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Beneficial Effect of Spaceflight Experience on Producing of Endoproteinase Lys-C by *Lysobacter enzymogenes*

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Endoproteinase Lys-C is an important tool enzyme in industrial produce, mainly gained from Lysobacterenzymogenesat a low efficiency and a long fermentation period. The special environmental factors of outer space may stimulate engineering bacteria to bear high-yield mutant strains. In this study, we sent L.enzymogenes into space via the Chinese Shenzhou-10 spacecraft for approximate 15 days,finding that the total cell number reduced to (24 ± 3.5) ×105cfu/ml after spaceflight, only 13.5% of that in the ground control sample. However, Lys-C activityin ground control bacteria was 0.087 U/ml after fermentation for 120 hours, which was its optical fermentation time for maximal yield, while that in spaceflight sample increased by 17.2%-40.2%,with theoptical fermentation time shortened by 24-48 hours. Further investigatingthe genetic stability of spaceflight sample revealed that both enzyme-producing ability and optical fermentation time after 1, 3, 5, 7, 9, 11 and 15 passages didn't change,revealing good stability of the spaceflight mutant strain. In summary, spaceflight experience could stably increase the yield of Lys-C by L.enzymogenesand shorten the optical fermentation time of Lys-C, which will benefit its industrial produce.

Key words: Endopeptidaselys-C; Bacteria; Spaceflight;Fermentation.

Endoproteinase Lys-C, a member of the serine protease family, with molecular weight of approximately 30 kD, can specifically hydrolyze protein or the C-terminus of amino acid residue in peptides. And it can also be used for protein identification through peptide mass fingerprinting or mass spectrometry (MS)/MS spectral matching. Therefore, Lys-C has good application prospects in proteomic analysis1. In addition, Lys-C can be used to cleave precursors in recombinant protein industrial production (like insulin and its analogues). Compared with the commonly used trypsin, it has several remarkable advantages, such as high specificity, efficiency, and stability.

Currently, *Pseudomonas aeruginosa, Xenorhabdusluminescens* and *Lysobacter enzymogenes* are recognized to synthesize Lys-C, and *L.enzymogenes* are the main producing strains for Lys-C2. However, its efficiency for producing Lys-C is not high, and the fermentation period is relatively long, which prevents the further application of Lys-C. Therefore, it's an urgent to

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improve the technology for high-efficiency fermentation of *L.enzymogenes*.

Space breeding, also known as space mutation breeding, primarily utilizes comprehensive space environment factors such as strong radiation, microgravity and high vacuum to induce genetic mutation of biological materials.The biological materials are usually sent into space via spaceships, recoverable satellites or high altitude balloons. The mutated materials are then used for breeding new varieties once they are returned to the ground. Space breeding, asa new breeding technology, has some advantages such as getting more beneficial mutation, large variation and good stability[3]. Some achievements have already been made by utilizing the special space environmental resources. In 1996, American scientists sent Humicolafuscoatrainto space for 10 days; they discovered that the yield of antibiotic monorden increased from 8.2 ± 2.2 μg (the yield on the ground) to 23.8 ± 3.3 μg4. In 2011, our team sent engineered Escherichia coli into outer space via the Chinese Shenzhou-8 spaceship for 17 days; after the space flight, its yield of interferon α1b was found to increase significantly[3]. In the present study, we sent L.enzymogenes into outer space via the Chinese Shenzhou-10 spaceship lasting about 15 days to explore effect of spaceflight experience on its production of Endoproteinase Lys-C.

MATERIALSAND METHODS

Strains and culture

Lysobacterenzymogenes (ATCC27796) were inoculated by puncture into two 500 μl plastic tubes which contained 220 μl Luria Bertani (LB) semisolid medium. After cultivation in a stationary incubator at 30°C for 48 hours, one plastic tube was sent into outer space by placing in the cabin of the Shenzhou-10 spaceship for 15 days (the spaceship was launched on June 11, 2013 and returned on June 26, 2013). The other plastic tube was kept stationary for 15 days on the ground at the same environmental temperature as that in the cabin of the spaceship for a control.

Culture medium

The LB semisolid culture medium consisted of 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl, 0.9% (w/v) agar and purified water. The fermentation culture medium (pH=7.2±0.1) consisted of 1% (w/v) polypepton, 1% (w/v) sucrose, 0.01% (w/v) potassium phosphate dibasic, 0.01% (w/v) potassium dihydrogen phosphate, 0.02% (w/v) magnesium sulphate and purified water. The tryptic soy broth (TSB) culture medium consisted of 1.5% (w/v) tryptone, 0.5% (w/v) soya peptone, and 0.5% (w/v) NaCl; the TSB culture medium was prepared using distilled water, and its pH was adjusted to 7.2±0.1.

Cell number analysis

After the cabin was returned from space, bacteria samples of spaceflight and ground control were immediately gradienS-diluted and smeared on plates. After 48 hours of cultivation, the colony forming units (CFU) in two samples were respectively assessed by plate clone count. The experiment was repeated three times.

Enzyme activity measurement

180 μl of 1 μM Tris-HCl (pH=8.5) buffer solution was added into a 96-well plate. Next, 10 ?l of 2 mM substrate, N-(p-tosyl)-Gly-Pro-Lys-4-nitroanilide acetate salt (Sigma, United States), was added to form a reaction system. Then, 10 ?l of bacterial broth sample was added into the reaction system, and mixed well. Each sample was performed with duplicate. A standard sample of pure Lys-C (Sigma, United States) was added into the reaction system to replace the bacterial broth sample as a positive control. Sterilized purified water was added into the reaction system to replace the bacterial broth sample as a blank control. A micro-plate reader was used to determine the kinetic reaction curve at 405 nm under 30°C; the reaction duration was 30 min. The enzyme activity was calculated and defined as follows: at 30°C (reaction temperature) in one minute, the amount of enzyme needed to catalyze the hydrolysis of 1 ?M substrate, N-(p-tosyl)-Gly-Pro-Lys-4-nitroanilide acetate salt, represented the same amount of enzyme activity unit[5].

Screening of spaceflighS-treated bacteria

From spaceflight sample, 300 single colonies were randomly selected and then were transferred onto the LB agar medium slant; they were then cultured in a stationary incubator at 30□°Cfor 48 hours. Subsequently, the colonies were inoculated into the fermentation culture medium with an inoculation amount of 5%. In addition, the strains of the ground control bacteria

were inoculated for comparison. All strains were cultured in a shaking table at 220 rpm under 30?C for 120 hours. Afterwards, the enzyme activity was measured. The enzyme activity of the ground control bacteria was used as the baseline. Colonies in spaceflight sample with greater than 110% of the baseline enzyme activity were defined as positive mutant strains, which were then thoroughly screened at least three times again for enzyme activity at the 72th, 96th, and 120thhours of fermentation, aiming to verify the positive mutant strains and investigate their optical fermentation time.To investigate the genetic stability of the positive mutant strain, its enzyme-producing ability and optical fermentation time after 1, 3, 5, 7, 9, 11 and 15 passages were measured, and then were compared with the data of primary generation.

Identifying the purification of Lys-C from flight bacteria

An amount of L.enzymogenes was added into TSB culture medium and was cultured on a shaking table with 220 rpm at 30±1?C for 22 hours. The culture medium was added to a 50 L fermenter for fermentation based on an inoculation amount of 5%. The stirring speed was 150 rpm, and the aeration rate was maintained at 3 mL/min; the fermentation temperature was controlled to be 30?C. After the fermentation was complete, Lys-C was isolated and purified. A bicinchoninic acid assay (BCA, United States) protein assay reagent kit (Pierce, United States) was used for quantitative determination. In addition, SDS-polyacrylamide gel electrophoresis (PAGE) detection was performed, the standard Lys-C was used as a positive control.

Statistical methods

The data were expressed as the mean ± standard deviation. Student's S-test was used as the statistical method. Difference was considered to be statistically significant when $p<0.05$.

RESULTS

Effect of the space environment on the growth of the strains

After the strains were returned from space to the ground, two samples were immediately diluted and smeared on plates and counted. The CFU of Lysobacterenzymogenes in ground control sample was (178 ± 20.5) ×105cfu/ml, while in spaceflight sample was (24 ± 3.5) ×105cfu/ml, approximately 13.5% ± 1.9% of ground control sample. The results (shown in Fig. 1) indicate that the space environment significantly inhibited the growth of Lysobacterenzymogenes.

Optimal fermentation time for *L.enzymogenes*

To verify the optimal fermentation time for maximal yield of Lys-C, the enzyme activitiesafter 72 hours, 96 hours, 120 hours, and 144hours of fermentation were measured. As shown in fig.2,the Lys-c activity reaches highest(0.87 U/ml) at the 120thhour. So we chose 120 hours as the optimal fermentation time for further studies. And 0.87 U/ml was used as the baseline of Lys-c activity for selecting mutant strain in spaceflight sample.

Effect of spaceflight on Lys-C yield

From 300 strains randomly picked from spaceflight bacteria, 10 positive mutant strains were obtained. After 120 hours of fermentation, the enzyme activity of the L.enzymogenesinground control sample was 0.087 U/ml; while thatin the positive mutant strains ranged from 0.102 U/ml to 0.122 U/ml, increased by 17.2% to 40.2% ($p < 0.05$, Table 1). To evaluate the optical fermentation time of the positive mutant strains, we also measured their enzyme activities at 72thand 96th hours (Table 2). From the data, optical fermentation time of two strains (S-022 and S-088) shortened by 24 hours, and two strains (S-041 and S-105) shortened by 48 hours. Combining data of the most raised enzyme

Table 1. Lys-C activity of ten positive mutant strains for 120 hours of fermentation

Colonies	Lys-C activity (U/ml)	
Ground	0.087	100.0%
S-009	0.102	117.2%*
S-022	0.108	124.1%*
S-041	0.121	139.1%*
S-054	0.110	126.4%*
S-061	0.112	128.7%*
S-088	0.122	140.2%*
S-099	0.109	125.3%*
S-105	0.111	127.6%*
S-107	0.108	124.1%*
S-149	0.117	134.5%*

Data was shown as the mean of three independent experiments.Ground is the *L. enzymogenes* cultivated as a control. "S-" means the strain was isolated from spaceflight sample. * $p<0.05$.

Table 2. Lys-C activity of positive mutant strains at specified time of fermentation

Colonies	Enzyme activity of Lys-C (U/ML)		
	72h	96h	120h (control)
Ground	0.058(66.7%)*	0.074(85.1%)*	0.087 (100.0%)
S-009	0.069(67.6%)*	0.078(76.5%)*	0.102 (100.0%)
S-022	0.080(74.1%)*	0.108(100.0%)**	0.108 (100.0%)
S-041	0.120(99.2%)**	0.118(97.5%)**	0.121 (100.0%)
S-054	0.078(70.9%)*	0.089(80.9%)*	0.110 (100.0%)
S-061	0.077(68.8%)*	0.100(89.3%)*	0.112 (100.0%)
S-088	0.109(89.3%)*	0.120(98.4%)**	0.122 (100.0%)
S-099	0.069(63.3%)*	0.080(73.4%)*	0.109 (100.0%)
S-105	0.110(99.1%)**	0.112(100.9%)**	0.111 (100.0%)
S-107	0.080(74.1%)*	0.099(91.7%)*	0.108 (100.0%)
S-149	0.087(74.4%)*	0.110(94.0%)*	0.117 (100.0%)

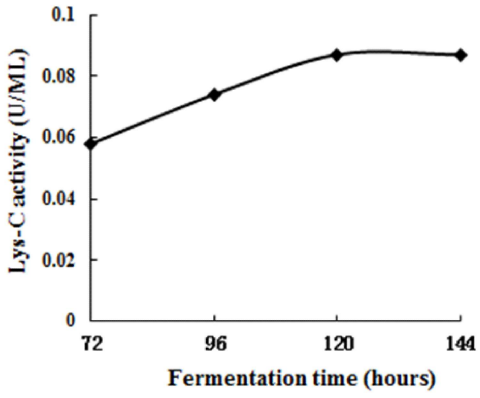
Data was shown as the mean of three independent experiments. For each line, data in brackets was the relative Lys-C activity compared with that of 120h, the control. Data in bold means the difference was not significant when compared with respectivecontrol.
* $p<0.05$; ** $p>0.05$.

activity and the shortestoptical fermentation time, strain S-041was selected for further studies, whose Lys-C activity increased by 39.1%. And it had been sent to China General Microbiological Culture Collection (CGMCC) for preservation (CGMCC8328). After S-041and ground control strains were fermented in a 50 L fermenter for 72 hours, Lys-C was isolated and purified. The quantitative determination revealed that 390 mg Lys-C in S-041, and 206 mg Lys-C in control sample were obtained; the yields were 7.8 mg/L and 4.12 mg/L respectively, indicating spaceflight apparently raised the yield of Lys-C in L.enzymogenes. In SDS-PAGE analysis, the Lys-C isolated from S-041 and ground control strains locatedthe same as standard Lys-C, indicating the purification was good(Fig. 3).

Table 3. Analysis of the passage stability of S-041

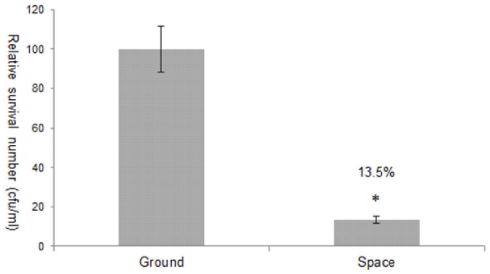
Subculture times	Activity of Lys-C (U/ML)		
	72 h	96 h	120 h(control)
0(control)	0.118	0.120	0.121
1	0.118*	0.121*	0.121**
3	0.117*	0.122*	0.120**
5	0.115*	0.119*	0.118**
7	0.121*	0.119*	0.122**
9	0.118*	0.121*	0.120**
11	0.116*	0.117*	0.119**
13	0.118*	0.119*	0.120**
15	0.120*	0.119*	0.119**

Data was shown as the mean of three parallel samples. "0" in the "subculture times"column mean the primary passage of S-041, a control for enzyme-producing ability evaluating after passaging (**). Activity of Lys-C in 120h was used as a control for optical fermentation time evaluating in each passage (*). * $p< 0.05$; ** $p < 0.05$.



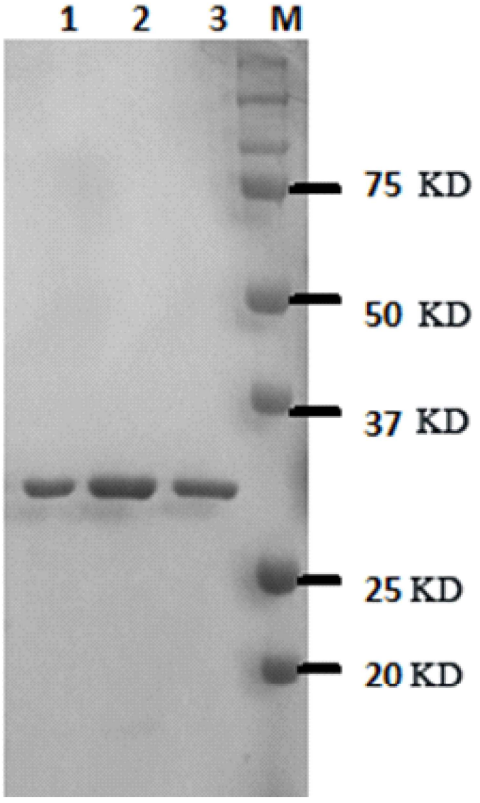
Data was shown as the mean of cell number (±SD) in two samples for three independent experiments. * $p< 0.05$.

Fig. 1. Relative cell number of *L.enzymogenes* in two samples.



Lys-C activities at 72th hour, 96th hour, 120th hour, and 144th hour of fermentation were 0.58 U/ml, 0.74 U/ml, 0.87 U/ml, and 0.87 U/ml separately. This experiment represented three independent experiments.

Fig. 2. Lys-C activities at specified times of fermentation



"1" represented standard pure Lys-C; "2" was Lys-C isolated and purified from spaceflight sample; "3" mean Lys-C isolated and purified from ground control sample; while "M" was marker. This result represented three independent experiments.

Fig. 3. SDS-PAGE image of purified Lys-C

Passage stability of space flight strain

To further elucidate the effect of spaceflight experience on the enzyme activity of L.enzymogenes, we passaged the space mutant strain S-041 in triplicate and investigated its genetic stability. We discovered that after 1, 3, 5, 7, 9, 11 and 15 passages, the enzyme-producing ability and the optical fermentation time of this strain didn't change from those of the primary passage ($p < 0.05$, Table 3), manifestinggood stability of the mutant strain.

DISCUSSION

The outer space contains some special environmental factors that are not present in the ground environment, including microgravity, high vacuum, strong radiation, weak magnetic field and extreme temperature differences. These factors impose complicated impacts on the growth, biological characteristics and hereditary metabolism of bacteria. Generally, these impacts are detrimental to the survival and growth of microorganisms6, 7. One study showed that microorganisms in a spaceship grew more slowly than those on the ground8, indicating that the space environment is a severe test for the survival and growth of bacteria. When facing a hostile environment, bacteria adapt to the changes of the environment viamodifying their physiologies and morphologies9; during this process, the bacteria's genetic mutation process will accelerate and diversify. In the present study, we discovered that the total amount of bacteria after the space flight was only 13.5% of the total bacteria cultivated on the ground; however, the yield of Lys-C was raised and the optical fermentation time was shortened,probably resulting from a series of genetic changes in L.enzymogenesfor surviving in the space environment.

Exploring the environmental conditionsduring spaceflight as special mutation sources, such as space breeding could benefit human beings. Space breeding has so far produced impressive results. Space products such as space mutation vegetables have been promoted to general consumers and are being industrially produced on a large scale. In addition, quite a few studies have reported that high-yield strains with significantly increased secondary metabolites and hereditary stability

have been gained from various microorganisms through space mutation and screening. For instance, after space flight,the yield of tylosinin Streptomyces fradiaeincreased by 91.5%10; the yield of actinomycin D (an antibiotic) in Streptomyces plicatusincreased from 0.47 ?g/ml, the levelon the ground, to 1.02 ?g/ml8. In our study, L.enzymogeneswas sent into space via the Shenzhou-10 spaceship for space mutation, with a pleasing result of Lys-C activity increasing by 17.2% to 40.2%, and of optical fermentation time shortened by 24 to 48 hours. Further investigation revealed that Lys-C yield and optical fermentation time remained stable after passaging. The collective data revealed that spaceflight could stably increase the yield of Lys-C in L.enzymogenes, and thus could in some degree solve the omnipresent issue of high cost using L.enzymogenes in industrial production of Lys-C with alow enzymeproducing abilityanda long fermentation periodat present[1, 5, 11].

The mechanism of space mutation is relatively complicated; microgravity and space radiation are considered to play major roles in space mutation12-14. These factors significantly increase the mutation frequencies of some genes in microorganisms. Wilson et al.15 discovered that after Salmonella typhi were exposed to the space environment for a period of time, 167 gene expression levels changed, such as the regulator,Hfq. And these changes caused the lethal dose 50 (LD50) of bacteria for mice to decrease sharply, which mean the toxicity of these bacteria significantly increased. A study on Enterococcus faecium that had been sent into space for 17 days via the Chinese Shenzhou-8 spaceship revealed that there was a significant difference between the metabolic profiling of the bacteria that had been sent into space and the bacteria of the control group on the ground; further analysis showed that two genes (dprA and arpU) changed in the genome, transcriptome and proteome16. In the present study, due to an insufficient amount of the original samples, we did not conduct an analysis on the genetic changes; additionally, we believed that genetic material analysis after culturing could not properly explain the effect of space factors on the phenotype and genotype of these bacteria. However, we were convinced that mutations occurred to the genetic materials of these bacteria under the influence of the space environment factors. It will be an interesting and important issue to research in the future.

Although we currently understand the mechanism of space mutation to some degree, we cannot control the mutation direction and thus can only let mutations develop randomly. In addition, we can only obtain beneficial mutant strains through large-scale screening after the samples returned to the ground. How to use of space resources for directed or even site-directed mutation remains a scientific unknown that should be further explored by scientists.

CONCLUSIONS

AfterL.enzymogenes was sent into space via the Chinese Shenzhou-10 spacecraft for 15 days, its total cell number was only 13.5% of the ground cultivated sample, but the yield of Lys-C improved by 17.2%-40.2%. And itsoptical fermentation time shortensby 24-48 hours. Further investigation revealed that both the enzyme-producing ability and optical fermentation timefor spaceflight sample remain stableafter passages. In summary, the space environment can increase the yield of Lys-C and shorten the optical fermentation time of L.enzymogenes, which will be beneficial for industrial produce.

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