Research Paper

Development of an Inquiry Laboratory Experiment for Upper Secondary School Biology – A Simple Preparation Method for *Bacillus natto* Cells from a Traditional Japanese Food "Natto" for Gram Staining and DNA Extraction –

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Abstract

In the present study, we designed a student laboratory experiment for a DNA extraction. At the first step, *Bacillus natto* cells were isolated from a traditional Japanese food "natto." By using the isolated bacterial cells, Gram staining was carried out to confirm the Gram stain type. Then, DNA was extracted from the bacterial cells after lysing the cells with lysozyme. The laboratory experiment developed in the present study was tested in some in-service training courses for biology teachers in Osaka Prefecture, and positive evaluation was obtained from the attendees. The laboratory experiment was implemented in a senior high school advance biology class which was offered as an elective subject to the third-year students whose majors were science and mathematics. The implementation was successful. Therefore, the laboratory experiment developed is suitable for an inquiry activity in advanced biology classes.

Key words: Bacillus natto, DNA extraction, Gram staining, inquiry laboratory experiment, senior high school advanced biology

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INTRODUCTION

Many students in the upper secondary schools are in favour of science laboratories. They want to do special technological experiments such as "DNA Extraction and DNA Analysis." Using new technologies may enhance their interest in learning. The motivation is also very important in studying science. If they can derive satisfaction for their inquisi-

tive minds from the successful completion of laboratory experiments, they will come to study science harder. However, teachers are too busy to prepare these experiments. For such biology teachers, we need to develop some simple inquiry laboratory experiments.

For student laboratory use, the method of DNA extraction from plant and animal tissues has been simplified (Aihara 1986, Nakayama and Maekawa 1998, Maekawa 1999, Kusumoto et al. 1999). Shibazaki (1986) tried to introduce an experiment for DNA extraction from a bacterium, *Bacillus natto*, into student laboratories. However, for ordinary biology teachers who are not familiar with bacterial culture, the procedures should be simplified further.

"Natto," a traditional Japanese fermented food, is made by storing boiled soybeans with *B. natto*, which serve as an agent for fermentation, under suitable conditions. *B. natto* is a kind of hay bacterium which commonly exists in hay, soil, rice stubble and even in the air. Traditionally, natto was made as follows: boiled soybeans were packed into a sack of rice straw and then the sack was stored at a temperature around 40°C. The boiled soybeans become very soft and sticky due to the proliferation of *B. natto* whose spores adhere to rice straw.

The bacterium produces various enzymes, vitamins, and amino acids during natto fermentation. Some of the unique compounds in natto are thought to be effective in preventing cancer, osteoporosis, obesity and intestinal diseases caused by pathogens. Many Japanese eat natto every day, so small packages of fresh natto can be purchased easily and cheaply at supermarkets and convenience stores. If we could isolate *B. natto* cells from natto, we would easily be able to use the bacterium in biology laboratory exercises without any equipment for fermentation.

In the present study, we developed a very simple method to collect *B. natto* cells from commercially sold natto. We also developed an inquiry laboratory experiment in which the isolated *B. natto* cells are used.

Materials and Methods

Preparation of B. natto cells

Materials and equipment used in the isolation of *B. natto* cells are shown in Figure 1. Fifty grams of fresh natto were transferred into a paper cup and mixed well with 30 ml of warm water (60°C). Then, the mixture was filtered with a sheet of nylon mesh. This filtrate with high viscosity contains a large number of *B. natto* cells. The viscous filtrate was then used for Gram staining and DNA extraction.

Figure 1 Small packages of fresh "natto," a traditional Japanese food, nylon mesh sheets and paper cups

Gram staining

Gram staining is a technique for staining bacteria and yeast (Okuhira et al. 1999). A drop of the viscous filtrate mentioned above was spread on a clean glass slide and air-dried. The specimen was fixed to the glass slide by

passing it a few times over a flame. Stock solution of crystal violet (crystal violet 0.3 g, ethanol 20 ml) was mixed with 80 ml of 0.1% ammonium oxalate solution to make a diluted crystal violet solution. The slide was flooded with the crystal violet solution for 1 minute and was rinsed with water. Then, the slide was flooded with Gram's iodine solution (iodine 2 g, potassium iodine 2 g, water 300 ml) for 1 minute and rinsed with water. The slide was decolorized by rinsing with ethanol for 10 seconds and then rinsed with water. The slide was flooded with a saffranin solution (saffranin 0.25 g / ethanol 10 ml, water 90 ml) for 1 minute, rinsed with water, and dried in the air. The slide was observed under a microscope at low magnification (x 100) to find the best observation area and then viewed at a higher magnification (x 1000) by oil immersion lens. Gram-positive bacteria and yeasts are stained purple and Gram-negative bacteria are stained pink.

DNA Extraction

The methods for DNA extraction described by Kainuma (1969a, 1969b) and Shibazaki (1986) and were modified partially in the present study. In a test tube 10 ml of the viscous filtrate mentioned above were mixed with 1 ml of 2% lysozyme solution and the same volume of an EDTA, ethylenediaminetetraacetic acid, solution (1 M EDTA, 1.5 M NaCl, Na-phosphate buffer pH 8.0). The mixture was incubated at 40°C for 15 min. Then, 2.5 ml of 20% SDS, sodium dodesylsulfate, solution and 1.5 ml of 30% trichloroacetic acid were added to the mixture and incubated again at 60°C for 5 min. After incubation, the mixture was poured into a glass centrifuge tube. Into the tube, 14 ml of a mixture of chloroform and octyl alcohol (99:1 v/v) was added and the tube was capped tightly. The capped tube was shaken well for 5 min and was centrifuged for 5 min at 2000 rpm (Fig. 2-A). The upper layer in the tube was removed to a beaker and 8 ml of 95% cold ethanol was poured carefully down along the side of the beaker to form a layer. DNA precipitate formed in the alcohol layer, and at the interface of the two layers a stirring rod was swirled, around which the thin threads of DNA were wound.

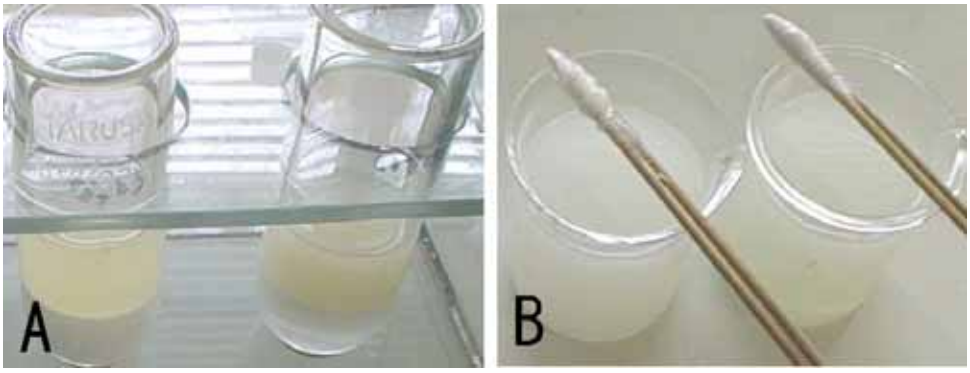

Figure 2 DNA extraction of *Bacillus natto* isolated from a commercially sold natto. A: A mixture in glass tubes centrifuged for 5 min at 2000 rpm; B: Natto DNA wound around the stirring rods

Results and Discussion

The viscous fluid obtained from a commercially sold small package of fresh natto (50 g) contains a large number of *B. natto* cells. Therefore, by the method explained in the present paper, one can easily obtain enough bacterial cells to use in a student laboratory exercise without using any other equipment for fermentation.

B. natto was shown to be a Gram-positive bacterium, because the bacterial cells were stained purple (Fig. 3). The chemical formula of crystal violet is shown in Figure 4 (Okuhira et al. 1999). In the case of a Gram-negative bacterium, crystal violet which binds with Gram's iodine is washed away with alcohol. On the other hand, in the case of a Gram-positive bacterium, crystal violet which binds with Gram's iodine is linked together with teichoic acid and Mg^{2+} (Okuhira et al. 1999). Teichoic acid is peculiar to the cell walls of Gram-positive bacteria (Sakurai 2000, Fig. 5).

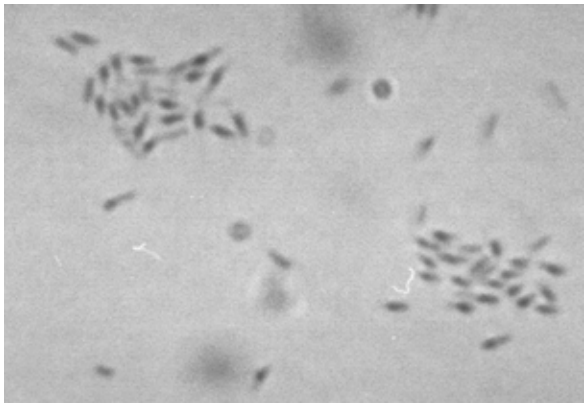

Figure 3 *Bacillus natto* cells stained purple with Gram staining (x 1000)

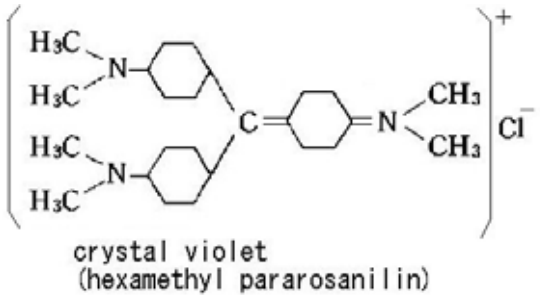

Figure 4 The chemical formula of crystal violet

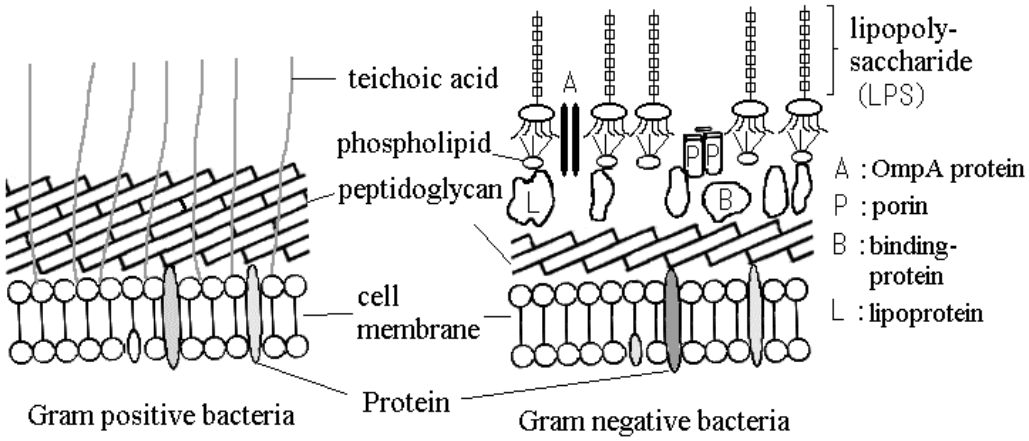

Figure 5 The molecular compositions of cell walls in Gram-positive bacteria and Gram-negative bacteria (partially modified from Sakurai 2000)

After confirming the Gram stain type of *B. natto*, students can decide the method of DNA extraction. Compared to Gram-negative bacteria, Gram-positive bacteria have a thicker peptidoglycan layer at the cell wall. In order to extract DNA from the Gram-positive bacteria, one must degrade the bacterial cell wall. Peptidoglycan can be broken down into *N*-acetylglucosamin and *N*-acetylmuramic acid with the enzyme, lysozyme. Students are expected to select a sound method of lysing *B. natto* cells.

By the methods explained above, DNA could be extracted from the isolated *B. natto* cells. As shown in Figure 2-B, bacterial DNA threads could be wound around a stirring rod. The bacterial DNA obtained had an absorption peak of 260 nm (data not shown). In a student laboratory one can confirm by staining with Schiff's reagent that the threads wound around a stirring rod contain DNA (Aihara 1986, Nakayama and Maekawa 1998, Maekawa 1999).

The laboratory experiment developed in the present study has been tested in some in-service training courses for biology teachers in Osaka Prefecture. The number of attendees in each course was 12-20. Biology teachers attending these courses could carry out the experiment without any trouble. Most of the attendees evaluated the experiment positively. The laboratory experiment was also implemented once in the inquiry activity of a senior high school biology class which was offered to science and mathematics majors. The implementation was successful. However, it was suggested that a large-scale centrifuge is required for the DNA extraction to obtain a good result and the number of students in a laboratory class is preferably less than 20. If the student number is 40, which is the ordinary number of students in one class in Japanese senior high school, the implementation of this laboratory experiment may be difficult.

Whenever students carry out inquiry laboratory experiments, it is very important for them to consider the best way to attain the purpose of their experiment and to consider the reason for each step of the method in the experiment. The experiment developed in the present study, therefore, is considered to be suitable for the inquiry laboratory experiment in the new biology course.

Not only in Japan, but also in other Asian countries, there are many sorts of products of fermentation from fungi such as *Rhizopus oligosporus*, *Mucor hiemalis*, and *Neurospora crassa* (Murao and Arai 1998). These, as well as the products of bacterial fermentation, may be used as the sources of experimental materials.

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