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Extracellular 5-Aminolevulinic Acid Production by *Escherichia coli* Containing the *Rhodopseudomonas palustris* KUGB306 *hemA* Gene

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The *Rhodopseudomonas palustris* KUGB306 *hemA* gene codes for 5-aminolevulinic acid (ALA) synthase. This enzyme catalyzes the condensation of glycine and succinyl-CoA to yield ALA in the presence of the cofactor pyridoxal 5'-phosphate. The *R. palustris* KUGB306 *hemA* gene in the pGEX-KG vector system was transformed into *Escherichia coli* BL21. The effects of physiological factors on the extracellular production of ALA by the recombinant *E. coli* were studied. Terrific Broth (TB) medium resulted in significantly higher cell growth and ALA production than did Luria-Bertani (LB) medium. ALA production was significantly enhanced by the addition of succinate together with glycine in the medium. Maximal ALA production (2.5 g/l) was observed upon the addition of D-glucose as an ALA dehydratase inhibitor in the late-log culture phase. Based on the results obtained from the shake-flask cultures, fermentation was carried out using the recombinant *E. coli* in TB medium, with the initial addition of 90 mM glycine and 120 mM succinate, and the addition of 45 mM D-glucose in the late-log phase. The extracellular production of ALA was also influenced by the pH of the culture broth. We maintained a pH of 6.5 in the fermenter throughout the culture process, achieving the maximal levels of extracellular ALA production (5.15 g/l, 39.3 mM).

Keywords: Extracellular production, 5-aminolevulinic acid, *Escherichia coli*, *Rhodopseudomonas palustris*, *hemA*

5-Aminolevulinic acid (ALA) is a precursor of the tetrapyrroles, which include heme, porphyrin, chlorophyll, and vitamin B_{12}, and are synthesized *via* porphobilinogen. ALA is a key metabolic intermediate in the regulation of tetrapyrrole biosynthesis [7, 27]. ALA can be biosynthesized through two major pathways [6]. One is the C_4 pathway, which is present in mammals, birds, yeast, some protozoa, and purple nonsulfur photosynthetic bacteria. In the C_4 pathway, the pyridoxal 5'-phosphate-dependent enzyme ALA synthase [ALAS; succinyl-CoA: glycine succinyl transferase (decarboxylating), E.C. 2.3.1.37] catalyzes the condensation of succinyl-CoA and glycine to yield ALA. The second route is the C_5 pathway, which occurs in higher plants, algae, and in many bacteria, including *Escherichia coli* [12] and the archaea. In the C_5 pathway, ALA is formed from glutamate *via* three enzymatic steps. Recently, ALA has received a great deal of attention, largely due to its potential use as an herbicide, insecticide, antimicrobial drug, and photosensitizer for photodynamic therapy [11, 14, 17, 18, 20]. However, until recently, mass production of ALA was a difficult proposition, owing to the numerous steps required for its chemical synthesis [25]. Therefore, many studies have sought to develop an improved method for the biological production of ALA. Using a *Rhodobacter sphaeroides* mutant selected after nitrosoguanidine treatment, ALA was obtained at 20 mM in 42 h *via* aerobic dark fermentation [16]. However, strain improvement by mutation and screening is inefficient and time-consuming. Recently, most researchers have preferred metabolic engineering to mutation for improving ALA production. Various recombinant strains of *E. coli*, which lacks native ALAS, harboring the *hemA* gene mainly from *R. sphaeroides* have been engineered, and their abilities to produce ALA have been investigated [1, 4, 23, 26, 28, 29] (Table 1). Using factorial design optimization to improve ALA synthase activity, the yield of ALA reached 5.2 g/l using recombinant *E. coli* containing the *R. sphaeroides hemA* gene [29].

Previously, we have cloned and sequenced the *hemA* gene encoding for ALAS from *Rhodopseudomonas palustris* KUGB306, and characterized the properties of the recombinant

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Table 1. Production of 5-aminolevulinic acid (ALA) by recombinant strains of *E. coli*.

Host strain	*hemA* origin	Extracellular ALA (g/l)	Reference
E. coli CGSC4676	*R. sphaeroides*	3.8	[28]
E. coli MG1655	*R. sphaeroides*	5.2	[29]
E. coli Rosetta (DE3)	*R. sphaeroides*	3.8	[4]
E. coli BL21(DE3)	*B. japonicum*	3.2	[1]
E. coli BL21	*R. palustris* KUGB306	5.15	This study

enzyme expressed in *E. coli* [2]. The sequence of this *hemA* gene showed 65% identity with the *hemA* gene from *R. sphaeroides*. This difference will possibly bring a marked difference in the kinetics of the enzyme. The recombinant ALAS from *R. palustris* KUGB306 exhibits more favorable characteristics than does the recombinant ALAS from *R. sphaeroides*: the former possesses higher specific activity than the latter [2].

We constructed an *E. coli* strain transformed with a plasmid carrying the *hemA* gene encoding for ALAS from *R. palustris* KUGB306. In this study, we report the extracellular production of ALA with recombinant *E. coli* harboring *R. palustris* KUGB306 *hemA*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The culture conditions for *R. palustris* KUGB306 were described previously [3]. *E. coli* DH5α was used as the host for the recombinant plasmids. *E. coli* BL21 and pGEX-KG [5] were used as the expression host and plasmid, respectively. *E. coli* strains were grown in Luria-Bertani (LB) medium [19] or Terrific Broth (TB medium) [19]. The growth medium was supplemented with the antibiotic ampicillin (100 μg/ml), whenever necessary. Cultures were grown at 37°C on a rotary shaker at 200 rpm. Shake-flask studies were conducted in 250-ml shake-flasks containing 50 ml of medium. The optimal shake-flask results were used to conduct a controlled fermentation (Fermenter KF-5L; KoBioTech, Korea) [10, 22, 24]. A 5-l fermenter contained 2 l of medium, and was inoculated with 100 ml of a shake-flask culture grown until the optical density at 600 nm (OD_{600}) was 0.8–1.0. The fermenter was operated at 400 rpm, 37°C, an air flow rate of 1 l/min, and a pH of 6.5 that was controlled using 5 N HCl and 5 N NaOH.

Chemicals

All chemicals were of analytical grade.

General Recombinant DNA Techniques

The genomic DNA from *R. palustris* KUGB306 was isolated using the QIAGEN Genomic-tip (Qiagen, Hilden, Germany). Plasmid DNA was purified from *E. coli* strains using a High Pure Plasmid Isolation Kit (Roche, Penzberg, Germany). DNA fragments were recovered from agarose gels with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, U.S.A.). Other general DNA manipulation procedures were carried out as described by Sambrook and Russell [19].

Plasmid Construction for Overexpression in *E. coli*

The complete coding sequence for ALAS was amplified by PCR using Cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The genomic DNA of *R. palustris* KUGB306 was used as a template. The 5' primer, 5'-CGG GAT CCA TGG ATT ACA CCA AGT TCT TCG-3', and the 3' primer, 5'-CGG AAT TCT CGG TTG AAC TTA TTC CGC AG-3', contained (underlined) BamHI and EcoRI restriction sites. PCR was carried out as follows; initial denaturation at 95°C for 1 min, followed by 25 cycles of amplification (95°C for 45 s, 50°C for 45 s, and 72°C for 2.5 min) and an additional extension step at 72°C for 10 min. The amplified PCR product was then digested with these two restriction enzymes, separated by agarose gel electrophoresis, and purified from the gel. A DNA fragment of approximately 1.23-kb, which contained the ALAS structural gene, was cloned downstream of the *tac* promoter of the BamHI-EcoRI-treated pGEX-KG expression vector. The constructed plasmid was then designated as pGEX-hemA.

Analysis of ALA

Extracellular ALA was analyzed by the method described by Mauzerall and Granick [15]. Specifically, 0.5 ml of either sample or standard was mixed with 0.5 ml of 1 M sodium acetate buffer (pH 4.7), 50 μl of acetylacetone (2,4-pentanedione) was added, and then the mixtures were boiled in a water bath for 15 min. After cooling, 1 ml of freshly prepared modified Ehrlich's reagent was added, and 15 min later, the absorbance at 555 nm was measured. All assays were carried out in triplicate.

RESULTS AND DISCUSSION

Effects of LB and TB Media on the Extracellular Production of ALA During Growth

We first attempted to determine the differences between LB medium and TB medium with regard to cell growth and the extracellular production of ALA, using *E. coli* BL21 transformed with pGEX-hemA. TB medium supports a great cell density, and in the case of *E. coli*, it maintains the growth in the logarithmic phase for a long time. As a result, it yields a greater number of recombinant proteins and plasmid DNA. Unlike LB medium, TB medium is buffered with 89 mM phosphate, which prevents cell death due to a drop in pH and contains glycerol as a carbon and energy source. As shown in Fig. 1, the use of TB medium resulted in significantly higher cell growth and ALA production than did LB medium. Therefore, TB medium was selected for subsequent studies.

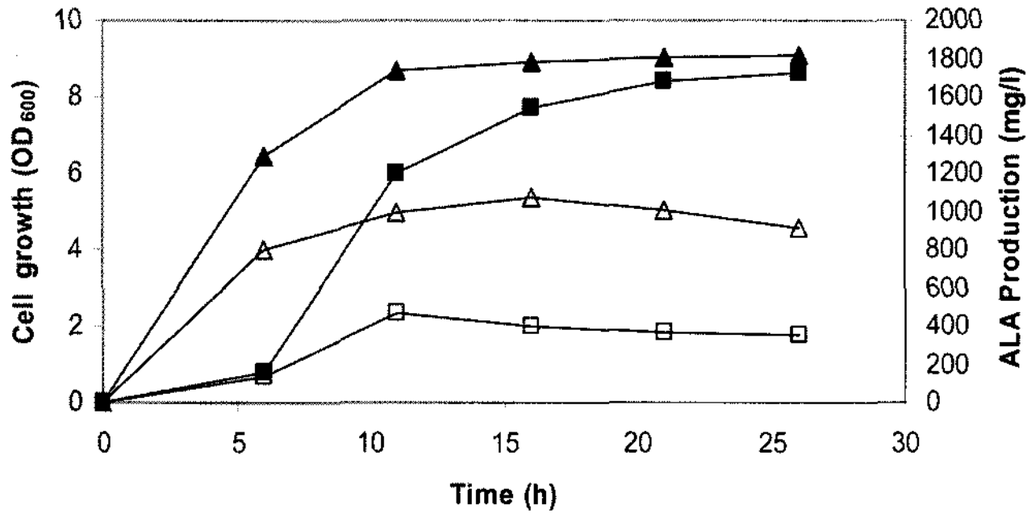

Fig. 1. Effect of different media on the extracellular ALA production of recombinant *E. coli*.
Glycine (30 mM) and succinate (90 mM) were added at the beginning of cultivation. Symbols ▲, cell growth in TB medium; △, cell growth in LB medium; ■, extracellular ALA production in TB medium; □, extracellular ALA production in LB medium. The values shown are averages of triplicate experiments.

Effects of Glycine and Succinate on the Extracellular Production of ALA During Growth

It has been reported in several studies that glycine and succinate enhance ALAS activity as precursor or growth substrates for ALA synthesis in *Rhodobacter*. In the C_4 (ALA synthesis) pathway, succinyl-CoA and glycine are condensed by ALAS. Therefore, we examined the effect of the concentration of growth substrates on ALA production levels with *E. coli* BL21/pGEX-hemA. A significant effect was observed on ALA production as the result of the addition of glycine, with succinate (60 mM) in the medium, but not with glycine alone (Fig. 2). Maximal ALA production was observed with 90 mM glycine in the medium, without a significant concomitant effect on cell growth. ALA production was also enhanced by the addition of 120 mM succinate, together with 90 mM glycine in the medium (data not shown).

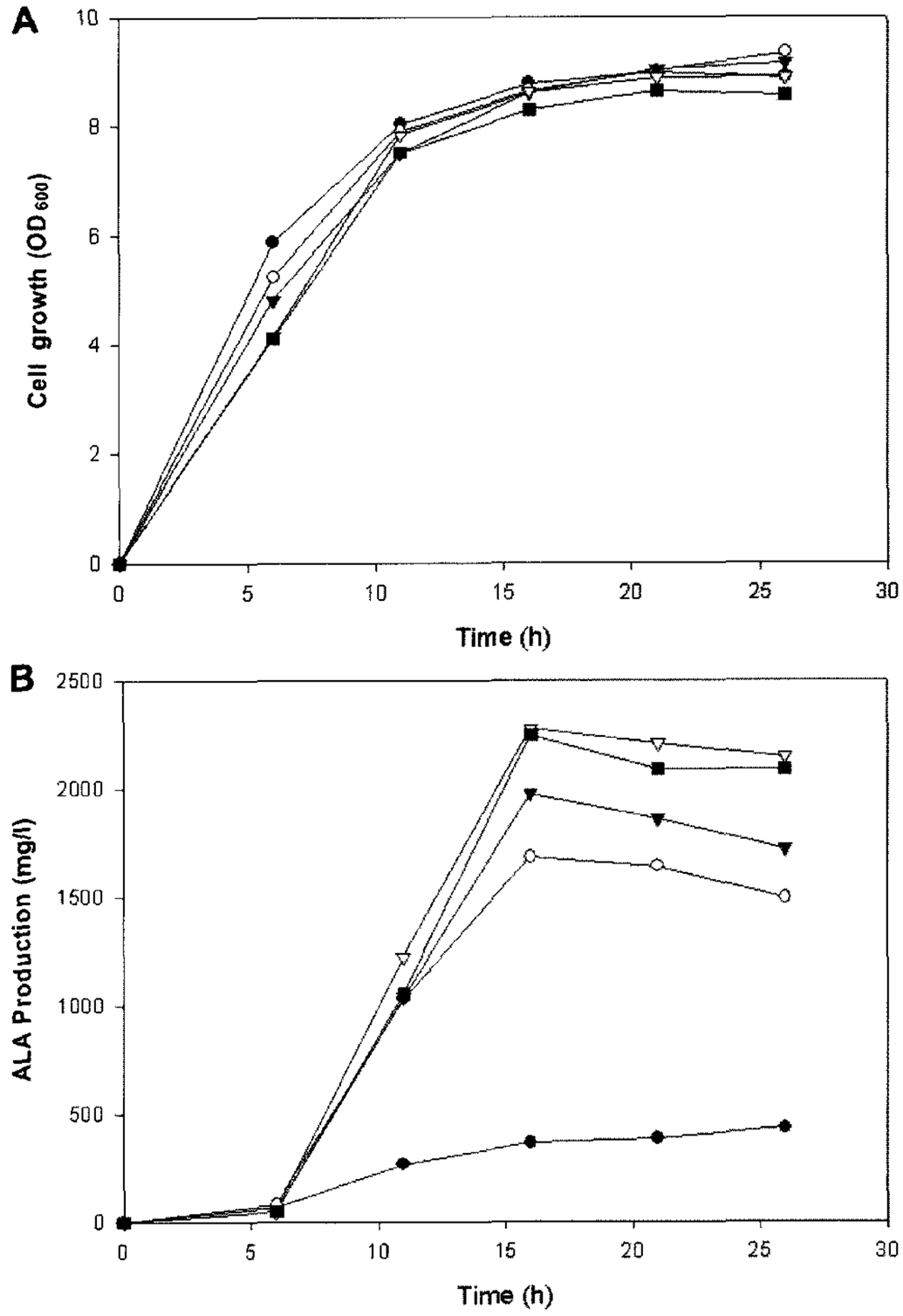

Fig. 2. Effect of glycine with succinate on the extracellular ALA production of recombinant *E. coli*.
A. Cell growth. **B**. Extracellular ALA production. Each concentration of glycine and 60 mM succinate were added at the beginning of cultivation. Symbols used for glycine concentration ●, no addition; ○, 30 mM addition; ▼, 60 mM addition; ▽, 90 mM addition; ■, 120 mM addition. The values shown are averages of triplicate experiments.

Effect of ALA Dehydratase Inhibitor on the Extracellular Production of ALA During Growth

For the high-yield production of ALA, the inhibition of ALA dehydratase (ALAD) is essential. ALAD is a metalloprotein enzyme, which converts ALA into porphobilinogen (Fig. 3). ALAD can be inhibited by using either substrate or product analogs [13]. Utilizing the most widely used substrate analog, levulinic acid (LA), high amounts of ALA could be produced from a variety of microorganisms [1, 21]. Enhanced ALA production by repeated additions of LA was also reported [20]. Recently, it was reported that ALAD could be inhibited using D-glucose in recombinant *E. coli* [8]. At the concentration of 10 mM, its inhibitory activity was over 90% on ALAD purified from *E. coli* containing the *Bradyrhizobium japonicum hemA* gene. The maximal ALA production, 3.8 g/l, was obtained by adding 28 mM of D-glucose at the late-log culture phase [8]. In another report of ALA production with *E. coli* Rosetta (DE3), a rare codon optimizer, containing the *hemA* gene from *R. sphaeroides*, the maximal ALA production was obtained by adding glucose at the late stage of fermentation [4]. When D-glucose was added in late-log culture phase, the recombinant *E. coli* could not keep transporting succinate, which would consequently reduce the carbon flow of succinate+glycine to ALA. However, the inhibitory effect of glucose on ALAD could be more essential to extracellular ALA production [4, 8]. It was also reported that the concentration of glycine in the medium was more important than that of succinate on ALA production [4]. D-Glucose is non-toxic and even less expensive than LA. Therefore, we assessed the effects of LA and D-glucose on the ALA production levels of *E. coli* BL21/pGEX-hemA. Similar ALA production was observed with the addition of LA (30 mM) and the addition of D-glucose (30 mM) (data not shown). Therefore, D-glucose was selected as an ALAD

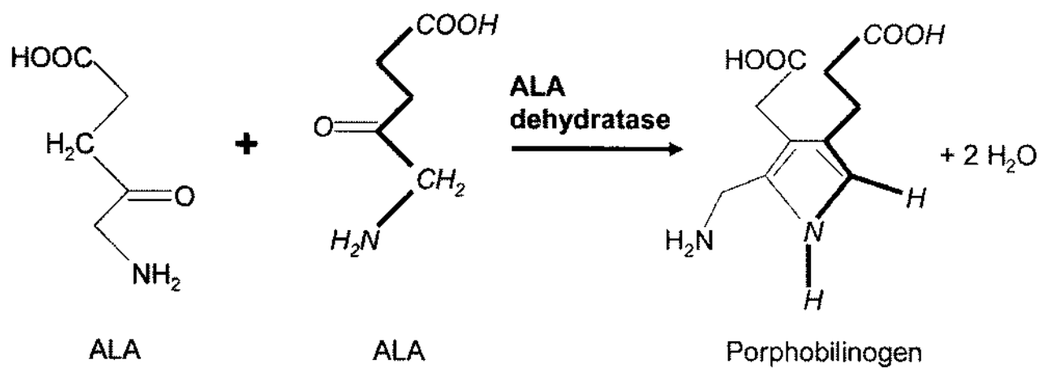

Fig. 3. Reaction mechanism of ALA dehydratase (ALAD).
ALAD catalyzes the asymmetric formation of one molecule of porphobilinogen from two molecules of ALA.

inhibitor for subsequent studies. Maximal ALA production (2.5 g/l) was observed upon the addition of 45 mM D-glucose in the late-log culture phase (Fig. 4).

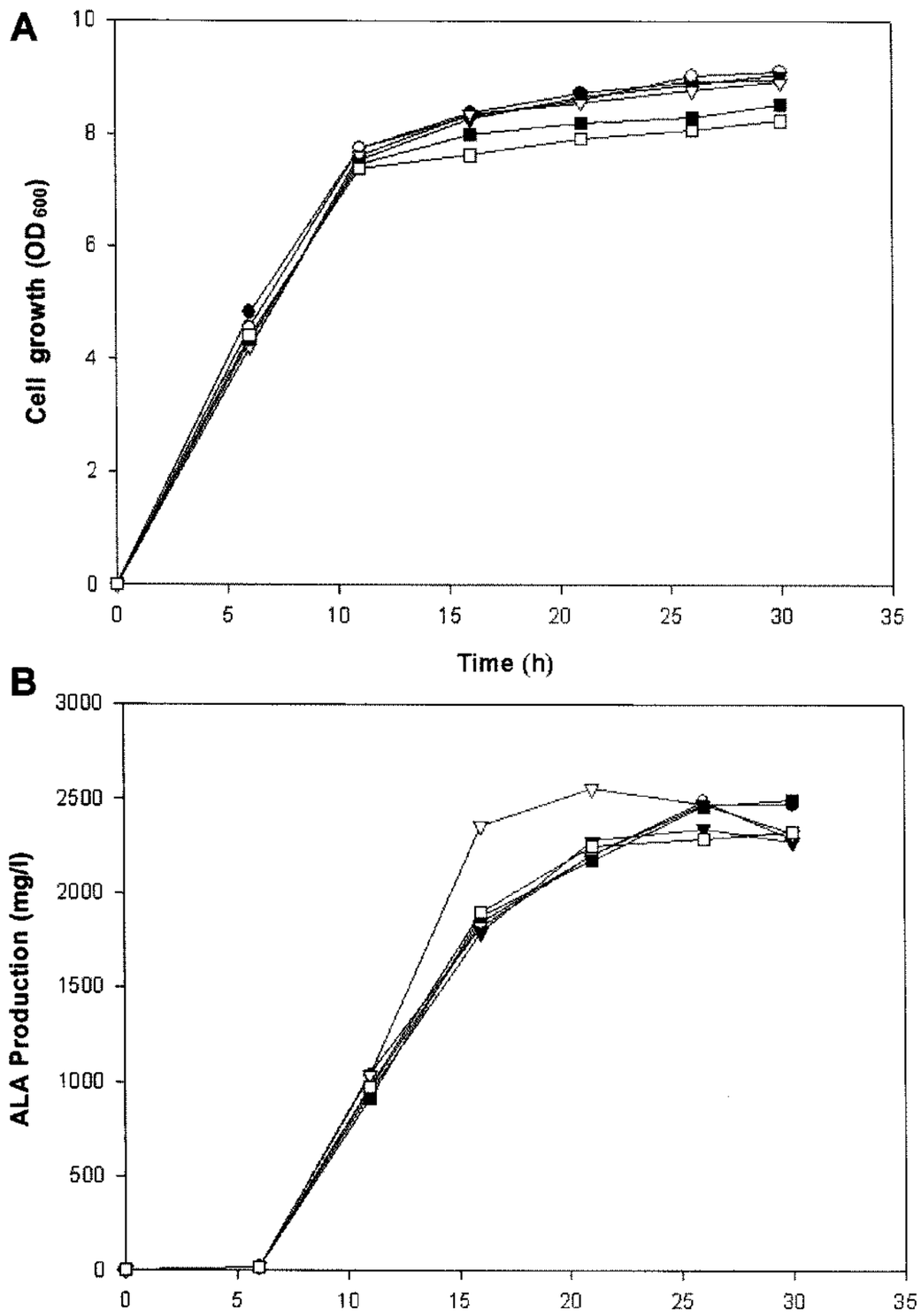

Fig. 4. Effect of glucose (ALAD inhibitor) on the extracellular ALA production of recombinant *E. coli*.
A. Cell growth. **B**. Extracellular ALA production. Glycine (90 mM) and succinate (120 mM) were added at the beginning of cultivation. Each concentration of D-glucose was added at the late-log culture phase (11 h). Symbols used for D-glucose concentration ●, no addition; ○, 15 mM addition; ▼, 30 mM addition; ▽, 45 mM addition; ■, 60 mM addition; □, 90 mM addition. The values shown are averages of triplicate experiments.

Effect of pH Control on the Extracellular Production of ALA During Fermentation

Based on the results obtained from the shake-flask cultures, fermentation was carried out using BL21/pGEX-hemA in TB medium, with the initial addition of 90 mM glycine and 120 mM succinate, and the addition of 45 mM D-glucose at the late-log phase. The ALA production is significantly affected by the pH of the culture broth. Extracellular ALA production with recombinant *E. coli* containing the *B. japonicum hemA* gene was significantly enhanced when the pH was maintained at 6.0–7.0 (maximal ALA production at 6.5) compared with that at lower (up to 5.5) or higher (up to 8.0) pH values [9]. At the pH of 6.5, maximal ALA production was reported with *E. coli* harboring the *R. sphaeroides hemA* gene [4, 29]. They all used LB as culture media. Unlike LB, the TB medium that we used is buffered with phosphate. When pH control was not performed in the fermenter, the culture pH was maintained in a range of 6.0 and 7.0 during the cultivation. When the pH was maintained at 6.5, ALA production was slightly enhanced compared with that of no pH control (Fig. 5). In this study, we maintained a pH of 6.5 in the fermenter during the culture process, achieving maximal extracellular ALA production levels (5.15 g/l, 39.3 mM) (Fig. 5). Table 1 shows the comparison of our yield with those in previous reports. Our yield is, in fact, similar to the highest level reported recently through media and process optimization, using a statistical design approach [29]. However, we achieved our maximal levels without the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), an expensive chemical inducer. Additional optimization studies may permit a more economically feasible process with regard to the industrial production of ALA.

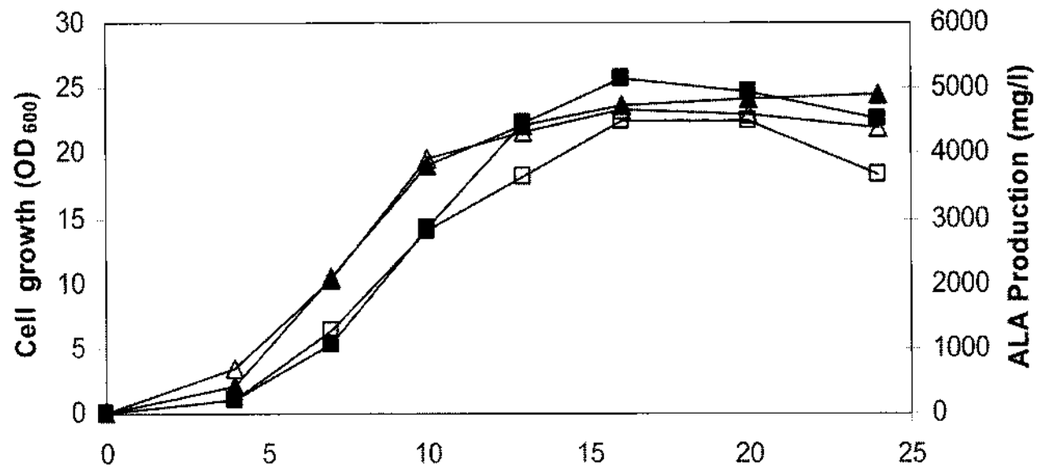

Fig. 5. Effect of pH control on the extracellular ALA production of recombinant *E. coli* during fermentation.
Glycine (90 mM) and succinate (120 mM) were added at the beginning of cultivation. D-glucose (45 mM) was added at the late-log culture phase (10 h). Symbols △, cell growth by no pH control; ▲, cell growth by pH control (6.5); □, extracellular ALA production by no pH control; ■, extracellular ALA production by pH control (6.5). The values shown are averages of triplicate experiments.

Acknowledgments

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References

1. Choi, C., B. S. Hong, H. C. Sung, H. S. Lee, and J. H. Kim. 1999. Optimization of extracellular 5-aminolevulinic acid production from *Escherichia coli* transformed with ALA synthetase gene of *Bradyrhizobium japonicum*. *Biotechnol. Lett.* **21:** 551–554.
2. Choi, H. P., J. W. Hong, K. H. Rhee, and H. C. Sung. 2004. Cloning, expression, and characterization of 5-aminolevulinic acid synthase from *Rhodopseudomonas palustris* KUGB306. *FEMS Microbiol. Lett.* **236:** 175–181.
3. Choi, H. P., H. J. Kang, H. C. Seo, and H. C. Sung. 2002. Isolation and identification of photosynthetic bacterium useful for wastewater treatment. *J. Microbiol. Biotechnol.* **12:** 643–648.
4. Fu, W., J. Lin, and P. Cen. 2007. 5-Aminolevulinate production with recombinant *Escherichia coli* using a rare codon optimizer host strain. *Appl. Microbiol. Biotechnol.* **75:** 777–782.
5. Guan, K. L. and J. E. Dixon. 1991. Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192:** 262–267.
6. Jordan, P. M. 1991. Biosynthesis of tetrapyrroles, pp. 1–24. *In* A. Neuberger and L. L. M. van Deenen (eds.). *New Comprehensive Biochemistry*, Vol. 19. Elsevier, Amsterdam, The Netherlands.
7. Lascelles, J. 1978. Regulation of pyrrole synthesis, pp. 795–808. *In* R. K. Clayton and W. R. Sistrom (eds.), *The Photosynthetic Bacteria*. Plenum Press, New York.
8. Lee, D. H., W. J. Jun, K. M. Kim, D. H. Shin, H. Y. Cho, and B. S. Hong. 2003. Inhibition of 5-aminolevulinic acid dehydratase in recombinant *Escherichia coli* using D-glucose. *Enzyme Microb. Technol.* **32:** 27–34.
9. Lee, D.-H., W.-J. Jun, D.-H. Shin, H.-Y. Cho, and B.-S. Hong. 2005. Effect of culture conditions on production of 5-aminolevulinic acid by recombinant *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **69:** 470–476.
10. Lee, S.-Y. and D.-I. Kim. 2006. Perfusion cultivation of transgenic *Nicotiana tabacum* suspensions in bioreactor for recombinant protein production. *J. Microbiol. Biotechnol.* **16:** 673–677.
11. Levy, J. G. 1995. Photodynamic therapy. *Trends Biotechnol.* **13:** 14–18.
12. Li, J. M., O. Brathwaite, S. D. Cosloy, and C. S. Russel. 1989. 5-Aminolevulinic acid synthesis in *Escherichia coli*. *J. Bacteriol.* **171:** 2547–2552.
13. Luond, R. M., J. Walker, and R. W. Neier. 1992. Assessment of the active site requirements of 5-aminolevulinic acid dehydratase: Evaluation of substrate and product analogues as competitive inhibitors. *J. Org. Chem.* **57:** 5005–5013.
14. Malik, Z., J. Hanania, and Y. Nitzan. 1990. New trends in photobiology: Bactericidal effect of photoactivated porphyrins – an alternative approach to antimicrobial drugs. *J. Photochem. Photobiol. B Biol.* **5:** 281–293.
15. Mauzerall, D. and S. Granick. 1956. The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. *J. Biol. Chem.* **219:** 435–446.
16. Nishikawa, S., K. Watanabe, T. Tanaka, N. Miyachi, Y. Hotta, and Y. Murooka. 1999. *Rhodobacter sphaeroides* mutants which accumulate 5-aminolevulinic acid under aerobic and dark conditions. *J. Biosci. Bioeng.* **87:** 798–804.
17. Rebeiz, C. A., J. A. Juvik, and C. C. Rebeiz. 1988. Porphyric insecticides. 1. Concept and phenomenology. *Pesticide Biochem. Physiol.* **30:** 111–127.
18. Rebeiz, C. A., A. Montazer-Zouhoor, J. M. Mayasich, B. C. Triphthy, S. M. Wu, and C. C. Rebeiz. 1988. Photodynamic herbicides: Recent development and molecular basis of selectivity. *CRC Crit. Rev. Plant. Sci.* **6:** 385–406.
19. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
20. Sasaki, K., T. Tanaka, and S. Nagai. 1998. Use of photosynthetic bacteria for the production of SCP and chemicals from organic wastes, pp. 247–291. *In* A. M. Martin (ed.), *Bioconversion of Waste Materials to Industrial Products*. Blackie Academic and Professional, London, England.
21. Sasikala, Ch., Ch. V. Ramana, and P. R. Rao. 1994. 5-Aminolevulinic acid: A potential herbicide/insecticide from microorganisms. *Biotechnol. Prog.* **10:** 451–459.
22. Seo, H.-P., K.-I. Jo, C.-W. Son, J.-K. Yang, C.-H. Chung, S.-W. Nam, S.-K. Kim, and J.-W. Lee. 2006. Continuous production of pullulan by *Aureobasidium pullulans* HP-2001 with feeding of high concentration of sucrose. *J. Microbiol. Biotechnol.* **16:** 374–380.
23. Shin, J.-A., Y. D. Kwon, O.-H. Kwon, H. S. Lee, and P. Kim. 2007. 5-Aminolevulinic acid biosynthesis in *Escherichia coli* coexpressing NADP-dependent malic enzyme and 5-aminolevulinate synthase. *J. Microbiol. Biotechnol.* **17:** 1579–1584.
24. Song, S. K., Y.-S. Jeong, P.-H. Kim, and G.-T. Chun. 2006. Effects of dissolved oxygen level on avermectin B1a production by *Streptomyces avermitilis* in computer-controlled bioreactor cultures. *J. Microbiol. Biotechnol.* **16:** 1690–1698.
25. Takeya, H., T. Tanaka, T. Hotta, and K. Sasaki. 1997. Production methods and applications of 5-aminolevulinic acid. *Porphyrins* **6:** 127–135.
26. Van der Werf, M. J. and J. G. Zeikus. 1996. 5-Aminolevulinate production by *Escherichia coli* containing the *Rhodobacter sphaeroides hemA* gene. *Appl. Environ. Microbiol.* **62:** 3560–3566.
27. Vladimir, Y. B., A. L. Demain, and N. I. Zaitseva. 1997. The crucial contribution of starved resting cells to the elucidation of the pathway of vitamin B12 biosynthesis. *Crit. Rev. Biotechnol.* **17:** 21–37.
28. Xie, L., M. A. Eiteman, and E. Altman. 2003. Production of 5-aminolevulinic acid by an *Escherichia coli* aminolevulinate dehydratase mutant that overproduces *Rhodobacter sphaeroides* aminolevulinate synthase. *Biotechnol. Lett.* **25:** 1751–1755.
29. Xie, L., D. Hall, M. A. Eiteman, and E. Altman. 2003. Optimization of recombinant aminolevulinate synthase production in *Escherichia coli* using factorial design. *Appl. Microbiol. Biotechnol.* **63:** 267–273.