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Improving *Aspergillus niger* Tannase Yield by N^+ Ion Beam Implantation

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ABSTRACT

This work aimed to improve tannase yield of Aspergillus niger through N^+ ion beam implantation in submerged fermentation. The energy and dose of N^+ ion beam implantation were investigated. The results indicated that an excellent mutant was obtained through nine successive implantations under the conditions of 10 keV and 30-40 ($\times 2.6\times 10^{13}$) ions/cm^2, and its tannase yield reached 38.5 U/mL, which was about five-time higher than the original strain. The study on the genetic stability of the mutant showed that its promising performance in tannase production could be stable. The studies of metal ions and surfactants affecting tannase yield indicated that manganese ions, stannum ions, xylene and SDS contained in the culture medium had positive effects on tannase production under submerged fermentation. Magnesium ions, in particular, could enhance the tannase yield by the mutant increasing by 42%, i.e. 53.6 U/mL. Accordingly, low-energy ion implantation could be a desirable approach to improve the fungal tannase yield for its commercial application.

Key words: *Aspergillus niger*, N^+ ion implantation, tannase

INTRODUCTION

Tannase (tannin acyl hydrolase) hydrolyzes tannic acid into one glucose and ten gallic acids (Lekha and Lonsane 1997) and it is extensively applied in the food (Belmares et al. 2004), beverage (Cantarelli et al. 1989) and brewing (Rout and Banerjee 2006), pharmaceutical and chemical industries (Lane et al. 1997; Kar et al. 2002; Yu et al. 2004; Yu and Li 2006). Tannase is usually present in the plants, animals, and microorganisms (Aguilar et al. 2007) and mainly produced by the microorganisms such as fungi due to its tolerance of relatively high concentration of tannic acid, e.g., *Aspergillus niger* (Rana and Bhat 2005; Treviño-Cueto et al. 2007; Aguilar 2009). However, the productivity by tannase-producing fungi is almost not satisfactory, especially under the submerged fermentation (SmF). Most of the previous studies have been carried out largely on the isolation of the microorganism and process development (Bradoo et al. 1997; Cruz-Hernández et al. 2006; Manjit et al. 2008; Enemuor and Odibo 2009; Selwal et al. 2010) in order to enhance the yield of microbial tannase, while few studies on mutagenesis of tannase-producing microorganism have been reported. Presently, not only have such mutagenic sources as ultraviolet rays, γ-rays, laser, and neutron been developed and successfully used to acquire various sorts of valuable strains , but also novel sources have still been making for a wider mutation spectrum with a higher ratio of

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mutation. Implantation of low energy of ion beam $(IB)^2$ an innovative mutation approach characterized by higher mutation frequency and wider mutation spectra for organism was firstly invented by Chinese researchers in 1986, which has played an increasing important role in breeding. Afterwards, underlying mechanisms such as energy absorption, mass deposition, and charge exchange were proposed (Yu 2000; Yu 2006). Further Studies have proved the mutagenic effect of IB implantation on breeding (Yuan et al. 2002; Yu 2002), gene transfer (Wang et al. 2002), and cell modification (Yu 2000), and biological efficiency of the implantation technique has drawn a considerable attention in recent years.

In this work, the technique of N^+ IB implantation to mutate *Aspergillus niger* TA9701was studied.

MATERIALS AND METHODS

Microorganism and culture medium

Aspergillus niger TA9701 was obtained from the industrial microbiology subdivision of key laboratory of ion beam engineering, Chinese academy of science (CAS), and conserved on the PDA medium containing 2.0% glucose, 20% potato extract, and 2.0% agar) at 4 ^oC. The screening plate contained (g/L): sucrose 1.5%; $NaNO_3$ 0.3%; K_2HPO_4 0.1%; $MgSO_4$ 0.03%; $FeSO_4 \cdot 7H_2O$; tannic acid 2%. The fermentation medium was composed of (%) rice flour, 5.0% (g/L); $(NH_4)_2SO_4$, 1.0%; $MgSO_4$, 0.03%; KH_2PO_4, 0.3%; $CaCO_3$, 0.5%; tannic acid, 2%.

Equipment and reagents

A special implantation source devised by Chinese Academy of Sciences, Institute of Plasma Physics was introduced to mutate the organisms. Figure1a sketches out the scheme of IB implantation (Song et al. 1999). The equipment shown in the Figure1b was applied in the present work. Tannic acid and ammonium sulfate were purchased from Sinopharm Chemical Reagent Co., Ltd. (SCRC), China. Rice flours were made of the hybrid rice grounded by a pulverizer in the laboratory.

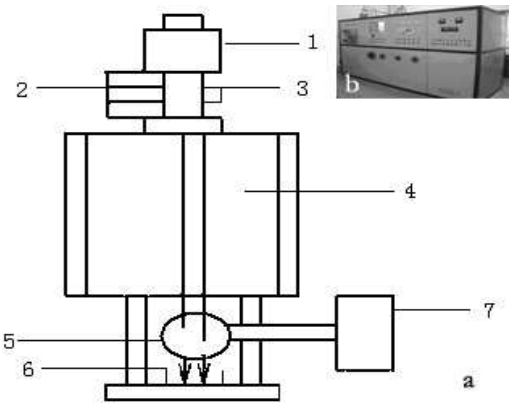

Figure 1 - Sketch of the ion beam implantation installation. Figure a is a framework of the installation of IB implantation. 1. Ion source, 2.Molecular pump, 3.Gas inlet, 4.Vacuum chamber, 5.Induction coil, 6.Targer chamber, 7.IB integrator. Figure b refers to a full-scale photo of the installation of IB implantation in our laboratory.

Ion beam implantation and screening

The spores of fungi were generally objectives implanted by N^+ IB. *Aspergillus niger* TA9701 was incubated on the PDA slants at 30 ^oC for 3-4 d, and then the new-born spores were picked up and shook up in 10 mL distilled water. One hundred microliters of the spore solution was smeared on the sterilized Petri disk, and air-dried in axenic workbench. Afterwards, it and the control group were implanted with the N^+ IB and without N^+ IB, respectively, in the target chamber under dry vacuum station. The control group aimed to evaluate the effects of vacuum on microorganisms.

Treated plates and control plates were washed with 10 mL antiseptic distilled water separately after

the ion implantation, and 1.0 mL washed sample was smeared on the screening plate and incubated at 30 °C. More than 24 h later, the maximal colonies with high tannase yield were transferred to PDA slants. The new-born spores on the slant were eluted and diluted by 10^{-4} fold with antiseptic distilled water after 3-4 d. Two milliliters of dilution (about 10^7 spores) was inoculated into a 250 mL flask containing 50 mL of the fermentation medium and cultured for 72 h at 200 rpm and 30□. The high yield tannase-producing mutants were screened by assaying tannase activity of the fermentation broth.

Tannase assay

The tannase activities of the fermentation broth were determined according to the method of Iibuchi (1967). One milliliter of crude tannase filtrate was added into 4.0 mL of substrate solution composed of 0.35 % (w/v) tannic acid dissolved in 0.05 M citrate buffer (pH 5.5), and incubated at 45 °C for 10-30 min. One hundred microliters of the enzymatic reaction solution was transferred to 10 mL 90% ethanol solution, and the optical density of the mixture at 310 nm was detected. Tannase activity (unit/mL) was calculated according to the following formula: $u = 114*(E_{t1}-E_{t2})/(t_2-t_1)$. Where E_{t1} and E_{t2} refers to the optical densities of the ethanol solution at 310 nm after t_1 and t_2 minutes reaction, respectively. One unit of tannase is defined as the amount of the enzyme hydrolyzing one micromole of the ester bond in tannic acid in 1 min.

Optimization of mutagenesis

The optimal mutagenesis parameters desired for N^+ IB implantation were determined for energy of N^+ implantation (5-30 kev) and dose of N^+ implantation ($20\text{-}100*2.6*10^{13}$ ions/cm^2). The effects of metal ions and surfactants on SmF were investigated. Metal ions (manganese sulphate, stannaic sulphate, ferrous sulphate, zinc sulphate, copper sulphate, and cobaltous sulfate) at 0.1% (w/v) and surfactants (xylene, SDS, Triton, and EDTA) at 0.1% (w/v) were analyzed. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Presently, most of tannase-producing microorganisms are not considered suitable for large-scale enzyme production due to low productivity of tannase under SmF. N^+ IB implantation was chosen as a desirable mutagen to improve tannase productivity of *A. niger* TA9701.

Optimum conditions of N^+ ion implantation

The energy and dose of N^+ implantation were optimized to obtain appropriate survival rate and mutation frequency of the microorganisms. Figure 2 indicated that the energy negatively correlated with the survival rate of microorganisms, which declined fast under the lower energy (less than 10 kev) and reached 20%-30% at 10 kev, while it decreased slowly above 10 kev. Generally, the positive mutants are sought out easily when the survival rate ranges from 20%-30% because the level of energy leading to 70-80% dead rate of microorganisms can result in the occurrence of the highest rate of positive mutation more easily. Therefore, 10 keV was chosen as the optimum energy to obtain a certain number of living cells, this was beneficial to screen the highest positive mutant.

Energy and dose of N^+ IB implantation were fixed at 10 kev and $40\times(2.6\times10^{13})$ ions/cm^2 when effects of dose and energy of the implantation on survival rate of *Aspergillus niger* TA9701 were investigated respectively.

The dose of N^+ implantation also affected the survival rate remarkably. As shown in Figure 2, the survival rate under different dose of N^+ implantation exhibited a typical “saddle shape”, which was used to sign the abnormal radiation damages of low energy implantation possibly due to the energy and momentum deposition effect in lower dose and the charge stimulating effect in higher dose (Song 1999). The survival rate decreased sharply from 0 to $20\times2.6\times10^{13}$ ions/cm^2, and then the rate increased in the range of 30-40 ($\times2.6\times10^{13}$) ions/cm^2. It restarts to decrease when the dose exceeded 40 ($\times2.6\times10^{13}$) ions /cm^2.

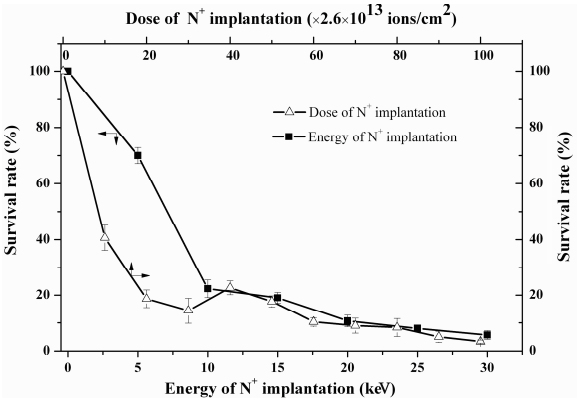

Figure 2 - Effects of dose and energy of N^+ implantation on survival rate of *Aspergillus niger* TA9701.

Moreover, N^+ implantation dose had a vital influence on the mutation rate of TA9701. Figure 3 showed that the frequency of the positive mutation was higher than that of the negative between the dose range of $30\times2.6\times10^{13}$ and $60\times2.6\times10^{13}$ ions/cm^2. Therefore, the range of 30-60 ($\times2.6\times10^{13}$) ions/cm^2 was selected for the next investigation owing to the proper survival rate in favor of high frequency of positive mutation.

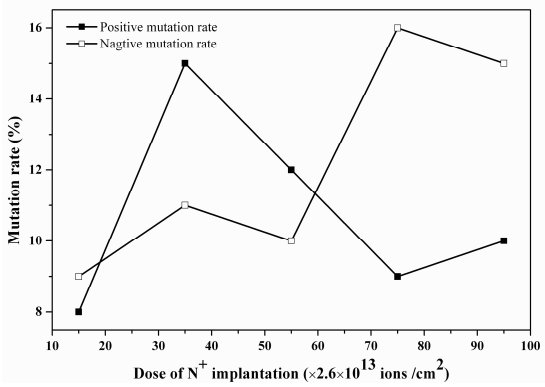

Figure 3 - Effects of the dose of N^+ ion beam implantation on the mutation rate of *Aspergillus niger* TA9701.

The energy and dose of ion implantation have close correlation with the survival rate and the mutation frequency of microorganisms. High level energy and dose of N^+ implantation can kill more cells, while both of the low level cannot create the desired mutants on account of lacking of interaction enough between the target microorganisms and the mutagen. Some ions, which could perforate in cell membrane and reach the inside of cells, may directly act with DNA and result in DNA strand break, base lesion, transition, and transversion which eventually contribute to various biological effects (Yu 2006). Consequently, IB is considered as a desired mutagenic method for microorganism breeding. Ions implanted into microorganisms give rise to obvious biological effects (such as microbe death and mutation).

Horizontal ordinate of N^+ implantation dose refers to a range of the dose, where 15, 35, 55, 75, and 95 refer to the following range: 10-20 (Choosing 110 colonies), 30-40 (Choosing 32 colonies), 50-60 (Choosing 43 colonies), 70-80 (Choosing 25 colonies), and 90-100 (Choosing 22 colonies) respectively. The mutant, which tannase productivity increases by or decreases by 2% than one of the control, is defined as a positive mutation or a negative mutation separately.

Mutagenesis and genetic stability

A single microbial mutagenesis is generally unavailable to obtain a perfect mutant which is presented inevitably by a few continuous mutagenic treatments. In this study, nine times of N^+ IB implantations of *Aspergillus niger* TA9701 were carried out continuously. Figure 4 showed that N^+ IB implantation could significantly enhance the yield of tannase. A desirable mutant (*A. niger* J-T18) was obtained, and its tannase yield increaseed up to 38 U/mL, about 5 times that of original strain (8 U/mL). The yield was significantly higher than 20.6 U/ml as found by Costa et al. (2008) and 13.65 U/mL by Selwal et al. (2010). However, the effect of the implantation on tannase productivity decreased slowly with implantation times growing. The increasing amplitude tended to be narrow especially after the sixth implantation. It could be due to this fact that the microorganism dealt with the same mutagen frequently and could tend to be immune to the mutagen.

An experiment determining the genetic stability of the mutant J-T18 was performed to examine whether the mutant could inherit with a steady productivity of tannase. The mutant was breed for eight generations consecutively. The data of the later five generations are shown in the Figure 5. This showed that the offsprings of the mutant almost had almost an equal performance in tannase production. This implied that the mutant J-T18 was remarkably stable in tannase synthesis.

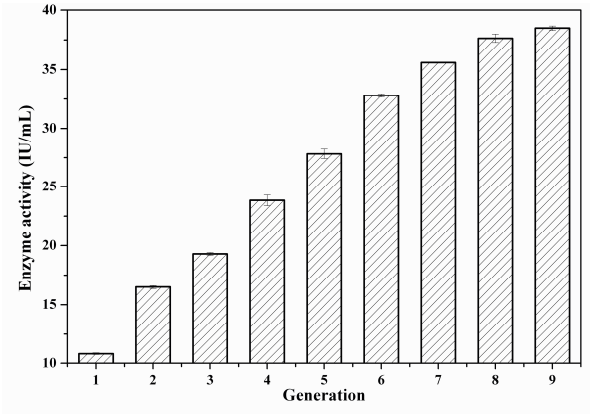

Figure 4 - Sketch of mutagenesis of *Aspergillus niger* by N^+ ion beam implantation.

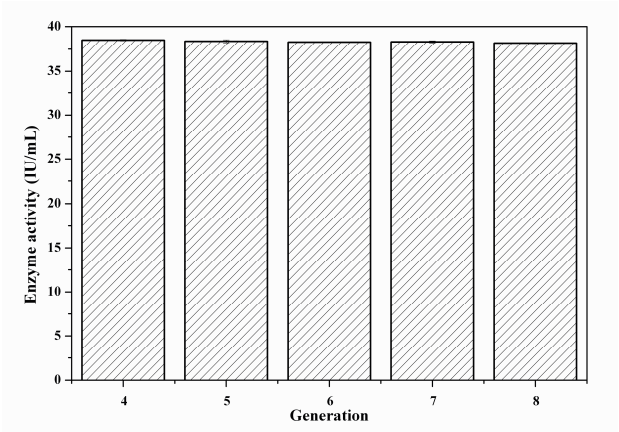

Figure 5 - Illustration of genetic stability of the mutant *Aspergillus niger* J-T18. The data about the tannase activity of the mutant *Aspergillus niger* J-T18 are from generation four to eight.

Characterization of growth and enzyme production of the mutant

The growth and enzyme-producing characteristics of the mutant J-T18 were explored, this is essential for the improvement of tannase productivity of the mutant. Figure 6 indicates that the biomass of the mutant rises with the incubation time prolonging, and reaches the peak at 72 h. Tannase activity arrived at the maximum at 80 h, less than 144 h (Enemuor and Odibo 2009) and 96 h (Kumar et al. 2007), but more than 24 h (Selwal et al. 2010). On the whole, the two characteristic curves seem to be synchronous, and thus the incubation time is chosen as 72 h for the next investigation. On the other hand, the tannase yield reaches the top when the pH value in fermentation broth is 5 between 4.5 and 6.6 consistent with the other reports (Barthomeuf et al. 1994; Bradoo et al. 1997). The trend of the pH curve goes reversely vs. that of the enzyme activity curve. The pH value has a gradual decline in 72 h, but afterward it tends to ascend possibly because the hydrolysis of tannic acid into gallic acid catalyzed by tannase results in the decline of pH value. Otherwise, the depletion of gallic acid and tannic acid as carbon source by microorganisms also leads to pH value increasing later. Satisfactorily, so is counteracted by the addition of 0.5% calcium carbonate to maintain a relatively constant pH value fit for tannase production.

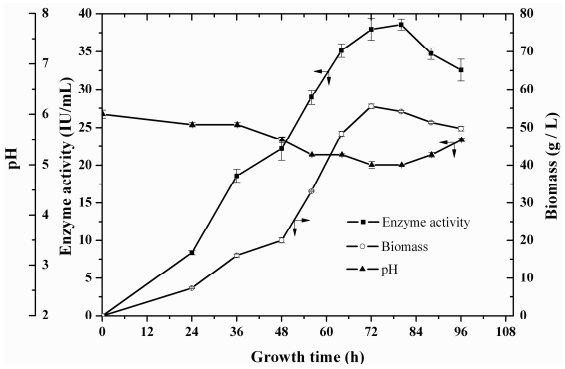

Figure 6 - Fermentation curve of the mutant *Aspergillus niger* J-T18 for tannase production.

Effects of metal ions and surfactants on tannase yield

Table 1 summarizes their effects of metal ions on tannase production. Manganese ions and stannum ions elevated the tannase productivity of the mutant. Manganese ions promoted the mutant to express the highest yield of tannase, about 53.6 U/mL, which was appropriate 6.7 folds than that of the original strain, while the other metal ions inhibited the mutant to synthesize tannase. On the contrary, Manjit et al. (2008) found that manganese ions inhibited the bacterial tannase production. Previously, it is contradictory to report effects of metal ions on fungal tannase synthesis.

Table 1 - Effects of different metal ions and detergents on the tannase-producing performance of *Aspergillus niger.*

Concentration of the ion (0.1%)	Enzyme activity (U/mL)	Concentration of the detergent (0.1%)	Enzyme activity (U/mL)
control	38.48	control	38.53
Mn^{2+}	53.58	xylene	42.93
Sn^{2+}	48.04	SDS	39.62
Fe^{2+}	18.43	Triton	29.07
Fe^{3+}	14.5	EDTA	14.82
Zn^{2+}	13.68		
Cu^{2+}	12.3		
Co^{2+}	9.12		

Kim et al. (1995). have reported that surfactants play a role in the catalytic activity of enzyme The results of the study on the effects of four different surfactants on fungal tannase production are shown in Table 1. The surfactants such as xylene and SDS stimulated the tannase productivity.
However, EDTA and Triton reduced the tannase production. Most of previous reports on the effect of surfactants on tannase synthesis were contradictory (Kim et al. 1995). Manjit et al. (2008) found SDS inhibited bacterial tannase production in contrast to the present results.

CONCLUSIONS

N^{+} IB implantation was a suitable method for mutating *A. niger* to increase the production of tannase. The mutant produced 38.5 U/mL tannase, which was approximate five times higher than the parent strain. Furthermore, manganese ions had great impact on the mutant for tannase production. The mutant under the optimum conditions produced 53.6 U/mL tannase, which was around 6.7 folds higher than that of the parent strain, and the yield was unavailable previously. Besides, rice flour, as the optimal carbon source was not costly in China. Therefore, it could be hoped to produce tannase in commercial scale by the mutant *A. niger* J-T18.

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