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Production of Antibacterial Violet Pigment by Psychrotropic Bacterium RT102 Strain

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Abstract The antibacterial action of violet pigment, a mixture of violacein and deoxyviolacein, isolated from phychrotrophic bacterium RT102 strain was examined, and the operational conditions for the effective production of violet pigment were studied. The antibacterial activity of the violet pigment was confirmed for several bacteria such as *Bacillus licheniformis, Bacillus subtilis, Bacillus megaterium, Staphylococcus aureus*, and *Pseudomonas aeruginosa*, and the high concentration of violet pigment, above about 15 mg/L, caused not only growth inhibition but also death of cells. The growth properties of RT102 strain were clarified under various incubation conditions such as pH, temperature, and dissolved oxygen concentration. The maximum violet pigment concentration, *i.e.* 3.7 g/L, and the maximum productivity of violet pigment, *i.e.* 0.12 g L^{-1} h^{-1}, were obtained in a batch culture of pH 6, 20°C, and 1 mg/L of dissolved oxygen concentration.

Keywords: antibacterial activity, growth inhibition, incubation operation, lethal effect, violet pigment

INTRODUCTION

The disinfection, sterilization, inhibition, and others are effective methods for preventing the septic action of food, and it has been reported that some pigments produced by a microorganism has a physiology activity for the prevention of bacteria contamination [1-4]. The natural pigments are comparatively often used as antibacterial reagents for food preservation because of their high decomposition nature and low toxicity compared with synthetic pigments [5,6]. Furthermore, organic materials such as sorbic acid, diphenyl, and others, and heavy metal ions such as zinc ion, copper ion, and others are also effective as antibacterial reagents for growth inhibition of cells in the food [7,8]. However, since some antibacterial reagents has a bad influence not only to a human body but also to environment, the usage of natural pigments with antibacterial activity is desired significantly as the prevention technology of bacteria contamination. The authors [9] have reported that psychrotrophic bacterium RT102 strain, which was isolated from the organic residua of a water tank keeping rainbow trout, was a new species very close to *Janthinobacterium lividum* and produced an antibac terial violet pigment consisted of violacein and deoxyviolacein.

In this work, the antibacterial activity of violet pigment produced by the bacterium RT102 strain and the incubation conditions of this bacterium for the efficient production of violet pigment were investigated. The growth inhibitory and lethal effects of violet pigment on various putrefactive bacteria were clarified and the antibacterial activity of violet pigment was estimated quantitatively. In addition, the optimal incubation condition for production of violet pigment was determined using the bacterium RT102 strain.

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MATERIALS AND METHODS

Microorganism

A psychrotrophic bacterium RT102 strain, which was a new species very close to *Janthinobacterium lividum*, was used in this research [9].

Antibacterial Activity of Violet Pigment

After the violet pigment was separated and purified from the culture, the chemical structure of the violet pigment was determined using absorption spectra qualitative analyses. The separation and purification of the violet pigment was carried out as follows: the culture was centrifuged at 8,000 rpm for 30 min at 4°C, and then the violet pigment was extracted from the supernatant with diethyleter and purified using high performance liquid chromatography (TSK gel DOS 80Ts column, Tosoh Co. Ltd., Japan). The antibacterial effect of violet pigment was examined using various bacteria such

as *Bacillus lichemiformis*, *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Flavobacterium balustinum*, *Escherichia coli*, and *Trichosporon cutaneum*. The incubation experiment for the antibacterial action of violet pigment was carried out by adding various violet pigment concentrations to the culture where the cells grew logarithmically in the medium containing 2.5 g/L yeast extract, 5 g/L polypeptone, and 1 g/L glucose. Incubation temperature and pH were 30°C and 7, respectively.

Incubation Method

A culture medium containing 10 g/L glucose, 10 g/L casein, 5 g/L yeast extract, 1 g/L K_2HPO_4, and 0.2 g/L $MgSO_4 \cdot 7H_2O$ was used for the production of violet pigment by the RT102 strain. The preculture was carried out by inoculating the cells into a 0.1 L medium in a 0.3 L conical flask. When the optical density of culture reached 1.0, the culture was poured into a 3 L jar fermentor (KMJ-3B, Mituwa Co. Ltd., Japan) containing 2 L of the fresh medium. For determining the optimal incubation condition for production of violet pigment, the culture experiments were carried out under various incubation conditions, *i.e.* at pH 5-7, 5-30°C, and a dissolved oxygen concentration of 0.5-5 mg/L. The pH value, temperature, and dissolved oxygen concentration were adjusted to constant values using a controller (Degital Controller TBC-3R, Mituwa Co. Ltd., Japan). Samples (3 mL) withdrawn from the culture at intervals were used for measuring cell concentration, glucose concentration, and violet pigment concentration.

Analytical Methods

After 3 mL ethanol was added to the sample (3 mL), the sample was centrifuged at 15,000 rpm for 3 min at 4°C. The cell concentration was measured from the dry weight of a precipitate after the centrifugation. The violet pigment concentration was determined by the extraction of violet pigment from a supernatant (a liquid containing violet pigment) using dimethyl ether. Glucose concentration was measured by the mutarotase GOD method (Glucose C-Test, Wako Pure Chemicals Co. Ltd., Japan). The viable cell number was measured by the agar layer method.

RESULTS AND DISCUSSION

Antibacterial Effect of Violet Pigment

The growth inhibitory and lethal effect of violet pigment for a putrefactive bacterium, *B. licheniformis*, was examined. Fig. 1 shows time courses in the ratio of viable cell number to initial viable cell number after the addition of various violet pigment concentrations (0-30 mg/L) in the logarithmic phase of cells. In the case where no violet pigment was used, the cells increased with the decrease in the glucose concentration and then

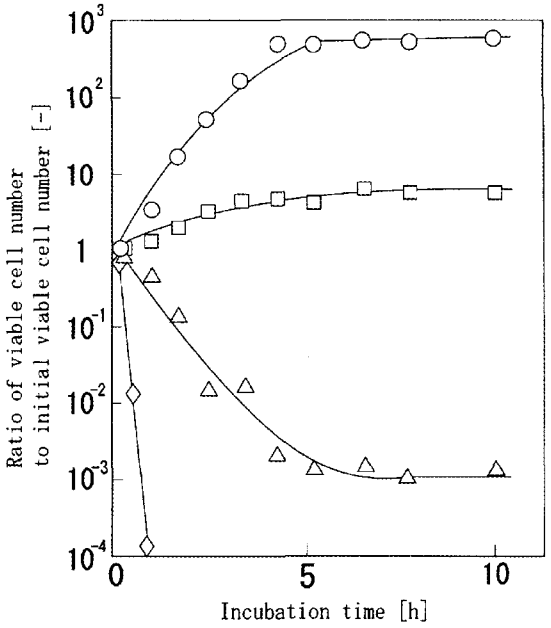

Fig. 1. Growth inhibitory and lethal effect of violet pigment on *Bacillus licheniformis*. Symbols: ○, 0 mg/L; □, 10 mg/L; △, 20 mg/L; ◇, 30 mg/L.

reached a constant value of about 7×10^2 after an incubation time of 5 h. In the case where 10 mg/L violet pigment was added, the rate of viable cell number was about 7 even at an incubation time of 10 h due to the growth inhibitory effect of the violet pigment, and it decreased significantly compared with the case where no violet pigment was used. In the case where 20 mg/L violet pigment was added, the ratio of viable cell number decreased logarithmically for an incubation time of 5 h and then reached a constant value after an incubation time of 7 h. In the case where 30 mg/L violet pigment was added, almost all cells were died after an incubation time of 1 h. As a result, it was found that the high concentration of violet pigment could kill *B. licheniformis* completely.

Table 1 shows the minimum inhibitory concentration (MIC) of violet pigment for various bacteria. MIC is defined as the lowest concentration of antibacterial material that can inhibit cell growth completely. 10-15 mg/L violet pigment showed antibacterial activity for gram-positive bacteria such as *B. licheniformis*, *B. subtilis*, *B. megaterium*, *S. aureus*, and that for gram-negative bacteria such as *P. aeruginosa*. However, it could not inhibit the growth of gram-negative bacteria such as *F. balustinum*, *E. coli* and that of yeast such as *T. cutaneum* even if more than 50 mg/L violet pigment was added. Since the antibacterial activity of a material depends on the destruction of the physical structure or the inhibition of the necessary metabolic reaction in a microorganism, it seems that the presence and the level of the antibacterial activity of the violet pigment varied significantly with the type of microorganism used. Furthermore, since the violet pigment caused not only growth

Table 1. Antimicrobial activity of violet pigment for various bacteria

Bacterium	MIC (mg/L)
Bacillus licheniformis IFO 12107	15
Bacillus subtilis IAM 1026	10
Bacillus megaterium IAM 1111	15
Staphylococcus aureus IAM 1011	15
Pseudomonas aeruginosa IAM 1054	15
Escherichia coli HB 101	> 50
Trichosporon cutaneum IFO 1198	> 50

All values are expressed as means derived from triplicate experiments.
MIC means minimum inhibitory concentration.

inhibition but also the death of bacteria as shown in Fig. 1, it suggests that the violet pigment can decompose essential materials for life maintenance of cells. Though the reason why the psychrotrophic bacterium produces two kind of violet pigments, *i.e.* violacein and deoxyviolacein, is not known, it may be considered that the antibacterial activity for the putrefactive bacteria is increased by the additive effects obtained by the two materials.

Optimal Incubation Condition for Effective Production of Violet Pigment

Fig. 2 shows the time courses of cell growth, violet pigment production, and substrate consumption in a batch culture of bacterium RT102 strain. In this culture the incubation pH, incubation temperature, and dissolved oxygen concentration were 6, 25°C, and 0.5 mg/L, respectively. The cell concentration increased with a decrease in the glucose concentration and reached a maximum value of about 15 g/L at an incubation time of 20 h when the glucose was exhausted. The violet pigment concentration appeared at an incubation time of 14 h and increased even when the cell growth ceased, reaching a maximum value of 3.5 g/L an incubation time of 30 h. Since the production of violet pigment was observed after the cell growth stopped, it was found that the violet pigment was a secondary metabolite. Furthermore, the color of the culture changed into a deep violet caused by the violet pigment produced for an incubation time of 30 h.

The growth properties of psychrotrophic bacterium RT102 strain were clarified by changing the incubation conditions in a batch culture. From the results of culture experiments under various pH values, temperatures, and dissolved oxygen concentrations, it was found that the maximum cell concentration and the maximum violet pigment concentration were obtained at pH 6 and 1 mg/L dissolved oxygen concentration regardless of temperature (data not shown). Fig. 3 shows the effect of temperature on the maximum cell concentration, the maximum violet pigment concentration, and the productivity of violet pigment (the maximum violet pigment concentration/the time required for reaching the maximum violet pigment concentration) in the incubation of RT102 strain at pH 6 and a dissolved oxygen concentration of 1 mg/L. The maximum cell concentration and the maximum violet pigment concentration varied significantly with the temperature. The maximum cell concentration increased with the increase of temperature reaching a maximum value, *i.e.* about 15 g/L, at 15-22.5°C and decreased rapidly beyond 22.5°C. The tendency in the change of maximum violet pigment concentration was almost the same as the maximum cell concentration. Since the cell growth and the production of

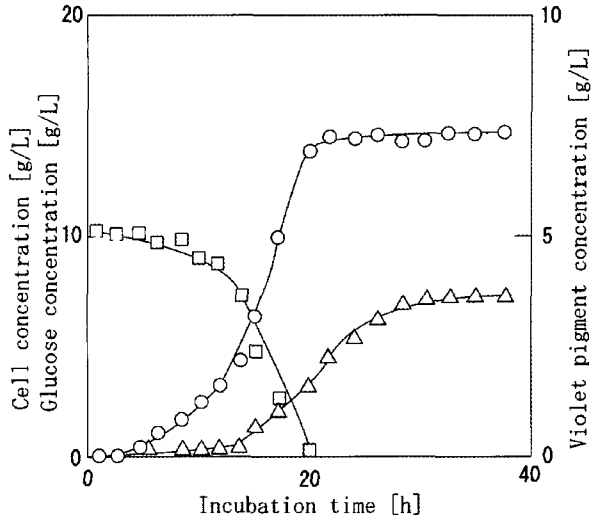

Fig. 2. Time courses of cell growth, substrate consumption, and violet pigment production using psychrotrophic bacterium RT102 strain. Symbols: ○, Cell concentration; □, glucose concentration; △, violet pigment concentration

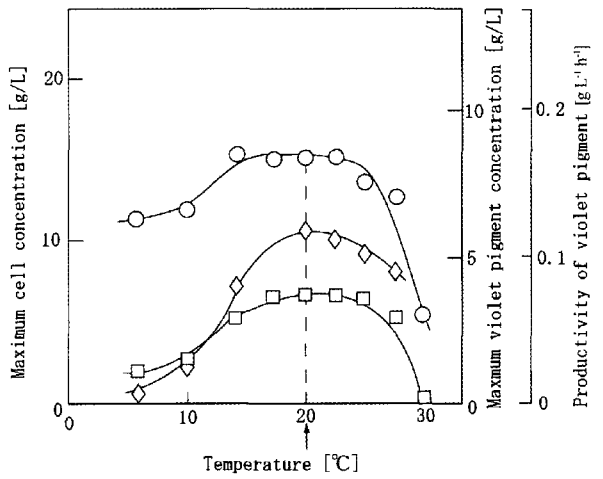

Fig. 3. Effect of temperature on maximum cell concentration, maximum violet pigment concentration, and productivity of violet pigment at pH 6 and a dissolved oxygen concentration of 1 mg/L. Symbols: ○, Maximum cell concentration; □, maximum violet pigment concentration; ◇, productivity of violet pigment.

violet pigment were not observed beyond 30°C, it was confirmed that RT102 strain is a microorganism which grows under comparatively low temperature. The maximum violet concentration and the maximum productivity of violet pigment, *i.e.* 3.7 g/L and 0.12 g L^{-1} h^{-1}, were obtained at 20°C. As a result, it was found that the optimal incubation condition for production of violet pigment was pH 6, 20°C, and 1 mg/L of dissolved oxygen concentration. Our findings will provide basic data for a feasibility study on the large-scale industrial production of antibacterial violet pigment using the bacterium RT102 strain.

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