Nuclear Instruments and Methods in Physics Research B 267 (2009) 99–102

Contents lists available at ScienceDirect

Nuclear Instruments and Methods in Physics Research B

journal homepage: www.elsevier.com/locate/nimb

# The selection and characterization of the mutants in bioflocculant bacteria C8 induced by 3 MeV helium ion irradiation

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ARTICLE INFO

*Article history:*
Received 20 August 2008
Received in revised form 5 November 2008
Available online 20 November 2008

*Keywords:*
Ion irradiation
Bioflocculant
Dehydrogenase
Survival ratio
Heavy ions
RAPD

ABSTRACT

A 3 MeV $He^{2+}$ beam was used to irradiate C8 (a flocculant-producing bacteria) with a fluence ranging from $10^{11}$ to $10^{13}$ ions/cm$^2$. The effects on the survival ratio, TTC-dehydrogenase activity, flocculating activity and RAPD analysis are reported. The survival ratio curve caused by irradiation is proved to be "saddle-shaped". Eleven mutants were obtained, all of which had a significant change in dehydrogenase activity and most showed a positive change in flocculating activity. RAPD measurements were used to analyse the DNA of mutants with a flocculating activity over 80%, which indicated that all their DNA had been changed by irradiation.



## 1. Introduction

The discovery of bio-effects by heavy ion irradiation of biological organisms has opened a new branch in the field of ion beam applications in life sciences [1]. Due to their wider mutation spectrum and high LET (linear energy transfer), the effects of heavy ion irradiation of organisms is obviously different from that of UV and γ-rays irradiation [2] and it has been used to induce mutation [3]. There have been many reports that keV heavy ions can produce designed physical changes [4,5], there have been also some findings of irradiation effects of ion irradiation on crop seeds, cells and microbes in recent years [6]. Meanwhile, there are some doubts on the bio-effects of keV heavy ion irradiation because of their short range and vacuum conditions which cause the death of most bacteria [7]. Compared with keV heavy ions, MeV ions can penetrate the whole bacteria which means their effect should not be limited to the surface. There have been some reports of MeV ions used to irradiate biomolecules and seeds [8,9]. With regards to microorganisms there are few reports [10], with MeV $He^{2+}$ ions seldom reported for use in bacteria mutation.

Random amplified polymorphic DNA (RAPD) has been extensively used for the identification of either species or cultivars. It is generally favored because of its sensitivity, simplicity and cost-effectiveness. RAPD does not require any specific knowledge of the DNA sequence of the target organism. Therefore, it is particularly attractive for determining genetic variations at the DNA level [11–14].

In this work 3 MeV $He^{2+}$ ions were used to irradiate one type of bioflocculant bacteria, C8, with the purpose of achieving mutation and other bio-effects such as survival ratio, TTC (2,3,5-triphenyltetrazolium chloride)-dehydrogenase activity, flocculating activity and DNA of mutants and the original strain were compared. RAPD analysis was used to detect DNA mutations [15,16].

## 2. Materials and methods

### 2.1. Sample preparation before $He^{2+}$ irradiation

An original flocculant-producing strain of C8 isolated from soil were stored in our laboratory. Before irradiation the original strains were cultured in a beef-protein fluid medium at 35 °C with a shaking frequency of 125 rpm for 10 h. Samples were placed in a centrifuge at 4000 rpm for 5 min, then washed three times with sterilized, distilled water. The concentrations of bacterial suspensions were modulated to $10^7$ cfu/mL. A 0.3 mL bacterial suspension which was made of normal saline containing glycerol in concentration of 10% was placed on a 34 mm diameter Petri dish and then dried at room temperature.

### 2.2. $He^{2+}$ ion irradiation

Irradiation was carried out in the Institute of Heavy Ion Physics of Peking University. 3 MeV $He^{2+}$ ions were produced with a

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doi:10.1016/j.nimb.2008.11.007

$2 \times 6$ MV tandem accelerator with a fluence ranging from $5 \times 10^{11}$ to $1 \times 10^{13}$ ions/cm$^2$. Ions were extracted from the vacuum chamber through a window made of 5 μm thick titanium foil film to irradiate the sample in the air at room temperature. The distance between the sample and the exit window was about 2 mm.

### 2.3. Methodology for survival ratio curves

Dried cells irradiated at different fluences were eluted with 1 mL sterilized saline water and evenly mixed, then a viable number was converted through a colony counting method. All the experimental conditions were strictly controlled in an asepsis condition. The percentage survival rate was calculated as $(A/B) \times 100$, where $A$ and $B$ are numbers of viable bacterium at each ion fluence and a blank control.

### 2.4. Mutation selection

After irradiation the samples were washed with sterilized, distilled water and diluted into serial dilutions in the ratio of 1:10. The mutated strains were selected and purified after a 1–2 day cultivation on a beef-protein agar medium at 35 °C according to the colony morphology in a asepsis condition.

### 2.5. Measurement of dehydrogenase activity

The TTC method [17] was used to determine the dehydrogenase activity of mutants and C8. Calibration curve of TTC's reduction product was made with 20, 40, 60, 80, 100, 120, 140 μg/mL TTC standard solution first.

A 10 mL fermentation broth was placed in a centrifuge at 4000 rpm for 5 min, then washed three times with sterilized, distilled water and completed with sterilized saline to 10 mL. The reaction volume was controlled at a constant temperature of 37 °C for 30 min. The 5 mL volume consisted of the sample 1.5 mL, Tris–HCl buffer 2 mL, $Na_2SO_3$ (0.36%) 0.5 mL, beef-protein fluid medium 0.5 mL and TTC (0.1%) 0.5 mL. A blob of sulphuric acid and butanol, 5 mL, were placed in the reaction volume after 30 min for terminating the reaction and extracting the TTC's reduction product at a temperature 90 °C for 5 min, then the volume was placed in a centrifuge at 4000 rpm for 10 min. Supernatants were measured on the $OD_{485}$ (optical density at the visible wavelength of 485 nm). The TTC's reduction product was calculated on the basis of the formula of calibration curve.

Synchronously, a dry weight of 2 mL of the sample was weighed. The dehydrogenase activity can be calculated as following:

$$\text{TTC-dehydrogenase activity } [\mu g/(g \text{ dry weight h})] = \frac{2 \times A \times B}{C},$$

where $A$ is the calculated TTC's reduction product, $B$ is dilution multiple of supernatants, $C$ is the dry weight of the 2 mL samples.

### 2.6. Measurement of flocculating activity

The flocculating activity of the bacteria was measured with Kurane's method [18]. It was cultivated on a beef-protein agar medium and innoculated into a 150 mL fermentation medium to harvest the bioflocculant sample. The culture temperature was kept at 30 °C for 66 h with a shaking frequency of 150 rpm. The fermentation medium contained (per litre): glucose 10 g, urea 0.5 g, yeast extract 0.6 g, NaCl 0.6 g, $K_2HPO_4$ 6 g, $KH_2PO_4$ 3 g, water 1000 mL and the pH value was adjusted. 8 mL of bioflocculants and 10 mL of $CaCl_2$ solution were mixed with 800 mL of kaolin solution in a 1000 mL beaker and the pH value was adjusted to 4 with HCl or NaOH. Sampling was performed in the middle of the beaker and $OD_{550}$ (optical density of turbidity at the visible wavelength of 550 nm) were measured. The test beaker was stirred for 1 min at 200 rpm and then 2 min at 80 rpm, then left to stand for another 5 min. The flocculating activity can be calculated as following:

$$\text{flocculating activity } (\%) = \frac{A - B}{A} \times 100,$$

where A and $B$ are the optical densities of the control and measured sample at 550 nm.

### 2.7. RAPD analysis

The method of genomic DNA extraction was used according to [16] and the RAPD was measured as in [19]. Amplifications were carried out in a 25 μL system. Each primer in Table 1 was detected and S28 was selected as the best primer in this experiment. DNA amplification was performed for 40 cycles, for 1 min at 94 °C, 1 min at 36 °C, 1 min at 72 °C, followed by a final extension cycle of 10 min at 72 °C. The RAPD products were resolved on agarose gel (1.4%). To confirm reproducibility of the method, all amplifications were repeated at least three times.

## 3. Results and discussion

### 3.1. Survival rates

Bacteria survival ratios as a function of ion fluence are shown in Fig. 1. Compared with keV ion irradiation where the fluence can be as high as $10^{15}$ ions/cm$^2$ [20], the use of 3 MeV $He^+$ ions is more efficient in damaging the bacteria. Even at a fluence of $5 \times 10^{11}$ ions/cm$^2$, only 6% of the bacteria survived. With increasing fluence the survival ratio drops exponentially until a fluence of $8 \times 10^{12}$ ions/cm$^2$ where it increased a little, then dropped again. Regarding the fluence dependence, the survival rate when irradiated by heavy ions differs from that using UV, γ-rays or high energy particles which follow an exponential decrease. This relationship between fluence and survival ratio can be explained by the hit model [21]. A molecular model for irradiation which assumes that the mass deposit effects can produce molecules to repair the damage of DNA in

**Table 1**
Primers for RAPD analysis.

| Serial number | Primer sequence | Serial number | Primer sequence |
|---|---|---|---|
| S11 | GTAGACCCGT | S15 | GGAGGGTGTT |
| S17 | AGGGAACGAG | S19 | TGGGGGACTC |
| S21 | CAGGCCCTTC | S24 | AATCGGGCTG |
| S28 | GTGACGTAGG | S42 | GGACCCAACC |

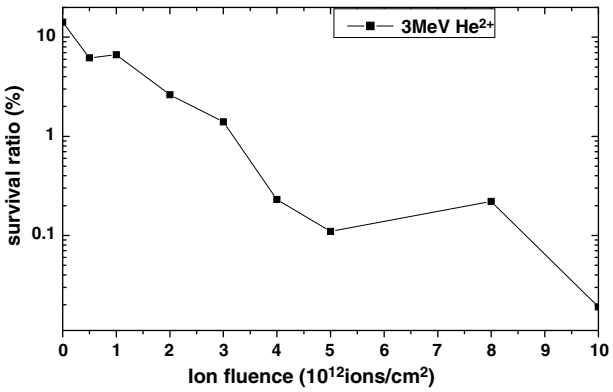


**Fig. 1.** Survival ratio of C8 bacteria as a function of ion fluence of 3 MeV $He^{2+}$.