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Production of a herbicide, 5-aminolevulinic acid, by *Rhodobacter sphaeroides* using the effluent of swine waste from an anaerobic digestor

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Summary. For the production of a herbicide, 5-aminolevulinic acid (ALA), from anaerobic digestion liquor, the utilization of the photosynthetic bacterium, *Rhodobacter sphaeroides* was examined. This bacterium could produce ALA extracelularly from this liquor with the addition of levulinic acid (LA), an inhibitor of ALA dehydratase (ALAD), and glycine, a precursor of ALA biosynthesis in the Shemin pathway. Succinate (another precursor) addition was unnecessary for ALA production. When repeated additions of LA were made together with glycine ALA production was significantly enhanced. However, above three additions of LA, ALA production was not further enhanced. The maximum value of ALA production attained was 4.2 m*M* (0.63 g/l), which was over double that of other ALA producers such as *Chlorella vulgaris*. Propionic acid was predominantly utilized compared with other lower fatty acids, suggesting that this might be converted to ALA via succinyl-coenzyme A (CoA) in the methylmalonyl-CoA pathway.

Introduction

Anaerobic digestion (methanogenesis) of swine waste is frequently performed to treat the waste and to produce biogas in energy recycling (Hobson and Shaw 1973; Aubart 1983; Cooney and Wise 1975; Fisher 1981). The effluent of an anaerobic digestor still contains relatively high amounts of organic matter (3-5 g/l as biological oxygen demand [BOD]) such as lower fatty acids and ammonia, and secondary treatment is necessary before discharge. Conventional activated sludge treatment is applied to treat this digestion liquor after 10-20-fold dilution with water (Inno et al. 1987). However, dilution increases the volume of waste water and requires high running costs. This liquor can be used as a fertilizer, but, the amount in the total volume of waste is quite small. An activated sludge treatment process is then essential (Inno et al. 1987). In place of such treatment, *Chlorella* sp. (Inno et al. 1987) and photosynthetic bacteria (Kobayashi and Kurata 1978; Vrati 1984) have been applied to the digestion liquor to convert valuable biomass to an animal feedstock and agricultural fertilizer, at the same time reducing BOD or chemical oxygen demand (COD) without dilution.

Regarding the application of photosynthetic bacteria, we observed previously (Sasaki et al. 1987a) that *Rhodobacter sphaeroides* produces 5-aminolevulinic acid (ALA) extracellularly when levulinic acid (LA), an inhibitor of ALA dehydratase (ALAD) in tetrapyrrole biosynthesis, was added in the culture broth. ALA has been paid attention as a new herbicide that damages weeds but does not harm humans and other animals (Rebeiz et al. 1984). If ALA can be produced by *R. sphaeroides* using digestion liquor as the culture medium, it would be advantageous because after cultivation of *R. sphaeroides* this liquor could be used directly both as a fertilizer and herbicide.

In this work, ALA production from digestion liquor using *R. sphaeroides* was studied. Effects of addition of LA and illumination on the production of ALA were examined. In addition, the role of organic components contained in the digestion liquor, such as lower fatty acids, for ALA production is discussed.

Materials and methods

Digestion liquor. Effluent from an anaerobic digestor (digestion liquor) was prepared as described previously (Sasaki et al. 1987b). Swine waste (faeces:urine:tap water = 1:2:2, w/w) was digested in a 1.5-l fermentor (liquid volume, 1 l) for 5-7 days at 35° C. After digestion, the broth was centrifuged (12000 *g*, 30 min) to remove the solid materials. The supernatant contained 0.5-1.0 g/l each of lower fatty acid such as acetic, propionic and butyric acids and 2-3 g/l NH_4^+-N as the organic substrates. The supernatant obtained from several digestions was collected in one vessel and mixed completely. Lower fatty acids were supplemented up to 1.0 g/l because these acid concentrations were slightly different

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in each digestion. This supernatant was stored in a freezer (−16° C) until use as a digestion liquor. The chemical composition of this liquor after addition of lower fatty acids was as follows (g/l): COD, 5.0; BOD, 3.8; NH_4^+-N, 2.5; acetic acid, 1.0; propionic acid, 1.0; *n*-butyric acid, 1.0.

Culture of photosynthetic bacteria. Rhodobacter sphaeroides IFO 12203 was cultivated in glutamate-malate medium (Co^{2+} and Fe^{2+} was eliminated) under anaerobic-light conditions (5 klx, two tungsten bulbs, 200 watt Leff lamp, Toshiba Electric, Tokyo) at 30° C for 2 days using a 1.5-l Roux bottle (1 l liquid volume) as described previously (Sasaki et al. 1987a). The cells were harvested by centrifugation (30000 *g*, 30 min) at late log phase (about 40 h culture) and were used immediately for ALA formation.

ALA formation. Fresh cells were resuspended in the digestion liquor and the cell concentration was adjusted to ca. 2 g/l. This suspension was transferred into a 70-ml test tube (liquid contents, 50 ml) or 1.5-l Roux bottle (liquid contents, 1 l), and cultivated for 4–7 days at 30° C under static-light conditions (light intensity: 3–10 klx).

Levulinic acid (LA, 5-60 m*M*) and glycine (0–60 m*M*) and/or succinate (0–60 m*M*) were added in various combinations. The initial pH of the culture broth (or suspension) was adjusted to 6.5; during culture, the pH was manually controlled in the range 6.5–7.5 using 4 *N* HCl and 4 *N* NaOH solutions.

Analysis. Cell mass, ALA, LA and lower fatty acids in the culture broth were measured as described elsewhere (Sasaki et al. 1978, 1987a). Glycine in the broth was measured by a colorimetric method using ninhydrin solution since digestion liquor itself (glycine not added) did not respond to ninhydrin reaction after being diluted 10–20 times with deionized water.

Results and discussion

Effects of precursor additions

Glycine and succinate (succinyl-CoA) are the precursors of ALA biosynthesis in *R. sphaeroides* (Shemin pathway, Lascelles 1978). Therefore, the effect of adding these precursors into digestion liquor on ALA formation was examined in the presence of added LA. This bacterium did not produce ALA extracellularly during culture without the addition of LA, while with 15 m*M* LA, the optimal level that inhibited ALA dehydratase (ALAD), cell growth was retarded as observed previously (Sasaki et al. 1987a) and a small amount of ALA was formed as shown in Fig. 1a (cell growth is omitted).

When glycine and succinate were added simultaneously (Fig. 1b), significant amounts of ALA were formed depending on the amount of precursors added in the range 20–60 m*M*. Above 80 m*M*, addition of precursors resulted in a negative effect on ALA formation.

With glycine alone (20–60 m*M*, Fig. 1c), ALA formation was almost the same as in Fig. 1b (both glycine and succinate added). On the other hand, with succinate alone (60 m*M*, Fig. 1d), ALA formation was not enhanced. In other experiments, adding succinate alone (20, 40 and 80 m*M*), yielded almost the same results as shown in Fig. 1d (data not shown). It is suggested that the supply of succinate was sufficient but that glycine supply might limit ALA formation in this culture system. In addition, ALA formation by this cultivation using LA reached a maximum value in about 2 days and then decreased (see, Fig. 1b, c). This phenomenon has been observed previously (Sasaki et al. 1987a) and might depend on the conversion of ALA to tetrapyrroles by residual ALAD activity in the cells.

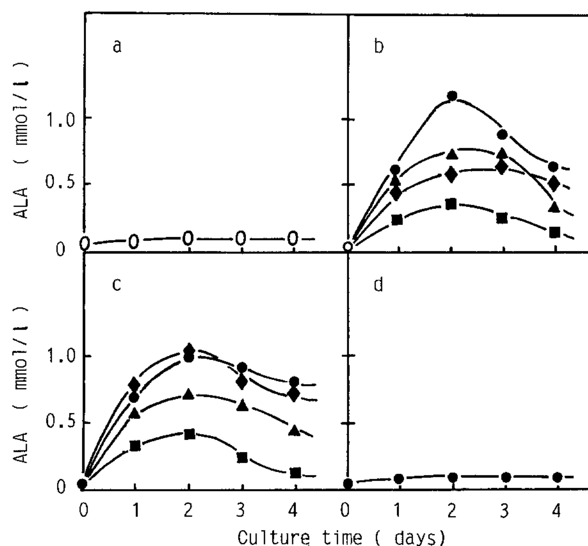

Fig. 1 a–d. Effect of precursor (glycine and succinate) supplements with levulinic acid (LA) on 5-aminolevulinic acid (ALA) formation from digestion liquor by *Rhodobacter sphaeroides*. Initial cell concentration was 2.0 g/l. LA (15 m*M*) and precursors (0–80 m*M*) were added at the beginning of cultivation. Illumination intensity was 5 klx. **a** LA. **b** LA plus glycine (20–60 m*M*) and succinate (20–60 m*M*). **c** LA plus glycine (20–60 m*M*). **d** LA plus succinate (60 m*M*). ○, no precursor; ■, 20 m*M* of each precursor; ▲, 40 m*M* of each precursor; ●, 60 m*M* of each precursor; ◆, 80 m*M* of each precursor

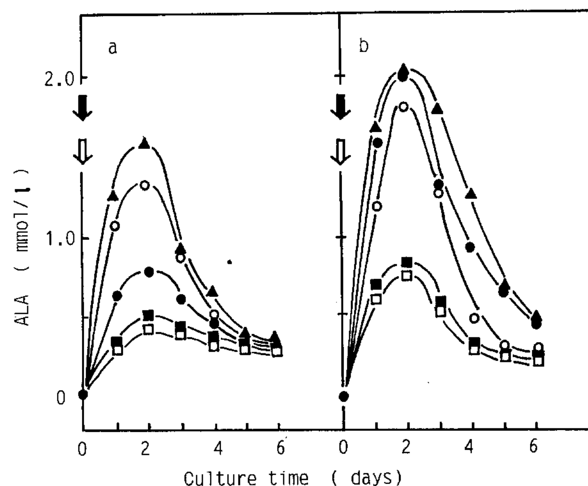

Fig. 2 a, b. Effect of LA concentration plus added glycine on ALA formation from digestion liquor by *R. sphaeroides*. Initial cell concentration was 2.0 g/l. Illumination intensity was 5 klx. *Solid* and *open arrows* indicate the addition of LA and glycine, respectively. **a** Glycine (20 m*M*) plus LA (5–60 m*M*). **b** Glycine (60 m*M*) plus LA (5–60 m*M*). □, LA = 5 m*M*; ■, LA = 15 m*M*; ●, LA = 30 m*M*; ○, LA = 45 m*M*; ▲, LA = 60 m*M*

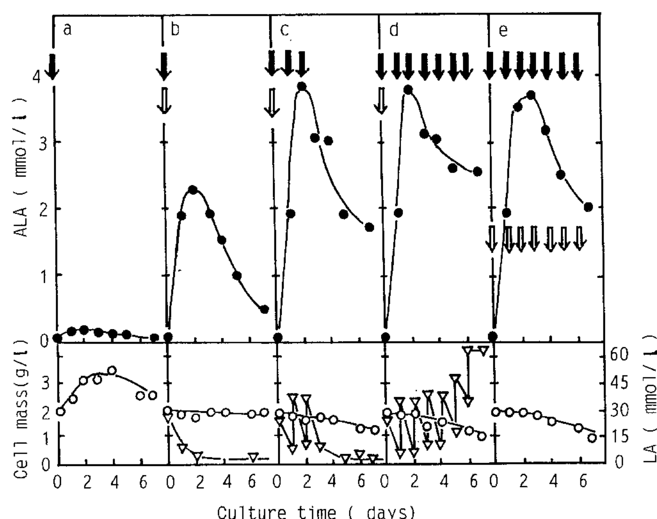

Fig. 3 a–e. Effect of repeated additions of LA and glycine on ALA formation from digestion liquor by *R. sphaeroides* at 5 klx. *Solid* and *open arrows* indicate additions of LA (30 m*M*) and glycine (60 m*M*), respectively. **a** LA = 30 m*M* × 1. **b** LA = 30 m*M* × 1, glycine = 60 m*M* × 1. **c** LA = 30 m*M* × 3, glycine = 60 m*M* × 1. **d** LA = 30 m*M* × 7, glycine = 60 m*M* × 1. **e** LA = 30 m*M* × 7, glycine = 60 m*M* × 7. ●, ALA; ○, cell mass; ▽, residual LA

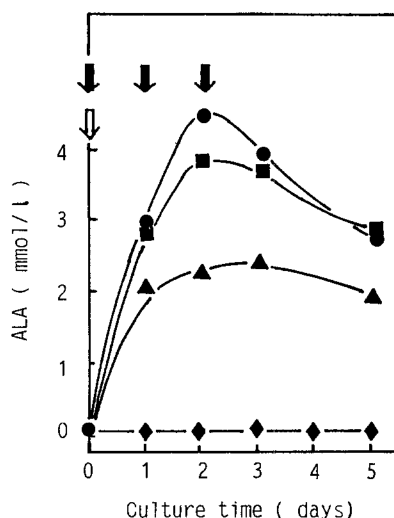

Fig. 4. Effects of light intensity on ALA formation from digestion liquor by *R. sphaeroides*. Initial cell concentration was 2.0 g/l. *Solid* and *open arrows* indicate the addition of LA (30 m*M*) and glycine (60 m*M*), respectively: ◆, 0 klx; ▲, 3 klx; ●, 5 klx; ■, 10 klx

Effect of LA concentration

The effect of LA concentration on ALA formation was examined at fixed concentrations of glycine (20 and 60 m*M*). At 20 m*M* glycine (Fig. 2a), the maximum values of ALA formation at 48 h depended on the LA concentration, while at 60 m*M* glycine (Fig. 2b), they remained at almost the same level when the LA concentration was above 30 m*M*. If ALA formation at 30 m*M* LA is compared (Fig. 2a, b), ALA formation in 60 m*M* glycine was ca. double (maximum 2.0 m*M* ALA) that in 20 m*M* glycine. The amount of LA should be as small as possible since LA is expensive compared with glycine.

Repeated addition of LA and glycine

Previously (Sasaki et al. 1987a), it was observed that LA added to the medium decreased and this decrease seemed to be related to the recovery of ALAD. For example, ALAD activity was rapidly decreased by 30%–50% within 3 h on addition of LA, but this activity returned to the original level after 60–90 h culture in relation to a decrease in LA (Sasaki et al. 1988). Therefore, to maintain ALAD activity at a low level, repeated addition of LA was applied. In addition, glycine was also repeatedly added to avoid limitation of the substrate supply for ALA formation.

As a control experiment, LA was added once at the beginning with and without glycine; the results are shown in Fig. 3a and 3b, respectively. With addition of LA alone ALA formation proceeded at quite a low level, but was enhanced up to 2.2 m*M* after 2 days cultivation by adding glycine, even though cell growth was more strongly retarded by glycine supplementation. This suggests that limitation of a precursor (glycine) was eliminated and that generation of energy for ALA biosynthesis was maintained in the cells even though cell growth was retarded. However, within 2 days, the added LA was almost consumed and ALA started to decrease.

When LA was added three times and glycine was supplemented only once at the beginning of the culture (Fig. 3c), ALA formation was significantly enhanced up to 3.9 m*M*, although growth retardation was almost the same as in Fig. 3b. However, the decrease in ALA accumulated was observed to correspond with the decrease in LA. Therefore, to maintain ALAD activity at a low level for long period, LA was added seven times (Fig. 3d). However, ALA accumulation was not enhanced any more than in Fig. 3c. Even if LA was added repeatedly, ALAD was not completely inhibited, as observed previously (Sasaki et al. 1988), and a large amount of LA remained in the medium. Large amounts of LA residue might become a problem if this culture broth was sprayed on farms directly as a herbicide, because the toxicity of LA for plants and animals has not been established. Therefore, excess addition of LA should be avoided.

In Fig. 3e, glycine was also added seven times to check the limitation of substrate supply, but no effect was observed. It seems that supplement of 60 m*M* glycine to this digestion liquor was sufficient for ALA production under these conditions.

Regarding the rapid decrease in LA in Fig. 3b and 3c, it was reported recently that a photosynthetic bacterium, *Rhodopseudomonas* sp. no 7 could utilize LA as a sole carbon source, i.e., LA incorporated was metabolized to acetate, propionate and other unknown substances under anaerobic illuminated conditions (Okuyama et al. unpublished data).

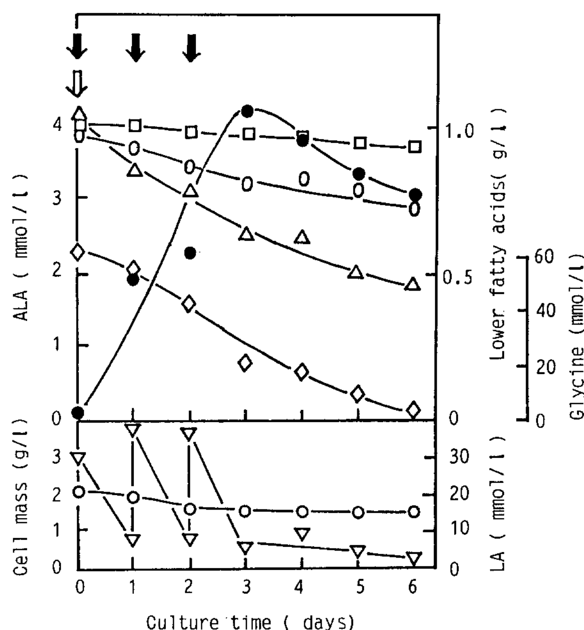

Fig. 5. Profiles of ALA, cell mass, LA, lower fatty acids and glycine in a culture of *R. sphaeroides* grown at 5 klx on digestion liquor. *Solid* and *open arrows* indicate additions of LA (30 m*M*) and glycine (60 m*M*), respectively: ●, ALA; ○, cell mass; ▽, residual LA; ◇, glycine; ○, acetic acid; △, propionic acid; □, *n*-butyric acid

Effect of light intensity

The effect of light intensity on ALA formation was examined under the same conditions as Fig. 3c (LA 30 m$M \times 3$, glycine 60 m$M \times 1$). As shown in Fig. 4, ALA formation increased with increase in light intensity. However, above 5 klx, ALA formation did not increase further. In the dark, no ALA formation was observed (Fig. 4). These results indicate that ALA formation by *R. sphaeroides* is light-dependent as reported previously (Sasaki et al. 1987a).

Profiles of ALA formation from digestion liquor

R. sphaeroides can utilize lower fatty acids such as acetic, propionic and butyric acid as carbon and energy sources (Sasaki et al. 1978). The digestion liquor used here contained abundant lower fatty acids. Therefore, the relationship between ALA formation and lower fatty acid utilization was examined. In addition, in place of test tubes, large-scale cultivation using a Roux bottle was used, considering the practical use of the digestion liquor for ALA production.

As shown in Fig. 5, up to 4.2 m*M* ALA was produced (0.63 g ALA/l) with three additions of LA in the presence of glycine. This was almost the same as the result obtained in Fig. 3c under the same conditions. It is suggested that the results obtained using test tubes and a Roux bottle are consistent with each other. It is worth noting that this concentration (ca. 4 m*M*) is almost twice that obtained with *R. sphaeroides* in glutamate-malate medium (Sasaki et al. 1987a) and that of *Chlorella vulgaris* (Beale 1970), which had a reported highest level of 2.0-2.2 m*M* ALA production. This concentration (ca. 4.2 m*M*) was at a level sufficient for practical use as a herbicide (3-5 m*M*; see Rebeiz et al. 1984).

In Fig. 5, propionic and acetic acids were utilized by this bacterium, but butyric acid was not. It must be emphasized that propionic acid was predominantly utilized compared with acetic acid in this cultivation, although *R. sphaeroides* utilized acetic acid and propionic acid simultaneously under normal growth conditions in aerobic-dark or anaerobic-light cultures (Sasaki et al. 1978). It was suggested that propionic acid contained in the liquor might play an important role in ALA formation as a source of succinyl-CoA via the methylmalonyl-CoA pathway (Maruyama and Kitamura 1975; Sasaki et al. 1978; Maruyama 1979).

In addition, for ALA production, we tried to use other propionate-containing media, such as digestion liquor from agricultural waste (mandarin orange peel) or acetate-propionate synthetic medium (Sasaki et al. 1978). However, the ALA production was quite small compared with that from swine waste. Some other elements in swine waste might enhance ALA production by *R. sphaeroides*.

The practical use of this culture broth as a herbicide was tested as reported by Rebeiz et al. (1984). The culture broth (ca. 10 ml, 4 m*M* ALA) was directly sprayed on the leaves and stems of cucumber. After 2-7 days, 50%-100% of the leaves and stems were withered, as also observed by Rebeiz et al. (1984). In addition, almost the same herbicide effects (50%-100% death) could be observed for *Artemisia princeps* P., *Commelina communis* L., *Trifolium repens* L. and *Oxalis corniculate* L., all common weeds in the field. However, only a small effect was observed on monocotyledonous weeds such as *Zoysia japonica* S. and *Eleusine indica* L.. ALA can therefore be used as a selective herbicide for dicotyledonous weeds mainly in cereal crop fields (Rebeiz et al. 1984).

From these results, it can be concluded that digestion liquor may be utilized for ALA production by *R. sphaeroides* with added LA and glycine, and that the culture broth containing ALA may be directly used as a herbicide.

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