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# Low-energy plasma immersion ion implantation modification of bacteria to enhance hydrolysis of biomass materials

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ABSTRACT

This report is on exploring low-energy plasma immersion ion implantation (PIII) as a novel bio-technique and an improved alternative to ion beams to induce bacterial mutation for enhancing the hydrolysis of biomass materials, eventually benefiting environmental protection and biofuel production. Cellulase-producing bacterial cells of *Bacillus amyloliquefaciens* were treated by argon or nitrogen PIII at a bias voltage of −2.5 kV with various fluences from $1 \times 10^{15}$ to $1 \times 10^{17}$ ions/cm$^2$ to induce mutation. The bacterial mutants exhibiting clear potentiality of enhanced cellulase activity which indicated improved hydrolysis capability were screened. Comparisons in the cellulase activity between the wild type as the control and the mutant under various buffer pH values and temperatures showed that the cellulase activities of the mutant were clearly higher than that of the control, particularly for neutral pH and lower and higher temperatures. The cellulase hydrolysis ability tests against various biomass materials including rice straw, corn stover and corn husk demonstrated the mutant possessing the higher hydrolysis activity, particularly for the corn husk. Physical and biological mechanisms involved in the bacterial cell modification induced by the low-energy PIII were investigated and discussed in terms of the ion stopping in the specific bacterial cell envelope and the modification of the DNA sequence.



## 1. Introduction

Ion beam modification of biological living materials has been developed as ion beam biotechnology for three decades [1]. The technology utilizes ion beams, particularly in a lower energy region, some tens keV, instead of the MeV higher energy region, to bombard biological cells to induce mutation or gene transfer. The technology has been demonstrated to be novel and very effective, but, on the other hand, not very convenient in operation and not cost effective either. Compared with accelerator-based ion beams, plasma-based ion implantation at relatively lower ion energy has been considered more convenient and cost effective as well and widely applied in various material surface modifications. However, it has not yet been well addressed by experiments in treating living materials for biotechnology applications due to the hesitation of its low energy character which might not be able to cause sufficient changes in the critical biological structures. Recently a few studies have emerged on applying plasma-based ion implantation to induce plant mutation [2] and DNA transfer [3]. This work was an attempt to explore low-energy plasma immersion ion implantation (PIII) for inducing bacterial mutation, aiming at enhancing the cellulase activity and thus the biomass processing capability.

Several chemical agents such as methylmethanesulfonate (MMS), ethylmethanesulfonate (EMS), diepoxybutane (DEB) and sodium azide are powerful carcinogens used to induce mutation in various living organisms including crop plants, fungi and bacteria, as well as physical irradiations including Gamma rays, X-rays, ultraviolet (UV) and fast neutrons. [4–6]. Furthermore, a combination of both chemical (EMS) and physical (UV) mutagenesis has been also applied to increase cellulase enzyme activity in bacterial cells [7]. All of the chemical agents and the radiations are powerful mutagens. In comparison with other physical mutagens, low-energy PIII technique is safer, more reliable and sustainable. On the other hand, evidence for induction of mutation in bacteria using the PIII technique has not yet been reported. Experimental evidences have shown energetic ion beam bombardment which is an environment-friendly technique capable of inducing broad-spectrum mutations of crops with high efficiencies owing to multiple physical agents involved compared with other mutation induction methods

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[1]. With many successes achieved in ion-beam-induced crop mutation breeding, PIII which is a non-line-of-sight ion bombardment process has rarely been tested in this application. This motivated us to carry out this study.

Lignocelluloses are the most abundant biomass providing carbon resources with a worldwide production of $1 \times 10^{10}$ MT per year [8]. Agricultural cellulosic wastes are enzymatically hydrolyzed into reduced sugar that can be converted to several chemical products such as alcohol, fatty acid, etc. [9]. One of the factors in limiting the converting process is inefficient hydrolysis of the enzymes. Cellulases are a group of hydrolytic enzymes capable of hydrolyzing cellulose to glucose and they can be produced by a large number of microorganisms. One of the approaches to develop higher cellulase activity is discovering new cellulolytic microorganisms and constructing mutants with higher expression [10]. In this work, an integration of PIII technology and biotechnology was conducted for enhancing the hydrolysis activity of the cellulase enzyme and eventually benefiting the biomass treatment.

## 2. Materials and methods

The target material was the cellulase producing bacteria, *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*), which were isolated from horse's feces. The bacterial strains were grown at 37 °C in Lysogeny broth (LB) with vigorous shaking at 120 rpm. *B. amyloliquefaciens* cells were harvested after overnight incubation by centrifugation at 6000 rpm for 5 min then resuspended with sterile distilled water. Bacterial suspensions were deposited in wells of a stainless steel sample holder and then placed in the PIII chamber [11]. A vacuum control was placed in one of the wells covered by carbon tape. The bacterial samples were treated by argon (Ar) or nitrogen (N) PIII with a bias voltage of $-2.5$ kV and to fluences of $1 \times 10^{15}$, $5 \times 10^{15}$, $1 \times 10^{16}$, $5 \times 10^{16}$ and $1 \times 10^{17}$ ions/cm$^2$, respectively, in a vacuum condition of $10^{-3}$ Pa at room temperature. The plasma was generated with 100-watt radiofrequency (RF) power and operated with a frequency of 50 Hz and a pulse length of 20 μs. After PIII, LB medium was added to each treated sample and incubated at 37 °C on a rotating shaker for 1 h. Then, 100 μl of each sample were spread on LB agar containing 1% of carboxymethyl cellulose (CMC) and incubated at 37 °C for 24 h. Both wild type control (without PIII treatment) and vacuum control were cultivated on the same agar media.

Mutants were screened based on the size of the hydrolysis halos detected by Congo red staining [12]. In the test, the bacterial colonies placed at the central area of the CMC-smeared plate would gradually digest the CMC to increase the central circle or halo size which could be detected by the dye staining so that the bigger the halo, the higher the activity. The qualitative cellulase activity of each bacterial colony was evaluated by the ratio between the hydrolysis halo diameter and the colony diameter (*H*/*C*). The isolate that showed a higher *H*/*C* ratio than that of the control was screened and selected for further analysis and tests. Genetic stability of the mutant with higher cellulase activity was confirmed by Congo red staining for 9 generations. All the experiments were carried out in triplicate repeats and calculated with statistical analysis at $p < 0.01$ (Duncan's multiple range test).

Tests of the cellulase activity of all bacterial samples were carried out against various pH and temperatures. All samples were inoculated in 50 ml of LB medium and incubated at 37 °C with vigorous shaking (120 rpm). To investigate the cellulase activity, the cured enzyme of all bacterial samples was harvested by centrifugation at 10,000 rpm for 15 min at 4 °C. The optimum pH of the cellulase activity was determined by incubation of the mixture containing 1% (w/v) CMC in various buffers of pH 2.0–10.0 with suitable amounts of enzyme at 50 °C for 30 min. To determine optimal temperature, cellulase activities were measured at different temperatures ranging from 20 to 100 °C. For investigation of the hydrolysis activity, releasing of reducing sugars was determined by the dinitrosalicylic acid (DNS) method [13]. As cellulose is an unbranched glucose polymer and cellulase degrades cellulose by cleaving a glycosidic bond of glucose polymer into reducing sugar [14], the more cellulase activity, the more the reducing sugar is released. The unit of hydrolysis activity was defined as the amount of enzyme releasing 1 mmol of reducing sugar/min. The protein concentrations of all samples were determined using Lowry's method with bovine serum albumin as the protein standard [15]. Cellulase hydrolysis activities of all bacterial samples against biomass materials were determined using lignocellulosic substrates including rice straw, corn stover and corn husk. The hydrolysis activities were investigated by separately incubating the crude enzyme in 5% (w/v) substrates in 50 mM citrate buffer (pH 5.0) at 50 °C for 7 days. Then the reaction mixtures were collected on day 0, 1, 3, 5 and 7. All measurements in the experiment were carried out in triplicate determinations with standard error.

## 3. Results and discussion

From the mutant screening, among 1000 colonies, mutants displaying the *H*/*C* ratio higher than that of the wild type were observed. The mutants showed various *H*/*C* ratios from 1.05 to 3 while the *H*/*C* ratio of the wild type was approximately only 1.45 (Table 1). The ratio data in Table 1 were obtained from Ar-PIII. Mutants under N-PIII were not detected. This was supposed to be due to the physical fact that Ar was nearly three times heavier than N and thus, to a cell-envelope mimetic target material, cellulose ($C_6H_{10}O_5$, 1.5 g/cm$^3$), the stopping power of 2.5-keV Ar was about 3.4 MeV/(mg/cm$^2$) while that of 2.5-keV N was about 1.6 MeV/(mg/cm$^2$), calculated from SRIM [16], namely, the linear energy transfer (LET) of Ar was about twice that of N. Therefore, at the same energy Ar should be more effective in inducing mutation than N. The mutant with the highest *H*/*C* ratio in the CMC plate was selected, as shown in Fig. 1, where the diameter of the halo of the mutant was seen more than two times that of the wild type, indicating the highest cellulose digestion capability. It was noted that most of the selected mutants were observed from the Ar-PIII treatment with the ion fluence of $1 \times 10^{16}$ ions/cm$^2$.

The qualified result on the enhancement of cellulose digestion capability was quantified. Figs. 2 and 3 display the results of the measured hydrolyzed enzyme which represented the cellulase activity of the bacterial samples as functions of the pH value and the temperature of the buffer, respectively. As shown in Fig. 2, an example of the cellulase activity with varied pH, the mutant obviously exhibited higher cellulase activity than the wild type with the highest enhancement around the neutral pH 4–6 by an increase of about 25–30%. Fig. 3, an example of the cellulase activity with various temperatures, shows that the mutant generally enhanced the cellulase activity over the tested temperature range compared to the wild type. Particularly in the low and high temperature ranges the enhancement was more pronounced, about 30–40% increase.

The hydrolysis activities of the cellulase produced from the bacteria on real cellulosic materials of rice straw, corn stover and corn husk showed continuous increasing with the time in the activity from the

**Table 1**
*H*/*C* ratios of the wild type (W) and the mutants (M1–M9).

| Bacterial sample | *H*/*C* ratio |
|---|---|
| W | $1.43 \pm 0.08^{b}$ |
| M1 | $1.68 \pm 0.14^{bc}$ |
| M2 | $1.05 \pm 0.09^{a}$ |
| M3 | $2.71 \pm 0.18^{d}$ |
| M4 | $2.50 \pm 0.10^{d}$ |
| M5 | $3.12 \pm 0.24^{e}$ |
| M6 | $2.53 \pm 0.15^{d}$ |
| M7 | $2.34 \pm 0.17^{d}$ |
| M8 | $1.81 \pm 0.11^{c}$ |
| M9 | $1.33 \pm 0.18^{ab}$ |

Values are means ± standard error. The letters indicate a significant difference ($p < 0.01$) as determined (examined) by Duncan's multiple range test.

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