Journal of Environmental Sciences Vol.17, No.1, pp.152—155, 2005

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2005)01-0152-04 **CLC number**: X703 **Document code**: A

Biological formation of 5-aminolevulinic acid by photosynthetic bacteria

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Abstract: In this study, 7 stains of *Rhodopseudomonas* sp. were selected from 36 photosynthetic bacteria stains storied in our laboratory. *Rhodopseudomonas* sp. strain 99-28 has the highest 5-aminolevulinic acid(ALA) production ability in these 7 strains. *Rhodopseudomonas* sp. 99-28 strain was mutated using ultraviolet radiation and a mutant strain L-1, which ALA production is higher than wild strain 99-28 about one times, was obtained. The elements affecting ALA formation of strain 99-28 and L-1 were studied. Under the optimal condition(pH 7.5, supplement of ALA dehydratase(ALAD) inhibitor, levulinic acid(LA) and precursors of ALA synthesis, glycine and succinat, 3000 lx of light density), ALA formation of mutant L-1 was up to 22.15 mg/L. Strain L-1 was used to treat wastewater to remove COD_{Cr} and produce ALA. ALA production was 2.819 mg/L, 1.531 mg/L, 2.166 mg/L, and 2.424 mg/L in monosodium glutamate wastewater(MGW), succotash wastewater(SW), brewage wastewater(BW), and citric acid wastewater(CAW) respectively. More than 90% of COD_{Cr} was removed in four kinds of wastewater. When LA, glycin and succinate were supplied, ALA production was dramatically increased, however, COD_{Cr} could hardly be removed.

Keywords: 5-aminolevulinic acid; photosynthetic bacteria; wastewater

ALA, essential precursor of tetrapyrrole biosynthesis, has received great attention as a new type of degradable herbicide and insecticide which damages weeds but does not harm crops, humans or the animals. ALA is excreted extracellularly by many kinds of microorganism, particularly in purple non-sulfur photosynthetic bacterium when LA, a competitive inhibitor of ALAD in tetrapyrrole biosynthesis was added(Mariet, 1990; Sasikala, 1994). An advantage with anoxygenic phototrophic bacteria (APB) is their ability to produce ALA from wastes (Sasaki, 1990). ALA thus produced was at a concentration level(4.2 mg/L) sufficient for practical use as an herbicide (3—5 mg/L; Rebeiz, 1984). In present study, we screened and selected a wild *Rhodopseudomonas* sp. strain which has high ALA production of ALA from photosynthetic bacteria stains in China. A mutated strain with much higher ability of ALA productivity was developed. The elements affecting ALA formation were studied. Furthermore, these strains were used to treat four kinds of wastewater to study ALA formation ability and COD_{Cr} removal efficiency.

1 Materials and methods

1.1 Organism

Photosynthetic bacteria stored in our laboratory, which were obtained from different water systems since 1997, were used to select a suitable strain with high ALA formation ability. Mutant L-1 was obtained by ultraviolet radiation mutating from wild stain 99-28.

1.2 Medium

Glutamate-malate (GM) medium without $CoCl_2$ and ferric citrate was used because it was reported that a large amount of ALA was produced extracellularly in the cobalt and ferric free medium, even though bacterial growth was slightly retarded(Sasaki, 1987). The composition of the medium is as follows: sodium-L-glutamate 4.83 g/L, DL-malic acid 2.7 g/L, KH_2PO_4 0.5 g/L, $K_2HPO_4 \cdot 3H_2O$ 0.65 g/L, $(NH_4)_2HPO_4$ 0.8 g/L($NH_4H_2PO_4$ 0.696 g/L), $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $CaCl_2 \cdot 2H_2O$ 0.053 g/L, $MnSO_4 \cdot 5H_2O$, 0.0012 g/L, nicotinic acid 0.001 g/L, thiamine-HCl, 0.001 g/L, biotin, 1.0×10^{-5} g/L, yeast extracts 0.2%. The pH of the medium was adjusted to 7.0—7.2 with 20% NaOH before autoclaving.

Four kinds of wastewater were derived from Hangzhou Monosodium Glutamate Factory (monosodium glutamate wastewater), Hangzhou Hongguang Succotash Factory (succotash wastewater), Hangzhou Brewage Factory(brewage wastewater), and Hangzhou Citric Acid Factory(citric acid wastewater) respectively(Table 1).

Table 1 Characteristics of four kinds of wastewater

	MGW	SW	BW	CAW
pH	3.2	4.5	4.0	3.0
COD_{Cr}, mg/L	67500	6800	7300	16000
BOD_5, mg/L	25000—30000	2500—3000	800—1000	2800—3000

1.3 Cultivation

Cultivation was carried out in a 100 ml serum bottle under anaerobic light condition(3000 lx) at 30℃. Cell mass collected by 3000 r/min centrifugation from 10 ml of 40 h cultures was added into 60 ml of liquid medium, incubated in dark for 12 h, then moved to illumination. For wastewater treatment, 4 kinds of wastewater were diluted differently to control the initiated COD_{Cr} at 4000—8000 mg/L. Cultivation was carried out in a 250 ml flask with 200 ml of diluted wastewater. 20 ml of 40 h cultures was inoculated. Other conditions were the same as described.

1.4 Mutant selection

Mutant selection was carried out as routine. The cells of

Foundation item: The National Natural Science Foundation of China(No. 50378082)

wild type strain grown under static-light (3000 lx) were harvested at logarithmic phase by centrifugation(3000 r/min, 40 min). The cells were re-suspended then radiated by ultraviolet for 3 min. The ultraviolet radiated cell suspension was inoculated on GM medium and cultivated for 2 d(static-light, 30℃). After centrifugation, cells were re-suspended in GM medium containing 100 μg/ml of penicillin, and incubated 2 h with mild shaking(30℃) according to the standard method of penicillin treatment(Neuberger, 1973). Mutated cells were concentrated by this treatment since non-mutated cells growing well on GM medium were killed by penicillin whereas some mutants (poor growth on GM medium) survived.

1.5 Analysis

Cell mass concentration was determined by optical density(OD_{660}) with spectrophotometer(UV-7500, made in Shanghai).

Extracellular ALA was measured calorimetrically according to the method of Mauzerall and Granick(Mauzerall, 1956). Ehrlich's reagent is as follows: 1 mg of *p*-dimethylaminobenzaldehyde(DMAB) is dissolved in 30 ml of glacial acetic acid, 8.0 ml of 70% of perchloric acid were added, then this solution was diluted to 50 ml with acetic acid. For measuring, 0.6 ml of acetyl acetone and 3 ml of sodium acetate buffer(pH 4.6) were added into 3 ml of supernatant in a 10 ml volumetric test tube. The test tube was heated in boiling water for 10 min, and then cooled to room temperature. Two ml of Ehrlich reagent was added into 2 ml of solution, and then mixed. After 15 min, the optical density at 553 nm was read against a blank(Sasaki, 1987; Tanaka, 1991).

Chemical O_2 demand(COD_{Cr}) is analyzed by standard methods.

2 Results and discussion

2.1 Preliminary screen and re-screen

Most of photosynthetic bacteria tested in this study had the ability of ALA production and excretion. Seven strains of *Rhodopseudomonas* sp. with the highest ALA production were selected from 36 photosynthetic bacteria strains. They were Shan 33-2, 99-21-5, 99-28, Hua 6-1, 7, NL, and 98-4-1. According to the growth condition and morphological and physiological characteristics of the cell, we finally determined 99-28 as target strain. Strain 99-28 was purple non-sulfur photosynthetic bacterium.

2.2 Morphological and physiological characteristics of strain 99-28

The cells of 99-28 strain was gram-negative, rod-shape with polar or sub polar flagella for motile. Daughter cells grew at the tip of a slender tube. Strain 99-28 can not utilize sulfur as a photosynthetic electron donor. Surface colonies on agar are 0.2—0.8 mm, circular, smooth, convex and red. The formation of rosettes and clusters in which the individual cells were attached to each other at their flagellated poles was characteristic for the organism in older cultures. Anaerobic liquid cultures were light pink at first, and then red to brownish red. Finally, old cultures were dark reddish brown.

2.3 Effect of the initial pH on the growth of strain 99-28

The effect of the initial pH was examined under the same condition as described. As shown in Fig.1, cells can grow under the pH range from 6.5 to 8.5. The optimal pH is 7.5. Cell growth was retarded in pH 5.5 and 9.5.

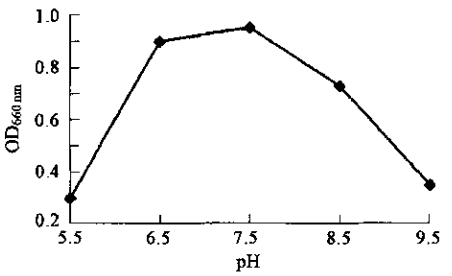

Fig.1 Effect of the initial pH on growth of strain 99-28

2.4 Effect of yeast extract on the growth of strain 99-28

Yeast extract did promote the growth of strain 99-28 (Fig.2). The effect of growth promoting was very weak when the concentration of yeast extract was from 0.15% to 0.25%. We use 0.2% of yeast extract in following experiments.

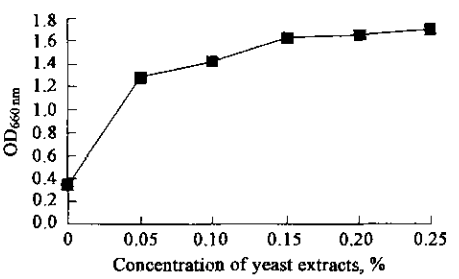

Fig.2 Effect of yeast extract on growth of strain 99-28

2.5 Effect of light density on the growth of strain 99-28

The effect of light density on cell growth under the same conditions is shown in Fig.3. The cell mass is increased with the increasing of light density. Under the present condition, we use 3000 lx as the optimal light density.

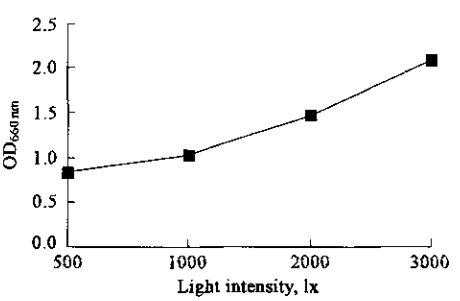

Fig.3 Effect of light intensity on strain 99-28

2.6 ALA accumulation of wild strain 99-28 and mutant

strain L-1 without LA

A mutant strain L-1 with higher ALA production was obtained from UV treated strain 99-28. When growing anaerobicly under illumination without LA, glycine and succinate, ALA productions of mutant strain L-1 were higher than that of wild strain 99-28 in all time point(Fig.4). The maximal ALA accumulation in mutant strain L-1 was 7.266 mg/L at 120 h, which was more than two times of that of wild type(3.145 mg/L at 96 h)(Fig.5 and Fig.6).

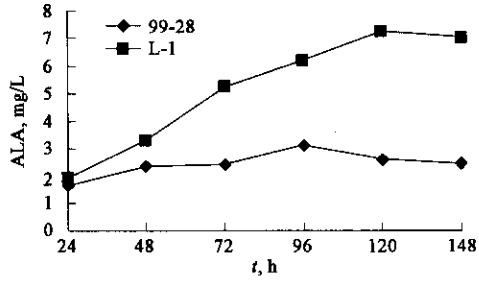

Fig.4 Compare the ALA formation capability between strain 99-28 and mutant L-1

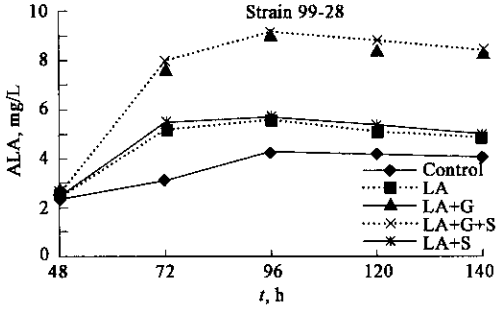

Fig.5 Effect of LA, glycine and succinate on ALA formation by wild-type strain 99-28

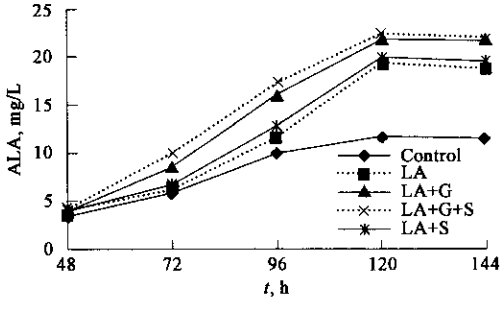

Fig.6 Effect of LA, glycine and succinate on ALA formation by formation in mutant strain L-1

2.7 Effect of LA, glycine and succinate to ALA accumulation

Glycine and succinate-CoA are the precursors of ALA biosynthesis in purple non-sulfur *Rhotosynthetic* bacterium. LA can inhibit ALA dehydratase activity (Sasaki, 1987; Tanaka, 1991). In this study, 30 mmol/L of LA, glycine and succinate was added to the culture medium during the middle logarithmic phases of the culture(48 h) to investigate the effects of LA, glycine and succinate on ALA formation in strain 99-28 and L1.

Strain L-1 accumulated much more ALA than strain 99-28 in all environments and time point. The effect of LA, glycine and succinate to extracellular ALA formation were the same in strain 99-82 and strain L-1. Extracellular ALA formation was significantly enhanced by adding LA. In the medium with LA, adding 30 mg/L of glycine greatly increased ALA production, whereas, there was almost no effect to ALA production when adding succinate. In strain 99-82, the effect of glycine was stronger than that of in strain L-1 relatively. ALA formation reached a peak at 96 h in strain 99-28 and 120 h in strain L-1 and then decreased. This phenomenon was related to the recovery of ALAD, which has been observed previously (Lin, 1989). When LA was added three times and glycine and succinate was supplemented only once at the middle logarithmic phase, ALA formation was about twice that of one time adding of LA. As previous study (Sasaki, 1990), even if LA was repeatedly added more than three times, ALA accumulation was not enhanced any more.

2.8 Effect of pH to ALA accumulation

pH is one of important factors for ALA formation. In this study, ALA formation is the highest in pH 7.5 in strain L-1(Fig.7).

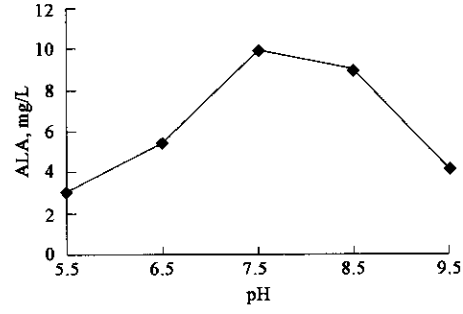

Fig.7 Effect of the initial pH on mutant L-1

2.9 Illumination intensity effects of ALA accumulation

The amount of ALA excreted was clearly affected by light intensity, reaching the maximum value at 3000 lx of light intensity (Fig. 8). It was reported that the strong illumination(over 5000 lx) was not effect to ALA formation and weak illumination below than 1000 lx inhibited growth rate and virtually formated no ALA(Sasaki, 1987).

2.10 Effect of initiate inoculate quantity on ALA formation

Inoculate quantity is also one of important factors for ALA formation. ALA formation is increased with the initiate innculate quantity increase(Fig.9). We selected 10 ml of 40 h cultured cell liquid as initiate inoculate quantity.

2.11 Strain L-1 producing ALA and removing COD_{Cr} in wastewater

Strain L-1 was used to treat wastewater to remove COD_{Cr} and produce ALA. After incubating 120 h, 91.6% of total

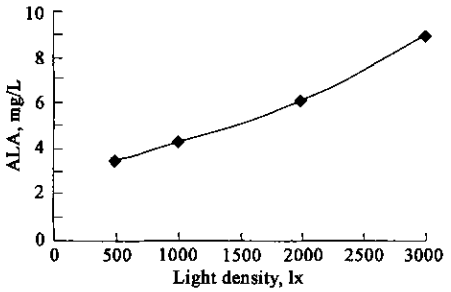

Fig.8 Effect of light intensity on mutant L-1

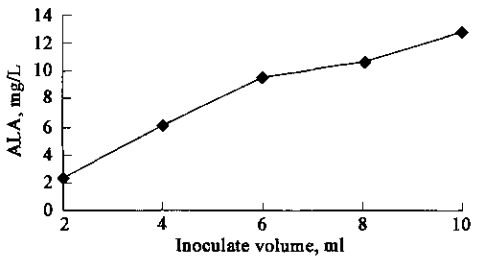

Fig.9 Effect of inoculate quanlity on ALA formation by mutant L-1

COD_{Cr} in MGW, 91.8% of total COD_{Cr} in SW, 91.5% of total COD_{Cr} in BW, and 73.4% of total COD_{Cr} CAW was removed(Fig.10). ALA production was 2.819 mg/L, 1.531 mg/L, 2.166 mg/L, and 2.424 mg/L in MGW, SW, BW, and CAW respectively(Fig.11).

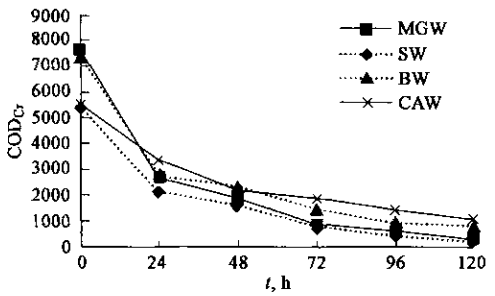

Fig.10 Strain L-1 removal COD_{Cr} in four types of wastewater

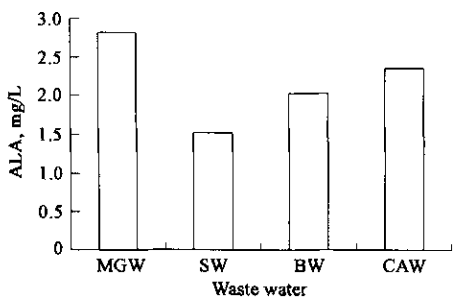

Fig.11 Strain L-1 ALA production wastewater

When LA, glycin and succinate were supplied, ALA production was dramatically increased in MGW, BW, and CAW. They were 6.493 mg/L in MGW, 1.994 mg/L in SW, 7.266 mg/L in BW, and 5.394 mg/L in CAW after 96 h incubation (Fig. 12). However, after LA, glycin and succinate were supplied, COD_{Cr} could hardly be removed.

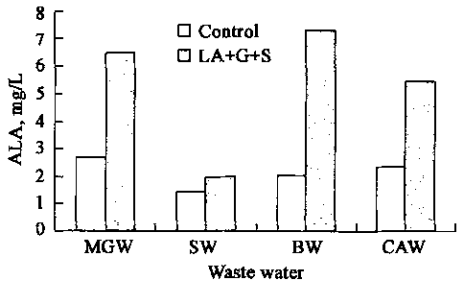

Fig.12 LA, glycin and succinate effect strain L-1 ALA production in four kinds of wastewater(culture 120 h)

3 Conclusions

In this study, 7 stains of *Rhodopseudomonas* sp. with the high ALA productive ability were primary selected. Purple non-sulfur photosynthetic bacterium strain 99-28 has the highest ALA production ability in these 7 strains. Strain 99-28 was mutated using ultraviolet radiation and a mutant strain L-1, which ALA production is higher than wild strain 99-28 about one times was obtained. The optimal conditions of ALA formation of strain 99-28 and L-1 were pH 7.5, supplement LA, glycine and succinat, 3000 lx of light density in present study. ALA formation of wild strain 99-28 and mutant strain L-1 were 9.15 mg/L and 22.43 mg/L respectively. Strain L-1 was used to treat wastewater to remove COD_{Cr} and produce ALA. ALA production was 2.819 mg/L(MGW), 1.531 mg/L(SW), 2.166 mg/L(BW), and 2.424 mg/L (CAW). More than 90 % of COD_{Cr} was removed. When LA, glycin and succinate were supplied, ALA production was dramatically increased, however, COD_{Cr} could hardly be removed.

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(Received for review February 16, 2004. Accepted March 22, 2004)