Photoproduction of Hydrogen from Acetate by *Rhodopseudomonas*: Effect of Culture Conditions and Sequential Dark/Light Fermentation

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Abstract

Rhodopseudomonas palustris P4 can produce H_2 either from CO by water-gas shift reaction or from various sugars by anaerobic fermentation. Fermentative H_2 production by P4 is fast, but its yield is relatively low due to the formation of various organic acids. In order to increase H_2 production yield from glucose, P4 was investigated for the photo-fermentation of acetate which is a major by-product of fermentative H_2 production. Experiments were performed in batch modes using both light-grown and dark-grown cells. When the dark-grown P4 was challenged with light and acetate, H_2 was produced with the consumption of acetate after a lag period of 25 h. H_2 production was inhibited when a nitrogen source, especially ammonium, is present. When the dark-fermentation broth containing acetate was adopted for photo-fermentation with light-grown cells, H_2 production and concomitant acetate consumption occurred without a lag period. The H_2 yield was estimated as 2.4 - 2.8 mol H_2/mol acetate and the specific H_2 production rate was as 9.8 ml H_2/g cell·h. The fact that a single strain can perform both dark- and light-fermentation gives a great advantage in process development. Compared to a one-step dark-fermentation, the combined dark- and light-fermentation can increase the H_2 production yield on glucose by two-fold.

Introduction

We have been studying an H_2-producing bacterium, *Rhodopseudomonas palustris* P4[1,2,3]. P4 was originally isolated for CO-dependent H_2 production, i.e. water-gas shift reaction, but also fermented various sugars. P4 was facultative anaerobe and could produce H_2 at a fast rate under anaerobic conditions. Similar to other H_2-producing bacteria, however, P4 produced extensive amounts of ethanol and acetic acid and their H_2 yield was low as 1.0 – 2.5 mol H_2/mol glucose.[1)]

Some *Rhodopseudomonas* sp. are photosynthetic and known to be able to utilize acetate for producing H_2 when light is available.[4,5] This implies that acetate, a major by-product of fermentative H_2 production from glucose, may drive H_2 production by the same microorganism of *Rps. palustris* P4 that is used for a dark-fermentation. The application of *Rhodopseudomonas* sp. to a separate photosynthetic[4,5] or non-photosynthetic[1,6,7] fermentation has been reported previously, but the application of a single strain to the sequential dark- and light-fermentation has not been reported yet to our best knowledge. If the same strain can be used for both a dark- and light-fermentation, it might be possible to increase H_2 production yield by operating a reactor in a sequential mode; under a dark condition and then under a photosynthetic condition.

In the present study, photo-fermentation of acetate was conducted with *Rps. palustris* P4. The freshly-prepared acetate medium and the spent broth from a dark-fermentation which contained acetate were examined for H_2 production with either light-grown or dark-grown cells.

Materials and methods

Microorganism and culture conditions: *Rps. palustris* P4 were cultivated anaerobically for 10 h under dark conditions (dark-grown cells) or 24 h under photosynthetic conditions of 2500 lux (light-grown cells) and used as inoculum. The PFN mineral salt medium[2] fortified with a phosphate buffer (pH 7.0) of 180 mM and containing 3 g yeast extract/l and 10 g glucose/l was used for the inoculum culture. The cells were washed twice with a phosphate buffer of pH 7.0 and the main fermentation broth when indicated.

For the main fermentation under the photosynthetic conditions, the phosphate-fortified PFN mineral salt medium containing acetate (acetate minimal salt medium, hereafter) or the spent medium from a dark-fermentation (dark broth, hereafter) was employed. For the dark fermentation of H_2, a fortified PFN medium containing 3 g yeast extract/l and 10 g glucose/l was employed. The dark broth was obtained by filtering cells through a 0.2 μm membrane. Both inoculum and main cultivations were performed in a serum bottle of 165 ml (working vol., 50 ml) at 30 $^{\circ}$C. White light at 2500 lux was used for photo-fermentation.

Analyses: Cell concentration was measured by a spectrophotometer. One unit of optical density at 600 nm corresponded to 0.38 g dried cell mass/l. Incident light intensity was measured with a light meter. Gas samples in the headspace of the reaction bottles were analyzed by a GC equipped with a TCD detector. The concentrations of acids and ethanol were analyzed by an HPLC equipped with a Shodex-SH1011 packed column after filtering the sample by a 0.45 μm disposable filter unit. A DAD detector was used for organic acids while a RI detector was for ethanol.

Results and discussion

Photo-fermentation of acetate by* Rps. palustris *P4: The cell growth and H_2 production by *Rps. palustris* P4 in the presence or absence of light was studied (data not shown). A light-grown inoculum and acetate minimal salt medium supplemented with 3.0 g yeast extract/l were used. In early stages, H_2 production and cell growth were almost identical regardless of light. After 36 h, however, H_2 production and cell growth were observed under photosynthetic conditions only. Acetate consumption was also observed only under photosynthetic conditions. These results suggest that P4 can utilize acetate as a carbon and an energy source for cell growth and H_2 production when light is available. Cell growth and H_2 production during the initial 12 or 36 h under dark conditions are attributed to the presence of yeast extract. When the yeast extract was omitted from the medium preparation, cell growth and H_2 production were almost negligible under dark conditions (data not shown).

Comparison between light-grown and dark-grown inoculum: Fig. 1 shows the effect of inoculum culture on photo-fermentation of acetate. An acetate minimal salt medium without yeast extract was used for the main fermentation. When light-grown cells were used as inoculum, H_2 production started without any delay. The H_2 production rates were higher initially and gradually decreased to a constant level which remained until the end of photo-fermentation. The volumetric and specific H_2 production rates with the light-grown inocula were estimated as 5.8 ml H_2/l·h and 7.2 ml H_2/g cell·h for 0.70 g cell/l and 3.9 ml H_2/l·h and 9.8 ml H_2/g cell·h for 0.35 g cell/l, respectively. The volumetric production rate was higher when the cell density was higher, but the specific rate was higher when the cell density was lower. The lower specific H_2 production rate with a higher cell density is attributed to the limitation of light. When

the dark-grown cells were used as inoculum, a lag period of about 25 h was observed. The lag seems to be required for synthesizing the cellular machineries for photosynthesis and light-dependent H_2 production. Cell growth was almost negligible during this period, but the color of the cells changed from yellow-white to red. The specific H_2 production rate with the dark-grown inoculum was estimated to be 7.0 ml H_2/g cell·h, which was 30% lower than that of the light-grown inoculum.

Effect of nitrogen sources: H_2 production by photosynthetic bacteria is catalyzed by nitrogenase which is strongly inhibited by the presence of nitrogen sources except for dinitrogen[5]. The effect of various nitrogen sources on H_2 production, cell growth and acetate consumption was studied by supplementing the acetate minimal salt medium with such nitrogen sources as glutamate, NH_4Cl and yeast extract (data not shown). Light-grown cells were used as inoculum. H_2 production at 220 h was high in the descending order of: None (control) > yeast extract > glutamate > NH_4Cl. The NH_4Cl was inhibitory most significantly and H_2 production stopped at an early stage approximately 50 h after the start of cultivation, although cell growth continued to a much later period. This experiment confirms the inhibitory effect of a nitrogen source on photosynthetic H_2 production and suggests that P4 might rely on its nitrogenase for the photosynthetic H_2 production as in other *Rhodopseudomonas* sp.[5] In addition, this result indicates that, in order to be used as the fermentation broth for photosynthetic H_2 production, the broth from a dark-fermentation should have low residual nitrogen content.

Effect of acetate concentration: The effect of acetate concentration (12 – 55 mM) on H_2 production was studied (data not shown) Yeast extract was not supplemented to the culture medium for the photo-fermentation. A light-grown culture was used as inoculum at an initial concentration of 0.17 g cell/l after being washed. The rate and yield of H_2 production were similar regardless of the initial acetate concentration as 1.6 ml H_2/l·h and 2.4 – 2.8 mol H_2/mol acetate, respectively. This indicates that a high acetate concentration up to 55 mM is not toxic to photo-fermentation of H_2 by *Rps. palustris* P4. Since approximately 0.5 mol of acetate is produced from 1.0 mol of glucose in a typical dark-fermentation[1], we can expect an additional 1.2 – 1.4 mol of H_2 by fermenting the dark broth under photosynthetic conditions. The amount of H_2 produced in the dark-fermentation is about 1.2 mol per mol of glucose, indicating that the combined dark- and photo-fermentation increases the overall yield of H_2 production

on glucose by two-fold compared to the dark-only process.

Photo-fermentation of dark-fermentation broth: Photo-fermentation of H_2 with a dark broth was performed. Light-grown cells were used as inoculum at an initial concentration of 0.23 g cell/l. Fig. 2 shows that the broth from the dark fermentation by P4 can be used for the photosynthetic H_2 production by the same strain. During the dark fermentation, large amounts of ethanol, acetate, and lactate were produced. Acetate was a major by-product and decreased most significantly during the photo-fermentation. However, the levels of other acids such as lactic acid also decreased during the photo-fermentation and we cannot exclude the possibility that these organic acids contributed to the photosynthetic H_2 production. The effect of ethanol was negligible, although it was another major by-product: Ethanol was not consumed, did it nor affect the H_2 production rate during photo-fermentation (data not shown). Fig. 2 also indicates that the level of yeast extract for the dark fermentation has a noticeable influence on photosynthetic H_2 production. When 0.5 g yeast extract/l was supplied, significantly more H_2 was produced than that with 3.0 g yeast extract/l. The H_2 conversion yield from acetate was estimated as 2.0 mol H_2/mol acetate with 0.5 g yeast extract/l, while only 0.8 mol H_2/mol acetate with 3.0 g yeast extract/l. An analysis of nitrogen was not attempted, but the dark broth with a large amount of yeast extract seemed to contain residual nitrogen at a high level enough to inhibit H_2 production under photosynthetic conditions. This result indicates that the nitrogen level in a photo-fermentation medium should be carefully controlled.

Implications for the process development: The fact that a single strain can carry out both dark-fermentation and photo-fermentation has an important implication to the process development for economic H_2 production. We can improve H_2 production yield by two-fold by simply combining the dark-fermentation and photo-fermentation. The reactor can be operated in a sequential mode; first, under a dark condition and, next, under a photosynthetic condition. For the application, however, there exist significant limitations with P4. First of all, the volumetric rate of H_2 production in the photo-fermentation with P4 is too low. Typically a dark fermentation with 10 g glucose/l was completed within 10 h[1)], while a photo-fermentation with 1.5 g acetate/l which is produced from dark fermentation with 10 g glucose/l took more than 250 h. The volumetric production rate might be improved by increasing cell concentration and light intensity, and this constitutes an important topic for a further study. Another limitation of two-stage fermentation with P4 is that many organic compounds including

ethanol and several acids are not converted to H_2 by photo-fermentation and remain in the fermentation broth. They are valuable resources, but at the same time, are pollutants that cannot be discarded without a proper treatment. Methane fermentation with theses organic compounds might be a solution, although was not attempted in this study.

Acknowledgements

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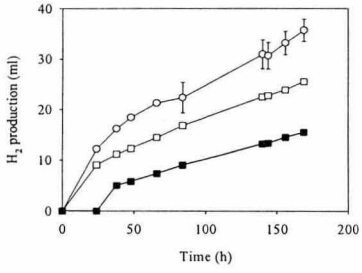

Fig. 1. Effect of initial cell concentration and inoculum culture on H_2 production. Initial cell concentration was at 0.70 g/l (○) or 0.35 g/l (□, ■). Open symbols represent light-grown cellsand closed symbols represent dark-grown cells.

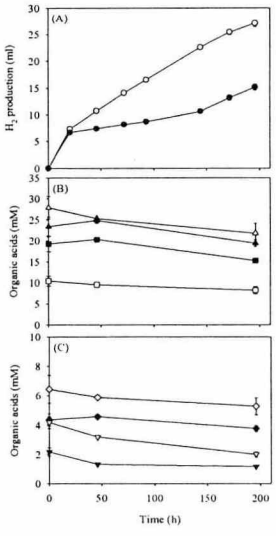

Fig. 2. Photo-fermentation of dark-fermentation broth by light-grown cells. Dark fermentation was conducted at 0.5 g yeast extract/l (open symbols) or 3.0 g yeast extract/l (closed symbols). Symbols: H_2 production (○,●), acetate (△,▲), lactate (□,■), formate (▽,▼), and succinate (◇,◆).