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Enhanced Cordycepin Production in *Cordyceps militaris* Mutated by Multifunctional Plasma Mutagenesis System

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SHORT TITLE: Multifunctional Plasma Mutagenesis System and Purification of Cordycepin

ABSTRACT： A multifunctional plasma mutation system (MPMS) method was used to create high cordycepin yielding mutations from wild *Cordyceps militaris*, which yielded many viable mutants, many of which produced more cordycepin compared to the wild strain. One particular mutant strain (GYS60) produced 7.883 mg/mL, which is much higher than those reported to date, over 20 times higher than that of the wild strain, while the cordycepin production of another viable mutant (GYS80) was almost zero. The extraction and purification of cordycepin, using the fermentation broth of *C. militaris* GYS60, was also investigated. Cordycepin was extracted by using AB-8 macroporous resin and purified by using reversed-phase column chromatography. When the sample was adsorbed onto the macroporous resin, 20% ethanol was used as the desorption solvent yielding various fractions. The fractions containing cordycepin were loaded onto a reversed-phase chromatography column packed with octadecyl bonded silica as stationary phase and ethanol (95%)/acetic acid solution (5%) at pH6.0 as the mobile phase. The combination of this two-step extraction-purification process yielded cordycepin at 95% purity with a total recovery rate was 90%.

Key words: Multifunctional Plasma Mutagenesis System, *Cordyceps militaris*, cordycepin, purification, mutant

ABBREVIATIONS: **BV**, bed volume; **CFU**: colony-forming units; **HPLC**: high-performance liquid chromatography; **LC-MS**: liquid chromatography - mass spectrometer; **MPMS**: multifunctional plasma mutagenesis system; **ODS**: octadecyl bonded silica; **PDA**: potato dextrose agar.

I. INTRODUCTION

As one of the most valued traditional Chinese medicines, *Ophiocordyceps sinensis* (Berk.) G.H. Sung et al. (syn. *Cordyceps sinensis*) is known as one of the tonics and medicinal mushrooms in East Asia.[1,2] It is an endoparasitoid which parasitizes insect larvae and grows in summer and autumn, and gradually turns into a mature fruiting body from the host insect's cadaver in the following summer. *O. sinensis* is commonly referred to as "winter-insect and summer-plant" in Chinese, or "Tochukaso" in Japanese. In traditional Chinese medicines, it is used to treat numerous illnesses, promote longevity, and relieve fatigue.[3,4] *O. sinensis* has become increasingly popular and important in both the public and scientific communities. Many bioactive constituents have been reported, such as various polysaccharides, mannitol, aminophenol, ergosterol and a number of unusual nucleosides such as inosine, hypoxanthine and cordycepin.[5-9]

Its unique medicinal value is causing an unsustainable demand and a marked decline of its presence in the wild. Unfortunately, the industrialized cultivation of *O. sinensis* fruiting bodies has not been realized yet. *O. sinensis* (Ophiocordycipitaceae) belongs to the same order of Hypocreales of Ascomycota as *Cordyceps militaris* (Cordycipitaceae),[10] and both possess some of the same highly desired bioactive constituents.[11] Because of *C. militaris* is easily cultivated, and the highly desired bioactive constituents are common in both *O. sinensis* and *C. militaris*, *C. militaris* is becoming a feasible surrogate for *O. sinensis*.[10-12] However, cordycepin yield from *C. militaris* is lower compared to its yield from *O. sinensis*,[13,14] hence investigations to achieve much higher yielding variants of *C. militaris* is imperative. *C. militaris* has long been demonstrated to possess many bioactive ingredients, including

cordycepin, mannitol (aka cordycepic acid), and cordycep-derived polysaccharides.[15,16] Cordycepin was first isolated from liquid cultures of *C. militaris* in 1951.[17] Since then, its structure, pharmacological actions, and its isolation and purification have attracted extensive attention. Cordycepin, 3'-deoxyadenosine, is a purine nucleoside analogue, which has a broad spectrum of biological activities, including: anti-cancer, antioxidant, anti-microbial, anti-inflammatory activities, anti-hyperuricemia and antidepressant effects.[18-25]

Cordycepin has long be produced by a range of methods, including chemical synthesis,[26,27] liquid fermentation technology,[28-32] solid-state fermentation for the cultivation of *C. militaris* fruiting bodies,[33-36] and subsequently recovered from *C. militaris* biomass.[37,38] There have been many attempts to promote cordycepin production by mutation technology,[39,40] and mating-based sexual reproduction.[41] However, despite the various efforts the improvements in cordycepin yield were limited to 30-100%;[39-41] while at the same time the demand for cordycepin remains high. This high demand for cordycepin is outstripping supply, which has severely inflated the price for cordycepin. Hence, continuous investigations into improving the yield of cordycepin can have great commercial appeal.

Recently, plasma (a partially or fully ionized gas) was successfully employed as a microbial mutation tool to generate functional mutants.[42] The plasma technique, which uses nonequilibrium discharge plasma sources at atmospheric pressure, has attracted much attention for its efficiency, convenience, and safety without polluting nature. Among different types of plasma sources, multifunctional plasma mutagenesis system (MPMS), using nitrogen gas, bare-metallic electrodes and driven by a high voltage power supply has shown promise in practical applications (Fig. 1).[43] However, until now, no reports have been published on the

use of MPMS as a mutation tool for *C. militaris*.

In this study, a nitrogen-based MPMS was applied to *C. militaris* protoplasts at various expose times, which yielded several functional mutants. Then a two-step extraction and purification process was employed in order to obtain concentrated cordycepin from the most productive mutant strain to a commercial purity and recovery rate.

II. MATERIALS AND METHODS

A. Chemicals

Cordycepin and adenosine, were purchased from Sigma (St. Louis, MO, USA). Methanol and acetic acid (HPLC grade) were purchased from Tianjin Shield Company (Tianjin, China). All other chemicals (analytical grade) were purchased from Beijing Chemical Reagents Company (Beijing, China). AB-8 macroporous resin was purchased from Tianjin Haoju Resin Company (Tianjin, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

B. Cultured Mycelia of *C. militaris*

Our wild-type *C. militaris* were cultivated from fruiting bodies purchased at a supermarket in Beijing, China. First, the mycelium of wild-type *C. militaris* was cultured at 28°C for 7-14 days on an enriched PDA slants (1000 mL of 20% potato extract liquid + 10% dextrose+ 2.5% peptone+ 1% yeast powder + 0.05% $MgSO_4$+ 0.1% KH_2PO_4+ 2% agar), followed by storage on PDA slants at 4 ºC in a refrigerator until the further experiment. Then, from the slant, the seed culture was transferred onto fresh PDA medium and incubated at 28°C for 10

days. Subsequently a submerged fermentation was carried out using an enriched potato peptone broth as fermentation medium (20% potato extract liquid + 4.0% dextrose + 0.5%+0.2% yeast powder + 0.2% KH_2PO_4 + 0.01% $MgSO_4$). The best condition for the fermentation of *C. militaris* was to use 500 mL Erlenmeyer flasks containing 250 mL liquid fermentation medium, incubated at 28°C and a rotatory shaker at 180 rpm for 15 days.

C. Operational Conditions of MPMS

Detailed descriptions on the experimental setup are referenced in previous publications.[41,42] Figure 1 illustrates the experimental setup for the treatment of *C. militaris* by the Nitrogen plasma jet. The gas, with purity of 99.999%, was employed as the plasma working gas at a constant flow rate of 10 standard liters per min, and a high voltage power supply input was maintained at 180 W. The material to be treated was transferred to a sterilized, stainless steel disk (8 mm diameter) by pipette, and then placed downstream of the plasma torch nozzle exit with a stand-off distance of 2.0 mm (Fig.1). Due to the low velocity (average 3.3 m/s) and small mass density (0.1784 kg/m^3) of the plasma jet, the corresponding dynamic pressure of the plasma jet was 1 Pa, which meant that the splashing effect of the plasma jet on the treated materials was negligible. The temperature of the plasma jet was kept below 40°C.

D. Experimental Protocol for *C. militaris* Mutagenesis and Cultivation

Following cultivation for 7-14 days, the protoplasts of wild-type *C. militaris* were prepared according to a modified method previously described by Guo and coworkers.[44] A 50 μl protoplasts suspension (10^6 cells /mL) was applied to a sterilized sample disk and exposed

downstream of the plasma jet. After the sample had been treated for various predetermined exposure times (either 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 sec), the treated protoplasts solution was spread onto solid PDA medium and cultivated at 28 ºC for about 2~3 days until an aerial mycelium appeared. The mycelium was then subcultured onto another solid PDA medium for 7-14 days under the same conditions. When the mycelium almost filled the entire Petri dish, several mycelium agar plugs were cut out from the agar using a 0.6 cm-diameter cork borer and six plugs were placed into each 500 mL fermentation vessel containing 250 mL enriched potato peptone broth and incubated at 28ºC, 180 rpm for 15 days. Because adenosine is a metabolic precursor to cordycepin in the same *de novo* pathway,[44] one set of cultures were spiked with 0.1 mg/mL adenosine to investigate whether enhanced cordycepin biosynthesis was due to a greater flux through the pre-adenosine part of the *de novo* pathway or the post-adenosine part of the *de novo* pathway. At the completion of the liquid fermentation a sample of the fermentation broth was centrifuged at 10,000 rpm for 5 min and the supernatant was analyzed by HPLC. All experiments (Fig. 2) were carried out in triplicate.

E. Determination of Lethality Percentage and Mutation Efficiency following MPMS

The lethality percentage and mutation efficiency of the *C. militaris* with different operation conditions by MPMS were evaluated. The lethality percentage provides an indication of the lethal impact of exposure to MPMS, which was determined using the following equation: Lethality % = (U-T)/U × 100%. Where U is the total colony counts of the samples not exposed to MPMS, and T is the total colony counts after the MPMS treatment. The mutation efficiency is an indication of the number of viable mutants with a cordycepin yield greater

than the wild strain, which was calculated by the following equation: Mutation efficiency = P/T × 100%, where P is the CFU of the mutants with a yield of cordycepin greater than that of the wild strain. Viable mutants from each time exposure (plus the wild strain) were continuously transferred of 10 generations on PDA. In order to assess long-term stability, each generation was followed by a liquid fermentation as mentioned before. At the completion of each of those fermentations, the productivity of cordycepin was evaluated by HPLC.

F. Determination of Cordycepin and Adenosine by HPLC

The concentrations of cordycepin and adenosine were determined using appropriate standard curves (range 0-100 μg/mL, with a coefficient of determination (R^2) of 0.999 for both compounds, on an Agilent 1200 HPLC fitted with an ODS-C18 Column (250 mm × 4.6 mm, 5 μm, Cosmosil, Nacalai Tesque Co., Kyoto, Japan). The column temperature was maintained at 30℃. Methanol or acetonitrile and phosphates are often used in HPLC as mobile phase components, however the salts are often harmful to the instrument and chromatographic column. In order to avoid any adverse effects of mobile phase salts to the instrument, methanol and water were used in this experiment. The samples and reference compounds were separated by using a gradient mobile phase which consists of water (A) and methanol (B). The gradient condition was as follows: 0−5 min, 95:5% A:B isocratic; 5−10 min, 95: 5−85:15% A:B gradient; 10−20 min, 85:15% A:B isocratic; 20−25 min, 85:15%−0:100% A:B gradient; 25−30 min, 100% B isocratic. The flow rate was set at 1 mL/min. The UV detection wavelength was set at 260 nm using variable wavelength detector (Agilent, model 1200, Shanghai, China) and the injection volume was 5 μL.

G. Purification of Cordycepin

In order to produce sufficient quantities of cordycepin for further analysis, the following purification procedure was used. The fermentation supernatant was first dried by means of a rotary evaporator and dissolved in methanol when used, then adjusted the concentration of cordycepin to at least 0.4 mg/mL according to the peak area of HPLC. This extract was first passed through an AB-8 column (10 mm × 300 mm) packed with a macroporous resin fitted with a UV detector (Agilent Technologies Inc., Shanghai, China) at 260 nm. The AB-8 column was initially prepared by passing distilled water (pH6) to balance the column, the 5 bed volumes (BV) concentrated supernatant was loaded and passed through the AB-8 column at a flow rate of 1 BV/h. Following the passage of 5 BV of concentrated supernatant, the column was washed by distilled water (pH6, acidified with acetic acid) to the equilibrium baseline, then the mobile phase was switch to 20% ethanol (pH6, acidified with acetic acid) to elute the semi-purified cordycepin from the column. This was then followed by 95% ethanol (pH6, acidified by acetic acid) to reclaim AB-8 resin. The eluate from AB-8 column was loaded on a semipreparative reversed-phase (Octadecyl bonded silica, ODS) C18 chromatography column. The C18 column was pre-equilibrated with 5% ethanol (pH6) and was gradiently eluted over a 50-minute period from 5 % to 95 % at a flow rate of 3 mL/min. Every elution peak was collected and analyzed by HPLC to confirm the presence of cordycepin. Purity of cordycepin is expressed as the mass of cordycepin as analyzed by HPLC divided the total mass of the material loaded onto a column.[45]

H. Identification of Cordycepin

To confirm the identity of the cordycepin produced by *C. militaris*, both pure standards and the extracted and purified cordycepin produced by *C. militaris* were analyzed by LC-MS using an Agilent 1200/6120 system (Agilent Technologies Inc., Shanghai, China) equipped with a diode array detector and mass spectrometer (MS). The HPLC system consisted of a G1311A solvent delivery unit, a G1379A degasser, a G1315B UV–Vis diode array detector operated at 260 nm, a sample injection valve (Model 7725i) with a 10μL loop, and an Agilent HPLC workstation (Agilent, Boblingen, Germany). An ODS column (4.6 mm × 50 mm, 1.8 μm) was used at a constant temperature of 40 °C. The mobile phase consisted of aqueous acetonitrile (acidified with 0.1% formic acid) at a flow of rate 0.5 mL/min commencing at 10% acetonitrile and increasing to 100% acidified acetonitrile over 4 minutes，which was then maintained at 100% acidified acetonitrile for 1 more minute. The analysis volume was set at 1 μL. Electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-QTOF MS) was used to analyze the ion cleavage of cordycepin with an ESI temperature parameter 400°C, in a positive ion mode, a capillary voltage 3500 V, and a nebulizer pressure at 0.24 MPa. The temperature of dry gas was 350 °C at a constant flow rate of 12 L/min. Spectra were scanned over a mass range of m/z 100-800. All data are the result of three independent, duplicate experiments, i.e. n=6.

III. RESULTS

A. Colony Morphology of *C. militaris*

Colony morphology may be an indicator of phenotypic variation. After the protoplasts of

wild-type *C. militaris* were treated by MPMS and cultivated on the PDA solid medium for 3 days, there were more than 200 single colonies growing on 20 plates across various time treatments of MPMS. All CFUs were treated as separate strains and transferred to a new PDA medium cultured at 28 °C for 7 days. The colonies that developed following subculturing had different appearances giving various colony morphologies. The strain that developed following 60 sec of plasma treatment appeared as white fluff with an abundance of filamentous mycelia surrounding the colony; (Fig 3A); while the strain that developed following 80 sec of treatment had more wrinkles (Fig 3C) than the wild strain (Fig 3B).

B. Lethality Percentage of *C. militaris* by MPMS

A high percentage of cell lethality is desirable for screening of effective mutants.[46] The lethality percentage of *C. militaris* treated by MPMS became quite significant when the exposure time exceeded 50 seconds with lethality percentages at 82.9%, 95.9% and 89.8% at 50, 60 and 80 seconds respectively (Fig.4). When the sample protoplasts were exposed for 100 sec with these conditions, no cells survived.

C. High Cordycepin producing *C. militaris* Mutants

The productivity of cordycepin of the wild strain *C. militaris* and selected mutant strains was determined following a 15 day submerged fermentation. Among the mutants, only those treated for 10, 40, 50, 60 and 70 seconds produced more cordycepin than the wild strain (Table 1), where the wild strain produced 0.381 mg/mL. One mutant, treated for 60 seconds (labeled as *C. militaris* GYS60) stood out from all the strains screened by having the highest

productivity of cordycepin, 7.883mg/mL, while another viable mutant treated for 80 seconds by MPMS, (labeled as *C. militaris* GYS80) had the lowest productivity of cordycepin, 0.154 mg/mL. The elevated productivity of cordycepin by GYS60 surpassed any of the other mutants screened in this study, and highlights the randomness of successful mutagenesis.

Adenosine is a metabolic precursor to cordycepin.[44] After 15 days of fermentation the wild strain accumulated 1.55 mg/mL adenosine, while the GYS60 and GYS80 mutants accumulated 1.64 and 3.32 mg of adenosine per mL. The addition of 10 mg/mL adenosine (at day 5) as the substrate for cordycepin resulted in an elevated level of cordycepin (Table 1), except in strain GYS80. The increase in productivity of cordycepin due to adenosine addition appears to be higher at the lower levels of exposure compared to the higher level of MPMS exposure. However, the observations that low exposure generates an increase in productivity in the presence of adenosine, might again be related to the inherent randomness of mutagenesis.[47] At the completion of the submerged fermentation with the addition of 10 mg/mL adenosine, the adenosine concentration of the wild strain was 10.21 mg/mL while the GYS60 and GYS80 mutants accumulated 12.57 and 5.88 mg of adenosine per mL These results indicate that the *de novo* adenosine pathway and the subsequent cordycepin pathway are tightly linked in the wild strain, and that this linked flux through the combined adenosine and cordycepin pathways was retained in the high producing GYS60 mutant.

D. Optimum Purification Conditions for Cordycepin

Cordycepin obtained from the GYS60 strain was purified using an AB-8 column, employing cordycepin at 0.4 mg/mL. Five column bed volumes (BV) of sample volume were loaded on

the column and eluted with 20% acidified ethanol (pH 6.0) as mobile phase at 1 BV/h. The third elution peak (at approximately 75 mins retention time) (Fig. 5A), was subsequently identified as cordycepin by HPLC (Fig. 5B) by means of retention time (11.038 min). Employing the AB-8 column allowed the purity of cordycepin to be increased from about 20% to 40%. The cordycepin peak from the AB-8 column was further purified on the C18 column, which reached a peak with ethanol concentration 59%, at an extraction time of 30 min (Fig. 5C). The C18-purified cordycepin was again confirmed as a single peak (at 11.038 min retention time) by HPLC (Fig.5D). The purity of cordycepin by C18 was increased up to 95% (Fig. 5C and 5D). By combination of those optimum conditions (AB-8 and C18), the total recovery rate was approximately 90%.

E. Identification of Cordycepin

In the positive ion mode for the LC, the retention time of authentic cordycepin was 3.275 min (Fig.6A), and the retention time of cordycepin LC produced by GYS60 was 3.278 min (Fig.6C). The mass spectra obtained from both compounds showed that *m/z* is 251.9 (Fig.6B and 6D), which is consistent with the molecular mass of cordycepin, while ion fragments of 3-hydroxyribose and adenine at 136 and 118 *m/z* respectively were also prominently present in both spectra.

IV. DISCUSSION

A variety of mycelial growth with multifarious morphologies were observed following the plasma treatment of *C. militaris* and subsequent cultivation on solid media for 15 days. Variation in colony morphology has previously been shown to reflect gene diversity.[46] In this

study, mutants with different morphologies produced cordycepin at greatly different productivities (Table 1). One treatment with an exposure time of 60 seconds had a high lethality percentage and yielded *C. militaris* GYS60, which achieved an exceptional cordycepin productivity (2070% increase compared to the wild strain). Similar lethality percentages (80+%) however, yielded quite different cordycepin productivities, ranging from an increase of 40% to a decrease of 40% compared to the wild strain. The large variation in viability and productivity of the mutants screened in this study highlights the randomness of successful mutagenesis.[46,47] However, despite the inherent randomness of the mutagenic method the plasma-induced mutagenesis treatment used in this study is shown to be an efficient method for creating functional mutants in *C. militaris*. Compared with the traditional mutation methods for *C. militaris* as described by Kang and coworkers,[41] the MPMS device is efficient, compact, with a low capital cost and flexible and safe operation process, it can be expected to be a useful tool for microbial mutation.

Both the wild *C. militaris*, and a series of mutants created following exposure to a range of MPMS periods retained the ability to synthesize adenosine. Adenosine ultimately accumulated in the growth media which implies that none of the genes involved in adenosine synthesis were negatively affected by the MPMS-induced mutation events. When a fixed amount of adenosine was added in the culture medium of the wild strains and the GYS60 mutants, adenosine accumulation was more pronounced, and caused an increase in the yield of cordycepin. Based on the highly elevated cordycepin production, we postulate that the flux through the adenosine pathway was significantly increased in the GYS60 mutant. However, the reduced conversion of adenosine to cordycepin in the GYS80 mutant suggests that the flux through the adenosine pathway reached saturation, and the conversion from adenosine to

cordycepin was also a major obstacle in proportionally lower accumulation of cordycepin.

Several works have been reported that production of cordycepin was increased to some extents through optimization of culture conditions.[28-41,48] Other studies showed that the yield of cordycepin produced by the mutagenic *C. militaris* strain increased significantly.[48] It has been reported that the productivity of cordycepin had been improved to some extents (Table 2). In this study, 7883 mg/L of cordycepin was produced by the *C. militaris* GYS60 mutant, showing a 2070% higher cordycepin production over the control (wild strain), in a liquid shake culture without optimization of culture conditions. Some previous reports have shown that cordycepin production in liquid cultures was much less productive compared to our present study (Table 2). A range of methods have been tried to optimize cordycepin productivity, including optimizing the amount of NH_4^+ during a fed-batch mode,[28] which generated a 70% increase in cordycepin compared to batch cultivation; while with optimizing the carbon/nitrogen ratio in the cultivation medium achieved a doubling of the cordycepin yield to produce 640 mg/l.[29] Exposure to blue light during cultivation caused a significant increase in cordycepin productivity and yielded 1580 mg/l compared to 750 mg/l under daylight conditions.[31] Others have investigated the most optimal oxygenation levels under different cultivation conditions, as such they found that hypoxic conditions were more productive in liquid surface cultures compared to the submerged cultures.[48] Through the addition of adenine, cordycepin production was improved by 4.6-fold to 6200 mg/l.[32] Using a mutant strain, the cordycepin production reached 6840 mg/l by optimizing the amount of C-source and N-source.[30] Hence, the mutant obtained in our study, *C. militaris* GYS60, will be a promising strain for the large-scale production of cordycepin, especially following optimization of cultivation conditions.

In order to carry out a definite identification of the cordycepin produced in this study, a two-step purification process was undertaken. Firstly, a crude cordycepin fraction was extracted from the fermentation broth employing an AB-8 chromatography column, using 20% acidified ethanol as the mobile phase. This process yielded a fraction containing cordycepin at approximately 40%, meaning that 60% of the fraction contained solids that were not cordycepin. This crude cordycepin fraction was further purified employing a C18 column using a gradient elution of acidified ethanol, which ultimately yielded a fraction containing cordycepin at 95% purity. Overall, the combination of these two stages of the purification process had a recovery rate for cordycepin of more than 90%.

The preparation and purification method is simple, highly efficient, and energy-saving environmentally friendly, and has here been demonstrated to be effective for large scale preparations of cordycepin from fermentation broth.

The identity of cordycepin was confirmed by means of HPLC (by retention time), and the confirmation of molecular structure by LC-MS compared with authentic cordycepin. In the positive ion mode, the mass spectra of the relevant LC peaks at 3.275 min and 3.278 min from the authentic and purified cordycepin respectively were compared in the range of *m/z* 100–400. The main *m/z* peaks were observed at 251.9 in both spectra, which would corresponded to the protonated molecular ion $[M+H]^+$ peak of cordycepin.[29,49]

In conclusion, in this work, multifunctional plasma mutagenesis generated inherently random mutagenic events, where an increasing exposure time induced increasing lethality in *C. militaris*. Across a range of exposure times (10 – 80+ seconds), there was a great variation in mutagenic events that yielded mutants with a significantly increased cordycepin productivity. One of the mutants isolated in our study (GYS60) was capable of producing

cordycepin levels never reported before at 7.883 mg/mL, which represents a 2000+% increase compared to its wild strain. This study also developed a two-step purification step for a simple and efficient purification method that can be used for the effective purification for large scale preparations of cordycepin from fermentation broth.

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TABLE 1. Cordycepin production of different strains treated by MPMS

Strain	no added adenosine		0.1 mg/mL adenosine added	
	(mg/mL) AVG±SD	cordycepin yield (%) *cf* wild strain	(mg/mL) AVG±SD	change in yield due to adenosine addition (%)
Wild strain	0.3808 ± 0.001	Control	0.5756±0.001	+ 51.2
GYS10	0.5407 ± 0.008	124	0.6010±0.009	+ 11.2
GYS20	0.3399 ± 0.006	89	0.7936±0.017	+ 133.5
GYS30	0.3186 ± 0.009	84	0.5216±0.004	+ 63.7
GYS40	0.5554 ± 0.008	146	0.5765±0.015	+ 3.8
GYS50	0.4246 ± 0.014	112	0.6846±0.016	+ 61.2
GYS60	7.8827 ± 0.005	2070	8.6729±0.001	+ 10.1
GYS70	0.4729 ± 0.011	142	0.6022±0.006	+ 27.3
GYS80	0.1537 ± 0.006	40	0.0440±0.001	– 71.4

TABLE 2. Cordycepin production using *C. militaris* reported in the literature

References	**Methodology**	**Cordycepin production (mg/L)**	**Strains**
This study	Submerged culture	380 mg/L	Wild strain
Mao[28]	Submerged culture	421 mg/L	Wild strain
Masuda [29]	Surface liquid culture	640 mg/L	Wild strain
Dong[31]	Surface liquid culture	1580mg/L	Wild strain
Wu [33]	Submerged and static culture	2214 mg/L	Wild strain
Suparmin [48]	Surface liquid culture	4920 mg/L	Wild strain
Sari[32]	Surface liquid culture	6200 mg/L	Wild strain
Das [30]	Surface liquid culture	6840 mg/L	Mutant
This study	Submerged culture	7883 mg/L	Mutant

Figure captions:

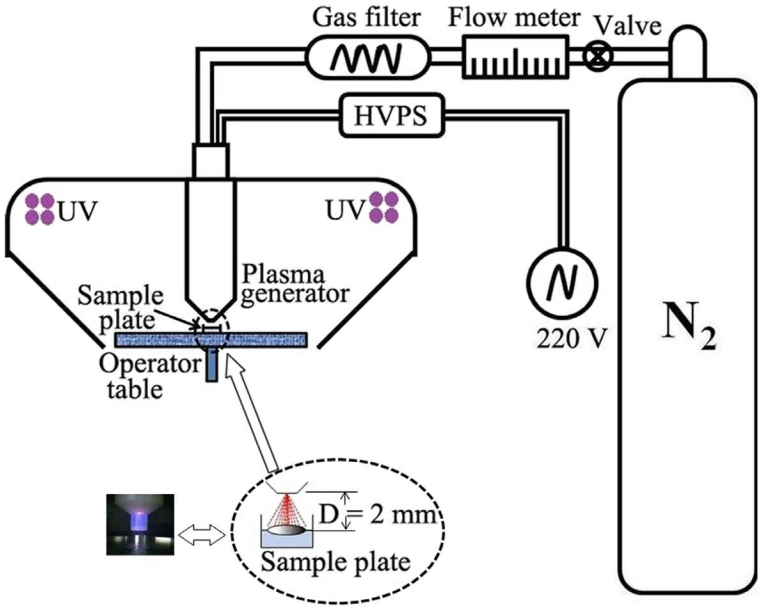

FIGURE 1. Schematic diagram of the multifunctional plasma mutagenesis system

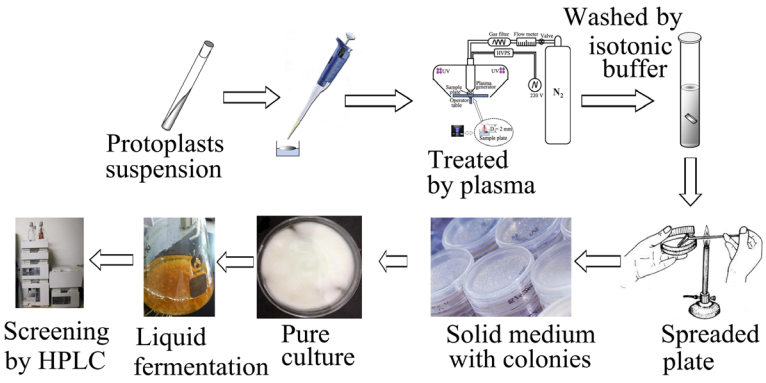

FIGURE 2. Schematic diagram of the mutation breeding and screening protocol for *C. militaris.*

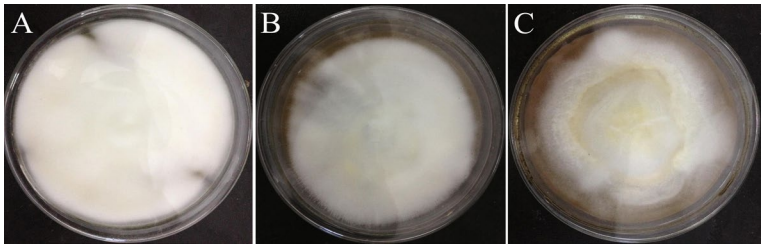

FIGURE 3. Colony morphologies of different *C. militaris* strains. (A) *C. militaris* GYS60 (exposed to MPMS for 60 sec); (B) wild strain *C. militaris* (no MPMS exposure); (C) *C. militaris* GYS80 (exposed to MPMS for 80 sec).

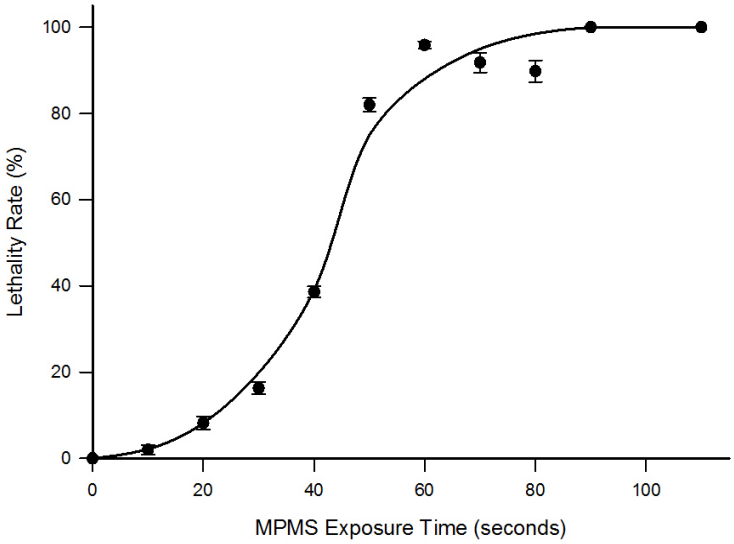

FIGURE 4. Lethality percentage with different exposure time, error bars indicate standard deviation (n=3).

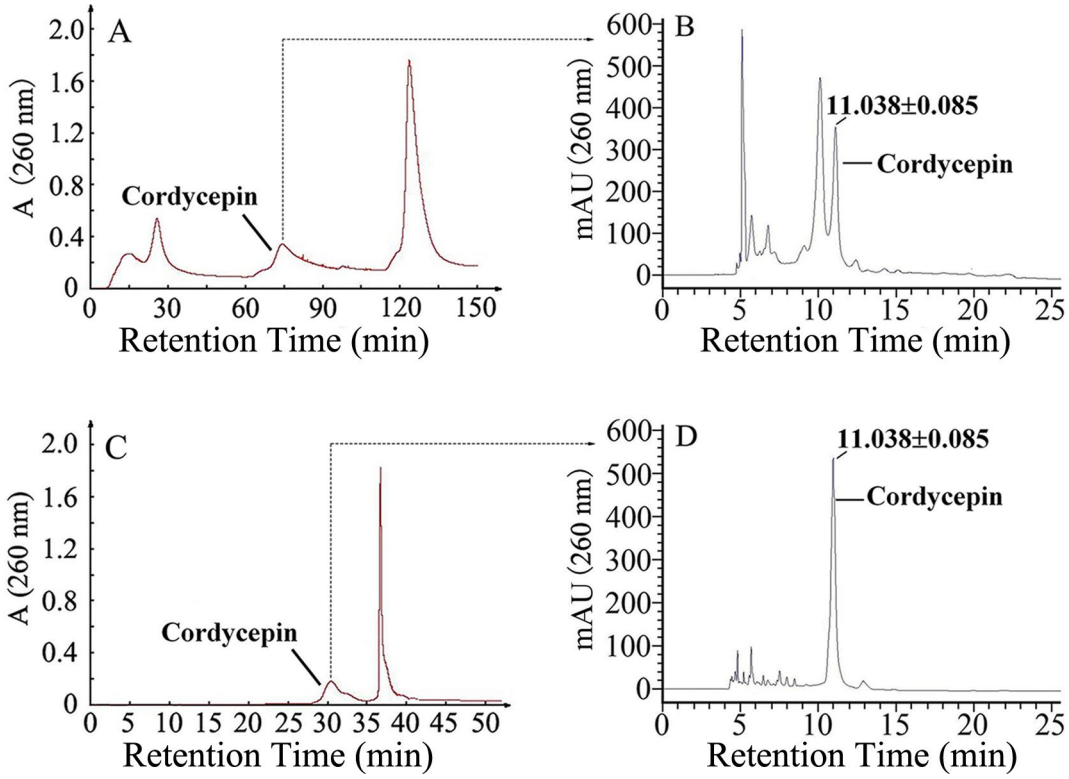

FIGURE 5. Sequence of purification conditions for cordycepin. (A) Cordycepin extraction from fermentation broth by AB-8 column; (B) HPLC chromatograph of cordycepin fraction following extraction on AB-8 column (see 5A); (C) Cordycepin purification on C18 column of fraction obtained after extraction from an AB-8 column; (D) HPLC chromatograph of cordycepin fraction following purification on C18 column (see 5C).

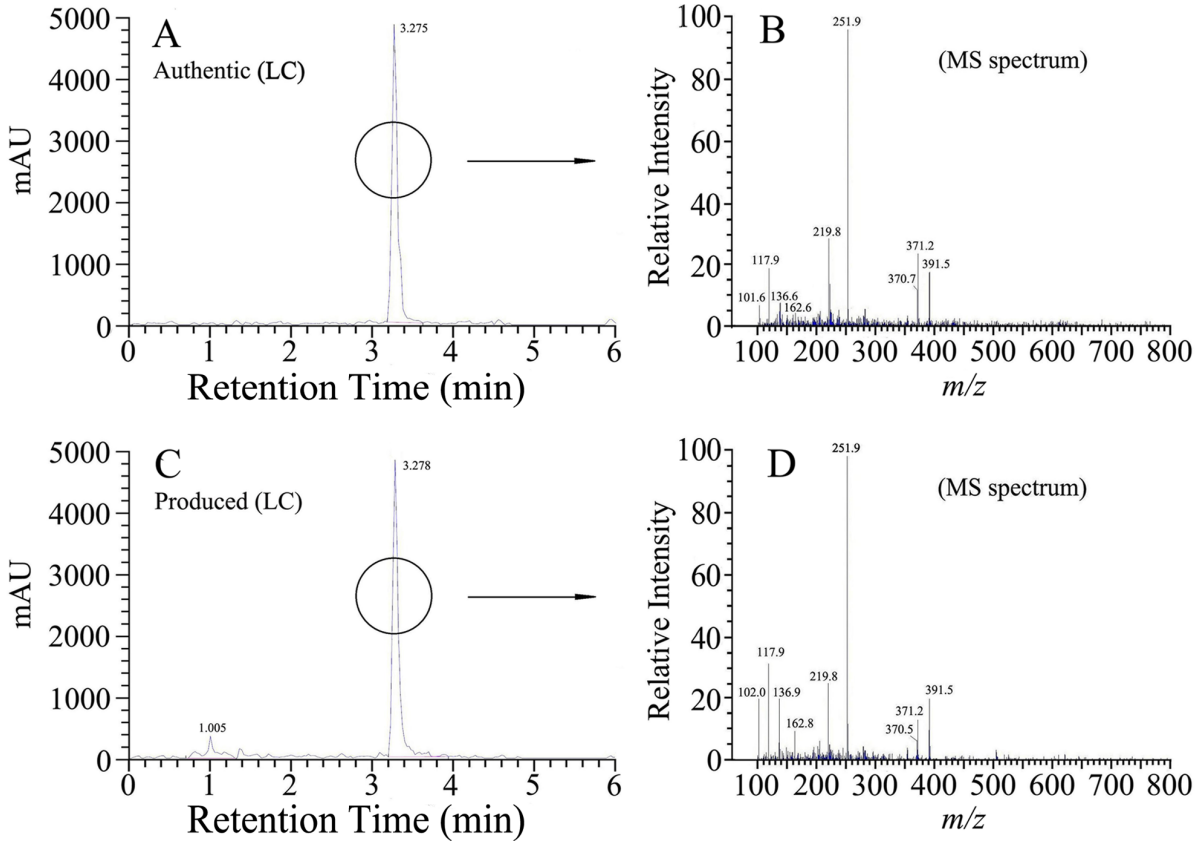

FIGURE 6. Identification of cordycepin by LC-MS (A) LC profile of authentic cordycepin; (B) Mass spectrum of authentic cordycepin; (C) LC profile of produced cordycepin; (D) Mass spectrum of produced cordycepin