

## OPTIMUM CULTURAL CONDITIONS FOR STRONG LIGHT PRODUCTION BY *PHOTOBACTERIUM* *PHOSPHOREUM*

NOBUYOSHI MAKIGUCHI, MASANOBU ARITA,  
AND YOSHIYUKI ASAI

*Research Center, Mitsui Toatsu Chemicals Inc., Totsuka-ku,  
Yokohama 247, Japan*

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Strong light intensity of the culture broth and industrially feasible cultural conditions are required in order to apply the bioluminescence of luminous bacteria to attracting fish. To obtain strong bioluminescence of the culture broth, studies of the optimum cultural conditions for strong light production by *Photobacterium phosphoreum* MT-10201 were carried out.

Strong light intensity of the culture broth was obtained at an initial NaCl concentration in the medium between 3% and 5.5%, starting pH 8.5, and cultivation temperature 20°. The addition of 0.1% malic acid to the medium resulted in enhanced light intensity of the culture broth but mannose, arginine, dodecylaldehyde, and phosphate buffer used for pH control during cultivation had no effect.

Cultivation of the luminous bacterium in 2-liter and 20-liter fermentors was carried out, and luminous raw bait was prepared successfully by using the culture broth directly as a luminous source.

In spite of the attracting light of luminous bacteria, little is known about the industrial application of bioluminescence. With a view to utilizing the light of luminous bacteria to attract fish, we have isolated many luminous bacteria that emit strong light intensity at various temperatures (1).

There have been reports concerning studies on the relationship between bioluminescence and luciferase synthesis (2–5), respiration (6, 7), and growth (8, 9). STREHLER and CORMIER (10) developed cell-free systems to examine the factors affecting bioluminescence from a luminous bacterium, *Achromobacter fischeri*, and SOLI (11) attempted to enhance the light emission by a photobacterium.

Although considerable literature on bioluminescence of luminous bacteria at the enzymatic or cellular level has been published, little is known about the optimum cultural conditions to obtain strong light intensity of the culture broth

*per se*. It would be advantageous industrially to use a culture broth directly as a luminous source to attract fish and the bioluminescence of the culture broth should be as strong as possible. To obtain a culture broth that emits a strong light intensity, studies on the environmental conditions and nutrient composition affecting the light intensity of *Photobacterium phosphoreum* MT-10201 and an attempt to render raw bait luminous by the culture broth were carried out.

This paper deals with the results concerning the effect of pH, temperature, NaCl concentration, and various substances on the light intensity of the culture broth, cultivation in 2- and 20-liter fermentors, and an attempt to prepare luminous raw bait by using the culture broth. A subsequent paper will describe the immobilization of luminous bacteria by which major problems hampering the commercial use of bacterial bioluminescence might be solved.

#### MATERIALS AND METHODS

*Luminous bacteria and medium.* *Photobacterium phosphoreum* MT-10201 isolated from the light organ of a small fish, *Physiculus japonicus*, was used (1). The basal liquid medium (BM medium) consisted of glycerol, 1 g; Polypepton, 10 g; meat extract, 3 g; NaCl, 30 g; deionized water 1,000 ml; pH 7.3. For solid medium 1.5% agar was added.

*Culture and maintenance.* Cultures of luminous bacteria were maintained on agar slants. Liquid seed cultures were grown at 15° on a reciprocal shaker for the time needed to reach maximum light intensity. One-tenth ml of the seed culture was inoculated into 100 ml of medium in a 500-ml flask and cultivated at 15° with shaking, unless otherwise stated. For the 2-liter fermentor, 10 ml of the seed culture were inoculated into 1.5 liters of medium and cultivated at 20° and 400 rpm for 22 hr with aeration. For the 20-liter fermentor, 50 ml of the seed culture were inoculated into 10 liters of medium containing 0.02% silicon oil as an antifoam reagent and cultivated at 20° with aeration.

*Measurement of light intensity.* Light intensity of the bacterial suspensions (3 ml) was measured at 20° with a photomultiplier of a Hitachi Model 124 spectrophotometer, the output being recorded on a chart. Each sample was aerated by bubbling before measurement of light intensity and light intensity was presented in arbitrary units.

*Determination of cell concentration.* The cell concentration was estimated by the optical density of the culture broth at 660 nm with a Hitachi Model 101 spectrophotometer.

*Preparation of luminous raw bait.* A slice of raw mackerel or squid was dipped in culture broth for 30 min at 20° and allowed to stand in air for 30 min at the same temperature.

## RESULTS AND DISCUSSION

*Effect of NaCl concentration on light intensity*

*Photobacterium phosphoreum* MT-10201 was cultivated at various NaCl concentrations and the maximum light intensity of the culture broth at each NaCl concentration was determined. The maximum light intensity and cell concentration were plotted against NaCl concentration, and the results are shown in Fig. 1. Strong light intensity of the culture broth was obtained at initial NaCl concentrations between 3% and 5.5%, while good growth was observed between 2% and 4.5%. The cell concentration decreased with increasing NaCl concentration from 4.5% to 5.5%, but the light intensity of the culture broth maintained a high level. These results indicate that the light intensity of individual cells is stronger at NaCl concentrations between 4.5% and 5.5% than between 3% and 4.5%. The light intensity of individual cells is not very important for our purpose in utilizing a culture broth directly as a luminous source and a relatively wide range of initial NaCl concentrations, 3–5.5%, can be used.

*Effect of pH on light intensity*

The effect of the initial pH on the light intensity of the culture broth was investigated in the same manner and the results are shown in Fig. 2. Fairly good growth was observed in the pH range between 6 and 9, and the light intensity of the culture broth peaked at pH 8.5. The time course of light intensity and cell concentration of the culture broth when the pH was controlled at about 7.3 with

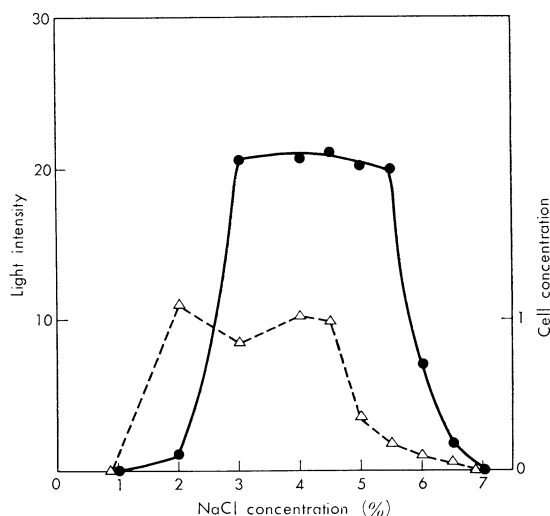


Fig. 1. Effect of NaCl concentration on light intensity of culture broth.

The composition of the culture medium was the same as that of BM medium except for NaCl concentration. Light intensity is plotted as the value of maximum light intensity at each initial NaCl concentration. ●, light intensity; △, cell concentration.

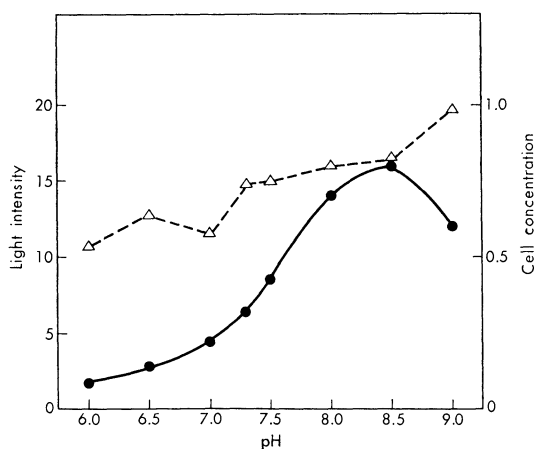


Fig. 2. Effect of pH on light intensity of culture broth. Light intensity is plotted as the value of maximum light intensity at each initial pH. ●, light intensity; △, cell concentration.

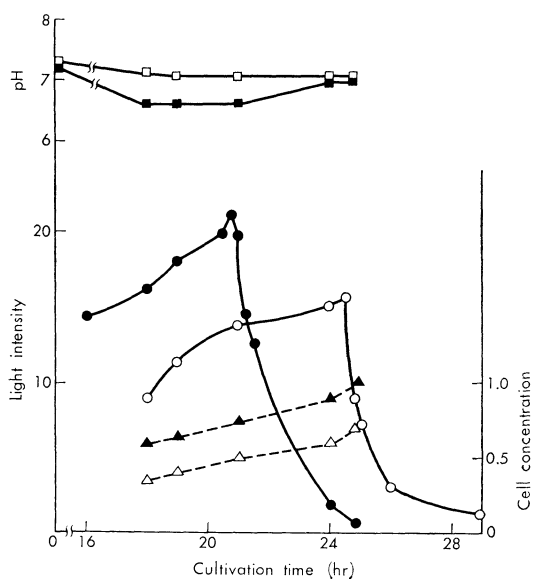


Fig. 3. Effect of pH control on light intensity of culture broth. Cultivation was carried out at 20°. ●, ▲, ■, light intensity, cell concentration, and pH without pH control; ○, △, □, light intensity, cell concentration, and pH with pH control.

0.2 M phosphate buffer is shown in Fig. 3. Both light intensity and cell concentration under controlled pH were lower than those without pH control, suggesting that phosphate buffer inhibited growth, resulting in decrease in light intensity of

the culture broth. As shown in Fig. 3, the light intensity decreases so rapidly from a maximum value that it should be monitored continuously during cultivation to obtain a culture broth emitting the strongest light intensity for industrial use.

#### *Effect of temperature on light intensity*

The time course of light intensity of the culture broth at different cultivation temperatures, 15°, 20°, and 25°, is shown in Fig. 4. Maximum light intensity was found at about 17 hr at 20° and 25°, but at 29 hr at 15°. Among the three cultivation temperatures, the strongest light intensity was obtained at 20°. The optimum cultivation temperature for luminous bacteria is fairly low compared with that of other bacteria, so a relatively high cultivation temperature like 20° is industrially advantageous because of the high growth rate as well as the low cost of cooling.

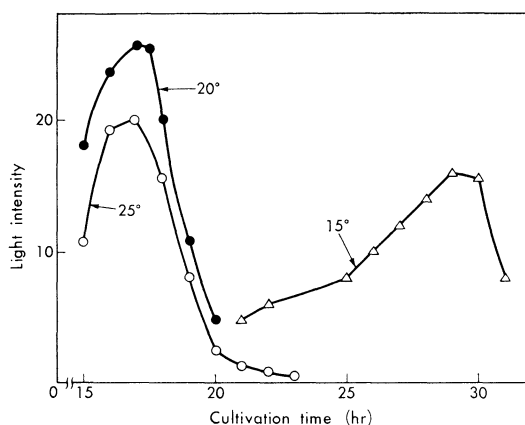


Fig. 4. Effect of cultivation temperature on light intensity of culture broth.

#### *Effect of various substances on light intensity*

The effect of malic acid, mannose, and arginine added to the BM medium at concentrations of 0.1%, 0.1%, and 0.04%, respectively, on the light intensity of the culture broth was examined and the results are shown in Fig. 5. Only malic acid enhanced the light intensity, while arginine showed no effect and mannose inhibited the light production of the culture broth. In addition, it was interesting that the time required to reach maximum light intensity was almost the same for all three substances. Subsequently, the optimal concentration of malic acid for strong light production was investigated and the results are summarized in Table 1. The strongest light intensity was obtained by adding 0.1% malic acid to the BM medium. As shown in Fig. 6, cultivation with 1 mM dodecylaldehyde in the medium failed to enhance the light intensity of the culture broth.

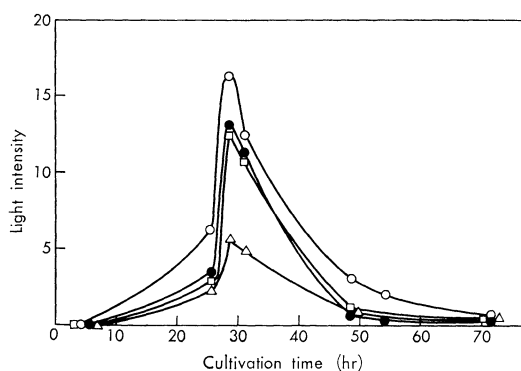


Fig. 5. Effect of various substances on light intensity of culture broth.

●, BM medium; ○, BM medium + 0.1% malic acid; △, BM medium + 0.1% mannose; □, BM medium + 0.04% arginine.

Table 1. Effect of malic acid concentration on light intensity of culture broth.

| Malic acid concentration (%) | Maximum light intensity | Cultivation time <sup>a</sup> (hr) | Cell concentration |
|------------------------------|-------------------------|------------------------------------|--------------------|
| 0.05                         | 12.2                    | 21.0                               | 0.79               |
| 0.10                         | 15.6                    | 21.0                               | 0.83               |
| 0.30                         | 13.3                    | 21.0                               | 0.82               |
| 0.50                         | 12.7                    | 24.5                               | 0.85               |
| 1.00                         | 10.4                    | 24.5                               | 0.85               |
| 1.50                         | 10.9                    | 24.5                               | 0.56               |
| 2.00                         | 9.7                     | 24.5                               | 0.42               |

<sup>a</sup> Cultivation time signifies the period of cultivation required to reach maximum light intensity.

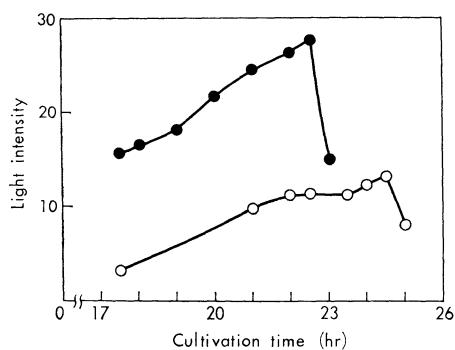


Fig. 6. Effect of dodecylaldehyde on light intensity of culture broth.

●, light intensity without dodecylaldehyde; ○, light intensity with dodecylaldehyde.

### *Cultivation in a 20-liter fermentor*

The time course of light intensity and cell concentration of the culture broth when cultivated in a 20-liter fermentor at different agitation speeds, 250 rpm and 500 rpm, is shown in Fig. 7. The maximum light intensity of the culture was higher at 500 rpm than at 250 rpm, while the time required to reach maximum light intensity was almost the same for the two speeds. Successful cultivation in a 20-liter fermentor shows the possibility of cultivating the luminous bacterium commercially on a large scale and of utilizing the resulting culture broth directly as a luminous source for attracting fish.

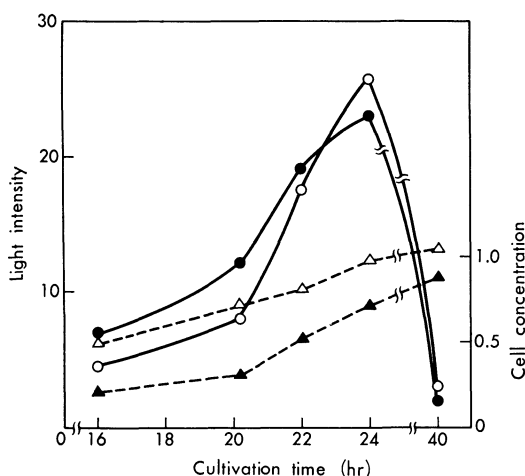


Fig. 7. Cultivation in a 20-liter fermentor

Cultivation was carried out at 20° and an aeration rate of 1 vvm. ●, ▲, light intensity and cell concentration with agitation speed 250 rpm; ○, △, light intensity and cell concentration with agitation speed 500 rpm.

### *Preparation of luminous raw bait*

The luminous bacterium was cultivated in a 2-liter fermentor, as shown in Fig. 8, and luminous raw bait was prepared successfully by using the culture broth as a luminous source. A photograph of the luminous raw bait, a slice of raw mackerel and a slice of squid, taken by their own light is shown in Fig. 9. Luminous cells attached to the surface of raw bait did not detach easily even in the agitated water and continued to emit light. This luminous raw bait can be prepared very easily before fishing, for example on a fishing boat, and could be used successfully to attract fish.

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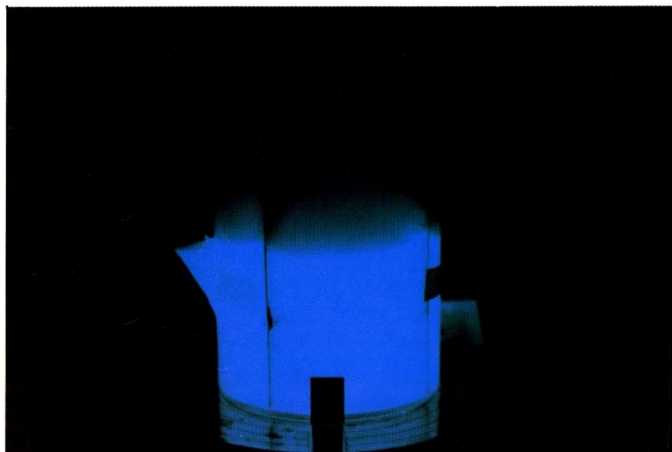


Fig. 8. Photograph of the luminous bacterium grown in a 2-liter fermentor.

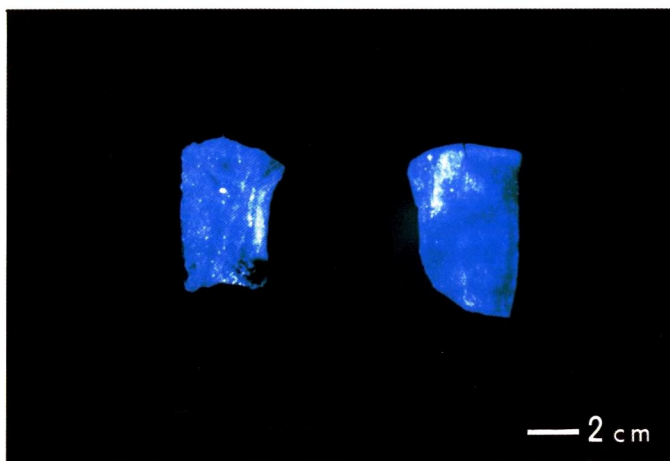


Fig. 9. Photograph of the luminous raw bait with luminous cells attached. left, a slice of luminous mackerel; right, a slice of luminous squid.



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