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Hyper-CMCase-Producing Mutants of *Bacillus* sp. 79-23 Induced by Gamma-Radiation

YOON, KI-HONG*, IN-KYUNG SHIN, KYUNG HWA JUNG[1], AND SEUNG-HWAN PARK[1]

School of Food Biotechnology, Woosong University, San 7-6, Jayang-Dong, Dong-Gu, Taejon 300-100, Korea
[1]Bacterial Molecular Genetics R.U., KRIBB, P.O. Box 115, Yusong, Taejon 305-600, Korea

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Abstract *Bacillus* sp. 79-23 spores were irradiated with ^{60}Co gamma-rays at doses ranging from 0.5 to 5 kGy. Following gamma-irradiation, seven mutant strains were isolated by scoring the halo sizes formed around the colonies grown on LB agar plates containing 4% carboxymethylcellulose (CMC) and trypan blue. The mutant strains showed a 1.5 to 2-fold increase in carboxymethylcellulase (CMCase) activity over the parent strain. Wheat bran acted as an effective inducer for CMCase production in the parent and mutant strains. Mutant strains 68 and 70 were identified as exhibiting higher CMCase activities than those of other mutants in LB media both with and without 3% wheat bran. In addition, these strains seem to produce substantially lower amounts of capsular materials, whereas the parent strain produced large amounts of them in both liquid and solid LB media. In flask cultures, the CMCase production by mutants 68 and 70 reached maximum levels of 17.5 unit/ml and 15.7 unit/ml, respectively, in an LB medium containing 3% wheat bran.

Key words: *Bacillus* sp. 79-23, CMCase, mutagenesis, gamma-radiation, wheat bran

Cellulose is the most abundant and renewable carbon source in the world. Accordingly, cellulases have been studied mainly with respect to their potential industrial use for the exploitation of biomass resources [2, 3, 4, 14, 16]. However, the purpose of this study is primarily focused on improving the texture as well as their bleaching efficiency in the textile industry. A neutral action is essential for enzymes in the textile industry, because an acidic pH can make blotches on fabric. However, most known fungal and bacterial cellulases operate at an acidic pH [4, 15, 16]. Fortunately, a *Bacillus* strain, named *Bacillus* sp. 79-23, isolated from soil, produces a neutral cellulase that has potential use in the textile industry [20].

*Corresponding author
Phone: 82-42-630-9742; Fax: 82-42-636-2676;
E-mail: ykh@lion.woosong.ac.kr

Bacillus sp. 79-23 has also been named *B. subtilis* based on its biochemical properties. This isolate exhibits about a 4-fold higher CMCase activity in a culture supernatant than other *B. subtilis*-type strains. Furthermore, wheat bran greatly increases the level of CMCase activity in the culture supernatant of this isolate. When the isolate is grown in an LB medium containing 3% wheat bran, it exhibits about a 13-fold higher CMCase activity than that grown only in LB medium [20]. This CMCase level is comparable to that of the recombinant *B. subtilis* containing a replicative plasmid harboring the *Bacillus* sp. 79-23 CMCase gene modified with a strong promoter [7].

To maximize this CMCase production of *Bacillus* sp. 79-23 for industrial use, two strategies can be considered: (i) classical mutagenesis and (ii) recombinant DNA manipulations. There are earlier reports on the cloning of the *Bacillus* sp. 79-23 cellulase gene (*celS*) and examining of this *celS* gene expression on a replicative plasmid and on the *B. subtilis* 168 chromosome [6, 7]. However, the CMCase production levels of these genetically engineered strains were not sufficient for a large-scale production. For developing a hyper-CMCase-producing strain, the classical mutagenesis of *Bacillus* sp. 79-23 seems to be sufficient since CMCase production by the isolate grown on wheat bran was comparable to that of the genetically engineered strains [6, 7]. This paper describes the isolation of the γ-radiation-induced mutants of *Bacillus* sp. 79-23 and the CMCase production of these mutant strains.

Materials and Methods

Bacterial Strain and Media

Bacillus sp. 79-23 was isolated from soil in this laboratory [20] and maintained on an LB medium (bacto-tryptone, 10 g; bacto-yeast extract, 5 g; sodium chloride, 10 g; per liter). An HCO medium [11] was used for the preparation of the spore suspension of *Bacillus* sp. 79-23. For the selection of hyper-CMCase-producing mutants, the

mutagenized cells were plated on LB agar supplemented with 4% (w/v) carboxymethylcellulose (CMC; low viscosity, Sigma Co.) and 0.008% (w/v) trypan blue. For quantitative CMCase assay, the bacterial strains were cultured in either LB medium or LB medium containing 3% (w/v) wheat bran.

Spore Preparation

Bacillus sp. 79-23 cells grown in the LB medium were inoculated into 2 l of the HCO medium and then cultured with vigorous shaking at 37°C for 5 days until most of the cells sporulated. The spores were collected by centrifugation, washed twice with sterilized-deionized water, and finally suspended in 30 ml (ca. 5×10^{10} CFU/ml) of sterilized-deionized water. The spore suspension was heated at 65°C for 30 min and stored at 4°C.

Mutagenesis

Five-ml aliquots of the spore suspension were irradiated with γ-rays in a ^{60}Co γ-source for 2 h. The total amount of irradiation given ranged from 0.5 to 5 kGy. The irradiated spores were diluted serially with LB medium and then an appropriate volume was spread on LB agar plates containing 4% CMC and trypan blue. Following overnight incubation at 37°C, the colony-forming units (CFU) were counted at a dilution, giving 100-200 colonies per plate to determine cell survival. The irradiated spores were used for the selection of the hyper-CMCase-producing mutants.

Enzyme Assay

The CMCase activity was determined by measuring the amount of reducing sugars using dinitrosalicylic acid (DNS) [13]. One-ml reaction mixtures containing 1% (w/v) CMC (medium viscosity, Sigma Co.) in a 50 mM sodium phosphate buffer, pH 6.5, were incubated at 55°C for 15 min. The reaction was stopped by adding 3 ml of DNS solution, and the reaction mixtures were boiled for 5 min. Absorbance at 540 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min.

RESULTS AND DISCUSSION

Gamma-Irradiation of *Bacillus* sp. 79-23

The classical mutagenesis of living cells has been utilized to improve the production of a certain target protein by industrial microorganisms [1, 9, 10]. Chemical and physical agents, and various types of radiation have been used to increase mutation frequencies. Because of its high effectiveness in inducing mutations, ionizing γ-radiation was selected as the mutagenic agent for the development of the hyper-CMCase-producing mutants of *Bacillus* sp. 79-23.

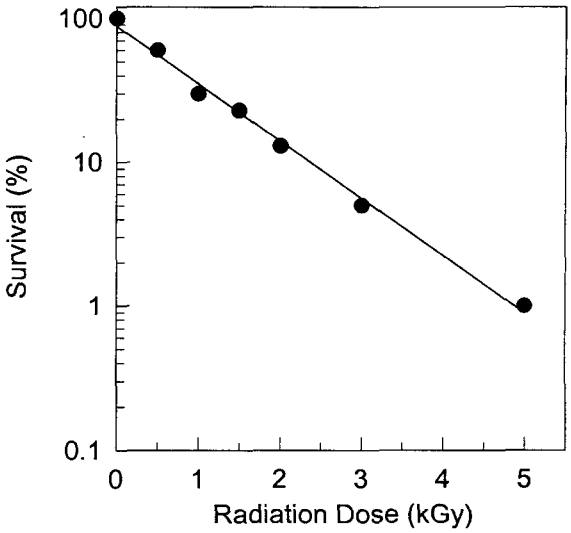

Fig. 1. Survival of *Bacillus* sp. 79-23 spores after gamma-irradiation.
The *Bacillus* sp. 79-23 spores (ca. 5×10^{10} CFU/ml) were irradiated at doses ranging from 0.5 to 5 kGy. The survival (%) was determined by estimating the ratio of the CFU of the irradiated samples to the CFU of a non-irradiated sample.

It has been known that ionizing γ-radiation causes many types of mutation in cellular DNA [5, 8, 17]. These mutations are the results of not only direct energy disposition into cellular DNA (direct effect) but also reactions with reactive radicals formed in the vicinity of DNA (indirect effect) [12]. γ-Irradiation primarily induces DNA damages such as strand breaks and base alterations, whereas it causes much less damage to proteins, lipids, and RNA [8, 17, 18, 19]. The survival of bacterial cells following irradiation depends upon certain intrinsic factors, such as the physiological condition of individual cells and their potential for repair. Prior to the selection of mutant strains, it is desirable to obtain a survival curve for the parent strain, because the lethal effect of γ-radiation varies from strain to strain. Therefore, *Bacillus* sp. 79-23 spores were gamma-irradiated with increasing doses from 0.5 to 5 kGy, followed by a determination of cell viability. As shown in Fig. 1, the survival rate of irradiated spores exponentially decreased in the dose range of 0.5 to 5 kGy. Based on this result, it was decided that spores irradiated at doses of 3 and 5 kGy would be used for the selection of *Bacillus* sp. 79-23 mutants. At these doses, the cell viability was approximately 5% and 1%, respectively.

Mutant Selection

For the selection of hyper-CMCase-producing mutants, the γ-irradiated spores were diluted with LB medium, and then an appropriate volume giving 100-200 colonies per plate was spread on LB agar plates containing CMC (4%) and trypan blue. To discriminate the hyper-CMCase-producing mutants, a high concentration (4%) of CMC was added to

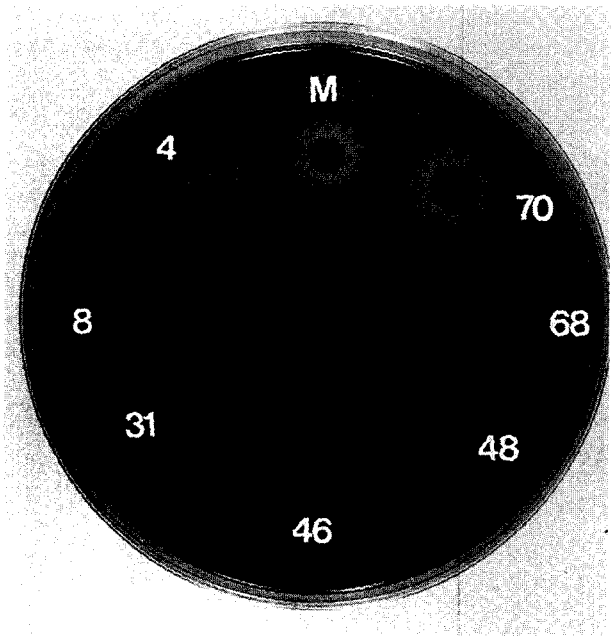

Fig. 2. γ-Radiation-induced mutants of *Bacillus* sp. 79-23 grown on LB medium containing 4% CMC and 0.008% trypan blue.
Symbols: M, the parent strain; 4-70, mutant strains.

the LB agar plates. The use of a high content of CMC facilitated observation of the differences in the halo sizes produced by the various hyper-CMCase-producing strains. The screening of the mutant strains which produced an increased amount of CMCase was based on the ability of colonies to form a halo on selective plates. About 400 colonies with large halos were first screened from approximately half a million colonies grown on selective plates. All the strains selected from the primary screening were grown overnight in 10 ml of LB medium and were subjected to quantitative CMCase assay with their culture supernatants. Finally, seven mutant strains that significantly increased the CMCase production were selected (Fig. 2).

It is worth noting that γ-irradiation caused the parent strain to change its colony morphology with respect to size and smoothness as well as CMCase productivity. In order to verify that the selected seven strains were actually derived from *Bacillus* sp. 79-23, a polymerase chain reaction (PCR) was performed using *celS*-specific primers to amplify the *Bacillus* sp. 79-23 *celS* gene. An agarose gel electrophoresis of the PCR-products showed that all seven strains produced one prominent band corresponding to the *celS* gene, indicating that the possibility of contamination during the selection procedure was negligible (data not shown).

CMCase Production by Mutant Strains

It was previously reported that wheat bran served as an effective inducer for CMCase production in *Bacillus* sp. 79-23 without any increase of cell growth [20]. However, wheat bran was not effective for CMCase production in *B. subtilis* 168 and DB104 strains, as shown in Table 1, even though these strains had a CMCase gene identical to that of *Bacillus* sp. 79-23 [7]. CMCase induction by wheat bran might be a characteristic of *Bacillus* sp. 79-23 among various *B. subtilis* strains. Recently, Yu *et al.* [21] also reported on the cellulase induction of *Trichoderma reesei* in wheat bran-containing media.

Table 1. CMCase productivity of the mutant strains of *Bacillus* sp. 79-23.

	CMCase activity (units/ml)*	
	LB medium	Wheat bran
B. subtilis 168	0.14	0.3
B. subtilis DB104	0.15	0.3
Bacillus sp. 79-23	0.61	7.6
Mutant strain 4	0.98	13.0
8	0.98	12.9
31	0.86	8.8
46	0.79	15.2
48	1.04	7.1
68	1.10	17.5
70	1.11	15.7

**Bacillus* strains were cultured at 37°C on LB media either with or without 3% wheat bran. After cultivation for 11 h, the CMCase activities were determined using the culture supernatant.

To examine the CMCase productivity of the seven mutant strains, all the strains were grown in 200 ml of LB medium either with or without 3% wheat bran. The CMCase activities were then assayed with the culture supernatant (Table 1). The mutant strains exhibited a 1.5 to 2-fold increase in CMCase activity over the parent strain. In particular, the two mutant strains 68 and 70 were superior to the others in CMCase productivity in LB media both with and without wheat bran. In addition, it was found that their colonies became rough and their liquid cultures were less viscous than the parent strain, suggesting that the production of slime materials dramatically decreased in these mutants. This property makes them more advantageous for large-scale cultivation and genetic manipulations such as a transformation with recombinant DNA.

As shown in Fig. 3, the γ-radiation-induced mutation did not inhibit the growth of mutants 68 and 70 (Fig. 3A), and CMCase production was associated with cell growth (Figs. 3B, 3C). The maximum CMCase activity was achieved within 11 h of cultivation. The increase in CMCase activity was 10 unit/ml for mutant 68 and 8 unit/ml for mutant 70 on LB media containing wheat bran, in comparison with the activity of the parent strain. Since the strain 68 showed a high level of CMCase activity with 17.5 unit/ml in spite of it being a batch culture, its CMCase production can be improved up to a level applicable for a large-scale fermentation by optimizing culture conditions. Therefore, it is expected to be a promising neutral cellulase producer.

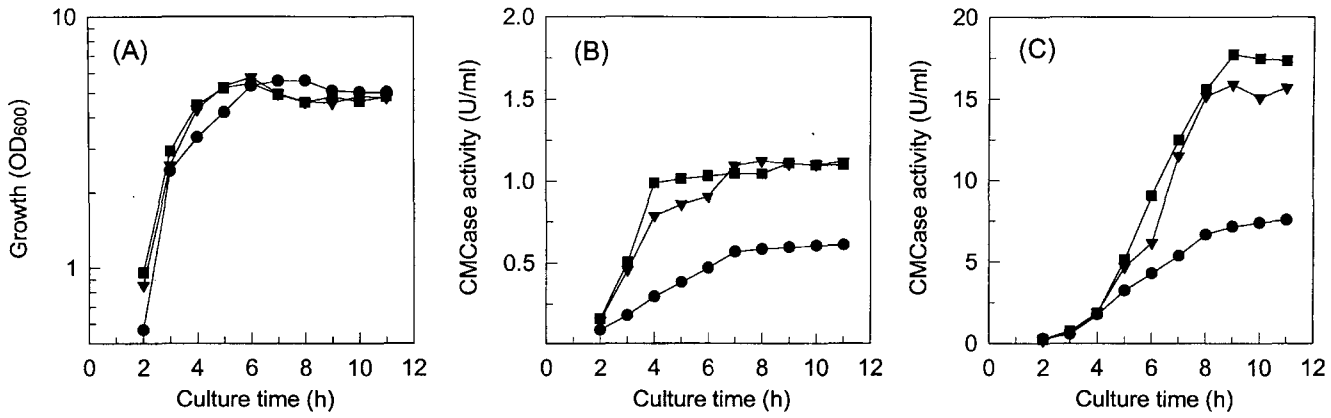

Fig. 3. Growth and CMCase production by the parent and mutant strains in LB medium either with or without 3% wheat bran.
(A), Growth on LB medium; (B), CMCase activity in LB medium; (C), CMCase activity in LB medium containing 3% wheat bran. Symbols: ● , parent strain; ▼ , mutant 70; ■ , mutant 68.

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