Note

Physiological Characteristics of *Bacillus* sp. HR6 in the Process of Decomposing Bean Curd Refuse

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***Bacillus* sp. HR6 and *Bacillus subtilis* ATCC6051 were compared in terms of their growth, activity of α -amylase, and pattern of heat evolution. *Bacillus* sp. HR6 grew faster than *B. subtilis* ATCC6051 at temperatures higher than 45°C. The maximum activities of α -amylase were 304 and 280 U/ml for *Bacillus* sp. HR6 and *B. subtilis* ATCC6051, respectively. The highest activity of α -amylase from *Bacillus* sp. HR6 was observed at 70°C, and more than 60% of that was still seen at 75°C. In case of *B. subtilis* ATCC6051, the maximum activity was observed at 65°C, and 30% of that was seen at 75°C. A rapid heat evolution was found for *Bacillus* sp. HR6 in comparison with that for *B. subtilis* ATCC6051 when cultivation was initiated in bean curd refuse at 50°C. However, there was no obvious difference in the heat evolution patterns between two strains at the starting temperature of 27°C. These results suggest that *Bacillus* sp. HR6 has a higher activity in decomposing bean curd refuse than *B. subtilis* ATCC6051 at higher temperatures.**

Key words : *Bacillus* sp. HR6/ α -Amylase/Growth/Bean curd refuse/Heat evolution.

There is great interest in the microbial decomposition of waste materials due to concerns regarding the preservation of the natural environment and the conservation of resources. A bacterial strain that we isolated showed a high efficiency and usefulness in the decomposition of some food wastes such as bean curd refuse and fishes (Maeda et al., 1998; Mimura et al., 1995, 1999). The strain was taxonomically classified into the genus *Bacillus* (Mimura et al., 1995), and the physiological and biochemical characteristics of the strain, *Bacillus* sp. HR6, were similar to those of *Bacillus subtilis*. When the strain HR6 was mixed with 30 kg of bean curd refuse and incubated in the organic waste decomposition machine (BIO COSMO 100A, Sanyo Techno Co., Ltd., Kakogawa), the temperature in the chamber rose to 73°C after 9 h. The net weight was reduced by about 70% after a 24-h decomposition period (Mimura et al., 1995). In case

of *B. subtilis* ATCC6051, which was employed as a representative strain of *B. subtilis*, the temperature did not reach 55°C, and about 25 kg (83%) of bean curd refuse remained after a 24-h decomposition process. Therefore, in this study, we tried to characterize *Bacillus* sp. HR6 closely in relation to the microbial decomposition of bean curd refuse.

Bacillus sp. HR6 was originally isolated from the soil. The procedure to select this strain from among 30 strains was as follows. First, we incubated each isolated strain in a nutrient broth containing 5 g of yeast extract (Difco, Detroit, Mi., USA) and 8 g of polypeptone (Nippon Seiyaku Co., Ltd., Tokyo) per liter. Second, 20 ml of the cell suspension was adsorbed in some amount of sawdust, and then mixed with fresh fish meat with bones. Third, the mixture was kept at 35°C in an incubator for 3 days to observe its state of decomposition. Consequently, *Bacillus* sp. HR6 was chosen as the candidate to be used for food waste decomposition.

Strains of *Bacillus* sp. HR6 and *Bacillus subtilis*

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ATCC6051 were preincubated at 30°C for 24 h in a medium containing 5 g of yeast extract and 8 g of polypeptone per liter. Then 0.5 ml of cell suspension was inoculated into a medium containing (per liter) 5 g of yeast extract, 8 g of polypeptone, 5 g of starch, which were autoclaved separately, and sodium chloride was added at concentrations of 0 to 87.7 g (1.5 M). The medium was adjusted to pH 7.5 by tetramethylammonium hydroxide.

The bacterial growth was monitored photometrically by measuring the turbidity of the cell suspension at 650 nm with a spectrophotometer (DU 640, Beckman, Fullerton, Calif., USA). The incubation temperature varied from 30 to 50°C in the absence of NaCl and it was fixed at 45°C in the presence of NaCl. The growth rates, doublings per h, were calculated from the growth curve. The turbidity used for the analysis was from 0.05 to 0.6 in optical density, be-

cause there was linearity in this range on a logarithmic scale.

The growth rates of both strains were shown in Fig. 1A. The doublings per h for both strains were almost the same below 37°C. A difference was observed at temperatures higher than 45°C, i. e., the values and their S. D. at 45°C were 3.16 ± 0.10 and 2.86 ± 0.11 (doublings/h) for *Bacillus* sp. HR6 and *B. subtilis* ATCC6051, respectively. The doublings per h were reduced with an increase of the temperature from 45 to 50°C. Since bacteria in the environment are sometimes exposed to stresses such as salt, heat, pH change and UV radiation, we examined the bacterial growth in the presence of NaCl (Fig. 1B). With increasing NaCl concentrations from 0.5 to 1.5 M, the growth rate of *Bacillus* sp. HR6 became smaller than that of *B. subtilis* ATCC6051. In the presence of 1.5 M NaCl, the doublings $\pm$ S. D. per h for *Bacillus* sp. HR6 and *B. subtilis* ATCC6051 were 1.2 ± 0.10 and 1.43 ± 0.03 , respectively.

In the microbial decomposition of bean curd refuse, attention must be paid to the possibility that many types of bacteria might prevent the efficient decomposition of waste materials by *Bacillus* sp. HR6. A large value of the doublings per h at a high temperature is one of the critical factors for a strain to be dominant in such a bacterial community. The growth of *Bacillus* sp. HR6 was enhanced by increasing the incubation temperature in the absence of NaCl, but repressed with an increase of NaCl concentration, compared to that of *B. subtilis* ATCC6051. These results indicate that *Bacillus* sp. HR6 is useful in decomposing food waste material containing lower salt concentrations.

The extracellular α -amylase activities of *Bacillus* sp. HR6 and *B. subtilis* ATCC6051 were measured by a modified DNS method (Park et al., 1997). The DNS solution contains 300 g of potassium tartrate, 10 g of 3, 5-dinitrosalicylic acid, and 32 g of NaOH (per 1.3 liter). After a 24-h incubation at 30°C in the medium which was the same as that used for the measurement of the growth rate, the cell suspension was centrifuged twice ($12,000 \times g$, 5 min) at 4°C and the cell free supernatant was stored at 4°C before use. Starch (Nacalai Tesque Co., Ltd., Kyoto), the substrate of α -amylase, was dissolved in 50 mM of potassium phosphate buffer, pH 7.5, to make a 6% (w/v) solution. A mixture containing 250 μ l of the starch solution and 1 ml of the supernatant was incubated at given temperatures. Two hundred and fifty μ l of aliquot in the reaction mixture was taken out at certain intervals and mixed with 250 μ l of the DNS solution. After being boiled for 5 min, the sample was mixed with 5 ml of distilled water, and the absorbance at 540 nm was measured. The α -amylase activity was

FIG. 1. Growth rates for *Bacillus* sp. HR6 and *Bacillus subtilis* ATCC6051 at given temperatures in the absence of NaCl (A) and at 45°C in the presence of various concentrations of NaCl (B). The growth experiments for *Bacillus* sp. HR6 (○) and *B. subtilis* ATCC6051 (●) in experimental conditions were carried out three times independently. Each point represents the mean of the doublings per h, and the standard deviations are represented by the error bars.

calculated from the slope of the linear regression curve using more than three points of data, with the correlation coefficient over 0.96 for every experiment. One unit of enzyme activity was defined as the amount of enzyme to make 1 nmol reducing sugar as maltose from starch per min.

When *Bacillus* sp. HR6 was cultivated with bean curd refuse in a Dewar flask for 24 h, the reduction percentage of carbohydrates was 19.4%, which was larger than those of proteins and lipids (Mimura et al., 1995). During the microbial decomposition of bean curd refuse in an organic waste decomposition machine, the temperature in bean curd refuse became over 70°C (Mimura et al., 1995). Thus, we expected that the extracellular α -amylase of *Bacillus* sp. HR6 would possess a higher activity at an increasing temperature or be produced in larger amounts than that of *B. subtilis* ATCC6051.

Figure 2 shows the effect of temperature on the activity of α -amylase. The activity of α -amylase from *Bacillus* sp. HR6 was at the maximum, 304 U/ml at 70°C, over 60% of which remained at 75°C. On the other hand, the maximum activity for *B. subtilis* ATCC6051 was 280 U/ml at around 65°C, which was reduced by 70% when the reaction temperature was increased to 75°C. The optimum temperature for the extracellular α -amylase activity of *Bacillus* sp. HR6 was 5°C higher than that of *B. subtilis* ATCC6051. In addition, the activity at 75°C was much higher than that of *B. subtilis* ATCC6051. These results indicate that α -amylase of *Bacillus* sp. HR6 reacts better than

FIG. 2. Effect of temperature on the extracellular α -amylase activity of *Bacillus* sp. HR6 and *Bacillus subtilis* ATCC6051. The supernatant of *Bacillus* sp. HR6 (○) and *B. subtilis* ATCC6051 (●) grown in the medium containing 0.5% (w/v) of starch was used to measure the initial α -amylase activity at given temperatures. Each point represents the mean of the three replications, and the standard deviations are represented by the error bars.

that of *B. subtilis* ATCC6051 at higher temperatures in the decomposition of bean curd refuse. Since the dry weights of *Bacillus* sp. HR6 and *B. subtilis* ATCC6051 strains were 3.7 and 3.6 mg/ml, respectively, the specific activity of α -amylase (U/mg of dry weight cells) for each strain showed the same pattern as in Fig. 2 (data not shown), indicating that the amount of enzyme produced from *Bacillus* sp. HR6 was almost equal to that from *B. subtilis* ATCC6051.

We also measured the heat evolution in bean curd refuse after being mixed with the strain of *Bacillus* sp. HR6 or *B. subtilis* ATCC6051. Cells grown in 500 ml of the medium containing 5 g of yeast extract and 8 g of polypeptone (per liter) were washed with a centrifuge and suspended in 50 ml of distilled water. The cell suspension was mixed with 700 g of bean curd refuse (Ogura Food Co., Ltd., Kakogawa) which had been previously autoclaved. The mixture was placed in a Dewar flask (ϕ 11.5 x 20 cm), the surface of which was covered with autoclaved cotton to prevent thermal loss and bacterial contamination. The temperature change in the flask was measured with a thermocouple (E52-CA20C, Omron, Kyoto) connected with a chart recorder (E55A-C3, Omron, Kyoto).

When the strains were cultivated in the bean curd refuse product at the initial temperature of 50°C, the temperature was once reduced within 3 h of incuba-

FIG. 3. Heat generation patterns for *Bacillus* sp. HR6 (—) and *Bacillus subtilis* ATCC6051 (·····) cultivated in the bean curd refuse product at the initial temperature of 50 and 27°C, respectively. Bean curd refuse was stored in a Dewar flask which was kept at 30°C in the incubator.

tion. Thereafter, the temperature of the bean curd refuse mixed with *Bacillus* sp. HR6 began to increase and reached to 57.5°C at 9 h of the incubation. A similar pattern was observed for the heat evolution by *B. subtilis* ATCC6051. The ratios calculated from the slopes (drawn in Fig. 3) of the heat evolution curves for *Bacillus* sp. HR6 and *B. subtilis* ATCC 6051 were 2.50 and 2.15°C per h, respectively. The decrease of the temperature observed during the initial stage seems to be related with the heat outflux from the Dewar flask. When the starting temperature was 27 °C, the heat evolution patterns of the strains were almost the same.

The heat evolved in the microbial decomposition of food waste caused the thermal death of other contaminating bacteria (Hamamatsu et al., 1993). Thus, a rapid growth followed by the heat evolution is primarily required when bacteria are used for microbial decomposition. *Bacillus* sp. HR6 showed a more accelerated rate of heat evolution (Fig. 3) and grew faster (Fig. 1A) than *B. subtilis* ATCC 6051 at the increased temperatures. These results support the likelihood that *Bacillus* sp. HR6 is superior to *B. subtilis* ATCC6051 in terms of food waste decomposition.

Mata-Alvarez and Llabrés (1992) have proposed that the anaerobic decomposition of the organic wastes can be used to produce methane gas which in turn can be used to produce electricity. In our system, we have observed the high temperature of 73°C in the chamber when a bean curd refuse was decomposed with aeration and stirring (Mimura et al., 1995). Thus, the heat produced by microbial decomposition can be possibly and efficiently utilized for a variety of ways to save energy.

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