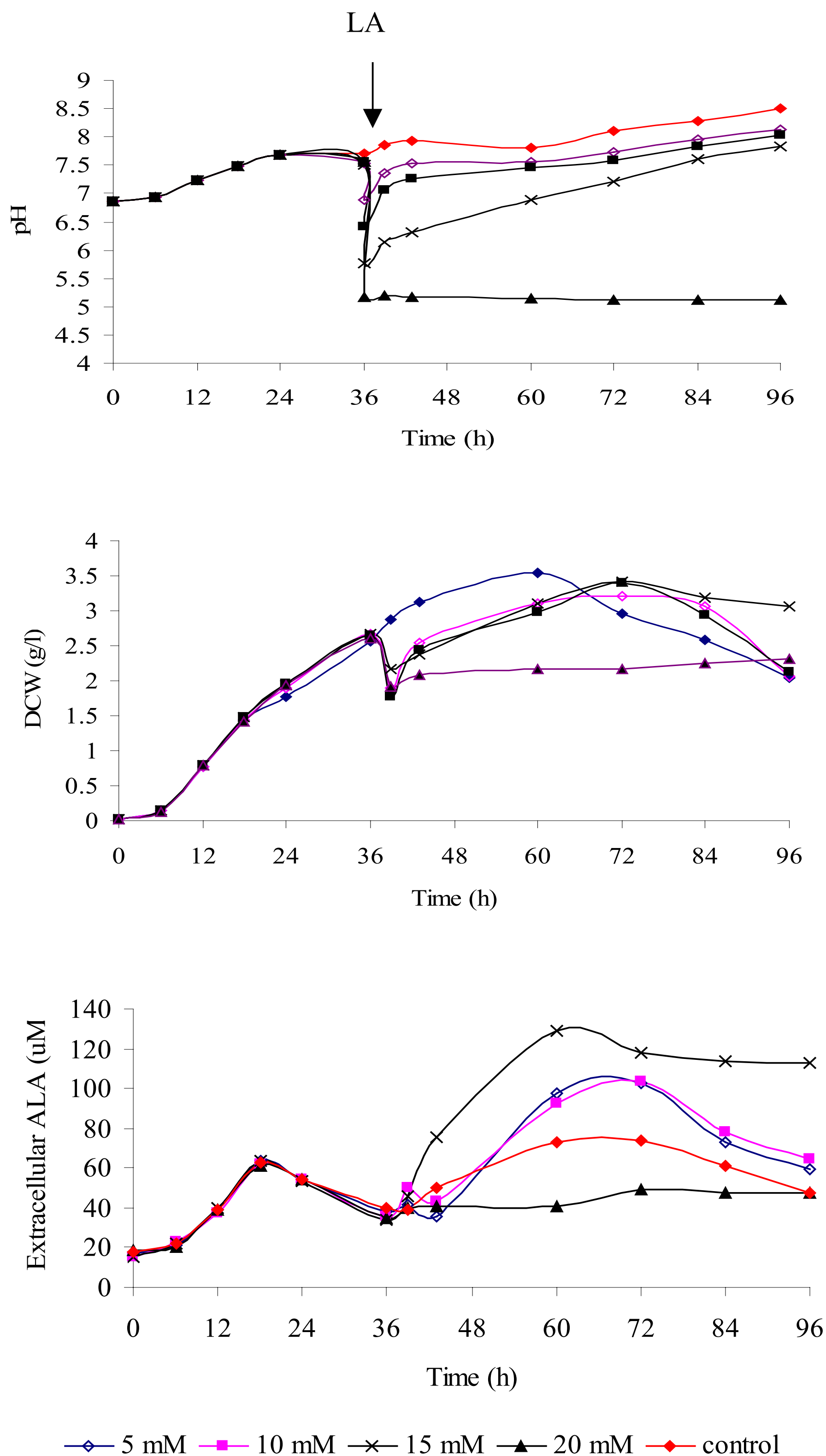


Figure 13 Effect of levulinic acid concentrations on pH, growth and extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 under aerobic-dark condition at 37º C in GG medium with the addition of levulinic acid (LA) at 36 h

Table 10 Activity of enzyme ALA synthase (ALAS) and ALA dehydratase (ALAD) from *Rhodobacter capsulatus* SS3 and *R. capsulatus* SN28 cultivated in GG medium +3% NaCl under aerobic-dark condition at 37 C

Time (h)	ALA synthase (nmol /h/ mg protein)		ALA dehydratase (nmol /h/ mg protein)	
	Wild type (SS3)	Mutant (SN28)	Wild type (SS3)	Mutant (SN28)
18	3.42	6.11	73.73	24.68
24	2.76	3.25	142.01	41.26
36	5.34	8.59	163.73	74.64
48	7.59	10.70	156.22	170.13
60	8.19	14.21	287.15	200.01
72	8.88	10.77	293.52	211.81
84	12.09	8.46	235.84	76.24
96	12.39	8.12	203.41	35.33

Preliminary study with the addition of 5 mM LA into the GG medium at 18 h, the results was shown in Table 11. From these, It was found that the addition of 5 mM LA could keep low ALA dehydratase activity after addition of LA (26.10 nmol/h/mg protein at 24h) then it was increase. Thus, to maintain ALA dehydratase activity at a low level, repeated addition of LA after addition of LA at the first time was added again every 18 h.

Table 11 Effect of levulinic acid addition on activity of enzyme ALA synthetase and ALA dehydratase from *Rhodobacter capsulatus* SN28 in GG+3% NaCl under aerobic-dark condition at 37 C

Time (h)	LA (5 mM) 3 times		LA (5 mM) 1 times	
	ALAS (nmol/h/mg protein)	ALAD (nmol/h/mg protein)	ALAS (nmol/h/mg protein)	ALAD (nmol/h/mg protein)
18	4.71	23.28	5.23	30.36
24	4.25	20.07	4.59	26.10
36	9.49	48.41	10.11	58.32
48	8.96	94.73	11.53	133.64
54	16.22	156.12	13.65	156.27
60	10.81	151.57	8.81	203.06

Repeated addition of LA two times at 18 and 36 h, three times at 18, 36 and 54 h with a total LA concentration of 15 mM and three times at 18, 36, 54 h with LA concentration of 15 mM each time were studied. The effect of repeated addition of LA on pH, growth and extracellular ALA production from *R. capsulatus* SN28 in GG medium under aerobic-dark condition was investigated (Figure 14 and Figure 15). Three times addition of each 15 mM LA after 18 h cultivation, cell growth and pH of the culture kept constant thereafter (2.61 g/l DCW and pH 5.12, after 24 h cultivation). Addition of 5-10 mM LA after 18 h cultivation, , cell growth increased rapidly (about 4.7 g/l DCW after 24 h cultivation and pH declined from about 7.45 to 6.60 and increased to pH 7.10 within 6 h. Total LA concentration of 15 mM (two times and three times additions) should not be added to the culture medium at the exponential growth phase (18 h) as it would suppress cell growth of *R. capsulatus* SN28.

The pH of the culture broth decreased to acidity rapidly at every LA concentrations added but they increased to neutral pH within 6 h after that except three times addition (15 mM LA each) in which the culture broth kept slightly constant at acidic pH. The maximum cell yields of 3.78, 3.02, 2.36, 2.68 and 1.276 g/l dry cell weight were achieved in the addition of LA one time at 36 h, two times at 18 h for 10 mM and at 36 h for 5 mM, two times at 18 h for 5 mM and at 36 h for 10 mM, three times at 18, 36 and 54 h with LA concentration of 5 mM in each time and three times at 18, 36 and 54 h with LA concentration of 15 mM in each time, respectively. However, dry cell weights in all repeated additions were lower compared to their control (no LA added) (3.54 g/l DCW) which was the same as one time addition of LA. These results confirmed that LA could excessively suppress growth.

The maximum extracellular ALA of 128.36 µM was achieved with only one time addition of LA at 36 h followed by the addition of LA two times at 18 h for 5 mM and at 36 h for 10 mM (102.95µM ALA), three times at 18, 36 and 54 h each with LA addition of 5 mM (90.40 µM ALA), two times at 18 h for 10 mM and at 36 h for 5 mM (81.78 µM ALA) and three times at 18, 36 and 54 h with each LA addition of 15 mM (69.83 µM ALA), respectively.

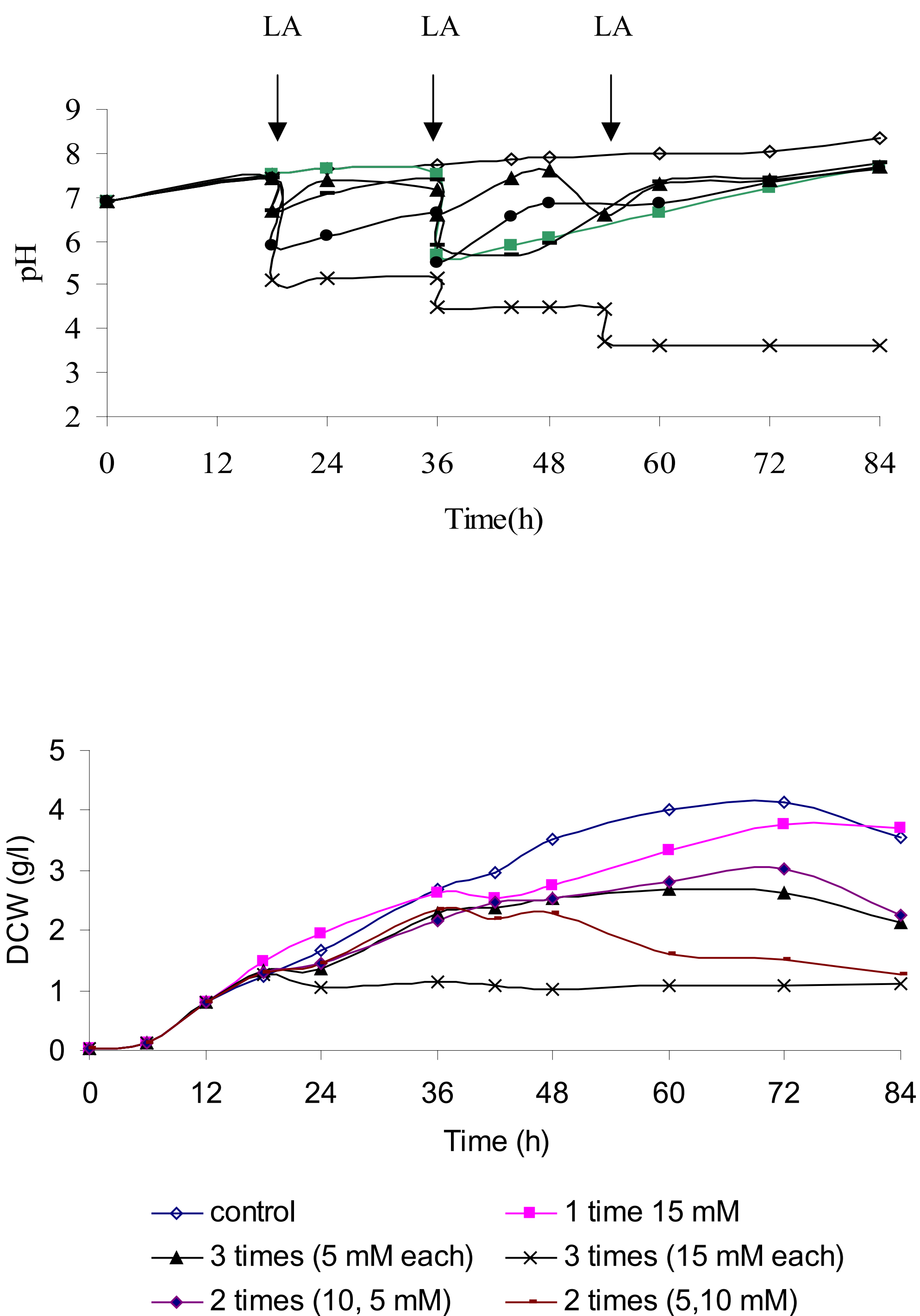


Figure 14 Effect of repeated addition of levulinic acid on pH and growth during cultivation of the mutant *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

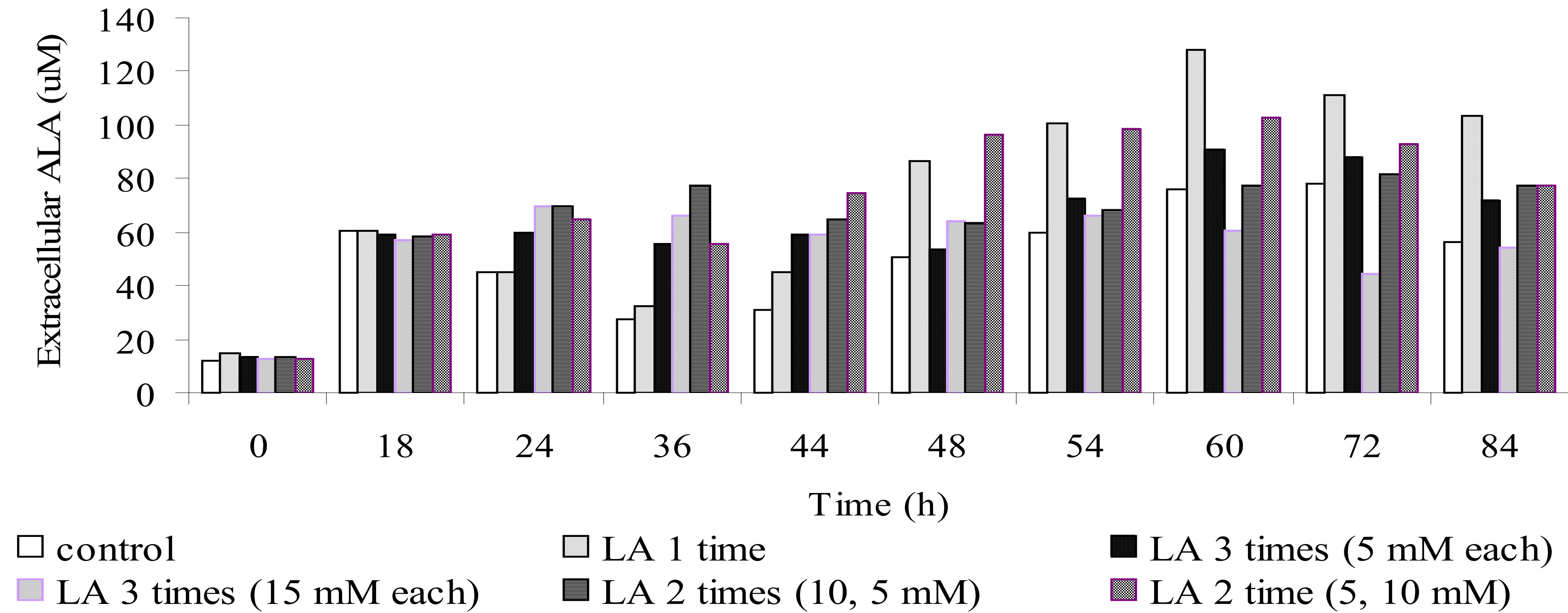


Figure 15 Effect of repeated addition of levulinic acid on extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

Three times addition of LA (15 mM) could not further enhance ALA accumulation (69.83 µM) which agreed with the previous observation at one time addition of 20 mM LA (48.92 µM). It might be emphasized that the excessive suppression of growth caused by the addition of LA is unfavorable for ALA excretion since the energy (ATP) supply is stopped within the cells (Sasaki *et al*., 1987).

2.3 Effect on the addition of C_5 pathway precursor

Glutamate and malic acid are precursors of ALA biosynthesis via C5 pathway, therefore the addition of these precursors into the medium may enhance ALA formation. A series of batch cultures in which different amount of precursors, the concentration of 2, 4, 6 and 8 g/l glutamate and the concentration of 1, 2, 3 and 4 g/l malic acid, on growth and extracellular ALA production from *R. capsulatus* SN28 were supplemented with a fixed amount of LA (15 mM) at 36 h cultivation were carried out under aerobic-dark condition. Results indicated that the 16 series of batch cultures at different amount of precursors, the pH of the culture increased from neutral to alkaline pH (6.92 to 8.54) within 36 h cultivation and decreased rapidly to neutral after adding 15 mM LA then increased within 6 h to pH about 7.9 at 42 h cultivation while to pH 6.18 for the control. In GG medium without glutamate, the pH declined rapidly after adding LA and kept slightly constant to pH 4.4 at 42 h cultivation (Figure 16).

Cultivation of *R. capsulatus* SN28 in 16 different combination of precursors gave significantly higher cell growth (4.2-5.7 g/l dry cell weight) than the control (3.45 g/l dry cell weight) ($p<0.05$) (Figure 17). Results the control were similar to those in GG medium without glutamate but gave lower cell yields. When the concentration of 2, 4, 6 and 8 g/l glutamate were supplemented to the GG medium with addition of 15 mM LA at 36 h cultivation, the maximum dry cell weights were 5.39, 5.71, 4.53 and 4.40 g/l, respectively, with 2 g/l malic acid while the control gave 3.45 g/l DCW. This result indicated that addition of glutamate enhanced cell growth but excess of glycine could moderately suppressed growth. This is contrary to the previous report of Sattayasmithstid (2001) that addition of glutamate was not effective to cell growth. Moreover, the cell growth at the concentration of 1, 2, 3 and 4 g/l malic acid were 6.91, 5.71,

5.52 and 5.20 g/l DCW, respectively, with 4 g/l glutamate. It could be suggested that higher concentration of malic acid gave lower cell growth. This optimum glutamate and malic acid concentration for cell growth was 4 and 1 g/l, respectively, which gave 6.19 g/l dry cell weight or about 2 folds higher than the control.

However, supplementation with these precursors resulted in a negative effect in extracellular ALA accumulation (42-107 μM) when compared to the control (131.16 μM) (Figure 18) at 60 h cultivation. The highest extracellular ALA production was found in GG medium without glutamate which gave 168.22 μM at 36 h cultivation and kept slightly constant thereafter. The pH of the culture was rather constant after addition of 15 mM LA (pH 4.4) which related directly to the stability of ALA at low pH (Gadmar *et al*., 2002). From this result, it may illustrate that glutamate might suppress ALA excretion. When no LA was added to the GG medium without glutamate, 246.57 μM of ALA was produced at 60 h cultivation (Figure 19) with the pH decreased from 6.91 to 5.35 in which low pH could suppress ALA production by reducing cell growth. In GG medium with the addition of 15 mM LA, the pH from pH 6.50 (at 36 h cultivation) to pH 4.35 after addition of LA and kept constant thereafter. From these results, the optimum medium for extracellular ALA production from *R. capsulatus* SN28 was GG medium without glutamate and without addition of LA.

2.4 Effect on the addition of C_4 pathway precursor

Biosynthesis of ALA proceeds from C4 pathway precursors, glycine and succinate through a combined action of the host enzymes, succinyl-CoA synthase and ALA synthase (Sasaki *et al*., 2002). Therefore, the addition of two precursors (glycine and succinate) should affect the production of ALA. The influence of a series of batch cultures in which different amount of precursors 2.5, 5, 7.5 and 10 mM glycine and 10, 20, 30 and 40 mM succinate, on growth and extracellular ALA production from *R. capsulatus* SN28 were performed in 100 ml GG medium without glutamate under aerobic- dark condition at 37 C (Figure 20-22). The changes of pH during this cultivation of were the same pattern in which pH increased from 6.92 to about 8.3 at the end of cultivation (84

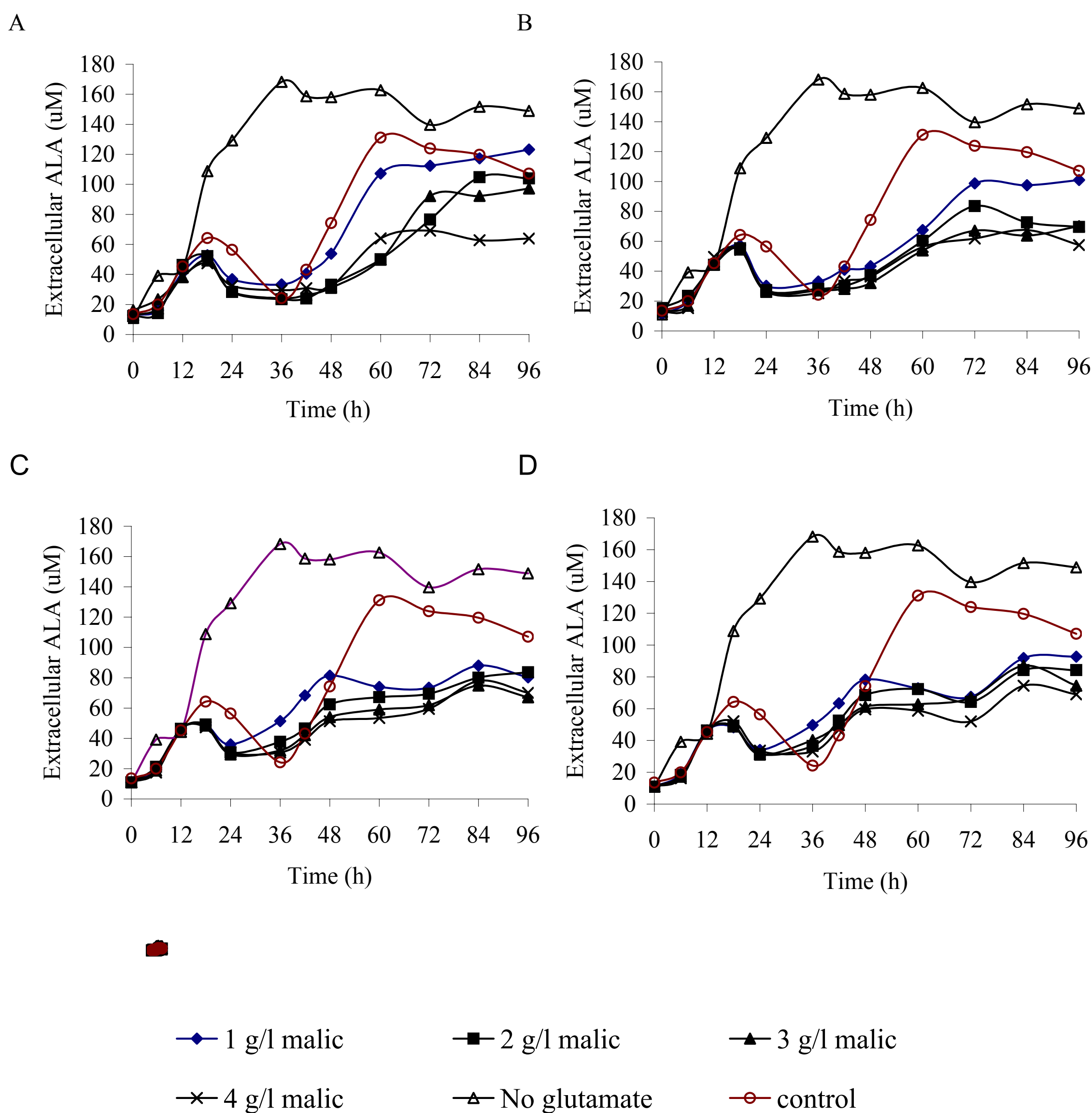


Figure18 Effect of supplementing C5 pathway precursors (glutamate and malic acid) on extracellular ALA formation during cultivation of the mutant *Rhodobacter capsulatus* SN28 under aerobic-dark condition at 37°C
(A) 2 g/l glutamate (B) 4 g/l glutamate (C) 6 g/l glutamate (D) 8 g/l glutamat

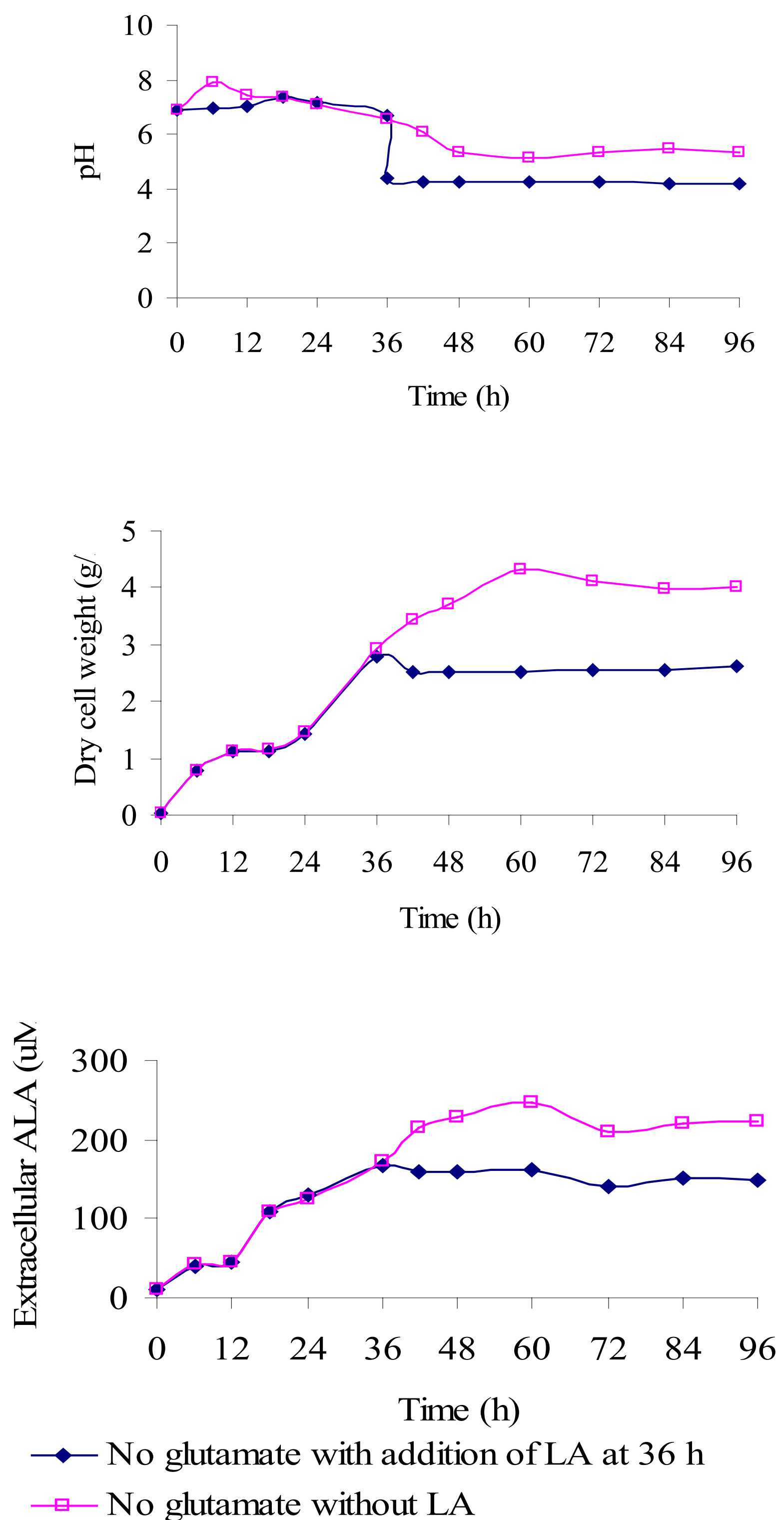


Figure 19 Comparison on pH, growth and extracellular ALA production during cultivation of the mutant *Rhodobacter capsulatus* SN28 in GG medium (no glutamate) with and without addition of LA at 36 h under aerobic–dark condition at 37°C

h) while the control (without addition of glycine and succinate) the pH declined from 6.92 to about 5.36 within 48 h, then kept constant thereafter.

The maximum cell growth was achieved from the control (4.38 g/l DCW) and significantly ($p < 0.05$) higher than of precursors addition. When the concentration of 10, 20, 30 and 40 mM succinate were supplemented to the GG medium without glutamate, the maximum dry cell weights were 4.12, 3.71, 2.93 and 2.31 g/l, respectively, with 5 mM glycine (Figure 21B). This result for *R. capsulatus* SN28 agreed with the report that the maximum cell growth decreased with increased concentration of succinate which the excessive amount could suppress on cell growth (Chung *et al*., 2004). At the concentration of 2.5, 7.5 and 10 mM glycine, the cell growths were 4.26, 4.12, 3.15 and 1.94 g/l DCW, respectively, with 10 mM succinate. This is in agreement as previous reports study of Sattayasmithstid (2001) in which *R. capsulatus* SS3 could not grow at high concentration of glycine (above 10 mM) and also agreed with the reported from Sasaki *et al* (1990 and 1991). Excess glycine addition suppressed cell growth both by glycine itself and by the ammonia produced as a result of glycine metabolism. In addition, ALA is chemically unstable under alkaline condition therefore, ALA production should be carried out at a slightly acidic pH (Sasaki *et al*., 2002).

The above experiments using factorial concentrations of glycine and succinate were significantly ($p < 0.05$) suppressed the extracellular ALA production to about 52-85 µM ALA compared with the control (241.37 µM). It was reported that high amount of glycine in the medium can inhibit porphyrin formation (Neuberger, 1961). Therefore, GG medium without addition of glycine and succinate should be used.

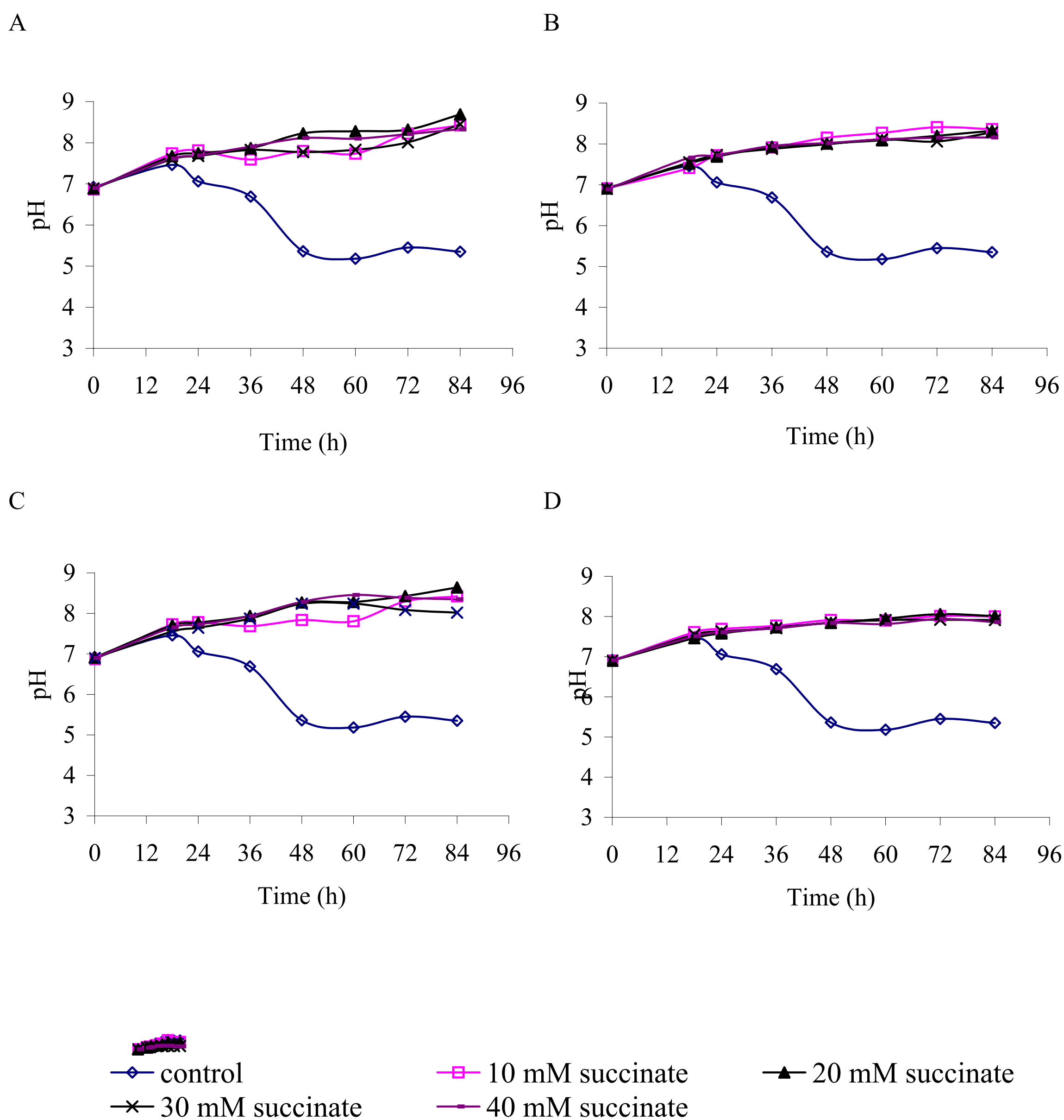


Figure 20 Effect of supplementing C4 pathway precursors (glycine and succinate) on pH during cultivation of the mutant *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition 37°C (A) 2.5 mM glycine (B) 5 mM glycine (C) 7.5 mM glycine (D) 10 mM glycine

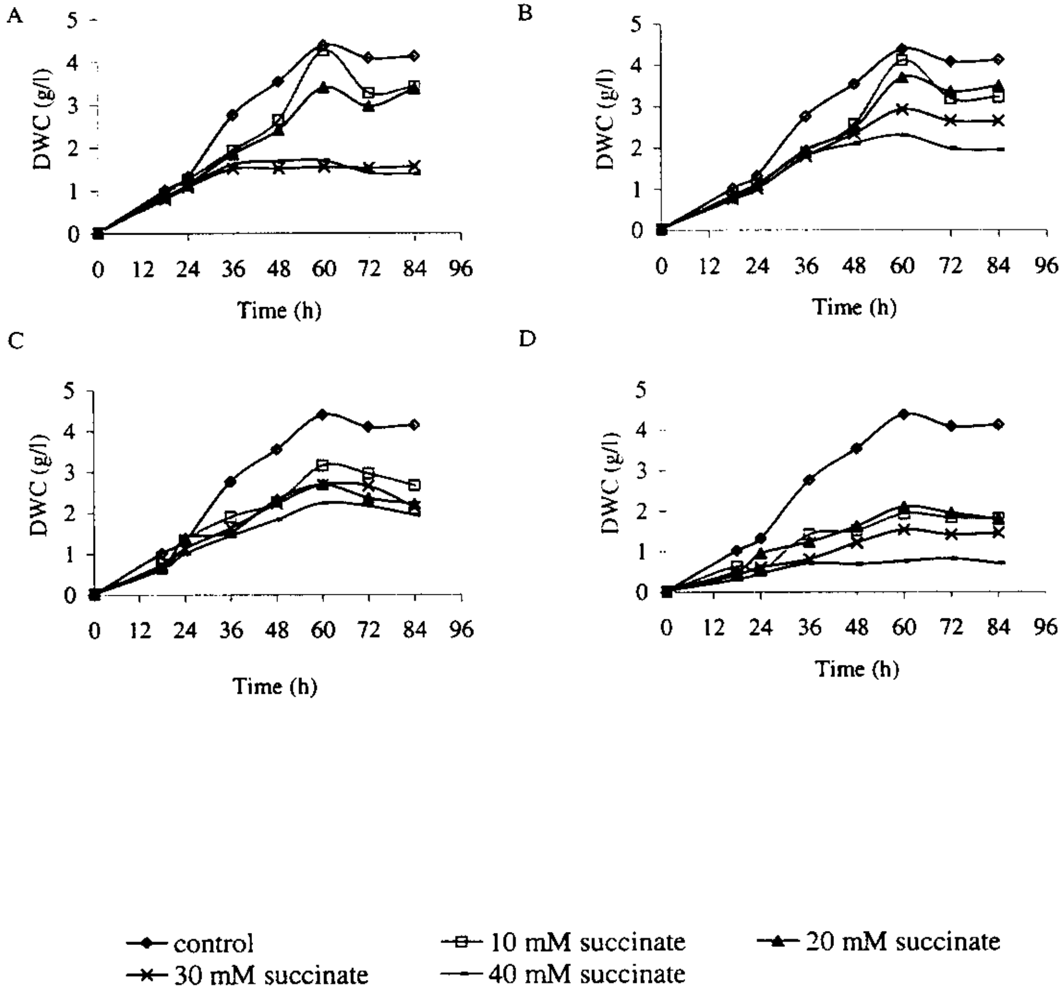


Figure 21 Effect of supplementing C4 pathway precursors (glycine and succinate) on growth during cultivation of the mutant *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C
(A) 2.5 mM glycine (B) 5 mM glycine (C) 7.5 mM glycine
(D) 10 mM glycine

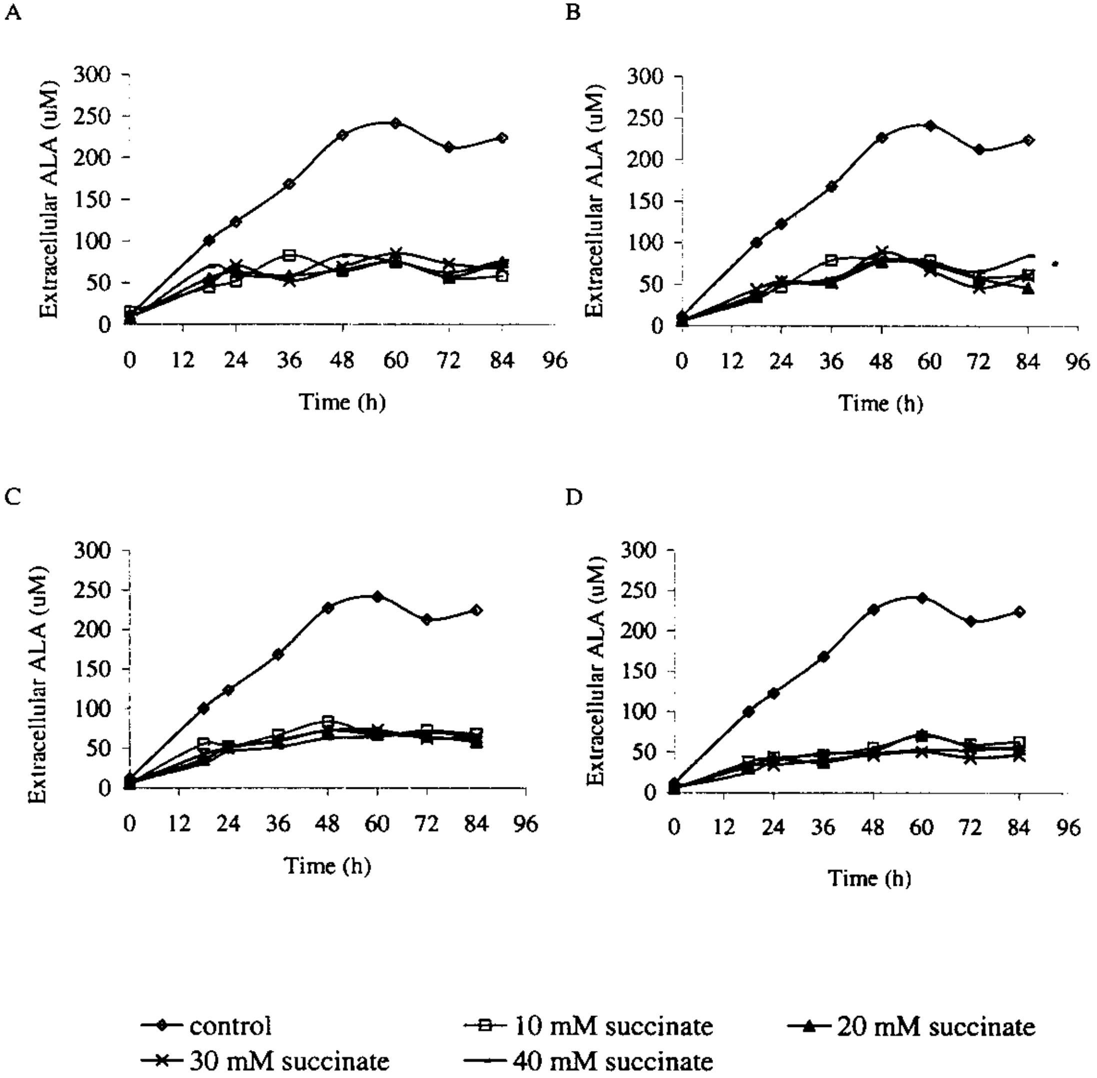


Figure 22 Effect of supplementing C4 pathway precursors (glycine and succinate) on extracelular ALA production during cultivation of the mutant *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

(A) 2.5 mM glycine (B) 5 mM glycine (C) 7.5 mM glycine (D) 10 mM glycine

2.5 Effect of initial pH

For the production of extracellular ALA by some micro-organism including *R. sphaeroides,* the intracellular balance of ALA synthetase and ALA dehydratase is important and the enzyme activities are influenced by the culture pH (Sasaki *et al*., 1993). The influence of initial pH (5.5-8.0)) on pH, growth and extracellular ALA production of *R. capsulatus* SN28 was investigated in 100 ml GG medium without the addition of glutamate + 3% NaCl cultivating under aerobic – dark condition (Figure 23). Growth was inhibited at the initial pH of 5.5. At initial pH of 6.0 and 6.5, growth increased within 48 h giving the dry cell weight of 1.83 and 2.99 g/l dry cell weight, respectively, and rather stable thereafter. However, at pH 7.0, 7.5 and 8.0 growth continued and reached 4.58, 4.32 and 4.17 g/l dry cell weight, respectively, at the end of the cultivation. It may be due to the fact that the culture pH of the initial pH 6.0 and 6.5 decreased more rapidly to pH 5 in the first 48 h after cultivation which growth of photosynthetic bacteria was suppressed at pH values below 6.5 (Noparatnaraporn *et al*., 2000).

For extracellular ALA production and the maximum values of 180.09, 178.41, 250.12, 235.52 and 225.60 µM, respectively, were achieved after 72 h cultivation. It is possible that the culture pH in the initial pH of 6.0, and 6.5 deceased to acidity quickly which bacteria can't not grow well while the culture pH in the initial pH of 7.0, 7.5 and 8.0 decreased more slowly than those initial pH of 6.0 and 6.5 so it caused to decrease the ALA production.

From these results, the optimum initial pH for extracellular ALA production from *R. capsulatus* SN28 was pH 7.0.

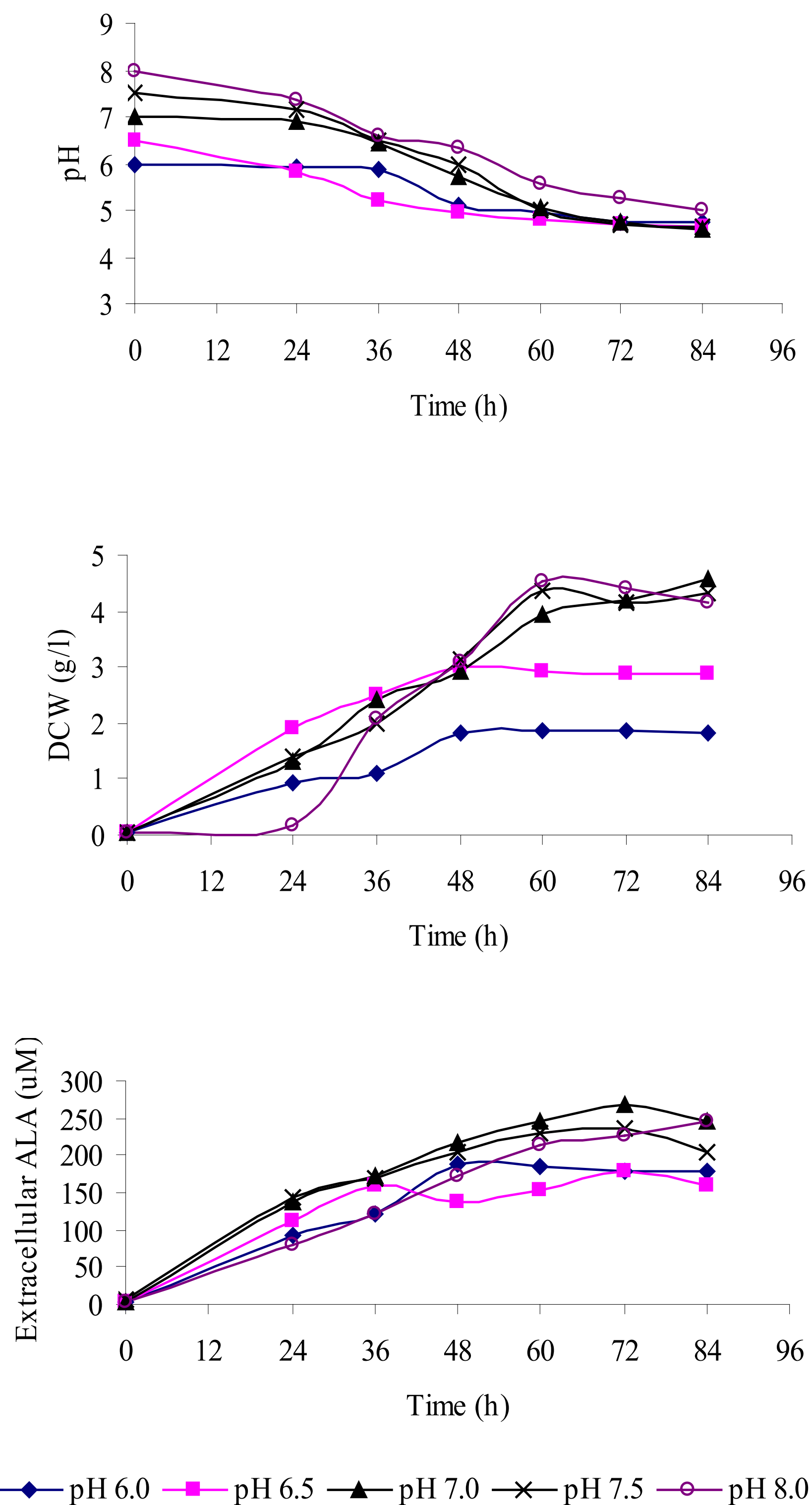


Figure 23 Effect of initial pH on pH, growth and extracellular ALA formation during cultivation of the mutant *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

2.6 Effect of volatile fatty acids

The production of ALA by the photosynthetic bacterium *Rhodobacter sphaeroides*, using volatile fatty acids (VFA) such as acetic, propionic, butyric acid was reported earlier (Sasaki *et al*., 1990, 1993) and *R. sphaeroides* can utilize lower acids such as acetic, propionic and butyric acid as carbon and energy sources (Sasaki *et al*., 1987). Therefore, the influence of VFA concentration (0 -3 g/l) on growth and ALA synthesis of *R. capsulatus* SN28 cultivating in GG medium without glutamate+3% NaCl with the initial pH of 7.0 under aerobic–dark condition is shown in Figure 24-26**.** For changes of pH during the cultivation (Figure 24), at 0.5 – 1% acetic acid, the pH increased within 24 h, then decreased slowly and kept rather constant (pH about 6.5 at 72 h). At 2 and 3 % acetic acid concentrations, the pH increased within 24 h then increased slowly to pH 8.5 at 96 h. At 1-3 % propionic and butyric acid concentrations, the patterns were the same from pH 7 to 7.8 within 48 h then decreased and kept slightly constant (about 6.8 at 96 h). However, the results at 0.5 % propionic and butyric acid concentrations were different as pH increased within the first 12 h, then decreased slowly from 7.40 and 7.22 to pH 6.18 and 5.48, respectively. Addition of acetic acid into the medium gave almost similar results to those of propionic acid addition in which higher concentration (2-3 g/l) decreased cell growth rate. The maximum cell yields was found at 1 g/l butyric acid (5.45 g/l DCW), followed by 0.5 g/l propionic acid (4.73 g /l) and at 0.5 g/l acetic acid (4.64 g/l), respectively, which were higher than the control (3.60 g /l). It should be noted that addition of 1-3 g/l butyric acid gave higher all concentrations of maximum dry cell weight (5.45, 5.31 and 5.29 g /l, respectively) than the 0.5 g/l of all VFA tested. High concentration of acetic and propionic acid gave lower maximum dry cell weight (0.95 and 1.39 g/l, respectively) than from low concentration of these VFA (4.63 and 4.73 g/l dry cell weight, respectively) Not only dry cell weight, the maximum extracellular ALA was also achieved with the addition of 0.5 g/l butyric acid (267.36 μM) at 60 h after cultivation which higher than the control (254.18 μM). This is contrary to the report that among volatile fatty acid in the medium, propionic acid was preferentially consumed by *R. sphaeroides* with the increase of ALA in contrast with other acids (Sasaki *et al.,* 1993). The remarkable propionic acid consumption

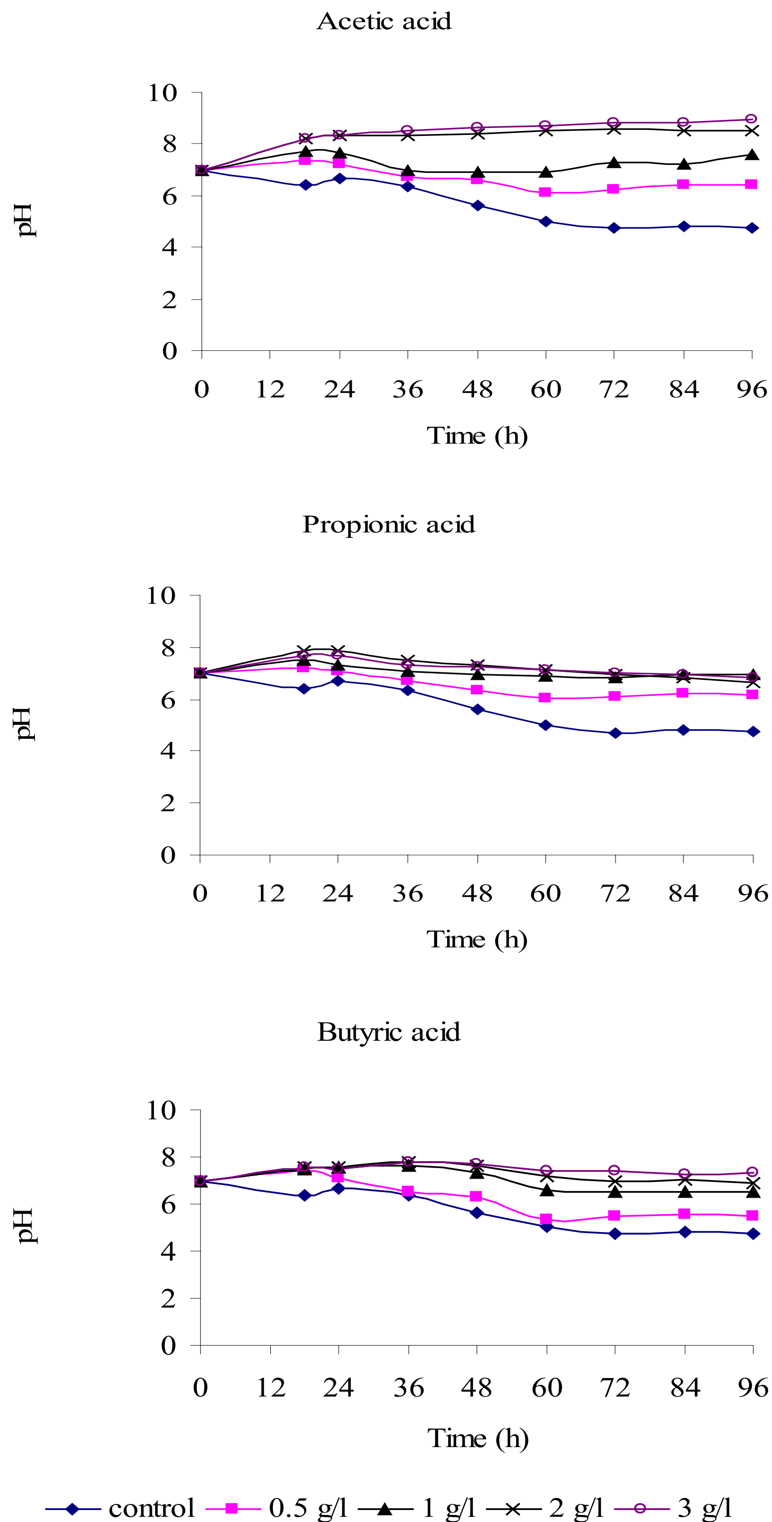


Figure 24 Effect of volatile fatty acid on pH during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

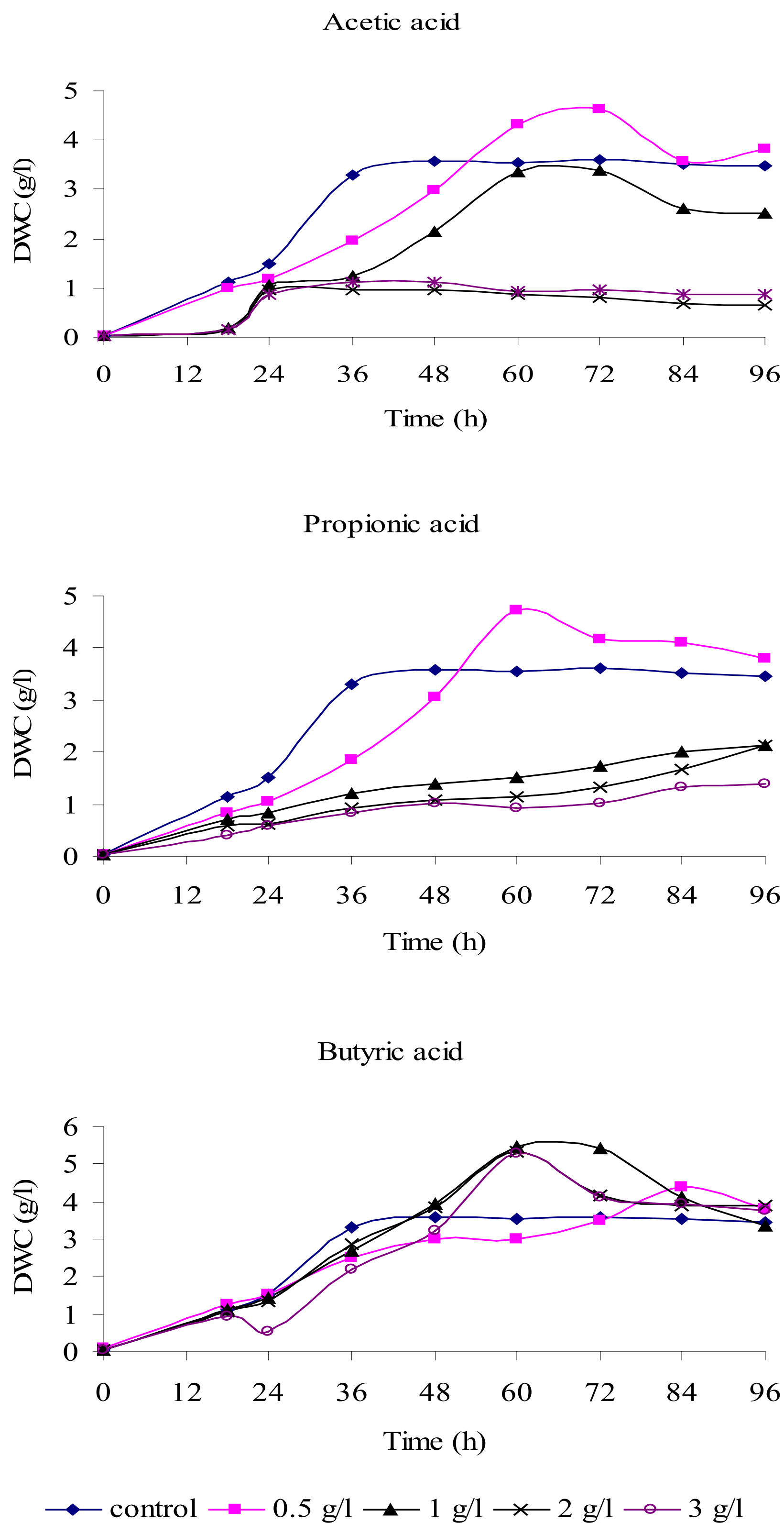


Figure 25 Effect of volatile fatty acid on growth during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

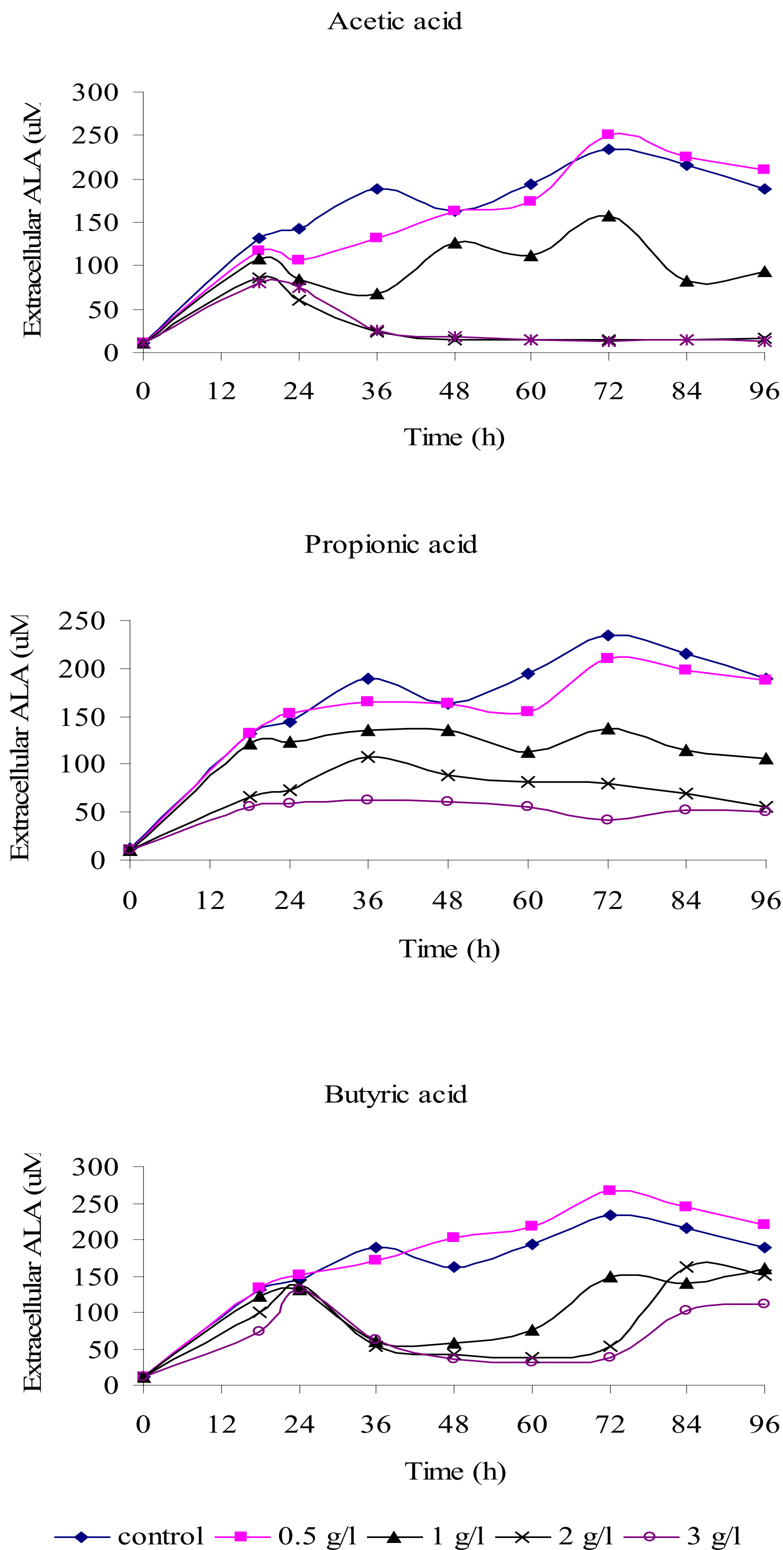


Figure 26 Effect of volatile fatty acid on extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

strongly suggested that ALA production might proceed via methylmalonyl-CoA pathway as follows: propionic acid → propionyl-CoA → methylmalonyl-CoA → succinyl-CoA → ALA (Sasaki *et al.,* 1978).

When studies on the effect of mix volatile fatty acid between butyric acid and propionic acid, cells did not grow under aerobic-dark condition. Therefore, the most suitable volatile fatty acid for extracellular ALA production from *R. capsulatus* SN28 was found to be 0.5 g/l butyric acid.

2.7 Effect of $MgCl_2.6H_2O$

The effect of $MgCl_2.6H_2O$ concentration (5-20 mM) on growth and ALA formation of *R. capsulatus* SN28 in GG medium without glutamate + 3 % NaCl with the of addition of 0.5 g/l butyric acid at the initial pH of 7.0 under aerobic–dark condition was investigated (Figure 27)**.** The pH of the culture broth decreased from 7.0 to 5.5 during the cultivation. Rapid growth occurred in the addition of $MgCl_2.6H_2O$ in the first 48 h of the cultivation and decreased with slower rate thereafter. The maximum dry cell weights of 5.33, 5.64, 5.46, 5.32 g/l were achieved in the presence of 5, 10, 15, 20 mM $MgCl_2.6H_2O$, respectively which were higher than the control (4.56 g/l dry cell weight). Magnesium is essential for sufficient bacterial growth and good metabolism. Availability of the Mg^{2+} ion plays a role, inter alia, in the phase and tempo of cell division of bacteria, as well as in the protection of the bacteria cell against negative factors such as heat shock (Getha *et al.,* 1998). Magnesium also plays a very important role in the stability and permeability of membranes (Nishio, 2002).

The most suitable $MgCl_2.6H_2O$ was found to be 15 mM in which the maximum extracellular ALA of 303.13 μM was achieved. The tetrapyrrole synthesis enzyme, ALA synthase, requires Mg^{2+} for catalytic activity in photosynthetic organisms. The addition of $MgCl_2.6H_2O$ at 0, 5, 10, 20 mM gave the ALA concentrations of 234.45, 249.94, 274.85 and 233.27 μM, respectively.

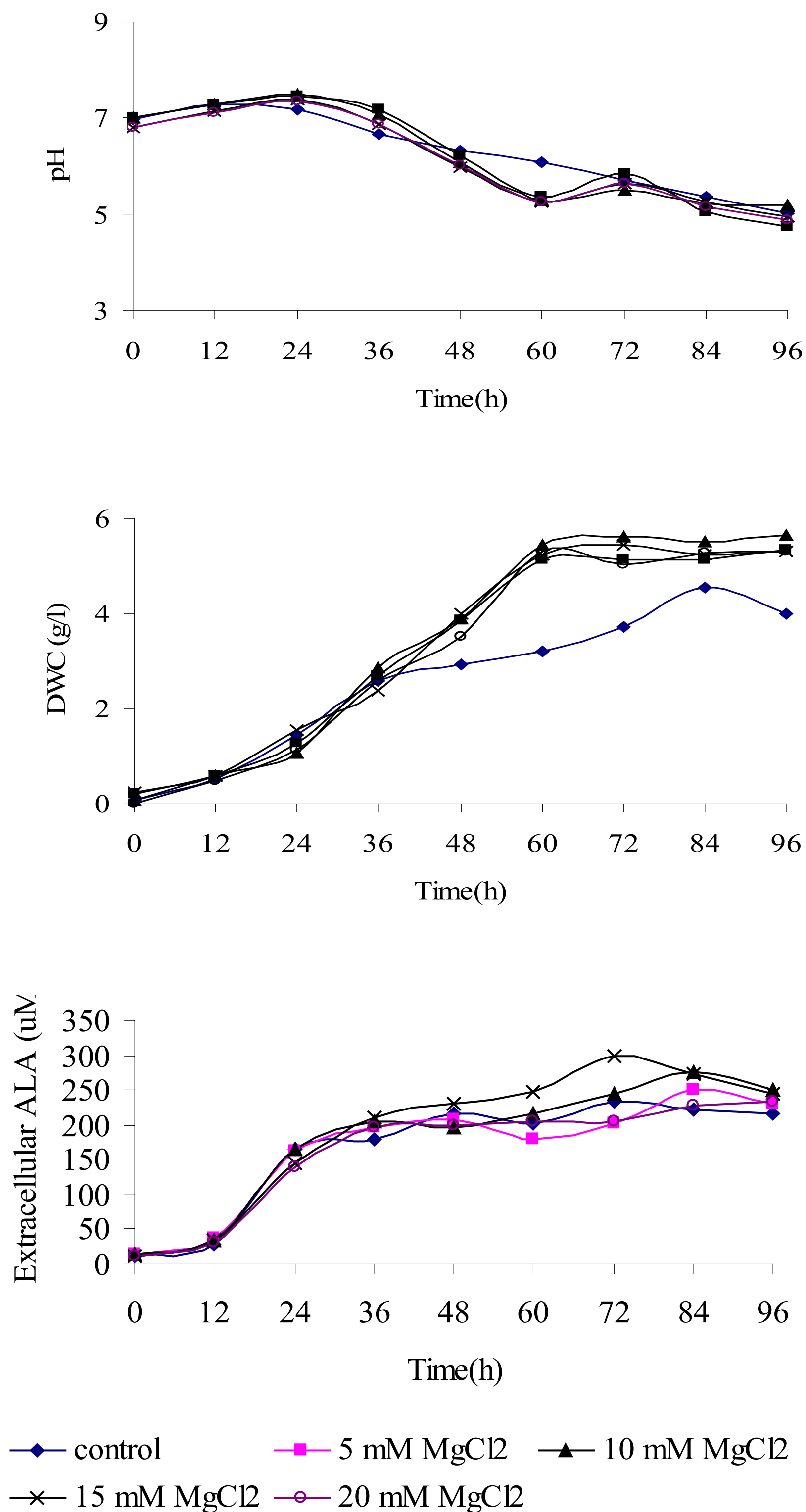


Figure 27 Effect of $MgCl_2$ on pH, growth and extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

2.8 Effect of pyridoxal phosphate

Pyridoxal phosphate (PLP) requiring enzymes act via the formation of a Schiff base between the amino acid and coenzyme in the enzymatic reaction of ALA synthase. The influence of pyridoxal phosphate concentration on growth and ALA formation of *R. capsulatus* SN28 in GG medium without glutamate + 3% NaCl with the addition of 0.5 g/l butyric acid and 15 mM $MgCl_2.2H_2O$ with the initial pH of 7.0 under aerobic–dark condition was studied (Figure 28). The results indicated that the pH of the culture broth in all pyridoxal phosphate concentrations gave the same pattern as pH decreased from 7.0 to 5.0 within 48 h cultivation and kept constant thereafter. The pyridoxal phosphate did not highly influence pH changes comparing with their control ($p < 0.05$). Cell growth at various concentrations of pyridoxal phosphate was almost the same as that with the control (5.7 g/l DCW). With the addition of 10, 20, 30 and 40 μM pyridoxal phosphate, the maximum dry cell weights were 5.70, 5.94, 5.68, 5.88 and 5.87 g/l. Whereas the maximum ALA production were 206.1, 197.1, 201.8 and 189.9 μM, respectively after 60 h cultivation which were all lower than the control (289.3 μM ALA). Thus, pyridoxal phosphate was not required to add into the medium. Moreover PLP is known to react with lysine residues and also an inhibitor of the active-site lysine of ALA synthase. Because the active site lysine residue is conserved in the primary structure of all ALA synthase (Zhang *et al*., 2002).

2.9 Effect of controlling pH

For the production of extracellular ALA by some microorganism including *R. sphaeroides,* the intracellular balance of ALA synthase and ALA dehydratase is important and the enzyme activities are influenced by the culture pH (Sasaki *et al*., 1993). Hence the influence of culture pH on growth, pH and extracellular ALA production from *R. capsulatus* SN28 was investigated in a 5 L bioreactor containing 3 L optimal medium with aeration rate of 0.5 vvm, aerobic speed of 200 rpm and incubation temperature of 37 C with and without control of pH (7.0) (Figure 29). When culture pH was adjusted to 7.0, *R. capsulatus* SN28 grew very well (6.96 g/l dry cell weight) and gave higher 107.5 μM extracellular

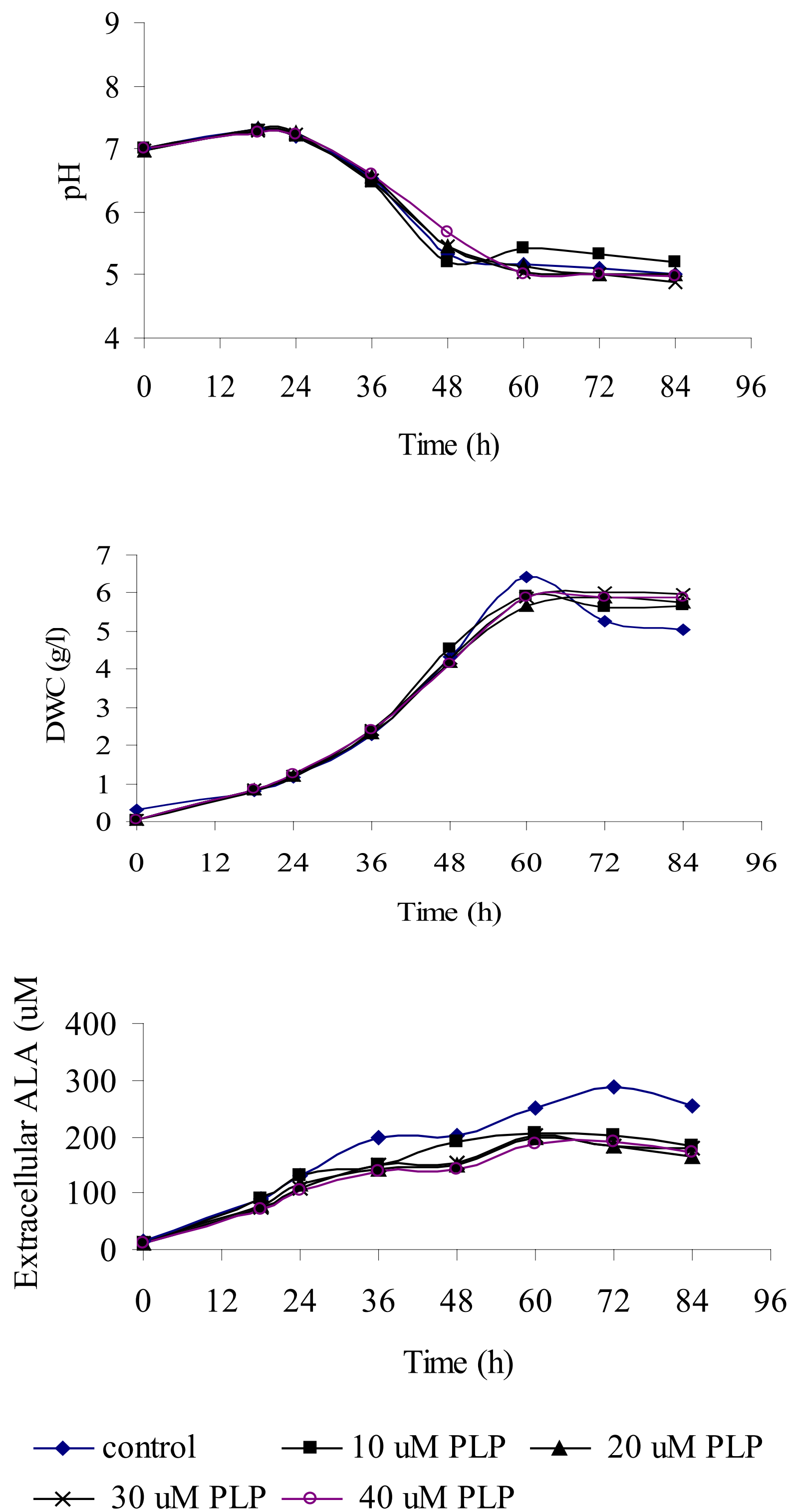
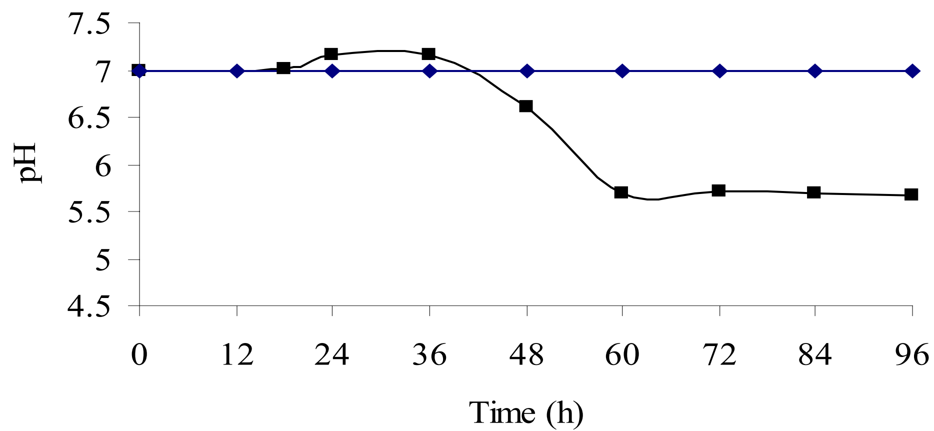
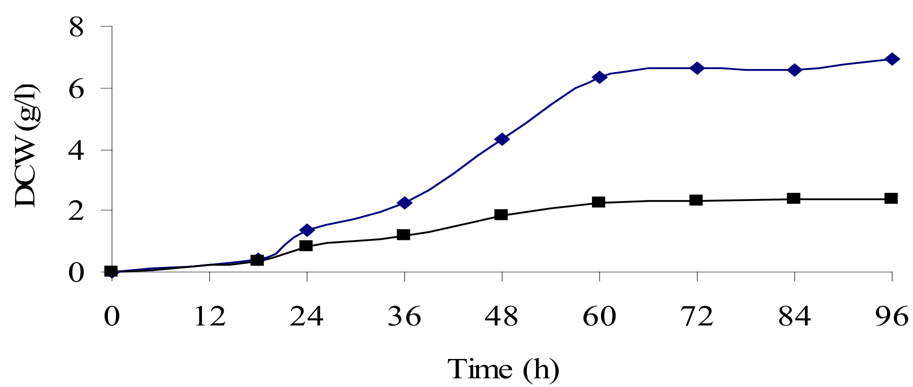
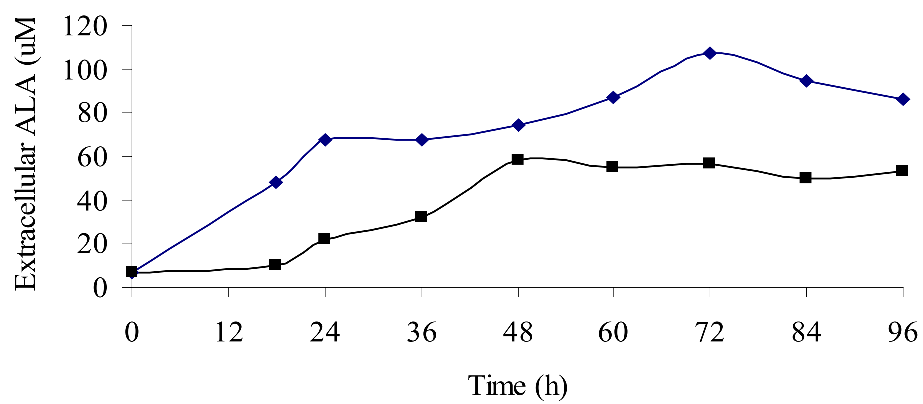


Figure 28 Effect of pyridoxal phosphate on pH, growth and extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C







control pH uncontrolled pH

Figure 29 Effect of controlling pH on pH, growth and extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

ALA concentration at 72 h after cultivation than the control. Without pH control in the bioreactor, the strain did not grow well (2.22 g/l dry cell weight) after the exponential growth phase as the culture pH decreased to 5.5. This directly affected the ALA production. ALA concentration (56.46 µM) was lower than that of the pH control, due to the culture pH in the bioreactor suppressed the secretion of ALA (Chung *et al.,* 2004).

The cultivation in 5 L gave the highest value of extracellular ALA which was 3 folds higher than that in the 250 ml flasks (289.35. µM) but dry cell weight in bioreactor was 1.7 folds (6.96 g/l dry cell weight) much higher than cultivation in the small scale (4.19 g/l dry cell weight). Moreover, the culture broth became red color but resulted in the red-brown color of the cultivation in the 250 ml flask. Thus, this results should be suggested that air supply strongly influences ALA production due to the inactivation of ALA synthase by oxygen, which is essential for cell growth (ATP formation) under aerobic conditions (Sasaki *et al*., 2002).

2.10 Effect of NaCl concentration

The influence of NaCl concentrations on growth, pH and extracellular ALA production from *R. capsulatus* SN2 was investigated using GG medium without glutamate with the addition of 0.5 g/l butyric acid and 15 mM $MgCl_2.2H_2O$ at the initial pH of 7.0 under aerobic–dark condition (Figure 30). Without addition of NaCl in the medium, cell did not grow. This was found to be abnormal in the cultivation of *R. capsulatus* SN28, so the cultivation were repeated, but *R. capsulatus* SN28 hardly grew without NaCl addition in GG medium. It could be suggested that its wildtype *R. capsulatus* SS3 was halotolerant, therefore, the strain preferred to grow in the culture medium containing 3%NaCl (30 ppt salinity nearly to seawater) (Sattayasmithstid, 2001). When addition of 1, 2 and 3 %NaCl in the medium, cells grew well and dry cell

weight were 5.10, 6.27 and 6.35 g/l , respectively and the pH of the culture broth in all NaCl concentrations gave the same pattern as pH decreased from 7.0 to 5.3

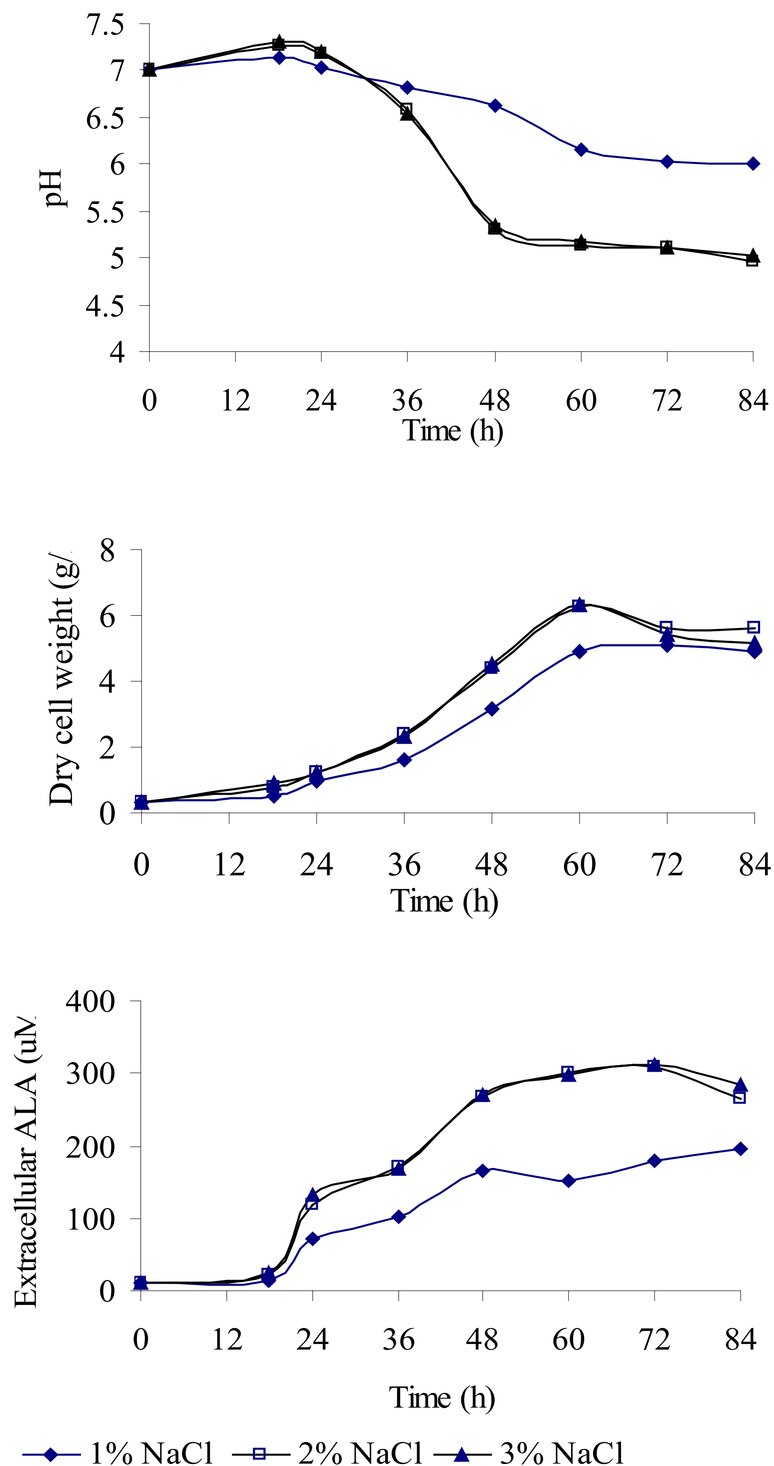


Figure 30 Effect of NaCl concentrations on pH, growth and extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 in GG medium under aerobic-dark condition at 37°C

within 48 h cultivation and kept rather constant thereafter. NaCl at 1% concentration gave decrease the pattern of pH slower than higher concentrations of NaCl (2 and 3% NaCl). However the lesser concentration of ALA, 196.34 µM, was produced in 1 %NaCl than using 2 and 3 %NaCl (308.74 and 312.15 µM, respectively) with no significant difference ($p<0.05$). Thus, the most suitable concentration of NaCl was found to be 2%.

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

Effect of six different media (GM medium, GG medium, MGG medium, MGS medium, MGSY medium and optimized medium) on pH growth and ALA production from *R. capsulatus* SN28 cultivated under aerobic-dark condition at 37 °C was investigated. The results were given in Figure 31 and kinetic parameters were presented in Table 12.

The pH of the culture broth in GM, GG, MGG and MGSY media increased from pH 7.0 to 8.5 except optimized medium which decreased from pH 7.0 to 5.5 after 96 h cultivation and MGS medium which increased slowly from pH 7.00 to 7.83 within 36 after cultivation, then decreased slowly to pH 7.51 at the end of cultivation. From this results, it could be suggested that GM GG, MGG and MGSY medium have contained glutamate which can transfer via TCA cycle to free ammonia (Pfenning, 1967), so pH of the culture tends to move to alkaline value while without glutamate in the optimized medium, pH of the culture tends to move to acidity.

The maximum growth rate (X_{max}) was 2.09, 3.92, 4.13, 0.415, 1.237 and 5.24 g/l, the maximum specific cell growth (μ_m) was 0.07, 0.09, 0.08, 0.03, 0.05 and 0.08 h^{-1}. The maximum extracellular ALA was 69.49, 98.14, 81.25, 5.49, 40.26 and 310.54 µM, respectively whereas the maximum productivity (R_m) was 0.97, 1.64, 1.52, 0.09, 0.42 and 6.45 µM ALA/h.

Extracellular ALA production from *R. capsulatus* SN28 in GG medium was compared with the modified medium, MGG medium under aerobic-dark condition. This modified medium used commercial monosodium glutamate (MSG) to replace glutamate, to reduce the cost of medium. The maximum extracellular ALA in

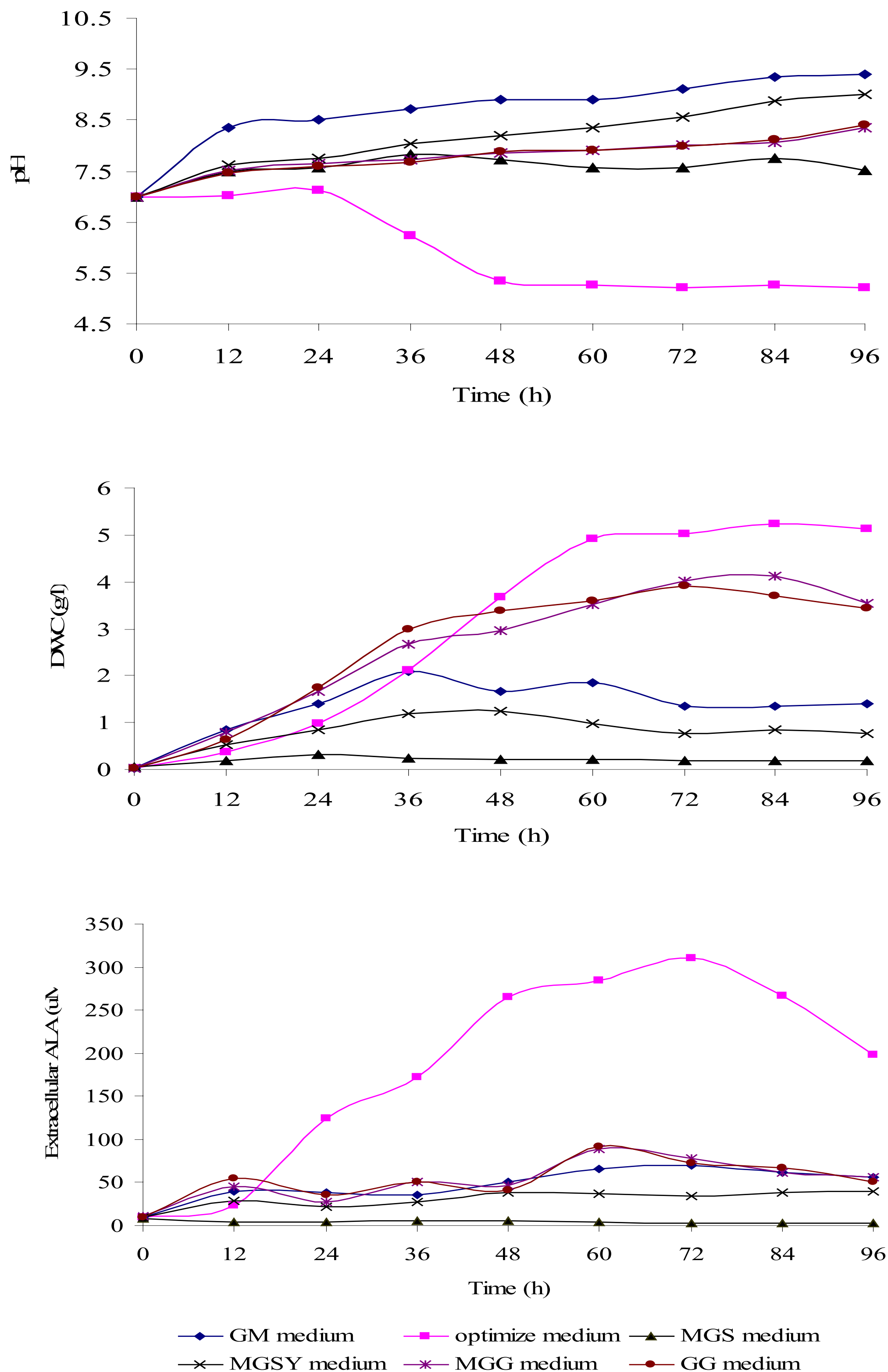


Figure 31 Effect of different medium on pH, growth and extracellular ALA formation during cultivation of *Rhodobacter capsulatus* SN28 under aerobic-dark condition at 37°C

Table 12 Comparison on kinetic parameters of *Rhodobacter capsulatus* SN28 during cultivation in analytical and commercial grade medium

Parameter	Unit	Medium					
		GM*	Opt**	GG***	MGG****	MGS*****	MGSY******
x_{max}	g/l	2.09	5.24	3.92	4.13	0.415	1.237
μ_m	h^{-1}	0.07	0.09	0.08	0.08	0.03	0.05
Extracellular ALA							
ALA_{max}	µM	69.49	310.54	98.14	91.25	5.49	40.26
R_m	µM ALA/h	0.97	6.47	1.64	1.52	0.09	0.42
ALA cost	Baht/ µM	1.08	0.19	0.71	2.72	1.16	0.41
Intracellular ALA							
ALA_{max}	µM/g cell	1.13	1.16	0.98	nd	nd	nd
R_m	µM ALA/h	0.047	0.041	0.048	nd	nd	nd
ALA cost	Baht/ µM	66.45	51.82	70.97	nd	nd	nd

GM* = Glutamate-malate medium, GG** = Glutamate-glucose medium, Opt*** = optimized medium,
MGG**** = Monosodium glutamate-glucose medium, MGS*****= (monosodium glutamate, sugar and commercial NaCl grade), MGSY****** = (MGS with the addition of yeast extract 2 g/l)

the GG medium was almost the same as that in the MGG medium (98.14 and 91.25 μM, respectively). Therefore, the commercial monosodium glutamate (MSG) gave the same result as analytical grade glutamate and could be used in optimal medium using commercial grade NaCl for large scale production. This would be beneficial in reducing the production cost as the price of MSG was 22.5 times lower than the analytical grade glutamate.

The specific growth and the maximum ALA productivity from the two types of medium; MGS medium (monosodium glutamate, sugar and commercial NaCl grade), and MGSY medium (MGS medium with the addition of yeast extract) were very low. These results suggested that *R. capsulatus* SN28 strongly requires yeast extract for growth and ALA production. This is similar to the report that yeast extract mainly contributed tothe cell growth under aerobic-dark condition for the purple non-sulfer bacteria (http:// www.diakvet.com/indiana-biolab/b410.htm). Furthermore, the color of the MGS medium gave pale pink color while the others gave deep red color. The yeast extract probably yielded a higher proportion of red pigment, relative to the medium which added yeast extract. On the other hand, cells grown on the medium which yeast extract added gave slightly higher production of red soluble pigments (Kilikian *et al*., 2001).

When comparison on the ALA cost in each medium, it was found that optimized medium gave only 0.19 Baht/μM for extracellular ALA production in optimized medium, while GM medium, GG medium, MGG medium, MGS medium and MGSY medium gave 1.08, 0.71, 2.72, 1.16 and 0.41 baht/μM, respectively. Thus, the optimized medium would be beneficial in reducing the production cost.