福井大学審査
学位論文　[博士（工学)]
A Dissertation Submitted to the University of Fukui for the Degree of Doctor of Engineering

Production of anti-cancer agent cordycepin from the medicinal mushroom *Cordyceps militaris*
（薬用キノコ ***Cordyceps militaris*** による
抗腫瘍性物質コルジセピンの生産）

2009, September

Shonkor Kumar Das

Dedicated to my beloved parents

CONTENTS

CHAPTER 1
General introduction

Medicinal chemistry is a field involving the study of chemistry of medicinal mushrooms as well as medicinal herbs and their application in the field of pharmacology, in order to prepare novel drugs by biotechnological means. This field is becoming more important day by day, and has been widely investigated due to its practical benefits. Synthetic drugs are available in the commercial market and are being used in daily life, but they are not safe enough as they leave some side effects and sometimes harmful for human health. On the other hand, there is still a lack in research on the chemistry and bio-constituents of the medicinal mushrooms those may provide more efficacious and safer drugs having no side effects. This thesis intends to fill this lack, focusing on the production of anti-cancer agent from the natural source like medicinal mushroom *Cordyceps militaris* to be used in the realm of pharmaco-industrial biotechnology.

1.1 Fundamentals of medicinal mushrooms

Cordyceps genus is the name given to the fungi on insects, and its existence has been known since in the era, 2000 B.C. The medicinal mushrooms including *Cordyceps* species are abundant sources of useful natural products with various biological activities [1]. One of the most important traditional Chinese medicines, *Cordyceps militaris* (an entomopathogenic fungus; Fig. 1-1), which belongs to the class *Ascomycetes*, has been used extensively as a crude drug and a folk tonic food in East Asia [2]. It contains many kinds of active components (such as cordycepin, ergosterol, mannitol and, polysaccharides), and due to its various physiological activities, it is now used for multiple medicinal purposes [3-5]. In the wide meanings of fungi parasitic to the insects, more than 350 types have been discovered until now. At present, the detailed research about its elements is being done in universities and other research institutions, this has increased greatly its medicinal value. There are over 2,500 mushroom varieties grown in the world today. It is estimated that more than 10 million metric tons of edible and medicinal mushrooms were produced last

year in various countries [6]. That is why mushrooms have recently received significant attention from medical and pharmacological researchers as rich sources of biologically active compounds [7]. The taxonomical data of *C. militaris* and popular names of *Cordyceps* are given below:

Fig. 1-1 Photograph of *Cordyceps militaris* (www.jscr.jp)

Taxonomical data of *Cordyceps militaris*:

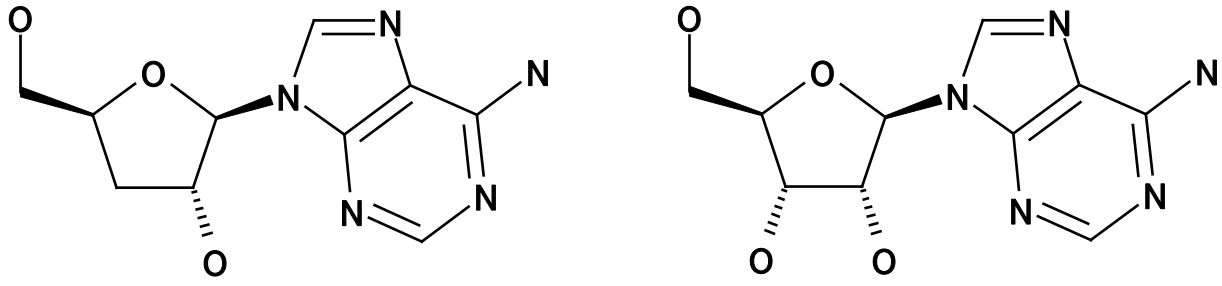
Kingdom	Fungi
Phylum	Ascomycota
Sub-phylum	Ascomycotina
Class	Ascomycetes/Pyrenomycetes
Order	Hypocreales
Family	Clavicipataceae
Genus	*Cordyceps*
Species	*Cordyceps militaris*

Popular names of *Cordyceps*:

Common name	Caterpillar fungus, *Cordyceps*, Cetepiller mushroom
Latin/English name	*Cordyceps militaris*, *Cordyceps* mushroom, Deer fungus, Caterpillar fungus
Chinese name	Dong Chong Xia Cao, Summer grass-winter worm, Hia tsao tong tchong
Japanese name	Tochukaso/Tochukasu, Totsu kasu
Korean name	Tong ch'ug ha ch'o
Nepali name	Yarsagumba, Jeebanbuti, Sanjivani, Kiraghans
Tibetian name	Yarchakunbu
Other names	Chong cao, Dong chong cao, Aweto

1.2 Applications of medicinal mushrooms

Cordyceps militaris is a potential harbour of bio-metabolites for many potential drugs and evidence is available about its applications for the revitalization of various systems of the body from ancient times. WHO estimated that about three-quarters of the world's population currently use natural drugs and other forms of traditional medicines like medicinal mushrooms to treat their diseases [8] including asthma (24%) [9] breast cancer (12%) [10], HIV (22%) [11], liver disease (21%) [12], and rheumatological disorders (26%) [13]. Simultaneously, the development of modern chemistry permitted the isolation of chemicals from medicinal mushrooms that have served as drugs or starting materials for the synthesis of many important drugs used today. Many modern drugs have been synthesized based on the structures and action mechanisms of chemicals isolated from medicinal mushrooms [7]. Thus, medicinal mushrooms have played a major role in the development of modern medicine and continue to be widely used in their original form [14].



Cordycepin (3′-deoxyadenosine) Adenosine

Fig. 1-2 Chemical structures of cordycepin and adenosine

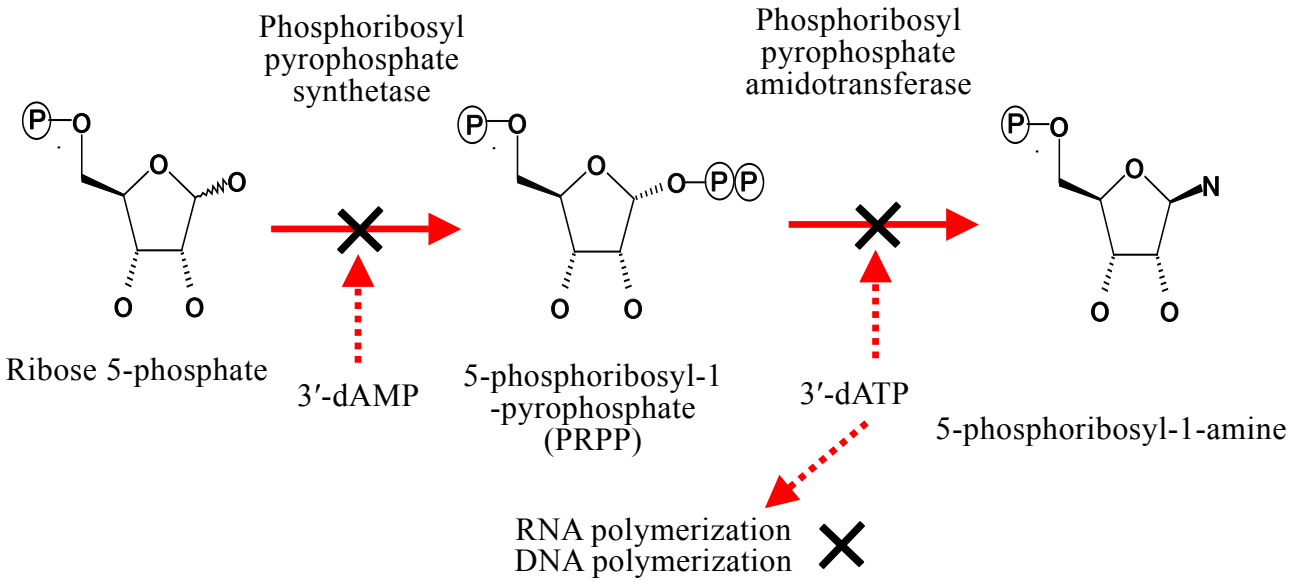


Fig. 1-3 Inhibition of purine and polynucleotide biosynthesis by cordycepin

The main active constituent of *C. militaris* fruiting bodies is cordycepin, which was first extracted from *C. militaris* and then found to be present in *Cordyceps sinensis* [15] and *Cordyceps kyushuensis* [16]. The cordycepin (3′-deoxyadenosine, $C_{10}H_{13}N_5O_3$, m.p. 225 °C, [α] D-47°) (Fig.1-2), a nucleoside analogue [15, 17], is considered as a nucleic acid antibiotic that might inhibit canceration of cells contributing to the normalization of cancer cells as one of the constituents of gene DNA [15]. In addition, it has been reported that the cordycepin is intracellularly converted into its 5′-mono-, di- and triposphates that inhibit the activity of several enzymes (Figs. 1-3) in the purine biosynthetic pathway [18]. The recent studies have demonstrated that the extracts of *C. militaris* have multiple pharmacological actions, such as inhibition of human glomerular mesangial cell proliferation [19], anti-fibrotic [20], anti-angiogenetic [21], improvement of insulin resistance and insulin secretion [22], anti-inflammatory [23], and growth inhibition of U937 leukemia cells [24]. Besides, it is also reported that cordycepin itself acts as an anti-tumour, anti-proliferative, anti-metastatic, insecticidal and anti-bacterial compound [4]. Therefore, the medicinal mushroom *C. militaris* is one of the most

important candidates that may be used as the medicinal base in the future.

1.3 Constraints and prospects of anti-cancer agent production from the medicinal mushroom *Cordyceps militaris*

As cancer remains the second leading cause of death in most of the countries, there is a need for effective medicinal compounds as its remedy. To synthesize and modify the structure of bioactive natural products is an effective approach to find some potential compounds with anti-cancer activity [25]. In the present state, the production of cordycepin from the fruiting body of *Cordyceps militaris* is not likely to reach commercial levels due to some practical limitations. For example, *Cordyceps militaris* is very scarce in nature due to the requirements of specific hosts and strict growth environments. On the other hand, it is necessary to note that the chemical synthesis of cordycepin requires a complicated process resulting a lower productivity, moreover, a large volume of organic solvents those are harmful substances to the environment may be discharged [26, 27]. The friendly production of cordycepin from the cultured mycelia of *Cordyceps militaris* in a large scale is currently an acute issue. Some experiments have already proven that the chemical components of natural and cultured *Cordyceps militaris* are similar [28, 29].

The ancient medicinal fungus *C. militaris*, which has been used as a crude drug for the welfare of mankind in old civilization, is now of a matter of concern due to its unexplored potentials. The anti-cancer agent cordycepin obtained from *C. militaris* have more than 21 clinically approved beneficial effects for human health. Especially, the anti-cancer agent cordycepin from *C. militaris* is expected to play evolutionary roles in the pharmacognosy sector, leading to create a viable base for pharmaceutical industries as some emerging diseases like CANCER, SARS, AIDS, SWINE FLU have no proper remedies yet.

Considering these facts, mutation of *C. militaris* by a high-energy ion beam irradiation to have a new potential mutant, medium optimization, use of suitable additives, and repeated batch culture technique might be vital tools to allow the

production of anti-cancer agent cordycepin reach to the commercial levels.

1.4 References

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CHAPTER 2

A new approach for improving cordycepin productivity in surface liquid culture of *Cordyceps militaris* using high-energy ion beam irradiation

2.1 Introduction

Cordycepin, one of the most bioactive compounds found in *Cordyceps* species (summer grass-winter worm) exhibits anti-microbial, anti-tumor, anti-metastatic, immunomodulating and insecticidal effects [1, 2]. Various studies of cordycepin production using *Cordyceps* species have been carried out to meet its increasing demand [3, 4]. In the present state, the production of cordycepin is not likely to reach a commercial level owing to some practical limitations regarding production, and therefore, the mutation using a new technique will be expected to allow its production to reach a commercial level.

Ion beams, among many mutation methods, are powerful and efficient mutagenic tools with high linear energy transfer (LET) radiation as they can generate mutations more frequently than low LET radiations such as electrons, X-rays and gamma rays [5], and the range of the ion beams on the target materials can be controlled [6]. Moreover, ion beams induce nuclear DNA alterations, such as inversion, translocation, transversion and large deletions rather than point mutations, and therefore, this random mutagenesis could produce various types of mutants [5, 7, 8]. There are some applications of low-energy ion implantation (keV) [9], but the penetration depth into a microorganism (~nm) is practically much lower than the MeV ion beam. Therefore, the probability that the keV ion beam reaches the nuclear DNA is much lower than that of the MeV ion beam. On the other hand, it is necessary to note that the chemical mutagenesis has potential health and environmental hazards.

The application of high-energy (~MeV) ion beam irradiation has been limited for micro-organism mutagen, and therefore, an unknown but strong possibility for the mutagen could be expected. In this study, the high-energy proton beam irradiation

was applied to get *Cordyceps militaris* mutants that could produce cordycepin at commercial level.

2.2 Materials and methods

2.2.1 Characteristics of mutant induction by ion beam irradiation

Ion beam irradiation is applied to various fields such as material science, biotechnology and medical sciences. In principle, any irradiation system usually change in the genetic sequences of targeted material resulting a new one having higher or sometimes lower production capacity. The characteristics of mutation induction by different irradiation systems are somewhat different regarding their point of action. In fact, many investigators, chiefly using mammalian systems, have reported that relative biological effectiveness (RBE) of DNA damage, lethality, mutation etc. of ion beams is much higher than that of low LET ionizing radiation. Also, it is still unknown whether ion beams can induce peculiar mutants that have never been found by low LET radiations and chemical mutagens in plants. Fig. 2-1 shows the comparative mutation induction types of gamma ray and ion beam irradiation.

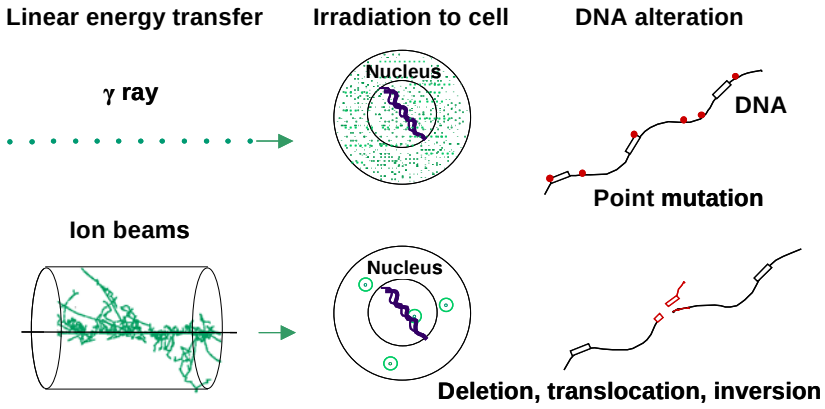

Fig. 2-1 Comparative mutation induction type of gamma ray and ion beam irradiation

2.2.2 Ion beam irradiation and screening

2.2.2-1 Irradiation protocol by carbon and proton beam

Cordyceps militaris NBRC 9787 (wild strain) used in the present experiments was purchased from the National Institute of Technology and Evaluation, Japan, and was stored at 5 °C as the control. It was inoculated on a PDA (Nissui Pharmaceutical Co., Ltd., Japan) plate with 3.5 cm diameter and incubated for 10 days at 25 °C. The carbon or proton beam irradiation to the plate culture was carried out using the Wakasa-wan Multi-purpose Accelerator with a tandem injector and a synchrotron (Hitachi, Ltd., Japan) at the Wakasa-wan Energy Research Center, Japan. Fig. 2-2 (a) is a photograph of the synchrotron accelerator, and Fig. 2-2 (b) is a schematic diagram of the ion beam irradiation apparatus for biological materials. The beam accelerated by a tandem injector and a synchrotron is transformed into an approximately 4 or 10 cm-square circle beam for the carbon and proton beam irradiations, respectively, with a uniform intensity distribution through the scatters, wobbler magnets and collimator. Fig. 2-2 (c) shows the irradiated areas of the carbon and proton beams, and the conditions for these irradiations are presented in Table 2-1.

Table 2-1 Characteristics of ion beam irradiations

Ion beams	Beam energy (MeV)	LET (keV/μm)	Penetration depth in water (mm)	Dose (Gy)
Carbon	660	42	9	200-2000
Proton	200	0.5	250	100-1000

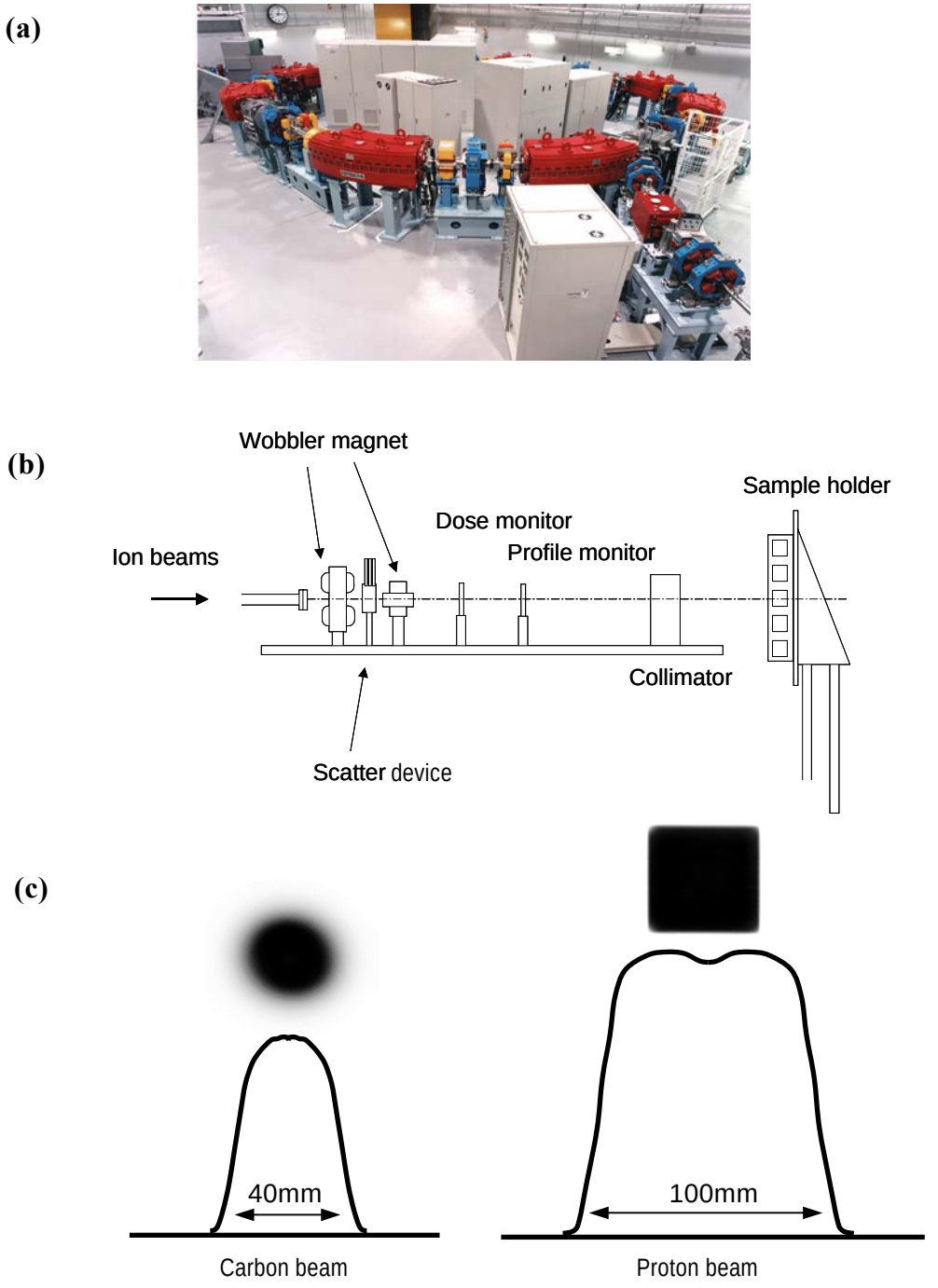

Fig. 2-2 (a) Synchrotron Accelerator at Wakasa-wan Multi-purpose Accelerator with Synchroton and Tandem (W-MAST); (b) Schematic diagram of ion beam irradiation apparatus for biological materials at W-MAST; (c) Ion beam irradiated areas of carbon and proton beams

2.2.2-2 Measuring the growth rate of irradiated strains

For the growth rate measurement in a plate, a loop of mycelia from the center of the irradiated plate was inoculated on the center of a fresh 9 cm PDA plate and incubated at 25 °C. The maximum and minimum diameters of the mycelial zone of each plate were measured using a vernier caliper every 3 days. The data from the two irradiated plates were averaged and compared to that of the control. The same procedure was applied for both the carbon and proton beam irradiations.

2.2.2-3 Selection of analogue (8-azaadenine or 8-azaguanine)-resistant mutants

To get higher cordycepin production, the irradiate mutants were grown in the presence of the analogue 8-azaadenine and 8-azaguanine to have 8-azaadenine- and 8-azaguanine-resistant mutants. Because, these mutants can remove the feedback inhibition cycle, and thus could increase the cordycepin production. The procedure followed in this experiment is described below:

A quarter of the mycelia from the irradiated and selected plate was suspended in 5 ml of physiological saline and filtered through double gauze and nylon mesh (250 μm). The filtrate was diluted to a 1/10 concentration, and then spread on Vogel's medium (using glucose instead of sucrose) agar plate supplemented with 200 mg/l of 8-azaadenine or 8-azaguanine [10]. Colonies that appeared on the plate after incubation at 25 °C (incubation time varied case by case) were isolated, and the selected colony was inoculated on an agar slant composed of the same medium as the stock culture of the mutant that was stored as 5 °C. In this way, the analogue resistant mutants were selected in order to observe the effects of the feedback inhibition cycle on the cordycepin production.

2.2.2-4 Evaluation of cordycepin production performance using anti-microbial test

A *Bacillus subtilis* NBRC 3134 suspension with 1.5×10^5 cells/ml was prepared for the anti-microbial test. A 0.1 ml portion of its suspension was seeded on an agar plate with Spizizen's minimum medium and 5 g/l casamino acids. Furthermore, an 8

mm disk was punched from the center of each plate on which the mutants and control strain of *C. militaris* had been cultured with the medium of the cordycepin production (the basal medium with 2 wt % agar in Table 2-2) for 30 days at 25 °C. Each disk from *C. militaris* was placed on the surface of the agar plate spread with *B. subtilis* and incubated for 24 h at 30 °C. The growth-inhibiting zone toward *B. subtilis* was compared between the mutant and control. The mutants having the more distinct and larger growth-inhibiting zone than the control were taken.

2.2.3 Surface liquid culture of analogue-resistant mutants and control

The stock culture for the control (*C. militaris*) was stored on a PDA slant at 5 °C. For the 8-azaadenine- or 8-azaguanine-resistant mutants, a Vogel's medium agar slant supplemented with 8-azaadenine or 8-azaguanine was used for the stock culture and also stored at 5 °C. The active PDA slant from the stock culture for the mutant and control was prepared by culturing for 8 days at 25 °C, and then the seed culture transferred from the active slant was grown on a 9 cm PDA plate for 13 days for the control and the 8-azaadenine-resistant mutant, and 20 days for the 8-azaguanine-resistant mutant at 25 °C. The inoculum was prepared by punching out 1 cm of the PDA plate culture using a sterilized cylindrical cutter. The surface liquid culture was started by inoculating the seed disk into a 500 ml culture bottle, which had a 8.5 cm diameter and 14.0 cm height with a bottleneck whose diameter and height were 4.5 and 4.0 cm, respectively. The working volume of the surface liquid culture medium was 100 ml for each bottle, and the bottleneck was fitted with a cotton plug during the culture. The prepared bottles were placed in an incubator maintained at 25±1 °C.

Table 2-2 Composition of basal and enriched medium

Components	Concentration (g/l)	
	Basal medium	Enriched medium
Nitrogen sources		
Peptone	2.5	0
Yeast extract	7.5	45
Carbon source		
Glucose	20	50
Others (diluted to 1/10 concentration of Vogel's medium) (Same for both basal and enriched medium)		
$NaOC(COOH)(CH_2COONa)_2 \cdot 2H_2O$	0.28	
KH_2PO_4	0.5	
NH_4NO_3	0.2	
$MgSO_4 \cdot 7H_2O$	0.02	
$CaCl_2 \cdot 2H_2O$	0.01	
Citric acid	0.46×10^{-3}	
$ZnSO_4 \cdot 7H_2O$	0.50×10^{-3}	
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.10×10^{-3}	
$CuSO_4 \cdot 5H_2O$	0.025×10^{-3}	
H_3BO_3	5.0×10^{-6}	
$MnSO_4 \cdot (4–5)H_2O$	5.0×10^{-6}	
$Na_2MoO_4 \cdot 2H_2O$	5.0×10^{-6}	

A 1 ml portion of the medium was mainly sampled at 3 day intervals through the sampling port and filtered through a 0.45 μm membrane filter in order to remove the

suspended mycelia. Just before the sampling, the medium was gently agitated for 5 s by a magnetic stirrer. The filtrate was analyzed for cordycepin, glucose and pH. All experiments were carried out at least in duplicate, and the results were then averaged. The composition of basal [4] and enriched medium for the surface liquid culture is presented in Table 2-2.

2.2.4 Analytical procedures

The cordycepin concentration was determined by an HPLC (LC-9A system, Shimadzu Corp., Japan) under the following conditions: TSK-gel ODS-80Ts (Tosoh Corp., Japan); mobile phase, methanol and 0.1% phosphoric acid (2/98, v/v); flow rate, 1.0 ml/min; column temperature, 40 °C and peak detection, UV at 260 nm. The cordycepin concentration shown in this experiment was re-estimated by considering the condensation of the medium in the culture bottle due to vaporization.

The glucose concentration was analyzed by the mutarotase-glucose oxidase method using the Glucose CII test Wako (Wako Pure Chemical Industries, Ltd., Japan). For the measurement of dry mycelia weight, at the end of each culture (glucose conc. <0.5 % of the initial concentration), the entire remaining content in the bottle was centrifuged at 16,000 g (10000 rpm) and 4 °C for 20 minutes. The precipitated mycelia were then sufficiently washed with distilled water, and dried at 105 °C for 24 h to measure the dry mycelia weight. The pH of the collected samples was measured by a pH meter.

2.3 Results and discussion

2.3.1 Comparison of growth rate of carbon and proton beam irradiated strains with control

Carbon beam irradiation to the plate culture of *C. militaris* with a dose range of 200-2000 Gy revealed that the irradiation doses up to 750 Gy had very little growth,

but over 1000 Gy, there was no growth. In spite of that, screenings of the carbon beam irradiated strains have been carried out for verification, resulting in the acquisition of some 8-azaadenine resistant mutants, but they did not show a higher production of cordycepin compared to the control (data not shown). These results indicated that the carbon beam irradiation was not effective on *C. militaris* as a potential mutagen.

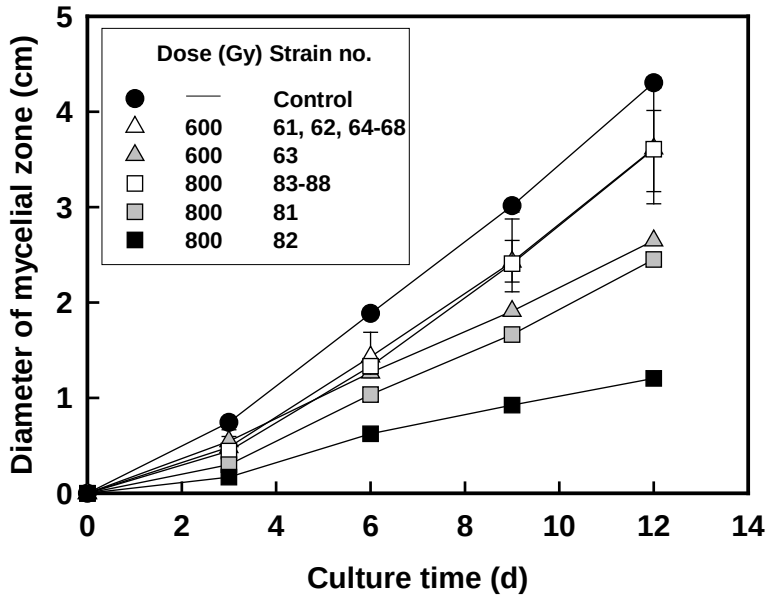

Fig. 2-3 Time courses of the mycelial growth after 600 or 800 Gy proton beam irradiations

Analysis showed that the mutation efficiency decreases (determined by the number of promising mutants obtained) as the growth rate (cm) decreases, but not linear. In fact, it is really hard to describe the relevance of the mutation rates to the changes of the growth rate due to irradiation. For example, the number of total promising mutants (azaadenine- and azaguanine-resistant mutants) obtained were 25, 16 and 17 and their relevant growth rates were respectively 0.22 (600 Gy), 0.19 (800 Gy) and 0.10 (800 Gy) cm, using 600 and 800 Gy irradiation doses. In contrast, the growth rate of the control was 0.34 cm in this experiment.

As DNA repairing after a break caused by ion beam irradiation affects the probability of inducing mutations, high LET carbon beam irradiation to *Cordyceps militaris* would cause dense spatial clustering of DNA damage, which might be difficult to repair [11]. These damages would become a significant impediment to the survival of the wild strain or mutants that might have lower cordycepin production. On the other hand, proton beam irradiation with relatively low LET would cause DNA damages to be capable of repairing and also there may be a higher degree of single gene mutation that occurs in the new mutant obtained by the proton beam irradiation than the carbon beam irradiation. In our preliminary experiment, it was revealed that the UV irradiation was not effective for cordycepin higher production.

After application of the proton beam irradiation to the plate culture at doses of 200, 400, 600 and 800 Gy, the growth rate of the irradiated strains were measured. The results of the irradiation doses of 200 and 400 Gy were almost the same level as that of the control (data not shown). Fig. 2-3 shows the time courses of the mycelial growth for the irradiation doses at 600 and 800 Gy. These results indicated that some of these, that included strain nos. 63, 81 and 82, were more significantly affected than the other irradiated strains. Therefore, these strains were selected for a subsequent screening. Hence, it was expected that a mutation might be induced in these strains whose growth rate was changed due to irradiation. It should be mentioned that the result of the irradiation at a dose below 200 Gy was like the control, while over 1000 Gy, was unsuccessful as there was no mycelial growth.

2.3.2 Selection and isolation of mutants that show preferable an anti-microbial effect

After successful irradiation by the proton beam, 30 classes of 8-azaadenine- and 28 classes of 8-azaguanine-resistant mutants were obtained from strain nos. 63, 81 and 82. As cordycepin inhibits the growth of *B. subtilis* [12], the *B. subtilis* growth inhibiting assays of 58 classes of analogue resistant mutants were performed to roughly estimate the capability of the cordycepin production. Of these, 4 mutants

(A63-7, A63-8, A81-2, A81-6) from the 8-azaadenine resistant mutants and 3 mutants (G63-8, G81-3, G82-4) from the 8-azaguanine resistant mutants were promising cordycepin producers. This was a qualitative indicating test prior to testing for the cordycepin production by the surface liquid culture since a quantitative test is not possible here.

2.3.3 Cordycepin production by surface liquid culture using selected mutants and control

Figs. 2-4 (a) and (b) show the time courses of the cordycepin production and glucose consumption by a surface liquid culture with basal medium using the 8-azaadenine and 8-azaguanine resistant mutants, respectively. It was observed that the cordycepin production performance of mutant nos. A81-2, A81-6, G63-8 and G82-4 was lower than that of the control. Mutant nos. A63-7 and A63-8 had a higher cordycepin production with a lower metabolic rate than that of the control. In contrast, the metabolic rate of glucose as well as the cordycepin production performance for mutant no. G81-3 was higher than those of the control. Moreover, an intra-strain comparison in 10 consecutive experiments for mutant no. G81-3 also showed similar results indicating the stability of the strain regarding its production.

Regarding the cordycepin production by the surface liquid culture, it was found that some of the 8-azaadenine and 8-azaguanine resistant mutants were better producers. Generally, the levels of the end products from the biosynthesis are regulated by the feedback inhibition, and thus the removal of the feedback cycle could lead to an accumulation of the end products. If a mutant can grow in the presence of the analogue, 8-azaadenine, the feedback inhibition by adenine-related compounds [adenosine monophosphate (AMP), adenosine, adenine] as well as 8-azaadenine is removed to some extent, and thus the adenine-related compounds may be accumulated in the 8-azaadenine-resistant mutant.

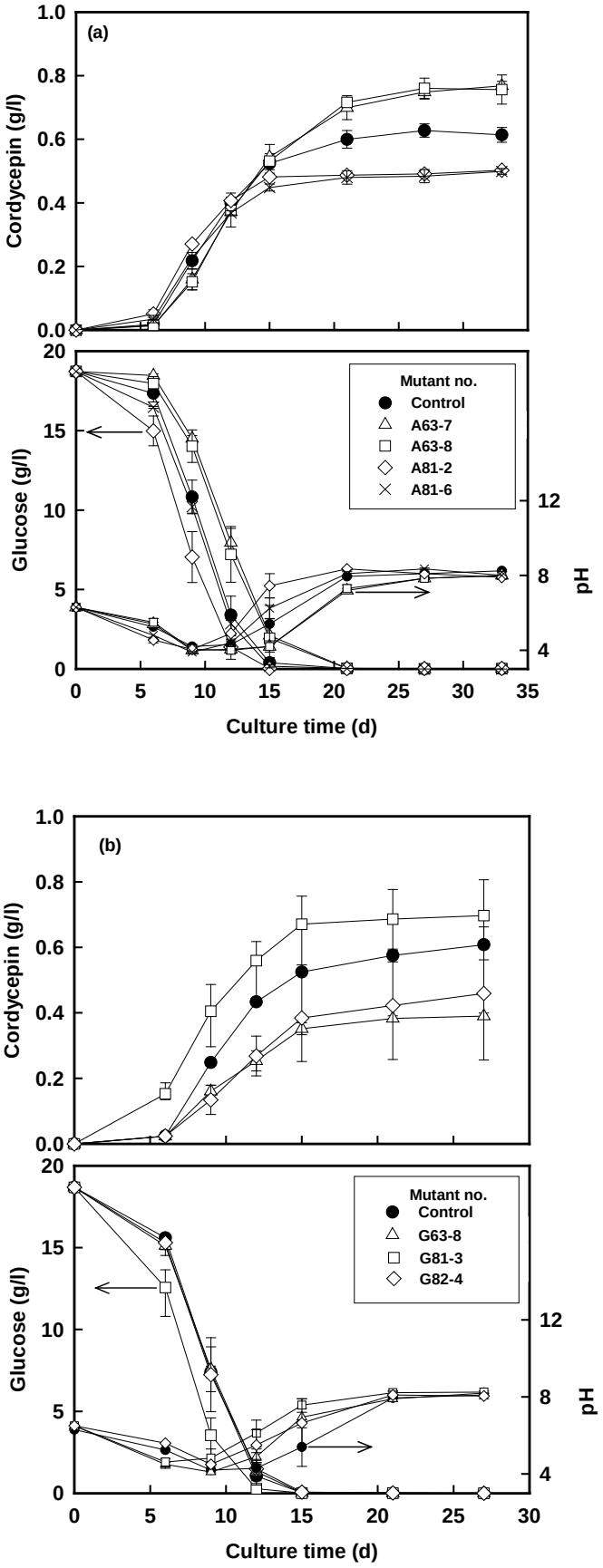

Fig. 2-4 Time courses of surface liquid culture using (a) 8-azaadenine resistant and (b) 8-azaguanine resistant mutants

Adenine or adenosine might be a direct precursor of cordycepin as reported in a previous study [13], and therefore, the accumulation of adenine or adenosine could result in an increased cordycepin production. In the same manner, the guanine-related compounds (guanine, guanosine, GMP) might be accumulated in the 8-azaguanine resistant mutant that could increase the codycepin production, because the production of cordycepin and guanine might be related to each other [13]. Although the increased production of the end products is due to a variety of reasons, the feedback mechanism may be one of the major explainable causes of the increased cordycepin production in this experiment.

The surface liquid culture was selected for this experiment, because the cordycepin was suppressed to 1/10~1/13 relative to the surface culture, probably due to shear stress [4].

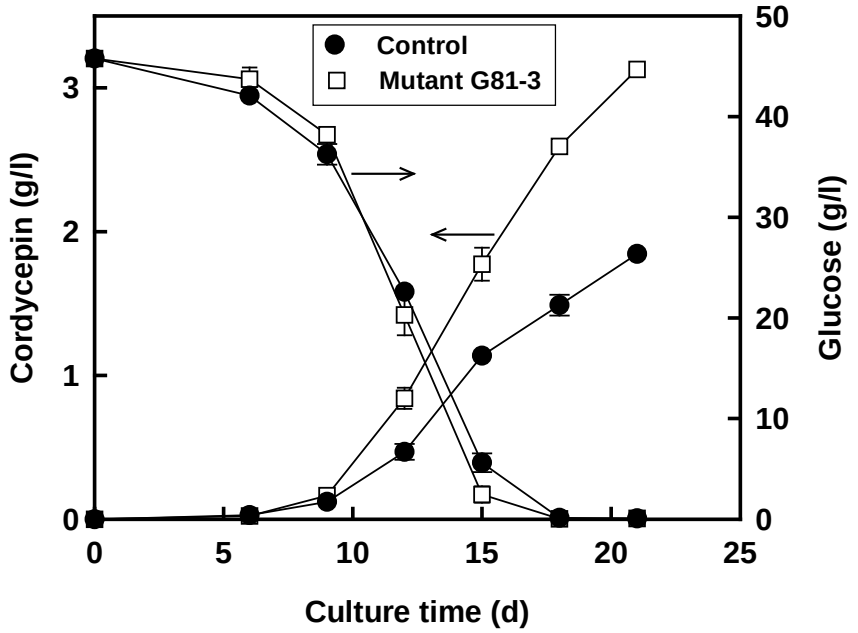

Fig. 2-5 Time courses of cordycepin production for surface liquid culture of mutant no. G81-3 and control in enriched medium

In primary optimization, among the 8-azaadenine and 8-azaguanine-resistant mutants, mutant no. G81-3 had the best productivity as 3.1 g/l, which was 72% higher than that of the control (Fig. 2-5). This is the highest value reported up to now

[14: 0.35 g/l; 15: 2.2 g/l], although the optimization of our present experiment was still in primary level. It is well known that the culture medium is very important to the yield of bioactive products like cordycepin because these nutrients are directly related to cell division and metabolic biosynthesis. Therefore, further optimization of the medium is necessary to obtain the highest production of cordycepin from the said mutant no. G81-3.

2.4 Conclusion

In the present experiment, the effects of the mutation induced by a high-energy ion beam, which would provide a higher mutation frequency and wider mutation spectrum, were studied with the aim of obtaining a higher cordycepin-producing mutant of *C. militaris*. After *C. militaris* NBRC 9787 was irradiated with a proton beam at 800 Gy, an 8-azaguanine-resistant mutant was selected from the irradiated strains. As a result, a promising mutant no. G81-3, which showed a better performance regarding the metabolic rate of glucose as well as cordycepin production than that of the control, was obtained. Preliminary optimization using the enriched medium also revealed higher productivity of the mutant no. G81-3 than that of the control (cordycepin production as 3.1 g/l *vs*. 1.8 g/l). As this mutant might have a different metabolic property from that of the control, further optimization of the medium components is suggested in order to reach a higher level of cordycepin production.

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CHAPTER 3

Optimization of culture medium for cordycepin production using *Cordyceps militaris* mutant obtained by ion beam irradiation

3.1 Introduction

The medicinal mushrooms are abundant sources of useful natural products with various biological activities [1]. Therefore, many researchers in the field of physiology, pharmacology and medical science, etc, are interested in the contents of mushrooms.

Among various mushrooms, *Cordyceps* spp. have been extensively used as the crude drug or folk tonic food in East Asia. The *Cordyceps* genus is a name given to the medicinal mushroom that parasitizes in insects and changes itself to fruiting bodies, and then comes out of the ground. As it appears like a plant growing out of an insect, it is given the self-explanatory name Tochukaso (winter insect-summer plant) in Japan. One of the most important species in *Cordyceps* genus is *Cordyceps militaris* (an entomopathogenic fungus), which belongs to the class *Ascomycetes*. *C. militaris* contains many kinds of active components, such as cordycepin (3′-deoxyadenosine), ergosterol, mannitol and polysaccharides, and due to its various physiological activities, is now used for multiple medicinal purposes [2, 3].

The cordycepin biosynthesized in *C. militaris* is a unique nucleoside analogue, which has a broad spectrum of biological activity, especially as an anti-cancer drug [4, 5]. In nature, *C. militaris* is very scarce due to the requirements of specific hosts and strict growth environments. Therefore, a large scale production of cordycepin by the cultivation of *C. militaris* is currently a significant issue.

To obtain a good productivity, a good strain and an optimized culture condition for it are necessary. As for the good producer of cordycepin, we previously reported a new approach for obtaining a novel mutant of *C. militaris* using high-energy ion beam irradiation [6]. The obtained mutant, G81-3, had 72% higher cordycepin production than that of control in preliminary optimization. As for the culture

condition, medium composition, especially carbon and nitrogen sources, is important in the yield of any fermentation products [7-10, 11]. However, as far as we know, there is limited knowledge about the nutritional requirement for cordycepin production by *C. militaris*, and there have been no reports on medium optimization to improve cordycepin production using surface liquid culture. Therefore, in this study, the effect of medium components on mutant strain was analyzed using the response surface method and the medium components for the cordycepin production was optimized.

3.2 Materials and methods

3.2.1 Surface liquid culture of the obtained mutant (G81-3) and control

The methodology of the surface liquid culture for *Cordyceps militaris* mutant (G81-3) and control used in the present experiment is as previously described in Chapter 2. The compositions of the basal and enriched media for the surface liquid culture are as shown in Table 3-1.

3.2.2 Application of statistical approach for surface liquid culture

3.2.2-1 Experiments on effects of initial carbon (C) and nitrogen (N) levels on cordycepin production

The effects of the initial carbon and nitrogen levels on the surface liquid culture of *Cordyceps militaris* were studied using glucose as the C source and yeast extract (Difco Laboratories, USA) as the N source. For investigation of the initial levels of the C and N sources for the better cordycepin production, the levels of glucose (35.86-128.28; 31.72-88.28 g/l for the mutant and control, respectively) and yeast extract (60.86-148.28; 31.72-88.28 g/l for the mutant and control, respectively) in the medium (Table 3-3) were changed, and a statistical approach was applied.

Table 3-1 Composition of basal & enriched media

Components	Concentration (g/l)	
	Basal medium	Media for optimized condition
Nitrogen sources		
Peptone	2.5	0
Yeast extract	7.5	60.86-148.28 (mutant)
		31.72-88.28 (control)
Carbon source		
Glucose	20	35.86-128.28 (mutant)
		31.72-88.28 (control)
Others (diluted to 1/10 concentration of Vogel's medium)		
(Same for both basal and enriched medium)		
$NaOC(COOH)(CH_2COONa)_2 \cdot 2H_2O$	0.28	
KH_2PO_4	0.5	
NH_4NO_3	0.2	
$MgSO_4 \cdot 7H_2O$	0.02	
$CaCl_2 \cdot 2H_2O$	0.01	
Citric acid	0.46×10^{-3}	
$ZnSO_4 \cdot 7H_2O$	0.50×10^{-3}	
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.10×10^{-3}	
$CuSO_4 \cdot 5H_2O$	0.025×10^{-3}	
H_3BO_3	5.0×10^{-6}	
$MnSO_4 \cdot (4–5)H_2O$	5.0×10^{-6}	
$Na_2MoO_4 \cdot 2H_2O$	5.0×10^{-6}	

3.2.2-2 Optimization using Central Composite Design (CCD)

In order to explore the effect of the medium components (glucose and yeast extract) and to optimize them, a statistical approach using the Box-Wilson central composite design (CCD) was conducted [12]. The variable levels for the CCD experiments were set according to the results of our previous experiments. The CCD experimental results were fitted with a second-order polynomial equation of Eq. (1) using a multiple regression technique.

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \qquad (1)$$

where y is the predicted response (maximum cordycepin concentration), β_0 is a constant coefficient, β_i is the linear coefficient, β_{ij} is the interaction coefficient, β_{ii} is the quadratic coefficient, and x_i and x_j are the actual values of glucose and yeast extract concentrations.

In this study, the experimental design consisted of 13 trials (1~8: star and factorial points, each has 2 replications; 9-13: center points), and the independent variables were studied at 5 different levels; as –1.414, –1, 0, +1 and +1.414 (Table 3-2). The analysis of variance (ANOVA) was performed to evaluate significance of the model and coefficients. This analysis included the Fisher's *F*-test (overall model significance) with the associated probability *p*-value, and its statistical significance was checked by the *F*-value. The second order polynomial coefficients were calculated and analyzed to have 3D response surface curves. All the above analyses were done using "Design Expert" software with the version 7.0 (Stat-Ease Inc., USA) statistical package.

Finally, the surface liquid culture with 7 replications was conducted for validation using the predicted concentration (optimized medium) in each culture bottle for both the mutant and control, and then the results were averaged.

Table 3-2 Experimental design of the central composite design

Trial	Coded levels	
	X_1	X_2
1	+1	+1
2	–1	+1
3	+1	–1
4	–1	–1
5	–1.414	0
6	+1.414	0
7	0	–1.414
8	0	+1.414
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0

3.2.3 Analytical procedures

3.2.3-1 Determination of cordycepin, glucose concentration, dry mycelial weight and pH

The concentrations of cordycepin and glucose, dry mycelial weight and pH were measured as previously described in Chapter 2.

3.3 Results and discussion

3.3.1 Optimization of cordycepin production by response surface methodology

The combined effect of the carbon source (glucose) and nitrogen source (yeast extract) was investigated using CCD, which can help to identify the interaction between the variables. The trace element except C and N sources were the same as the previous experiments [6, 13, 14]. In our preliminary experiments using CCD, that is, the center point was 40 g/l of glucose and 35 g/l of yeast extract and the intervals were 10 g/l for both the mutant and the control, the cordycepin productions by these strains increased as the glucose and yeast extract concentrations increased. The optimal point for the mutant was predicted in higher region than that for the control (data not shown). These results could not contradict with the previous report showing that the metabolic ability of the mutant was higher than that of the control [6].

Based on the above mentioned result, the CCD experiment shown in Table 3-3 (a-1) was first conducted for the mutant, and the predicted concentrations were higher than the experimental range. Then, the experimental ranges were shifted to high concentrations as shown in Table 3-3 (a-3). The results showed that there was no mycelial growth in some culture bottles having a high glucose or yeast extract content. Finally, the CCD experiment shown in Table 3-3 (a-2) was conducted to complement the range between Table 3-3 (a-1) and (a-3). All the three experiments were merged to make a single response surface plot in order to detect the optimized medium concentrations along with the prediction of maximum cordycepin production.

Table 3-3 Coded and actual values of design factors for investigation of medium components for the (a) mutant and (b) control

(a-1)

Coded levels	Actual factor levels	
	Glucose (x_1)	Yeast extract (x_2)
-1.414	35.86	60.86
-1	40	65
0	50	75
1	60	85
1.414	64.14	89.14

(a-2)

Coded levels	Actual factor levels	
	Glucose (x_1)	Yeast extract (x_2)
-1.414	36.72	56.72
-1	45	65
0	65	85
1	85	105
1.414	93.28	113.28

(a-3)

Coded levels	Actual factor levels	
	Glucose (x_1)	Yeast extract (x_2)
-1.414	71.72	91.72
-1	80	100
0	100	120
1	120	140
1.414	128.28	148.28

(b)

Coded levels	Actual factor levels	
	Glucose (x_1)	Yeast extract (x_2)
-1.414	31.72	31.72
-1	40	40
0	60	60
1	80	80
1.414	88.28	88.28

For the control, the predicted medium conditions and cordycepin production were obtained from the experiment having a center point concentration of 60 g/l for both glucose and yeast extract, with the range from 31.72-88.28 g/l as shown in Table 3-3 (b).

Table 3-4 Results of ANOVA for optimization of medium components [(a) mutant and (b) control]

(a)

Source	Sum of squares	df	*F*-value	*p*-value	Significance
Model	36.97	5	9.99	<0.0001	significant
X_1: Glucose	24.49	1	33.09	<0.0001	significant
X_2: Yeast extract	4.49	1	6.06	0.0184	significant
X_1X_2	1.06	1	1.43	0.2387	
X_1^2	10.19	1	13.77	0.0007	significant
X_2^2	5.73	1	7.75	0.0083	significant

Significant: $p<0.05$

(b)

Source	Sum of squares	df	*F*-value	*p*-value	Significance
Model	0.91	5	9.08	<0.0007	significant
X_1: Glucose	0.039	1	1.94	0.1874	
X_2: Yeast extract	0.56	1	28.05	0.0001	significant
X_1X_2	0.000032	1	0.001592	0.9688	
X_1^2	0.28	1	14.08	0.0024	significant
X_2^2	0.17	1	8.61	0.0116	significant

Significant: $p<0.05$

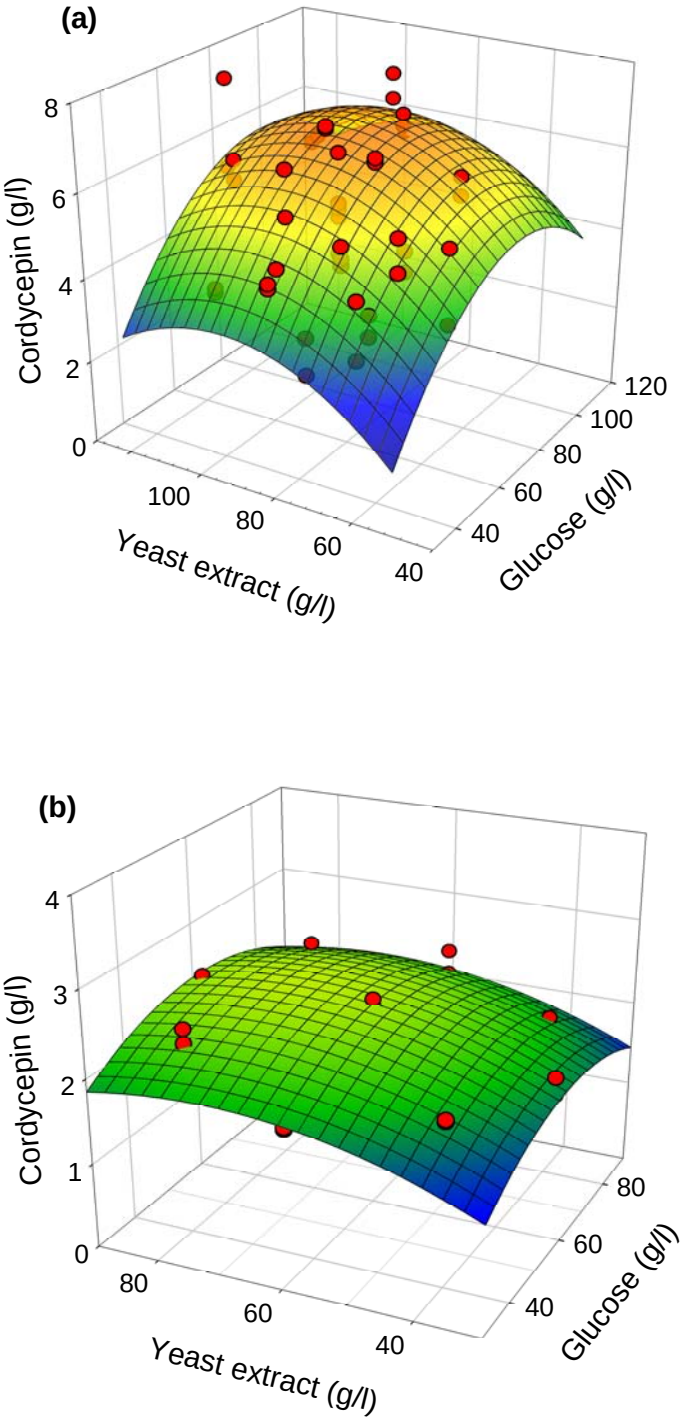

Fig. 3-1 Response surface plots of cordycepin production as a function of glucose and yeast extract concentrations for the (a) mutant and (b) control

A regression analysis was performed to fit the response (cordycepin production) with the experimental data. Based on the obtained variables, the responses were respectively expressed by Eqs. (2) and (3) for the mutant and the control, which presented the cordycepin production y (g/l) as a function of the glucose x_1 (g/l) and yeast extract x_2 (g/l) concentrations.

$$y = -10.14958 + 0.17365x_1 + 0.20110x_2 - 0.00101x_1^2 - 0.00107x_2^2 \quad (2)\ \text{(mutant)}$$

$$y = -1.37420 + 0.06007x_1 + 0.05443x_2 - 0.00048x_1^2 - 0.00038x_2^2 \quad (3)\ \text{(control)}$$

In both cases, there are no interactions between glucose and yeast extract concentrations. Results of the analysis of variance (ANOVA) showed that the regression model was statistically significant at a 95 % confidence level ($p<0.05$) [Tables 3-4 (a, b); where X is the coded value]. The response surface plots obtained from Eqs. (2) and (3) are shown in Figs. 3-1 (a) and (b). The model predicted a maximum response of cordycepin of 6.76 g/l at glucose 86.2 g/l and yeast extract 93.8 g/l for the mutant, and 2.48 g/l at glucose 62.6 g/l and yeast extract 72.5 g/l for the control. The optimized concentrations for the mutant was higher than that of the control. The cordycepin yield, which was defined as the cordycepin production divided by the initial glucose or initial yeast extract concentrations, for the mutant were 0.080 g-cordycepin/g-glucose and 0.073 g-cordycepin/g-yeast extract, respectively. On the other hand, those for the control were 0.040 g-cordycepin/g-glucose and 0.034 g-cordycepin/g-yeast extract, respectively. Both yields of the mutant are almost double that of the control. Therefore, one of the reason for higher production of the mutant was the increase in the yield, i.e., some metabolic or catabolic pathway might be deleted or suppressed by ion beam irradiation.

Although the cordycepin production increased with an increase in the glucose or yeast extract concentrations to a certain level, the results of our experiment showed that a high initial glucose (>100 g/l) or yeast extract (>120 g/l) concentration was unfavorable for the cordycepin biosynthesis (data not shown). Here, the osmotic

pressure caused by a high glucose concentration may be detrimental to the metabolic biosynthesis; although another possible reason is the carbon catabolite repression caused by glucose as reported in *Saccharomyces cervisiae* [15] and *Ganoderma lucidum* [10]. To avoid these negative influences of the high concentrations of carbon and nitrogen sources, the fed-batch culture may be effective for higher productivities.

3.3.2 Cordycepin production in optimized medium: validation of the model

Validation was carried out under conditions predicted by the model. Figs. 3-2 (a) and (b) shows the time profiles of the glucose consumption, pH and cordycepin production in the medium with the optimized carbon and nitrogen sources (as predicted) for the mutant and the control, respectively. The maximal cordycepin production reached 6.84 g/l and 2.45 g/l for the mutant and the control using the optimized medium, respectively. These values were almost the same with the predicted ones (6.76 g/l for the mutant and 2.48 g/l for the control). It is obvious that the cordycepin production by the irradiated mutant is much higher than that of the control. That is, the production obtained by the mutant was 179% (2.79 times) higher than the control.

In terms of culture methods of cordycepin production, it is also noteworthy that this new mutant showed a lower production in submerged culture than that in the surface liquid culture probably due to a shear stress as we previously reported for the control [13]. The cordycepin production of this mutant in submerged culture was suppressed to about 3% relative to the surface liquid culture; whereas, that of control was 80% using optimized media. As is predicted from the observation that the mycelial mat of the mutant was weaker than that of the control, the mutant might be more sensitive to shear stress. Therefore, the surface liquid culture was employed in this experiment.

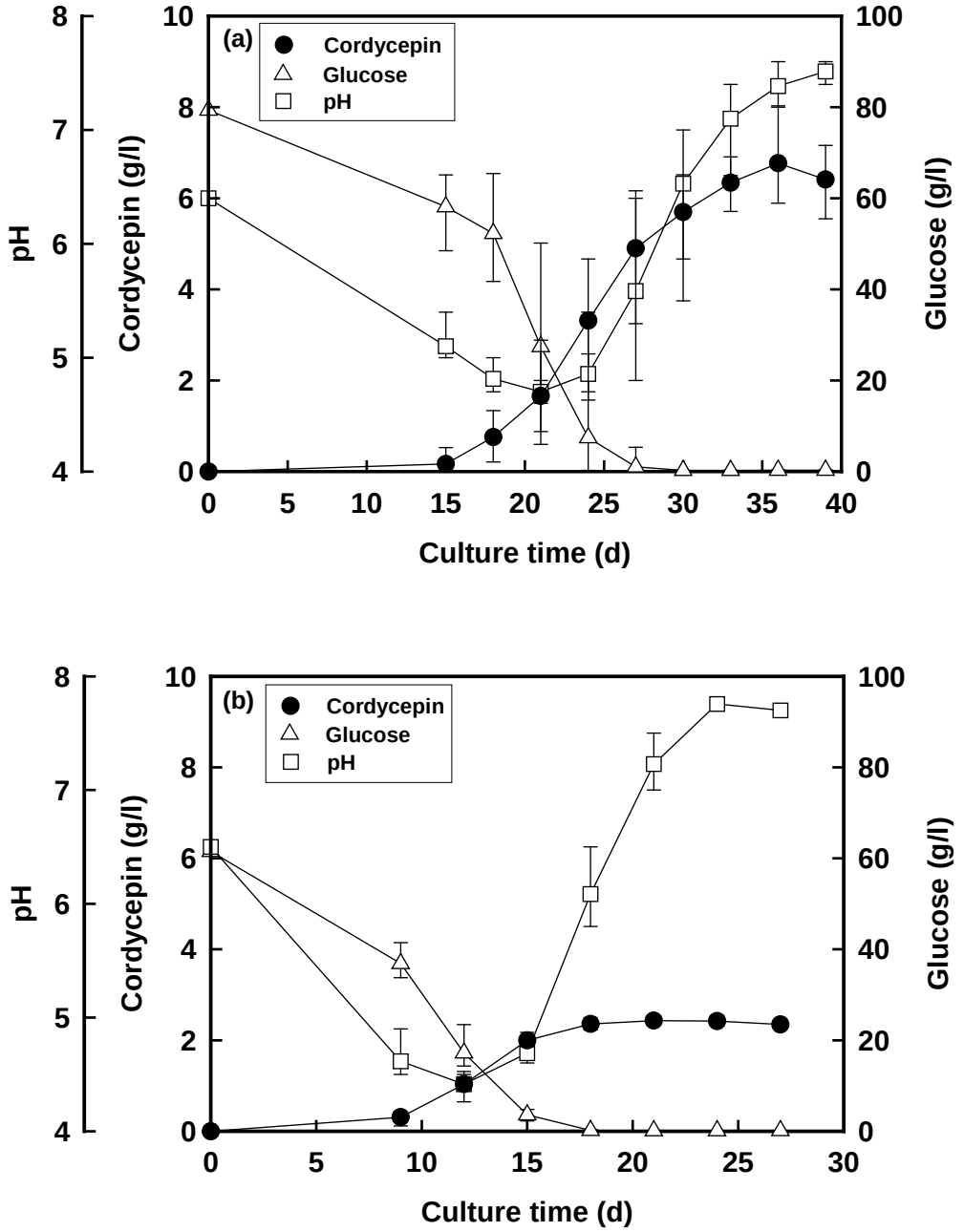

Fig. 3-2 Comparative study of production performance in optimized medium for the (a) mutant (G81-3) and (b) control

The biomass concentrations were 2.91 and 2.11 g-dry mycelia/l for the mutant and the control, respectively. For mycelial growth, it was revealed that the maximal cordycepin production/mycelia at the end of the culture for the mutant was much

higher than that of the control (2.35 *vs*. 1.16 g-cordycepin/g-dry mycelia) furnishing an additional proof of a higher cordycepin production by the mutant.

3.4 Conclusion

In this study, a process of surface liquid culture of *C. militaris* mutant for the production of a bioactive compound, cordycepin, was demonstrated. The effects of the major nutrients, i.e., the carbon and nitrogen sources on the cordycepin production were studied in order to obtain a suitable culture medium. According to the response surface analysis, the optimal concentrations of glucose and yeast extract for the mutant strain were 86.2 g/l and 93.8 g/l, respectively, and 6.84 g/l cordycepin was obtained. The irradiated mutant showed a 179% higher cordycepin production over the control (wild strain). It is necessary to mention that the cordycepin production reported by a number of researchers is much lower than that of our present study (Table 3-5). Therefore, the obtained mutant will be a promising strain for the large-scale production of cordycepin at pharmaco-industrial levels.

Table 3-5 Cordycepin production using *Cordyceps militaris* reported by several authors

References	Culture method	Cordycepin production (g/l)	Productivity [g/(l·d)]
Mao & Zhong, 2004 [16]	Submerged	0.201	0.015
Mao *et al*., 2005 [17]	Submerged	0.345	0.019
Mao & Zhong, 2006 [18]	Submerged	0.421	0.025
Masuda *et al*., 2006 [13]	Surface liquid	0.640	0.320
Shih *et al.,* 2006 [19]	Submerged & static	2.210	0.0.92
Masuda *et al*., 2007 [14]	Surface liquid	2.50	0.158[a]
This study	Surface liquid	6.84 (mutant)	0.190
		2.45 (control)	0.102[b]

The medium compositions of **a** and **b** are different. Additives other than C and N sources were used in **a**.

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CHAPTER 4

Further increased productivity of cordycepin by *Cordyceps militaris* mutant using additives and repeated batch operation

4.1 Introduction

It has been a difficult path to find a suitable method to increase the production of the anti-cancer agent cordycepin (3′-deoxyadenosine) from the medicinal fungus *Cordyceps militaris*. This novel bio-metabolite cordycepin has a number of valuable applications, not only against cancer, but also against some other diseases as reported by biotechnologists and medical researchers [1, 2]. In our previous experiments, a new mutant of the *Cordyceps militaris* was obtained using ion beam irradiation technology [3]. In the surface culture of that mutant, it was also evident that the biosynthesis of cordycepin can be regulated by the concentrations of the components used in the culture medium, especially of the C and N sources, and some additives that have been investigated in our previous studies [4].

Adenosine or adenine is one of the end products of purine-metabolic pathway and plays an important role as a component of DNA and RNA. In cordycepin production, the function of adenine and adenosine were the same, that is, the adenine moiety made a contribution to the cordycepin biosynthesis. Although adenine was more rapidly transported into the cells than adenosine [4], it was insoluble in higher concentrations during medium preparation.

Similarly, glycine is an amino acid that is used in the purine-metabolic pathway as L-glutamine and L-aspartic acid. Although the cordycepin production by glycine and L-glutamine were of the same level in our previous study, it was found that the effect of glycine was able to be superimposed on that of adenine, but those of adenine and L-glutamine did not posses such a property, using a combination of two such additives [4]. In addition, research is currently being conducted on the apparent anti-oxidant and anti-inflammatory properties of glycine, and it has been found in animal studies and could be useful in many disease states like certain cancers acting

through metabolic pathways [5, 6].

On the other hand, it is well known that the greatest gain in productivity can be achieved by a vicious culture system rather than one cycle cell culture technique, as the fungal cells grow substantially slower in such a time dependent culture system due to lack of required nutrition supply at the end of the each culture cycle. Therefore, the conventional culture system is time dependent and usually accompanied by a low productivity, whereas, the repeated batch operation is independent of time duration, may provide better productivity.

In a repeated batch culture technique, the un-useful medium is removed at the end of each batch and is replenished with a fresh medium having a same medium composition. The microbial mat can be used here more than one time. The addition of a fresh medium and elimination of an un-useful one at the end of each cycle/batch provides the cells with the environment they require actually to achieve a higher productivity.

In our previous experiment, the medium components were optimized for the surface culture of the obtained mutant (G81-3) as 86.2 g/l and 93.8 g/l for C & N sources, respectively and the relevant peak cordycepin production was 6.84 g/l. In an elaborative study to determine the strategies suitable for the extreme production of cordycepin using *Cordyceps militaris* mutant, it was revealed that the effective additive compounds for the cordycepin production were glycine, L-aspartic acid, L-glutamine, adenine and adenosine [4]. In addition, it was also revealed that the application of repeated batch operations had enhanced the productivities to 1.2-1.3 times higher than those by the batch operations [4]. Therefore, it was of great interest to investigate the effects of some additives like adenosine and glycine and repeated batch operation on cordycepin production in more detail.

4.2 Materials and Methods

4.2.1 Fungal strain, media and stock culture

Cordyceps militaris NBRC 9787 used in the present experiments as the control (wild strain) was purchased from the National Institute of Technology and Evaluation, Japan, and a prospective mutant of the said strain obtained by ion beam irradiation (G81-3) were stored on a PDA (Nissui Pharmaceutical Co., Ltd., Japan) slant at 5 °C.

4.2.2 Surface liquid culture using the prospective mutant (G81-3)

The surface liquid culture methodology is as previously described in Chapter 2, but the culture was started by inoculating two seed disks (instead of single disk per bottle) into a 500 ml culture bottle. All experiments were carried out at least in duplicate, and the results were averaged. The compositions of the optimized media for the surface liquid culture used in this experiment are shown in Table 4-1.

4.2.3 Experimental design for adenosine and glycine

In the culture medium using previously optimized conditions (Glucose: 86.2 and YE: 93.8 g/l) for the said mutant, 2, 4, 6, 8 and 10 g/l adenosine were separately added.

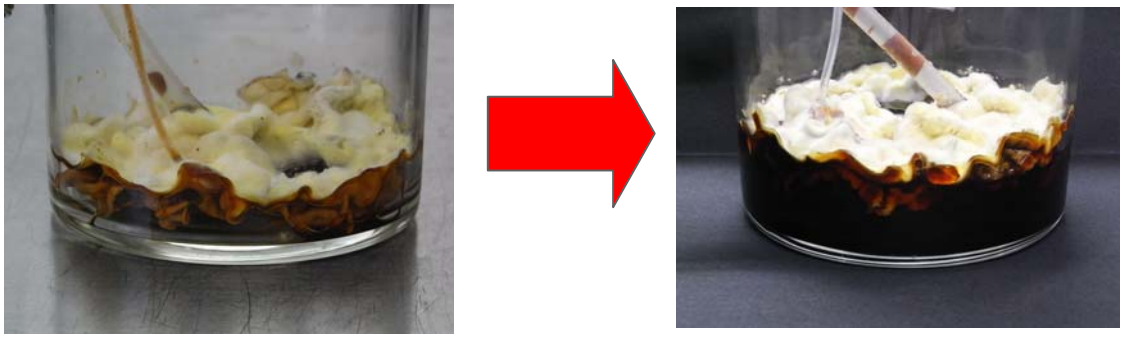
Glycine was used with the yeast extract in weight percent ratios (yeast extract/glycine) of 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10 under the condition that the total amount of yeast extract and glycine were fixed. Also, glycine was separately added as 10, 20 and 30 weight percents of the yeast extract in the culture medium having the same optimized conditions with a fixed yeast extract concentration.

Table 4-1 Composition of optimized media for repeated batch operation (mutant and control)

Components	Concentration (g/l)		
	A. Control	B. Mutant	C. Mutant +Adenosine
Nitrogen sources			
Yeast extract	72.5	93.8	93.8
Carbon source			
Glucose	62.6	86.2	86.2
Additive			
Adenosine			6
Others (diluted to 1/10 concentration of Vogel's medium) (Same for both mutant and the control)			
$NaOC(COOH)(CH_2COONa)_2 \cdot 2H_2O$	0.28		
KH_2PO_4	0.5		
NH_4NO_3	0.2		
$MgSO_4 \cdot 7H_2O$	0.02		
$CaCl_2 \cdot 2H_2O$	0.01		
Citric acid	0.46×10^{-3}		
$ZnSO_4 \cdot 7H_2O$	0.50×10^{-3}		
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.10×10^{-3}		
$CuSO_4 \cdot 5H_2O$	0.025×10^{-3}		
H_3BO_3	5.0×10^{-6}		
$MnSO_4 \cdot (4–5)H_2O$	5.0×10^{-6}		
$Na_2MoO_4 \cdot 2H_2O$	5.0×10^{-6}		

4.2.4 Repeated batch operation

A medium exchanging port was placed at the same position as the sampling port in the 500 ml surface liquid culture bottle. In addition, the supporter, which was made with stainless steel wire (ϕ =0.3mm) was placed in the bottle in order to prevent the biofilm from soaking in the liquid medium. The culture broth replaced with a fresh medium through the medium exchanging port using a syringe as soon as the glucose concentration tends to zero (glucose conc. <0.5% of the initial conc.) (Figs. 4-1 a, b). Three repeated batch operations were conducted simultaneously using the control, mutant, and mutant with the best adenosine concentration (6 g/l) attained in the preceding experiment and the optimized media were as shown in Table 4-1.



(a) Old Medium drawn **(b) With a fresh medium**

Fig. 4-1 (a, b) Replenishment of culture medium in a repeated batch culture

4.2.5 Preparation of extract from the mycelia of *C. militaris*

The cultured mycelia of *C. militaris* were separated from the medium and sufficiently rinsed with distilled water. The mycelia were dried in a vacuum drying oven at 40°C for 24 h and then ground into a powder. The dry powder (0.5 g) was suspended in 10ml of distilled water and sonicated for 30 min at 20KHz, and 100 W (XL2020, Heat System Inc., USA) in an ice bath. The supernatant obtained by centrifugation of this homogenate was used as the extract solution from the mycelia.

4.2.6 Analytical procedures

The concentrations of cordycepin and glucose, dry mycelial weight and pH were measured as previously described in Chapter 2.

The concentrations of the nucleic acid-related compounds other than cordycepin in the filtrate and the extract were also determined by HPLC monitored by UV absorbance at 260 nm, which was the same system as previously described in Chapter 2. The mobile phase was prepared from the two solvent systems (A, 2.0% MeOH in 0.01 M $(NH_4)H_2PO_4$; B, 20% MeOH in 0.01 M $(NH_4)H_2PO_4$). After solution "A" was used as the mobile phase for 20 min, the linear gradient was performed by decreasing the ratio of "A" from 100% to 0% for 10 min. For the next 10 min, 100% of "B" was used as the mobile phase. The overall elution time was 40 min. The flow rate was 0.8 ml/min, and the column temperature was 40 °C. The authentic standards were purchased from the Wako Pure Chemical Industries, Ltd., Japan.

4.3 Results and discussion

4.3.1 Effects of additives

4.3.1-1 Effect of adenosine on cordycepin production

In the culture medium using the previously optimized conditions (described in Chapter 2) for the said mutant, 2, 4, 6, 8 and 10 g/l adenosine were added separately. These results revealed that the highest cordycepin production was 8.57 g/l for the 6 g/l adenosine was 28% higher than that of the control (6.69 g/l) (Figs. 4-3 and 4-4). This is the highest cordycepin production reported to date. Similarly, the results of the other concentrations also superseded that of the control (Fig. 4-3). The time course of glucose showed that the glucose consumption for the 4 g/l adenosine was the fastest, while that of 10 g/l was the slowest with the longest culture time among all the treatments (Fig. 4-2).

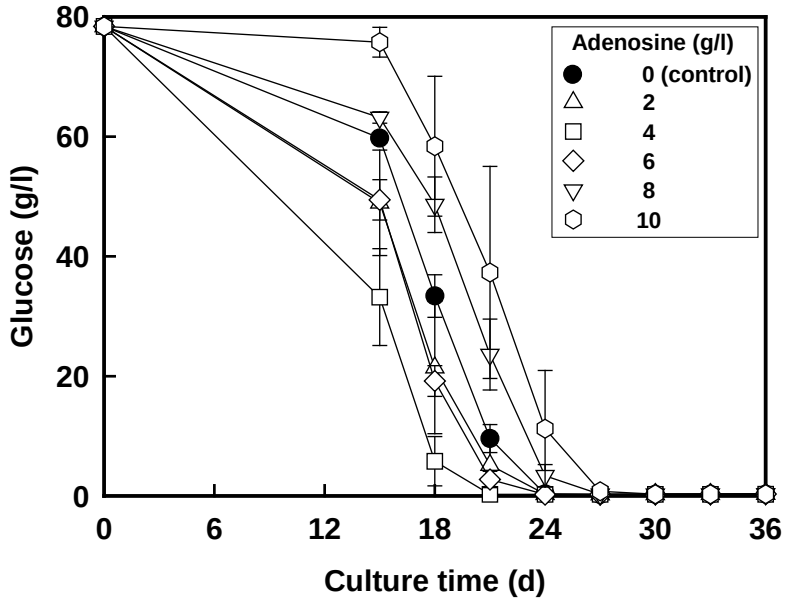


Fig. 4-2 Time courses of glucose consumption for surface liquid culture using adenosine as an additive

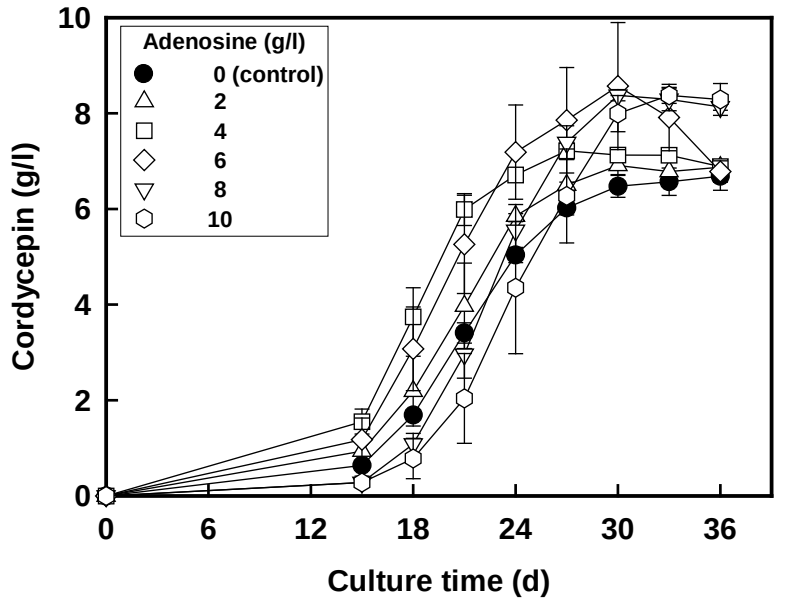


Fig. 4-3 Time courses of cordycepin concentration for surface liquid culture using adenosine as an additive

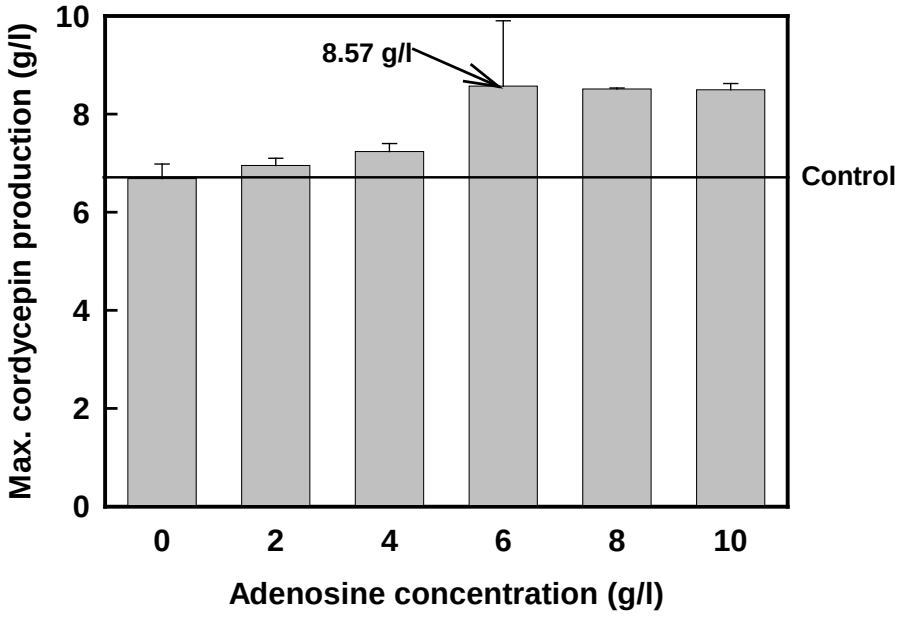


Fig. 4-4 Maximum cordycepin production in surface liquid culture using different concentrations of adenosine as an additive

As the structure of the nucleoside adenosine is very close to that of cordycepin (3′-deoxyadenosine), there might be a possibility of the metabolic conversion of adenosine to cordycepin by an unknown pathway, which needs to be investigated. This result is consistent with the findings reported by Masuda *et al.* [4].

4.3.1-2 Effect of glycine on cordycepin production

Glycine was used with yeast extract in weight percent ratios (yeast extract/glycine) of 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10 under the condition that the total amount of yeast extract and glycine were fixed. Also, glycine was separately added as 10, 20 and 30 weight percents of the yeast extract in the culture medium having the same optimized conditions with a fixed yeast extract concentration.

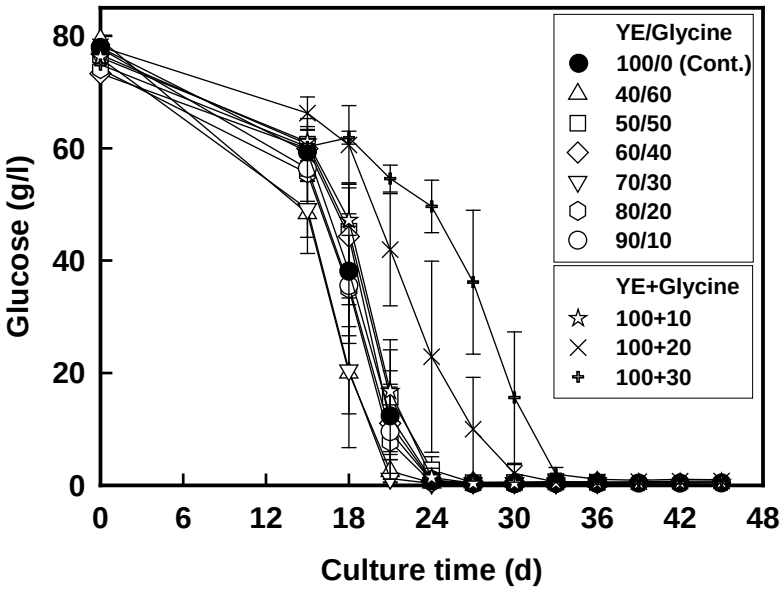


Fig. 4-5 Time courses of glucose consumption for surface liquid culture using glycine as an additive (100=93.8 g/l)

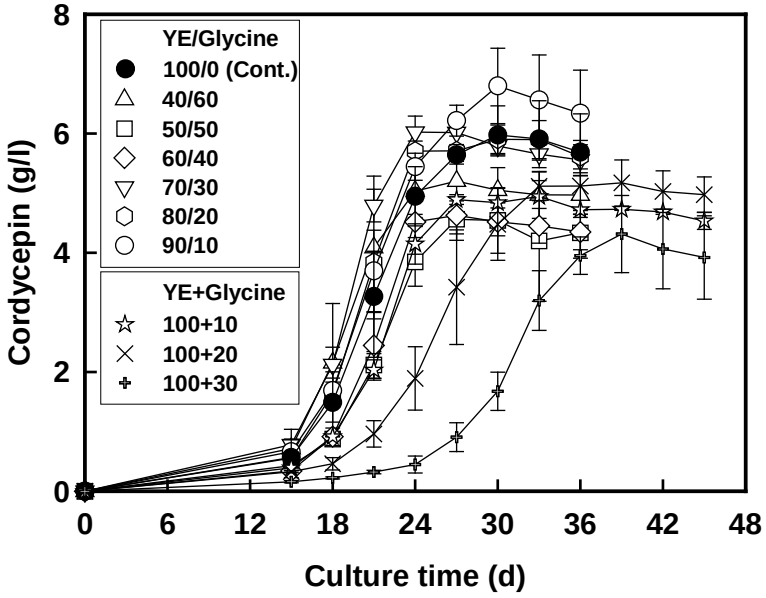


Fig. 4-6 Time courses of cordycepin concentration for surface liquid culture using glycine as an additive (100=93.8 g/l)

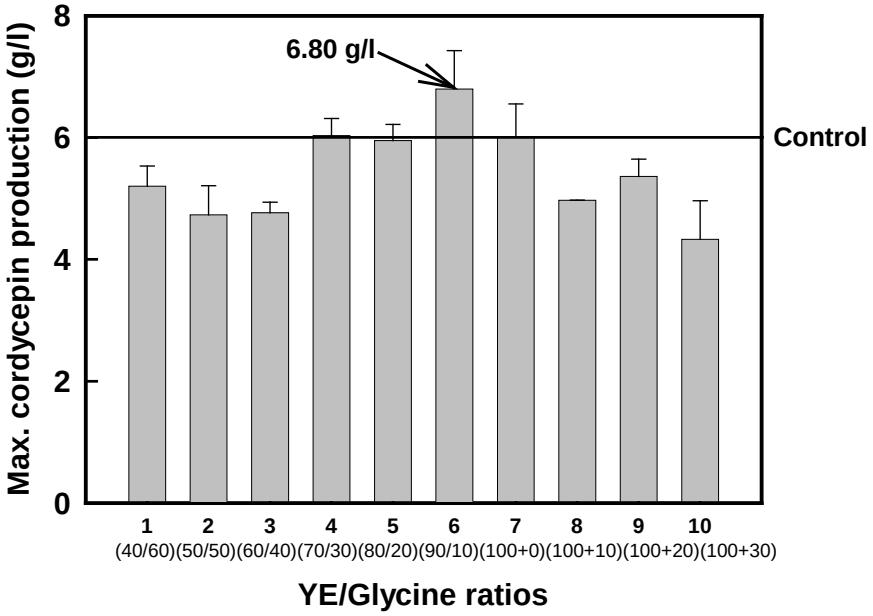


Fig. 4-7 Maximum cordycepin production in surface liquid culture using different concentrations of glycine as an additive

These results showed that the best cordycepin production was 6.80 g/l for the ratio of 90/10 was 12% higher than the control (6.05 g/l) (Figs. 4-6, 4-7). The cordycepin production for the ratios 70/30 and 80/20 were also higher than that of the control, while the others produced a lower cordycepin amounts compared to that of the control, especially the cordycepin production by the 10, 20 and 30 weight percents of yeast extract inversely decreased with the glycine concentration (Figs. 4-6, 4-7).

Regarding the time course, the glucose consumption for the 40/60 ratio was the fastest, while for the 30 weight percent yeast extract, it was the slowest with the longest culture time among all the treatments (Fig. 4-5). As the cordycepin production was lower in case of glycine, a minute investigation is needed to explore the constituents of glycine and yeast extract, which might furnish an important clue of decreased production.

4.3.2 Effects of repeated batch operation

4.3.2-1 Typical batch culture of C. militaris

Three repeated batch operations were performed using control and mutant using optimized medium for each. In addition, adenosine supplemented medium was used for the mutant. The surface of the liquid medium was covered with biofilm by 15 days for the mutant and 12 days for the control. Figs. 4-8, 4-9 & 4-10 show the typical time courses of cordycepin production, glucose, and pH by surface liquid culture using optimized medium for the control (wild strain), mutant, and mutant with adenosine each.

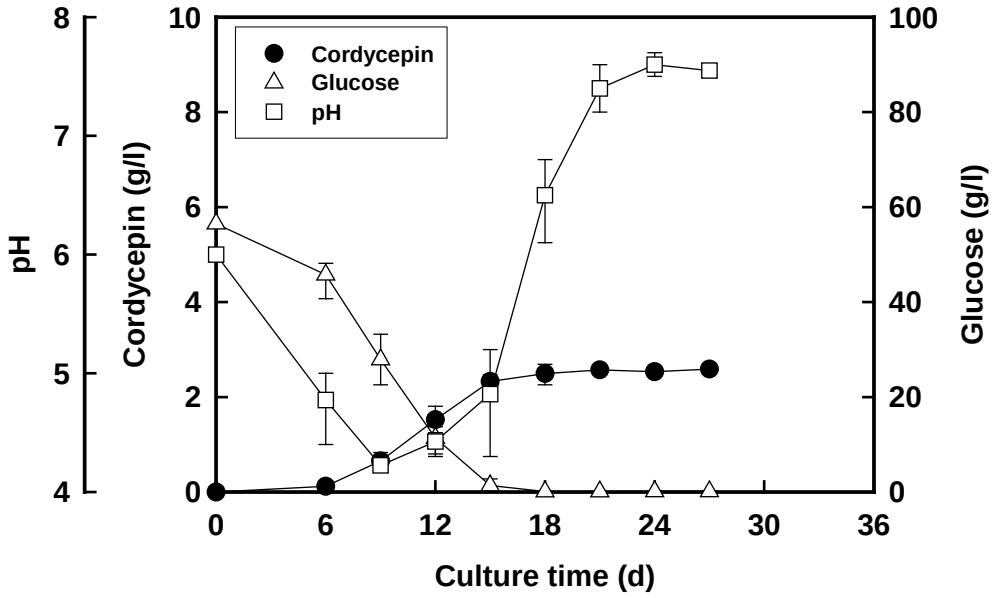


Fig. 4-8 Typical time course of the surface liquid culture using control

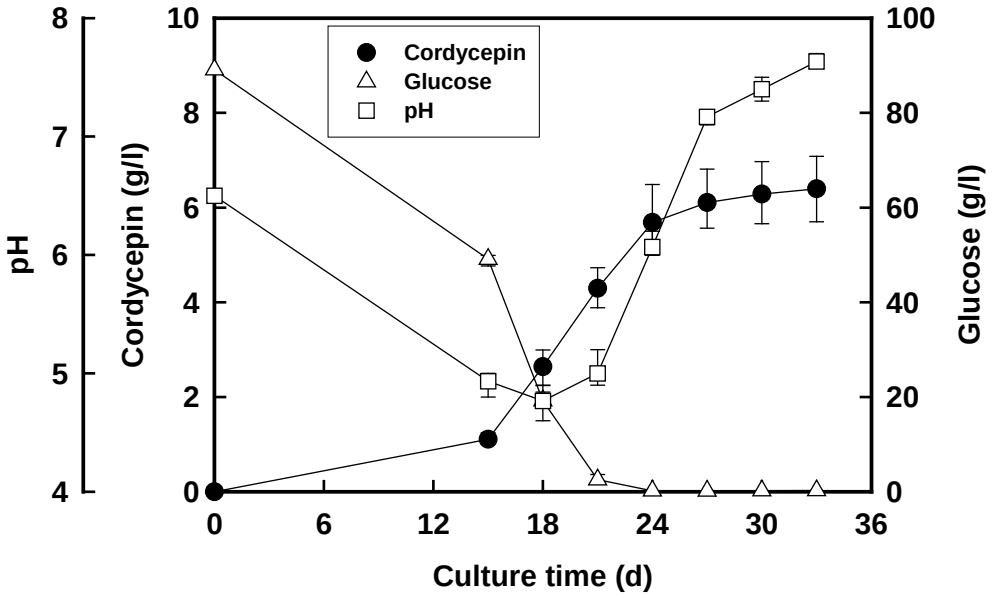


Fig. 4-9 Typical time course of the surface liquid culture using mutant G81-3

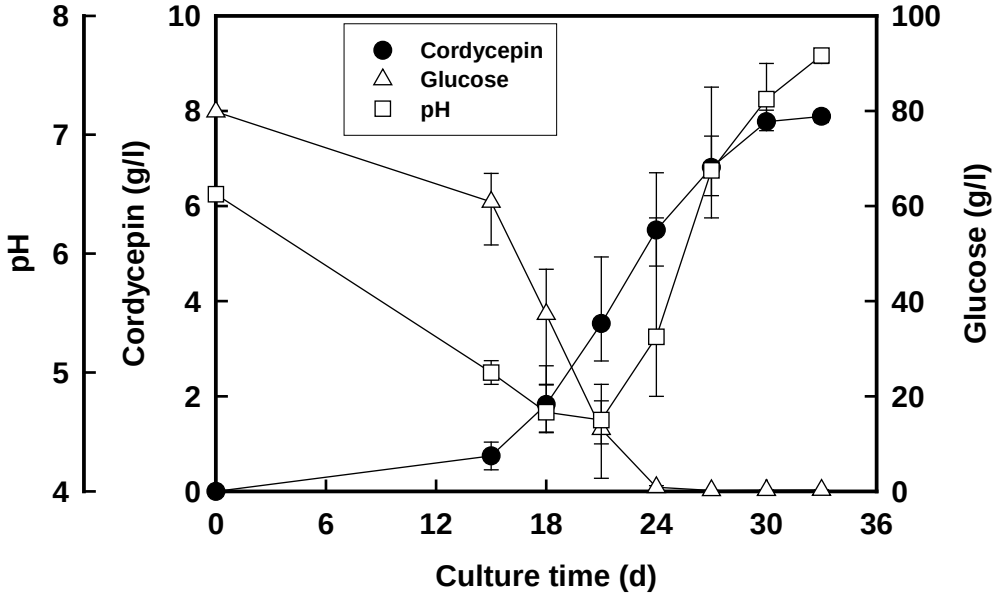


Fig. 4-10 Typical time course of the surface liquid culture using mutant G81-3 with adenosine

The cordycepin production started slightly after the starting of the cell growth and almost stopped when the glucose consumption was finished. When the residual glucose reached 0, there was a rise in pH due to autolysis leading to the maximum cordycepin production. The production and productivities of cordycepin were 2.33, 0.16; 5.68, 0.22 and 7.34 g/l, 0.26 g/(l· d) for control, mutant and mutant with adenosine in batch operations, respectively (Figs. 4-8, 4-9, & 4-10; Table 4-2). It is noteworthy that the present cordycepin productions were somewhat lower than those obtained in our previous experiment (Chapter 3, Table 3-5). In fact, cordycepin production was calculated in this experiment on the day having the highest productivity in each case in contrast to that of the control (Table 4-2), which is practically a few days before reaching the production peak level.

4.3.2-2 Repeated batch culture of control, mutant and mutant with adenosine using optimized media

For a further increase in cordycepin production, the feasibility of the repeated batch operation was examined. In our present technique, steel wires with special nets were used to replenish the medium without causing any harm to the surface biofilm. The glucose consumption was used as a criterion for the exchange of medium. Figs. 4-11 (a), 4-12 (a) and 4-13 (a) show the time courses of the cordycepin production and Figs. 4-11 (b), 4-12 (b) and 4-13 (b) show the glucose concentrations and pH of the control, mutant and mutant with adenosine, respectively, when an optimized medium was used for each.

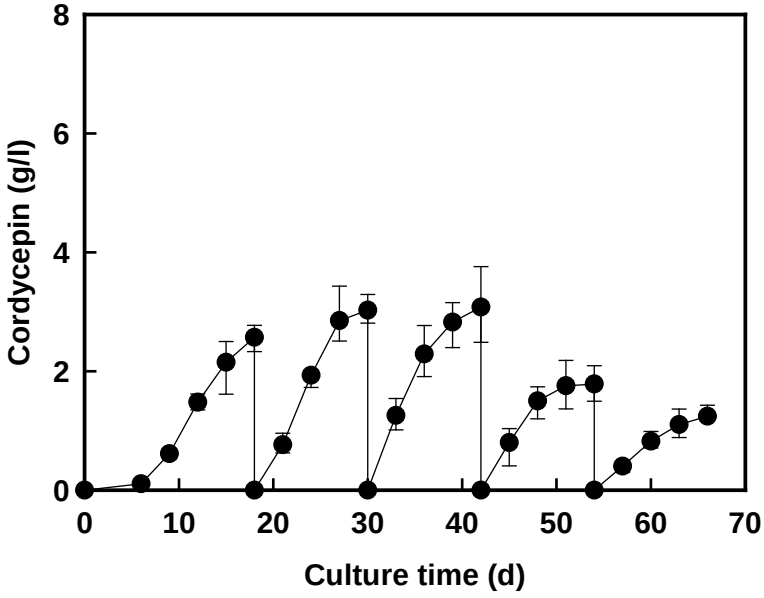


Fig. 4-11 (a) Time courses of the cordycepin production of repeated batch operation using control

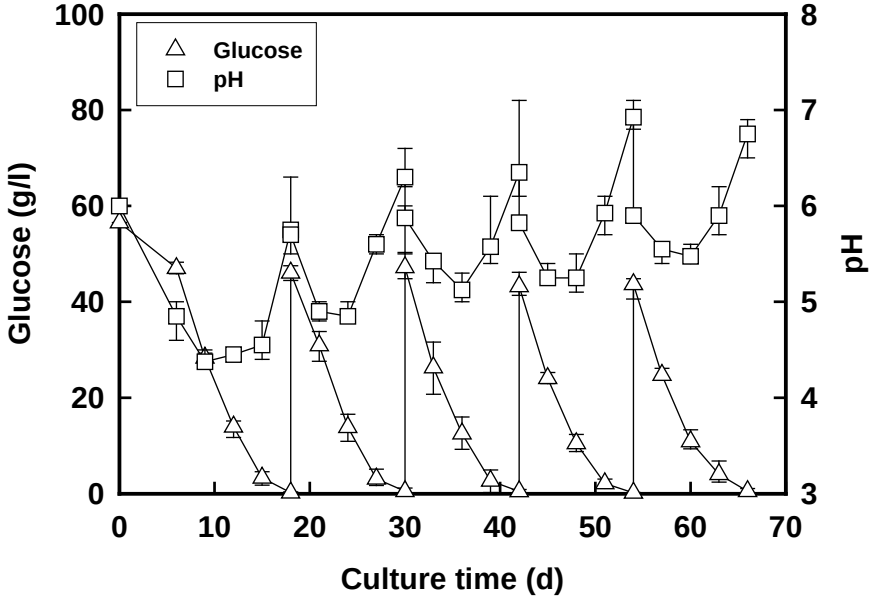


Fig. 4-11 (b) Time courses of the glucose concentration and pH of repeated batch operation using control

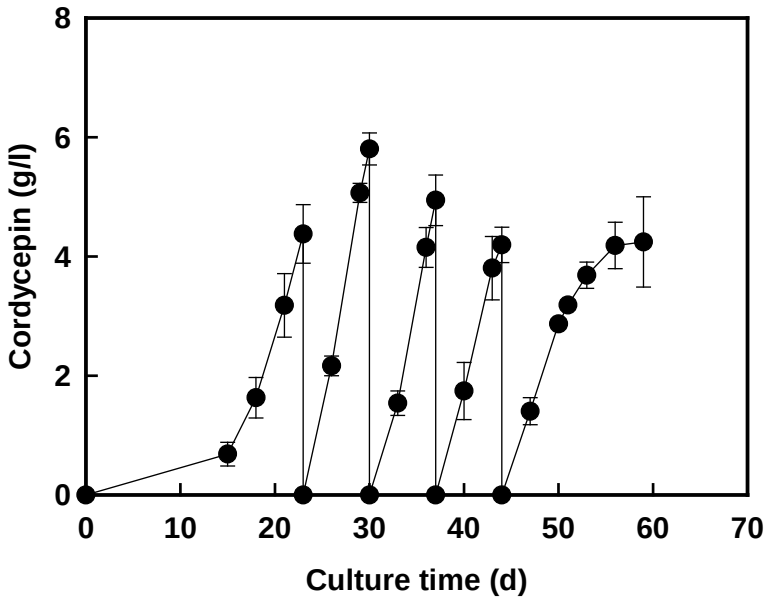


Fig. 4-12 (a) Time courses of the cordycepin production of repeated batch operation using mutant

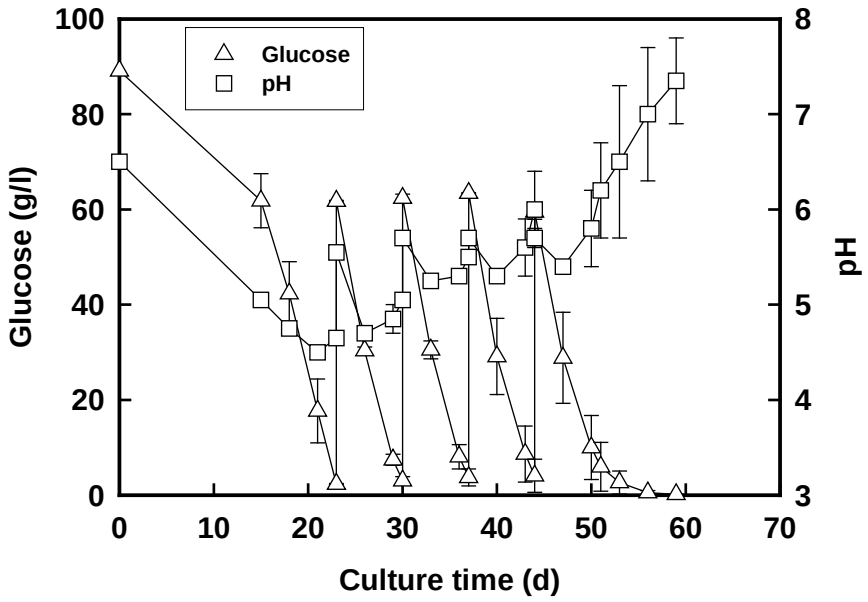


Fig. 4-12 (b) Time courses of the glucose concentration and pH of repeated batch operation using mutant

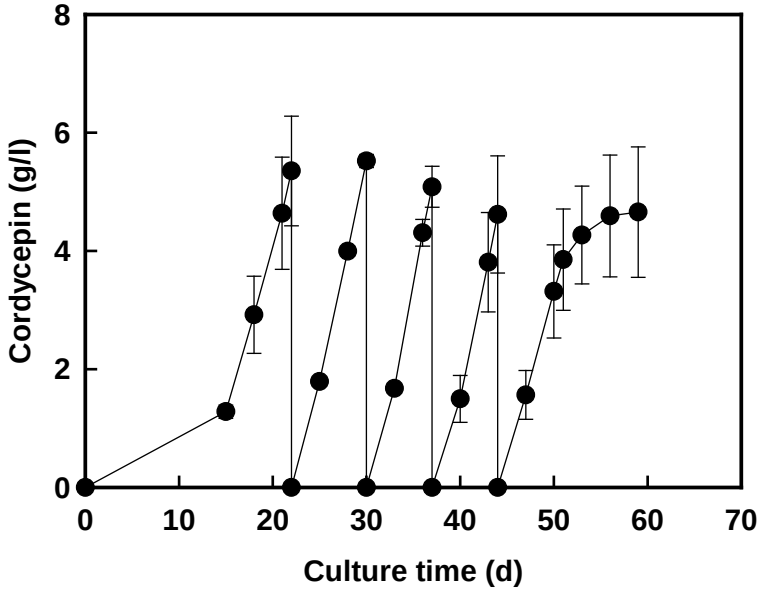


Fig. 4-13 (a) Time courses of the cordycepin production of repeated batch operation using mutant with adenosine

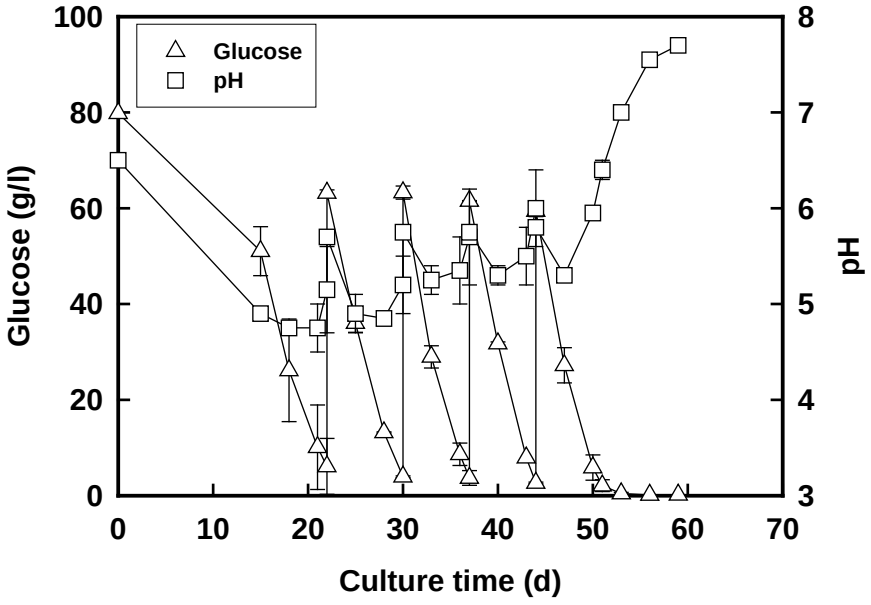


Fig. 4-13 (b) Time courses of the glucose concentration and pH of repeated batch operation using mutant with adenosine

In every case of repeated batch culture using control, mutant, and mutant with adenosine, the better production that was not less than the initial level was maintained up to the third cycle and later, they showed lower cordycepin productions as the whole operation was continued for five cycles/batches. On the medium exchanging day, sampling for both the last day of the succeeding batch and first day (0 day) for the preceding batch were done and between them the cordycepin production was considered as zero. As we mentioned about the mycelial autolysis, it is quite possible to prevent the mycelia from autolysis by the repeated batch operation resulting in an increased production and productivity. The productivity was calculated as the total cordycepin production (g/l) divided by the total time period needed for that. To have the highest productivity, this period is considered up to the day of the last batch after the glucose concentration became below 0.5% of the initial concentration (Table 4-2).

The results of cordycepin production and productivities of control, mutant and mutant with adenosine per batch calculated from the all sum of all the batches were summarized in Table 4-2. The productivities of the repeated batches were 34%, 86% and 82% higher than those of batches for control, mutant and mutant with adenosine, respectively; whereas, those of mutant and mutant with adenosine were 113% and 131% higher than that of the control, respectively. Also, productivity of mutant with adenosine was 9% higher than that of the mutant in repeated batch culture. Additionally, the productivities of batch operations of mutant and mutant with adenosine were 53% and 70% higher than that of the control. Hence, the effectiveness of the repeated batch operation is obvious in both the cases of mutant and control.

Table 4-2 Comparison of cordycepin productivity in batch and repeated batch cultures

Culture types	Control	Mutant	
	A. Optimized medium	B. Optimized medium	C. Optimized medium + Adenosine
Batch			
Period (d)	15	24	24-30
Production (g/l)	2.33	5.68	7.34
Productivity [g/(l·d)]	0.155	0.237	0.263
Repeated batch			
Period (d)	42[b]	51[a]	51[a]
Production [a] (g/l)	8.677[b]	22.50[a]	24.43[a]
Productivity [g/(l·d)]	0.207[b]	0.441[a]	0.479[a]

[a] Five batches, [b] Three batches

Although the production of repeated batch culture was higher than that of the batch culture, in a close investigation, it was observed that some parts of the mycelia did not touch the replenished fresh medium. It might be one of the major explainable causes (Fig. 4-14 a) for not reaching the production a further higher level. As the culture continued for a long time, the mycelia became curvy; also somewhere it sunk into the medium and some parts became cracked, these facts may be considered for decreased production of cordycepin (Figs. 4-14 b, c & d).

In this experiment, it was revealed that the application of repeated batch operation could increase the production and productivity significantly in contrast to the batch

operation. Therefore, this technique will be applicable for industrial production of cordycepin for future uses, leading to develop a continuous production protocol. These results are entirely consistent with previous work of our laboratory [4]. It is also mentionable that it could reduce the labour, time and energy.

(a) Untouched mycelia

(b) Curved mycelia

(c) Sunk mycelia

(d) Cracked mycelia

Fig. 4-14 Photographs of untouched, curved, sunk and cracked mycelia in repeated batch operation

4.3.2-3 Nucleic acid-related compounds

The contents of nucleic acid-related compounds in the extract from the mycelia and the filtrate of medium by the batch operation for the mutant and the control are summarized bellow in Table 4-3.

Table 4-3 Nucleic acid-related compounds obtained from *C. militaris*

Contents	Extract from mycelia (mg)	Filtrate of medium (mg)
C. militaris		
NBRC 9787 (18d)		
Mycelia	3.29×10^4	
Adenine	2.9	2.8
Guanine	18.5	175.2
Uracil	37.1	105.0
Adenosine	91.1	52.6
Guanosine	78.7	47.1
Uridine	106.8	81.1
Cordycepin	56.2	2.5×10^3
C. militaris		
G81-3 (24d)		
Mycelia	3.87×10^4	
Adenine	1.6	5.1
Guanine	23.2	271.7
Uracil	29.2	185.2
Adenosine	45.4	132.0
Guanosine	41.3	97.8
Uridine	45.4	122.6
Cordycepin	129.7	4.5×10^3

All values were estimated on the basis of a working volume of 1L.

Analyzing the results, it is evident that major portion of cordycepin existed in the medium, that is, the synthesized cordycepin was immediately excreted in the medium. This is consistent with the results reported by Masuda *et al.* [4]. In case of

the control, it was found that the concentrations of adenosine and guanosine decrease as the cordycepin concentration increase. On the other hand, in case of the mutant, the concentrations of adenosine and guanosine increase with an increase of cordycepin concentration. This result suggests that the production of cordycepin using mutant is linked to either adenosine and/or guanosine in the medium.

4.3.2-4 Measurement of dry mycelial weight

In a comparison of dry mycelial weight, it was found that in every case of mutant, regardless of culture technique, the dry mycelial weight was higher than that of the control. Table 4-4 summarized the comparison of dry mycelial weight of mutant and the control.

Table 4-4 Comparison of dry mycelial weight of mutant and the control

Culture types	Control	Mutant	
	A. Optimized medium	B. Optimized medium	C. Optimized medium + Adenosine
Batch			
Period (d)	18	36	36
Dry mycelial weight (g/l)	32.9	35.0	33.6
Repeated batch			
Period (d)	72	51	51
Dry mycelial weight (g/l)	113.2	126.5	130.2

It is evident that the dry mycelial weight of mutant is always accompanied by a higher cordycepin productivity showing a positive relationship with each other.

4.4 Conclusion

The results of the present experiments suggested that the additives had positive influences on cordycepin production in which the adenosine had a much better influence than that of glycine. It is necessary to mention that the cordycepin production reported by a number of authors [2, 4, 7-10] is much lower than that of our present research study (8.57 g/l). In this experiment, it was also evident that a higher concentration of both the adenosine and glycine negatively affected the cordycepin production.

Furthermore, the repeated batch operation presently investigated was also significantly effective in increasing the productivity of cordycepin as well as a continuous production protocol for industrial uses. In a course of searching a suitable culture technique, the repeated batch operations of control, mutant, and mutant with adenosine with an optimized medium for each were used. The cordycepin productivities of batches and repeated batches were 0.16, 0.20; 0.24, 0.44 and 0.26 g/l, 0.48 g/(l·d) for control, mutant, and mutant with adenosine, respectively. In fact, the productivities of the repeated batches were 34%, 86% and 82% higher than those of batches for control, mutant and mutant with adenosine, respectively; whereas, those of mutant and mutant with adenosine were 113% and 131% higher than that of the control, respectively. The nucleic acid-related compounds analysis revealed that the adenosine and guanosine were somehow linked to cordycepin production in case of mutant. Therefore, the mutant (G81-3) obtained by the proton beam irradiation with the best adenosine concentration (8.57 g/l) might be an effective combination for repeated batch operation in optimized medium to achieve a further higher cordycepin productivity. The present research will also provide valuable information for mushroom researchers, cancer and radiation biologists, chemical engineers, biotechnologists, medical practitioners, and personnel in the pharmaceutical industries.

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CHAPTER 5
General conclusion

5.1 Achievements

The author minutely investigated the different techniques that might be used for higher production of the potential anti-cancer agent cordycepin from the medicinal mushroom *Cordyceps militaris* to be used in the realm of pharmaco-industrial biotechnology. Mutation by ion beam irradiation (carbon and proton), medium optimization, use of suitable additives and finally repeated batch operations are the salient tools in this regard.

Chapter 2 describes a novel approach to obtain a prospective *Cordyeps militaris* mutant for higher cordycepin production using high-energy ion beam irradiation. Carbon beam irradiation revealed a little change in their growth rate, however, a higher production of cordycepin compared to the control was not accomplished. Following the successful proton beam irradiation, 30 classes of 8-azaadenine- and 28 classes of 8-azaaguanine-resistant mutants were obtained, of which 7 classes (A63-7, A63-8, A81-2, A81-6, G63-8, G81-3, G82-4) were selected as initially promising mutants using their antibacterial ability as an index of the cordycepin production. Results of a surface liquid culture revealed that the cordycepin production ability of mutant nos. A81-2, A81-6, G63-8 and G82-4 was lower than that of the control. Mutant nos. A63-7 and A63-8 had a higher cordycepin production with a lower metabolic rate than that of the control. On the other hand, mutant no. G81-3 was higher than that of the control regarding the metabolic rate of glucose as well as the cordycepin production ability. Therefore, for primary optimization using enriched medium, G81-3 was selected as a promising one that had 72 % higher cordycepin production than that of the control (3.1 vs. 1.8 g/l).

In Chapter 3, the optimization of medium components was depicted for the culture of *Cordyceps militaris* mutant obtained by ion beam irradiation. According to the response surface analysis using a central composite design the predicted maximum

response of cordycepin production was 6.76 g/l at glucose 86.2 g/l and yeast extract 93.8 g/l for the mutant no. G81-3, and 2.48 g/l at glucose 62.6 g/l and yeast extract 72.5 g/l for the control. The cordycepin production of the mutant was 2.79 times higher than that of the control. Validation was carried out under conditions predicted by the model that revealed a close correlation between the predicted and experimental values (6.84 and 2.45 g/l for mutant and control, respectively).

Chapter 4 describes the strategies for a further higher cordycepin production by employing the additives influenced biosynthesis of cordycepin and repeated batch operation using *Cordyceps militaris* mutant. In the culture medium under the previously optimized conditions for the said mutant, 2, 4, 6, 8 and 10 g/l adenosine were separately added. These results revealed the highest cordycepin production of 8.57 g/l when using 6 g/l adenosine was 28% higher than that of the control (6.69 g/l). This is the highest report of cordycepin production until now. Similarly, the results of other concentrations also superseded the control. For the same purpose, glycine was used with yeast extract in weight percent ratios (yeast extract/glycine) of 40/60, 50/50, 60/40, 70/30, 80/20 and 90/10 under the condition that the total amount of yeast extract and glycine were fixed. Also, glycine was separately added as 10, 20 and 30 weight percents of the yeast extract in the culture medium having the same optimized conditions with a fixed yeast extract concentration. These results showed that the 90/10 ratio had the best cordycepin production of 6.80 g/l that was 12% higher versus the control (6.05 g/l). The cordycepin production of the 70/30 and 80/20 weight percent ratios were also higher than that of the control, while the others had a lower cordycepin production compared to that of the control. Although, adenosine had a much better influence than glycine, it was also evident that a higher concentration of both adenosine and glycine negatively affected the cordycepin production. Later on, the repeated batch culture operations using optimized conditions for control, mutant and mutant with the best adenosine concentration (6 g/l) were performed in order to have a further higher cordycepin productivity for industrial uses. The cordycepin productivities of batches and repeated batches were 0.16, 0.20; 0.24, 0.44 and 0.26 g/l, 0.48 g/(l·d) for control, mutant and mutant with

adenosine, respectively. In fact, the productivities of the repeated batches were 34%, 86% and 82% higher than those of batches for control, mutant and mutant with adenosine, respectively; whereas, those of mutant and mutant with adenosine were 113% and 131% higher than that of the control, respectively. Also, productivity of mutant with adenosine was 9% higher than that of the mutant in repeated batch culture. Additionally, the productivities of batch operations of mutant and mutant with adenosine were 53% and 70% higher than that of the control. Hence, the effectiveness of the repeated batch operation is obvious in both the cases of mutant and control.

5.2 Future perspectives

Nature is the source of all the raw materials that we need. About 2-3 decades ago, most of the drugs were of herbal origin. A variety of reasons remain behind why people like to use natural medicines as it is evident that patients are getting even more distressed after using the chemically synthesized drugs, rather than natural means like medicinal mushrooms that can conquer life-claiming diseases, leaving no side effects on human health. To maintain proper growth, the pharmaceutical industries need innovation and access to high output rate on low-cost materials with reasonable safety. The combination of modern chemistry with bio-based starting materials, like bio-metabolites, offers the scope for revolutionizing mushroom based pharmaceutical industries. In the near future, bio-metabolites (cordycepin, polysaccharides etc.) extracted from medicinal mushroom like *C. militaris* will have a role that compares with that of oil and gas crackers today.

The ancient medicinal fungus *C. militaris*, which has been used as a crude drug for the welfare of mankind in old civilization, is now of a matter of concern due to its unexplored potentials. The anti-cancer agent cordycepin obtained from *C. militaris* have more than 21 clinically approved beneficial effects for human health. Especially, the anti-cancer agent cordycepin from *C. militaris* is expected to play evolutionary roles in the pharmacognosy sector, leading to create a viable base for

pharmaceutical industries as some emerging diseases like CANCER, SARS, AIDS, SWINE FLU have no proper remedies yet. In this regard, it needs further elaborative pharmacological investigation and clinical trials.

List of publications

A. In referred journals:

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B. Meetings/congress/conference publications:

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