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Improved thermostable α-amylase activity of *Bacillus amyloliquefaciens* by low-energy ion implantation

X.Y. Li, J.L. Zhang and S.W. Zhu

Key Laboratory of Crop Biology of Anhui Province, School of Life Science, Anhui Agricultural University, Hefei, China

Corresponding author: S.W. Zhu
E-mail: zhusuwen@126.com

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ABSTRACT. Thermostable α-amylase is of great importance in the starch fermentation industry; it is extensively used in the manufacture of beverages, baby foods, medicines, and pharmaceuticals. *Bacillus amyloliquefaciens* produces thermostable α-amylase; however, production of thermostable α-amylase is limited. Ion-beam implantation is an effective method for mutation breeding in microbes. We conducted ion-beam implantation experiments using two different ions, Ar^+ and N^+, to determine the survival rate of and dose effect on a high α-amylase activity strain of *B. amyloliquefaciens* that had been isolated from soil samples. N^+ implantation resulted in a higher survival rate than Ar^+ implantation. The optimum implantation dose was 2.08×10^{15} ions/cm^2. Under this implantation condition, we obtained a thermally and genetically stable mutant α-amylase strain (RL-1) with high enzyme activity for degrading α-amylase. Compared to the parental strain (RL), the RL-1 strain had a 57.1% increase in α-amylase activity. We conclude that ion implantation in *B. amyloliquefaciens* can produce strains with increased production of thermostable α-amylase.

Key words: Ion implantation; *Bacillus amyloliquefaciens*; Thermostable α-amylase

INTRODUCTION

Amylases are enzymes capable of hydrolyzing starch and are produced by animals, plants, and microbes (Hoj et al., 1989). These enzymes can specifically cleave the *O*-glycosidic bonds in starch, a storage polysaccharide present in the seeds and tubers of various plants. Starch is composed of α-1,4-linked α-D-glucose units from amylose and amylopectin; these starch components differ from each other in the degree of branching. α-Amylase is important in the metabolism of maltose and maltodextrins. The potential for commercial application of α-amylases is enormous, and its preparation accounts for approximately 25% of the enzyme production market (Azad et al., 2009).

Starch and glycogen are the most commonly used organic substrates in the fermentation industry. Currently, these substrates are largely degraded chemically, and the cost of this process is very high. Degradation of starch and glycogen using microbial fermentation (mostly at high temperatures) ensures optimal chemical degradation, low cost, and environmental protection. Because the thermostable α-amylase excreted by *Bacillus amyloliquefaciens* has considerably high thermal stability, it has been widely used in the production of beer, alcohol, foods, etc., since its isolation in 1973 (Tee and Kaletunc, 2009). However, very few natural strains produce thermostable α-amylases that can be used for the degradation of starch and glycogen. This feature of the natural strains is mainly attributed to the low enzyme activity of the α-amylases they produce.

Genetic variation in amylase activity and inducibility has been analyzed in *Drosophila melanogaster* (Yamate and Yamazaki, 1999). In fungi, there are 2 major classes of starch-degrading enzymes: α-amylases and glucoamylases. *Aspergillus awamori* and *A. oryzae* produce glucoamylases that can absorb and digest raw starch. In addition, the raw starch-degrading activity of *Rhizopus niveus* glucoamylase is stronger than that of *Aspergillus* sp glucoamylase (Fielden et al., 2002). However, little is known about the adaptation of enzymes other than glucoamylase in a starch-rich environment.

Previous studies on starch-degrading strains have mainly focused on the enzymatic properties and conditions of fermentation (Okita et al., 1979). Additionally, many studies on cloning and expression of thermostable α-amylases use genetic engineering and molecular microbiology techniques (Dong et al., 2008). Therefore, screening and improving the outstanding strains via new technology and/or new methods to produce a thermostable α-amylase that use starch more efficiently are intriguing prospects (Mollania et al., 2010).

The technique of ion-beam genetic modification has been widely applied in recent years. Previous research has shown that low-energy ion implantation into solid biological material has a unique mode of action. Thus, low-energy ion beams have many applications in the life sciences. During the 1980s, a scientific research group at the Institute of Plasma Physics, Chinese Academy of Science, first applied low-energy ion implantation to modify microorganisms (Xie et al., 2003). After years of development, low-energy ion implantation is now being widely used as a technique for inducing mutations in plants and microorganisms (Li et al., 2009), but few studies have reported the use of this technique to induce mutations in *B. amyloliquefaciens*. Thus, in this study, low-energy ion implantation was used to irradiate a *B. amyloliquefaciens* strain to screen for mutants producing highly active and thermostable α-amylase. We successfully isolated such a mutant. Here, we provide experimental evidence supporting the use of this mutant strain for efficient degradation of starch and glycogen.

MATERIAL AND METHODS

Bacterial strain

The RL strain with highly thermostable amylase activity was isolated from 100 soil samples. We collected the 100 soil samples from areas with high temperature, such as hot springs, flour mills, and breweries, in Anhui Province, China. The RL strain displayed amylase activity at high temperatures (70°C) and was identified as *B. amyloliquefaciens* on the basis of physiological and morphological analysis and 16S rDNA sequencing.

Culture media

The preliminary screening culture medium (1 L) contained 2.0 g soluble starch, 1.0 g peptone, 0.5 g NaCl, and 2.0 g agar. The slant medium (1 L; pH 6.0-7.0) contained 0.5 g NaCl, 0.5 g beef extract, 0.8 g maltose, 0.5 g soluble starch, and 1.8 g agar. The shaking flask culture medium (1 L; pH 7.0) contained 2.0 g soluble starch, 1.0 g peptone, 0.3 g beef extract, and 0.5 g NaCl. The enrichment culture medium (1 L; pH 7.2-7.5) consisted of 1.0 g peptone, 0.5 g beef extract, 0.5 g NaCl, and 0.5 g $CaCl_2$. All media were autoclaved at 121°C for 30 min before use.

Ion implantation

B. amyloliquefaciens spores suspended in liquid were removed aseptically from cultures of the parental RL strain grown in slant store culture medium for 72 h at 37°C. Next, 0.2 mL of the spore suspension was spread on an empty culture dish. The spores were air-dried at room temperature and irradiated with an LZD900 multifunctional implantation apparatus equipped with an ion-beam bioengineering instrument (Institute of Plasma Physics, Chinese Academy of Sciences). The spores were implanted with N^+ or Ar^+ ions at 10 keV, with doses of 0.52×10^{15}, 1.04×10^{15}, 1.56×10^{15}, 2.08×10^{15}, 2.6×10^{15}, and 3.12×10^{15} ions/cm^2. Briefly, the target chamber was evacuated to 1×10^{-3} Pa. The parental control spores were only exposed to the vacuum, but the experimental spores were exposed to 5-s pulses of implantation every 50 s. Next, the spores were washed aseptically with water and incubated at 37°C for 1-2 days. The cells were then prepared for screening after slant culture for 3 days.

Mutant screening

The monospores from the samples subjected to ion implantation were washed using 1 mL sterile water, and 0.1 mL of various dilutions of the monospore suspension was spread onto 3-5 culture dishes containing preliminary screening culture medium. The colonies were then incubated at 41.5°C for 36 h. Single colonies were selected on the basis of the greater ratio of the diameter of the colony to that of the transparent circle. The enzymatic activities of the selected mutant strains and the RL parental strain were tested by culturing in 250-mL flasks (containing the shaking flask culture medium) at 41.5°C for 48 h. The enzymatic activity of the mutant thermostable α-amylase was measured using a spectrophotometric method (Azad et al., 2009).

RESULTS

Dose effects of N^+ and Ar^+ implantation on the RL strain

To calculate the survival rate, the RL strain was implanted with different doses of either N^+ or Ar^+ at 10 keV. As shown in Figure 1, the survival rate of the strain decreased rapidly as the ion implantation dose increased to 1.5 x 10^{15} ions/cm^2. The survival rate increased slightly at doses of 1.5 and 2.0 x 10^{15} ions/cm^2. However, the survival rate declined as the implantation dose increased beyond 2.08 x 10^{15} ions/cm^2. This phenomenon is described as a saddle. Although N^+ and Ar^+ implantations resulted in similar survival trends in RL, lethality was slightly greater in Ar^+ than in N^+ at the same doses.

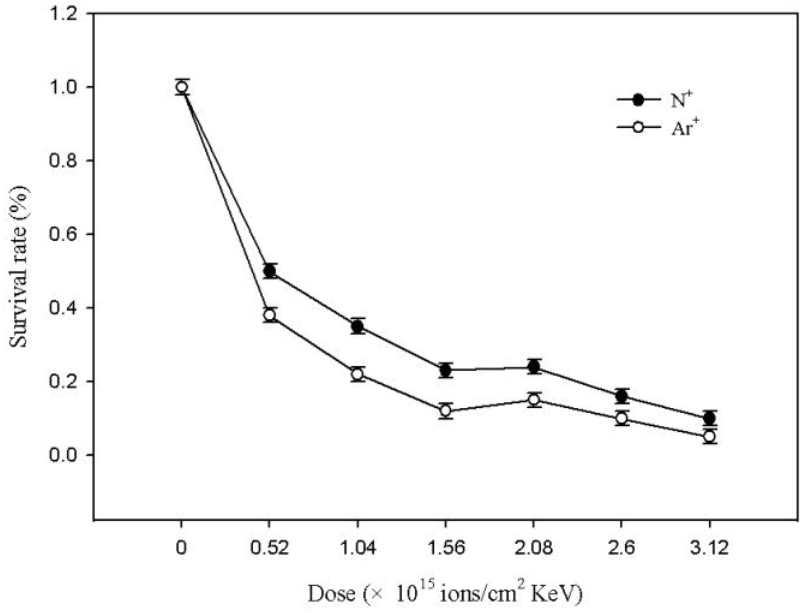

Figure 1. Survival rate of RL after N^+ or Ar^+ implantation.

Enzymatic activity was measured in liquid fermentation cultures of the mutant strains; 3 independent experiments were performed with consistent results. Mutant cultures with an absorbance at 395 nm (A_{395}) 10% higher or lower than that of the parental strain were defined as positive or negative mutants, respectively, whereas those with an A_{395} within 10% of the control were defined as insignificant.

The results shown in Figures 2 and 3 demonstrate that the mutation rate (calculated as the enzymatic activity of α-amylase) increased with increasing N^+ or Ar^+ implantation doses. The maximum mutation rate was obtained with a dose of 2.08 x 10^{15} ions/cm^2. In addition, the mutation rate caused by N^+ implantation was greater than that caused by Ar^+ implantation; this finding highlighted that mutation effects differ and depend on both the dose and type of implanted ions. On the basis of these results, an ion implantation dose of 2.08 x 10^{15} ions/cm^2 at 10 keV was used in the following implantation experiments.

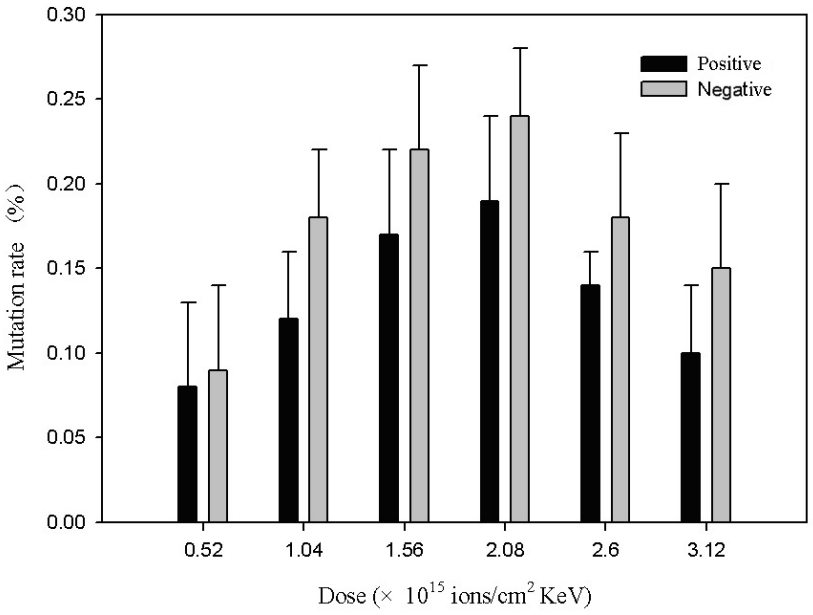

Figure 2. Mutation rates of RL implanted with N^+ at 10 keV. A positive value represents a 10% higher mutation rate in the mutant than in the parental strain; a negative value represents a 10% lower mutation rate in the mutant than in the parental strain.

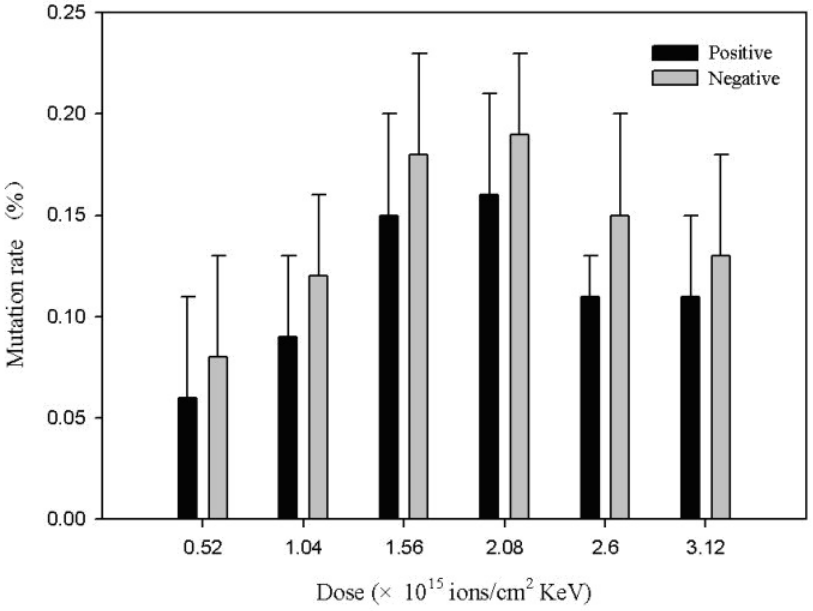

Figure 3. Mutation rates of RL implanted with Ar^+ at 10 keV. A positive value represents a 10% higher mutation rate in the mutant than in the parental strain; a negative value represents a 10% lower mutation rate in the mutant than in the parental strain.

Screening for highly active and thermostable α-amylase strains

N^+ ion implantation was used to mutate the parent RL strain as described above, and the mutants were screened for altered α-amylase activity. Of the colonies screened, 27 colonies had a larger halo diameter than the control. Further testing revealed that enzymatic activity in 6 of these mutants (RL-1, -2, -3, -4, -5, and -6) was constantly and significantly higher than that of the RL strain. The results shown in Table 1 indicate that the α-amylase activity and time for optimal enzyme activity varied among the 6 mutants. The greatest increase in enzymatic activity was found in the mutant strain RL-1 (57.1% greater than RL), in which the time required to reach optimal enzyme activity was decreased by approximately 4 h relative to the parental strain. When the strains were cultured under the same conditions, the starch degradation halo caused by RL-1 was visibly larger than that of the parental strain (Figure 4). Thus, the relative α-amylase activity of RL-1 was inferred to be greater than that of RL.

Table 1. Results of the mutant screen.

Strain	Highest enzyme activity (U/mL)	Time to optimal enzyme activity (h)	Increased activity of mutant compared to the parental strain (%)
RL	37.26 ± 0.09	40.5 ± 0.5	-
RL-1	58.51 ± 0.12*	36.6 ± 0.4	57.1
RL-2	45.31 ± 0.07*	48.3 ± 0.6	21.6
RL-3	50.60 ± 0.09*	36.5 ± 0.7	35.8
RL-4	52.17 ± 0.10*	54.4 ± 0.5	53.4
RL-5	41.62 ± 0.06*	48.7 ± 0.4	11.7
RL-6	39.20 ± 0.09	48.3 ± 0.3	5.2

*Enzyme activity of the mutant was higher than that of the parent strain with statistical significance ($P < 0.05$).

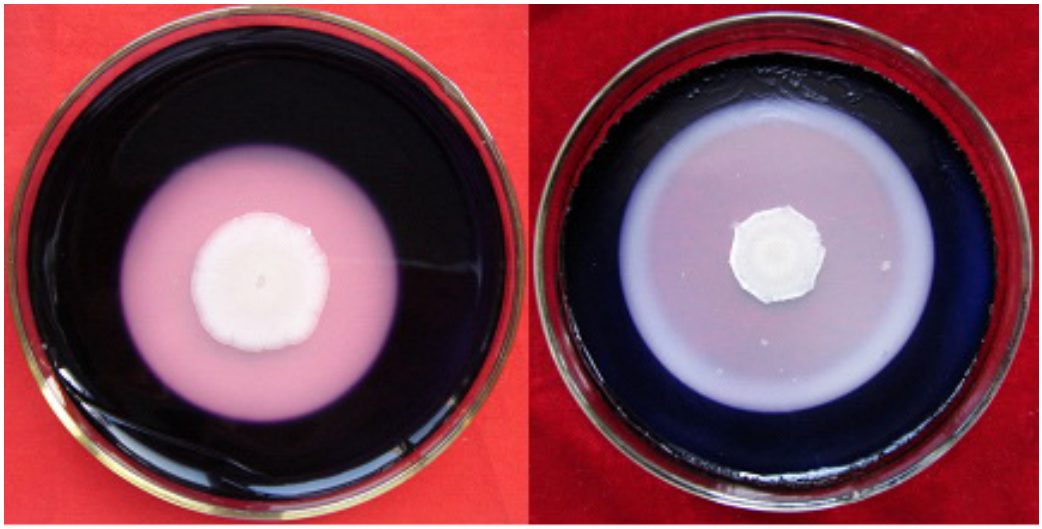

Figure 4. Starch degradation by RL **(A)** and RL-1 **(B)**.

Genetic stability of the RL-1 mutation

To determine the genetic stability of the α-amylase mutation(s), RL-1 was subcultured on solid media for 8 generations. As shown in Table 2, the enzymatic activity of the thermostable α-amylase in RL-1 was highly genetically stable. The mutant strain retained high enzyme activity even after culturing for 8 generations.

Table 2. Genetic stability of α-amylase production.

Generation	α-Amylase activity (U/mL)[a]
1	58.5 ± 0.11
2	58.2 ± 0.08
3	58.7 ± 0.09
4	57.9 ± 0.07
5	58.3 ± 0.12
6	58.1 ± 0.09
7	57.5 ± 0.10
8	58.5 ± 0.13

[a]Results are pooled from three independent experiments.

Identification of the extracellular enzyme

After culturing RL-1 in a flask with vigorous shaking for 24 h, the supernatant and cells were separated by centrifugation at 6000 rpm for 10 min. The RL-1 cells were lysed to allow for the measurement of the activities of enzymes in the cell supernatant and fermentation solution. Our results indicated that both the supernatant and fermentation solution contain α-enzyme activities that were largely lacking in the RL-1 cells. This indicates that the α-enzyme was secreted by RL-1 into the fermentation solution.

DISCUSSION

The sensitivity of various microorganisms to ion beams is different. *B. amyloliquefaciens* has been widely used in industry, agriculture, environmental reclamation, pest control, etc. (Machius et al., 1995; Igarashi et al., 1998; Asghari et al., 2004; Shareghi et al., 2007). In the past, X-rays and gamma rays were mainly used to mutate microorganisms; however, repeated use of these methods has led to an increase in the resistance of microbes, decrease in mutation efficiency, and narrowing of the mutation spectrum. To avoid such issues, low-energy ion implantation has been utilized as a novel mutation source, but the application of this technology has been very limited in *B. amyloliquefaciens* compared to *Escherichia coli*. As seen from the survival curves (Figure 1) and the amylase mutation curves (Figures 2 and 3) in this study, the *B. amyloliquefaciens* RL strain is sensitive to low-energy ions. However, the survival curve of RL is similar to that of *E. coli* (Fondy et al., 1989); both resemble saddles. Additionally, implantation of RL with different doses of N^+ and Ar^+ demonstrated that the α-amylase mutation rate of N^+ was higher than that of Ar^+, perhaps due to decreased cell damage caused by N^+ implantation. Interestingly, these same trends were also reported in *E. coli* and cotton.

The effects of low-energy ions depend on the type and radiation mass of the ion. The difference between ion beams and X- or gamma rays is that low-energy ions have larger energy, mass, and momentum effects. Ions with more mass have larger mass and momentum

effects, and therefore, they induce greater damage to the microbes. Indeed, in our experiments, the survival rate of recipients was lower than that of controls. However, different ions have varying mutational effects. For example, nitrogen is one of several important elements that form the nucleotide bases of DNA, and N^+ implantation can directly cause molecular structural changes in these bases. This can increase the DNA mismatch rate, which would manifest as an increased mutation rate during DNA replication. In contrast, Ar^+ is more likely to cause heavy damage via single- and double-strand breaks in DNA. The *in vitro* and *in vivo* effects of various ions on DNA breakage and mutation have been previously confirmed (Nordhoff et al., 1993; Sandstrom et al., 1994; Kiefer et al., 2002; Liu et al., 2003), but our results suggest that N^+ is the optimal ion for inducing mutations in microorganisms.

A highly active, thermostable α-amylase would be beneficial to the fermentation industry. In the present study, we used low-energy N^+ ion implantation to mutate the *B. amyloliquefaciens* strain RL, an important microbe (with roles in many aspects of industry, agriculture, environmental reclamation, pest control, etc.) that secretes a thermostable α-amylase, and screened for mutants with increased starch degradation activity (Declerck et al., 2000; Kim et al., 2003; Du et al., 2006; Khemakhem et al., 2009). We found that, compared to the parental strain, the mutant strain RL-1 excreted a highly thermostable α-amylase with 57.1% increased activity (58.51 U/mL).

The results reported in this paper demonstrate that low-energy ion implantation is a feasible mutation technique in *B. amyloliquefaciens* and that this technique could be used for improving the enzymatic activity of α-amylase. However, the mechanism underlying the increase in activity of this thermostable α-amylase requires further study.

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