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Full Length Research Paper

A new mutation breeding method for *Streptomyces albulus* by an atmospheric and room temperature plasma

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ε-Poly-L-lysine (ε-PL) is a novel food biopreservative with broad antimicrobial activity. To improve the fermentation efficiency of ε-PL, a plasma jet, driven by an active helium atom supplied with atmospheric and room temperature plasma (ARTP) biological breeding system, was used as a new method to generate mutations in *Streptomyces albulus.* After treating the spores with the ARTP jet, S-(2-aminoethyl)-L-cysteine plus glycine resistant mutants were derived. The plasma jet yielded high total (31.6%) and positive (26.0%) mutation rates on *S. albulus* and a mutated strain, designated as *S. albulus* A-29, showed a maximum productivity of 1.59 ± 0.08 mg/ml which was four times as much as that of the wild strain in the same culture condition. After 5-generation culture, the strain, *S. albulus* A-29 still maintained high productivity. This present study showed that the ARTP plasma jet has a strong mutagenic effect on *S. albulus*.

Key words: *Streptomyces albulus*, mutation, atmospheric and room temperature plasma, ε-PL yield.

INTRODUCTION

ε-Poly-L-lysine (ε-PL) is a homo-poly-amino acid characterized by the peptide bond between α-carboxyl and ε-amino groups of L-lysine. The polymer with 25 to 30 residues was discovered as a secreted product from a strain of *Streptomyces albulus* No.346 in culture filtrates (Shima and Sakai, 1977). ε-PL is water-soluble, biodegradable, and shows a broad-spectrum of antimicrobial activity (Shima et al., 1984; Hiraki, 2000) and antiphage action (Shima et al., 1982). Furthermore, the safety of ε-PL as a food additive was demonstrated by experiments using rats (Hiraki, 1995, 2000). ε-PL and its derivatives offer a wide range of unique applications such as emulsifying agent, dietary agent, biodegradable fibers, highly water absorbable hydrogels, drug carriers, anticancer agent enhancer, and biochip coatings (Yoshida and Nagasawa, 2003).

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ε-PL has generated increasing interests and usage in recent years. However, the conventional microorganism fermentation of ε-PL is inefficient, and the commercial ε-PL production is very costly due to the scarcity of ε-PL-producing strain source and limited production rates. These might be mainly attributed to the fact that ever since the first discovery of the *S. albulus* strain, no microorganisms producing ε-PL have been isolated until recently when a new screening method using an acidic dye, Poly R-478, succeeded in finding several ε-PL-producing species of Streptomyces and ergot fungi (Nishikawa and Ogawa, 2002). To date, many traditional mutagenesis strategies have been applied to improve the production of ε-PL. But the limited production rate is still a challenge to commercial production of ε-PL. Therefore, a new method for the breeding of mutations in *S. albulus* is required, especially in the ε-PL fermentation industry.

Atmospheric and room temperature plasma (ARTP) is a new kind of atmospheric pressure non-equilibrium discharge (APNED) plasma source (Li et al., 2007). Because of its lower breakdown voltages, more uniform

and large area discharges and low temperatures, many applications of ARTP concerning biological effects have been reported (Park et al., 2000). These researches have mainly focused on plasma-based sterilization or disinfection (Lerouge et al., 2002; Sato et al., 2006) and the decontamination of biological warfare agents (Herrmann et al., 1999; Uhm et al., 2006). However, there have been very few publications on the use of ARTP as a mutation tool for microorganisms until now. DNA can be destroyed by the chemically active species when treated by ARTP, rather than by heat, UV radiation, charged particles and intense electric field (Li et al., 2008a, b). This result implies that ARTP has the potential to be an effective method for microbial mutagenesis. An atmospheric pressure glow discharge was employed to generate mutations in *S. avermitilis* and a mutated strain with high productivity of avermectin B1a and the genetic stability was obtained (Wang et al., 2010).

In this study, a helium ARTP source was applied to *S. albulus* as a new microbial mutation tool for the first time to generate mutants via the treatment of spores. After the conditions of resistance screening were determined, mutants of *S. albulus* were generated. The productivity and genetic stability of the typical mutants were also examined.

MATERIALS AND METHODS

Microorganisms and culture conditions

The original ε-PL-producing strain, *S. albulus*, which was identified by Sherlock Microbial Identification System and 16S rDNA sequence analysis, was isolated from soil samples of northern China and bred in our laboratory. The strain was maintained on yeast malt agar medium (pH = 7.3) containing (g/L) yeast extract, 4; malt extract, 10; glucose, 4; and agar, 20 and stored at 4℃. The stock culture of the strain was activated on the yeast malt agar at 28 to 30℃. The mutants generated by the ARTP jet were grown on yeast malt agar medium, and subsequent fermentation was carried out using a liquid medium. The liquid medium (pH = 6.8) consisted of (g/L) glycerol, 50; yeast extract, 5; $(NH_4)_2SO_4$, 10; $MgSO_4$, 0.5; $FeSO_4$, 0.03; $ZnSO_4$, 0.04; KH_2PO_4, 1.36; $Na_2HPO_4.12H_2O$, 3.58. All the media were autoclaved at 121℃ for 20 min. The spore was transferred to a 250 ml shake-flask containing 50 ml liquid medium and cultivated at 180 rpm and 30℃.

Preparation of single spore suspension

A loopful of the spore from the stock culture was inoculated to a fresh solid medium at 30℃ for 4 to 5days. The mature spores were curetted with 0.1 M phosphate buffer (pH = 7.2) and transferred to a centrifuge tube with glass beads. Then spore chain was interrupted by oscillating the tube on a shaker, and a filter cloth was used to remove the hypha. The single spore solution was counted using a blood counting chamber and the concentration was adjusted to 10^6 CFU/ml.

Determination of the resistant screening conditions

In order to improve ε-PL yield, S-(2-aminoethyl)-L-cysteine (AEC) and glycine (Gly) were applied to the medium to screen resistant mutants (Hiraki et al., 1998). Dilute the spore solution to 10^5 CFU/ml. Then 0.2 ml spore solution was spread on the solid medium, which was added in AEC (5, 6, 7, 8, 9, and 10 mg/ml) and glycine (3, 4, 5, 6, 7, and 8 mg/ml), and cultivated to form colonies at 30℃ for 72 h. The concentration of AEC and Gly were determined according to the growth of colonies.

Operating method for the ARTP biological breeding system

The ARTP biological breeding system was supplied by Environment Biological Technology Laboratory, Department of Chemical Engineering, Tsinghua University, Beijing, China. In this study, pure helium was used as the plasma working gas. The operating parameters were as follows: the input voltage was 112 V, the gas flow was 2.5 L/min, the distance between the plasma torch nozzle exit and the sample plate (D) was 1 cm; the temperature of the plasma jet was <40℃ (Sun et al., 2007).

The spore solution (20 µl) was applied to a sterilized sample plate made of stainless steel (whose diameter is 1 cm) and exposed to the plasma jet downstream of the plasma torch nozzle exit. After the sample had been treated for predetermined time, the plate was placed into a new tube and washed with 1 ml 0.1 M phosphate buffer (Ph = 7.2) for the treated spore solution. Then the treated spore solution was spread on the solid medium and cultivated to form colonies.

Determination of the mutation dosage for the ARTP

In order to ascertain an optimum mutation dosage, a lethality rate of *S. albulus* using an ARTP biological breeding system was drawn. A high rate of cell lethality makes for effective mutation. The lethality rate of the spores under different mutation dosages was evaluated based on the following equation:

Lethality rate = (U-T)/U × 100%

Where U is the total colony count of the sample without treatment, and T is the total colony count after treatment with ARTP on the solid medium without AEC and Gly. All the colony numbers were obtained by the colony forming units (CFU) method on solid medium.

Evaluation of mutagenesis of *S. albulus* by ARTP jet

After the mutation dosage was determined, the treated spore solution was diluted properly and spread on the solid medium containing AEC and Gly. Then, many single colonies were observed after being incubated for 3 to 5 days at 30°C. Each colony was counted and isolated. A few isolates were selected as inoculants for fermentation in order to examine the production of ε-PL. The mutation rate and the positive mutation rate were calculated using the following equations:

Mutation rate (R_M) = (M/T) ×100%; Positive mutation rate (R_P) = (P/T) ×100%;

Where M is the total CFU of the mutant strains, and P is the CFU of the mutants with a yield of ε-PL which is 20% higher than that of the original strain, and T is the total colony count after treatment with ARTP on the solid medium without AEC and Gly.

Analytical methods

After 72 h of incubation, the mycelial biomass was harvested by

centrifugation at 4,500 × g for 10 min. The biomass was washed twice with phosphate buffer of pH 7.0 and dry cell weight (DCW) was determined by filtering, washing, and drying at 105℃ for 3 h. The final pH of fermentation medium was measured by pH meter (PB-10, Sartorius AG, Germany).

The ε-PL concentration was measured using the method of Itzhaki (1972) with slight modification. A total of 0.07 mM phosphate buffer (prepared by adjusting the pH of 0.2606 g/L $NaH_2PO_4.2H_2O$ solution to 6.9 with 0.1092 g/L $Na_2HPO_4.12H_2O$ solution) and 0.5 mM methyl orange solution were prepared. Then, 0.2 ml supernatant and 0.8 ml methyl orange solution were added to a 1.5 ml centrifuge tube. The mixtures were vigorously reacted on an incubator shaker at 30℃ for 30 min and then centrif uged. The optical density of the resulting supernatant was measured at 465 nm. The ε-PL concentration was calculated from the calibration curve.

Detection of the mutant's genetic stability

The genetic stability of the mutant was examined by subculturing five generations. First, the mutant was spread and cultivated on the solid medium for 3 to 5 days (the first subculture). Next, several single colonies were selected and streaked on to new plates for the second 3 to 5 days cultivation (the second subculture). The same procedure was repeated for a total of 5 subcultures. After each subculture, DCW, final pH and ε-PL yield were detected by fermentation.

RESULTS AND DISCUSSION

Lethality rate of *S. albulus* by ARTP jet

When the spore solution was treated with ARTP jet for 3, 4, 5, and 6 min, the lethality rates of the treated spores increased to 68.9, 77.03, 83.2 and 86.1%, respectively (Figure 1). When the exposure time was extended to 7 min, the lethality rate raised slightly (86.7%). Furthermore, if the exposure time is too long (over 6 min), experimental error would be increased, according to the operation feature of ARTP jet. As a result, the exposure time employed in this study was 6 min to obtain a desirable lethality rate.

Determination of the resistant screening conditions

The biosynthesis of ε-PL is nonribosoma peptide synthesis and is catalyzed by ε-PL synthetase and L-lysine is adenylated in the first step of ε-PL biosynthesis (Kawai et al., 2003; Yamanaka et al., 2008). In the metabolic pathway, L-lysine can cause partial repression of the synthesis of aspartokinase, a key enzyme for L-lysine biosynthesis, and Gly inhibits aspartokinase activity. Therefore, AEC and Gly can serve as resistant screening markers. Presence of 8 mg/ml AEC and 5 mg/ml Gly, the growth of *S. albulus* was not inhibited. However, the further addition of 1 mg/ml AEC or Gly inhibited cell growth. As a result, 9 mg/ml AEC and 6 mg/ml Gly was chosen as the resistant critical concentration (Table 1). In the course of the derivation of AEC (9 mg/ml) and Gly (6 mg/ml) resistant mutants from *S. albulus* in this study, the enhancement of aspartokinase activity was accompanied by a high productivity of ε-PL.

The mutation and screening of ARTP for *S. albulus*

After the spores solution was treated by ARTP jet for 6 min and cultivated on the solid medium (with 9 mg/ml AEC and 6 mg/ml Gly) for 3 to 5 days, there were about 226 single colonies growing on 10 plates. And then the colonies were transferred to solid medium respectively. After incubation on the plates for 5 days, ε-PL productivity of the original strain and 108 single colonies grew well in subculture and were determined by fermentation experiments. In order to avoid the effect of random error, only the strains with more than 20% increase (ε-PL yield > 0.5 mg/ml) in ε-PL yield compared with the original strain were considered as positive mutants. Table 2 showed that the mutation and positive mutation rate were 31.6 and 26.0%, respectively. The ε-PL yield of top 10 mutants were shown in Table 3, and the strain *S. albulus* A-29 showed the highest ε-PL yield (1.59 ± 0.08 mg/ml), which was 4 times as much as that of the original strain in the same culture condition.

Wang et al. (2010) demonstrated that ARTP jet has higher positive genotoxic response than conventional mutation methods, and the ARTP shows different genotoxic characteristics depending on the treatment dose and cultivation time of the strains, which indicated that the plasma has a different action pattern from other methods. This study made it the first time to use ARTP jet to generate mutants on ε-PL-producing strains and it showed that ARTP jet had a high positive mutation (26.0%) on *S. albulus*. Wang et al. (2010) also confirmed that radio frequency APGD plasma jet has a strong mutagenic effect on *S. avermitilis* and a mutated strain with high productivity of avermectin B1a and a genetic stability was obtained.

Genetic stability of mutants

The mutant *S. albulus* A-29 was isolated to study its genetic stability (Table 4). After a 5 generation culture, *S. albulus* A-29 still maintained high productivity (1.56 ± 0.02 mg/ml). Based on present data, we concluded that the ARTP treatment of spores improved aspartokinase activity, or probably changed the metabolic network in mutants of *S. albulus*. As a result of its high mutation rate, convenient operation, low capital costs, no side effects to human body, no pollution to environment, etc., ARTP jet might be an effective tool for microbial mutation.

Conclusion

This study made it the first time to use ARTP jet to

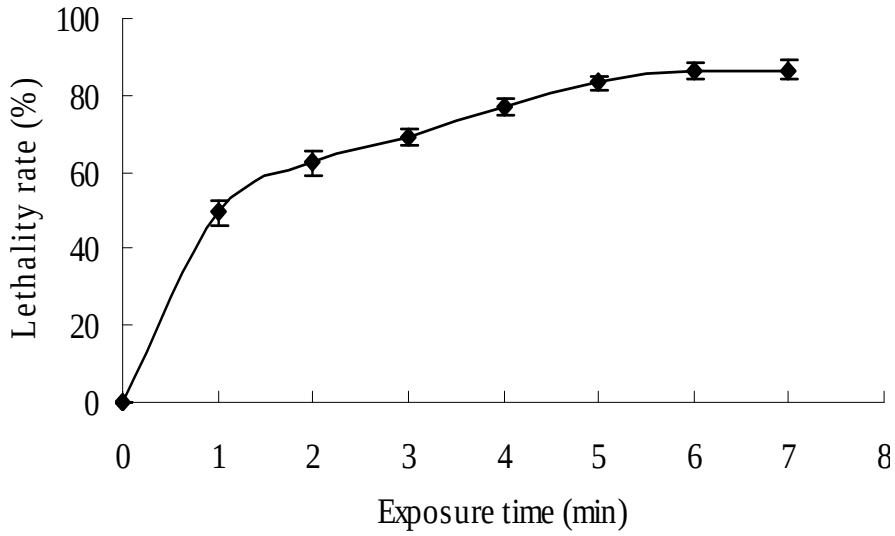

Figure 1. Variation of the lethality rate by the helium ARTP jet with different exposure time.

Table 1. Effects of AEC and Gly with different concentrations on *S. albulus* growth.

C (AEC/Gly) (mg/ml)	3	4	5	6	7	8	9	10
Growth on media added in AEC			+	+	+	+	-	-
Growth on media added in Gly	+	+	+	-	-	-	-	-

"+"presents that colonies could grow normally; "-"presents that colonies could not grow normally.

Table 2. The mutation and positive mutation rate of ARTP jet on *Streptomyces albulus.*

	T	T'	M	P
Colony number	342	226	108	89
R_M	31.6%			
R_P	26.0%			

T is the total colony count after treatment with ARTP on the solid medium without AEC and glycine; T' is the total colony count after treatment on the solid medium with AEC and glycine; M is the total CFU of the mutants; P is the CFU of the mutants with a yield of ε-PL 20% higher than that of the original strain; R_M and R_P represent mutation and positive mutation rate respectively, R_M = (M/T) ×100%; R_P = (P/T) ×100%.

generate mutants on ε-PL-producing strains. *S. albulus* A-29 exhibited the highest ε-PL productivity, which was 4 times as much as that of the wild strain in the same culture condition. The plasma jet shows a strong effect on mutation breeding of *S. albulus*. As a result of its high mutation rate, convenient operation, low capital costs, no side effects to human body, no pollution to environment, etc., ARTP jet might be an effective tool for microbial mutation.

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Table 3. The fermentation ability of original strain and mutants for *S. albulus.*

Strains	ε-PL yield (mg/ml)	R*
CK (original strain)	0.41 ± 0.05	
A-7	1.26 ± 0.09	3.1 ± 0.07
A-11	1.21 ± 0.04	3.0 ± 0.06
A-16	1.28 ± 0.04	3.1 ± 0.07
A-24	1.33 ± 0.07	3.2 ± 0.09
A-29	1.59 ± 0.08	3.9 ± 0.04
A-45	1.48 ± 0.05	3.6 ± 0.03
A-56	1.28 ± 0.12	3.1 ± 0.07
A-71	1.22 ± 0.09	3.0 ± 0.06
A-75	1.33 ± 0.07	3.2 ± 0.05
A-87	1.43 ± 0.02	3.5 ± 0.05

*R presents the percentage content of the ε-PL yield of mutants relative to the original strain. Values are the mean ± SD of data from three independent experiments.

Table 4. The genetic stablity of *S. albulus* A-29.

Indicator	Generation times				
	1	2	3	4	5
ε-PL yield (mg/ml)	1.59 ± 0.08	1.53 ± 0.07	1.62 ± 0.05	1.54 ± 0.03	1.56 ± 0.02
DCW (mg/ml)	4.78 ± 0.08	4.68 ± 0.12	4.59 ± 0.07	4.61 ± 0.10	4.73 ± 0.09
Final pH	3.20 ± 0.01	3.13 ± 0.02	3.18 ± 0.03	3.20 ± 0.01	3.11 ± 0.02

DCW represents dry cell weight. Values are the mean ± SD of data from three independent experiments.

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