EFFECT OF VEGETABLE MEDIUM NITROGEN SOURCES ON CORDYCEPIN PRODUCTION AND BIOSYNTHESIS PATHWAY OF *Cordyceps militaris* IN LIQUID SURFACE CULTURE

KRITSANA SRIPILAI

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biotechnology
Suranaree University of Technology
Academic Year 2022

ผลของแหล่งไนโตรเจนจากพืชในอาหารเพาะเลี้ยงต่อการผลิตคอร์ไดเซปิน และวิถีการสังเคราะห์ทางชีวเคมีของถั่งเช่าสีทอง ในการเพาะเลี้ยงพื้นผิวของเหลว

นายกฤษณะ ศรีพิไล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2565

EFFECT OF VEGETABLE MEDIUM NITROGEN SOURCES ON CORDYCEPIN PRODUCTION AND BIOSYNTHESIS PATHWAY OF *Cordyceps militaris* IN LIQUID SURFACE CULTURE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Areechun Sotthibundhu)
Chairperson

(Assoc. Prof. Dr. Parinya Noisa)
Member (Thesis Advisor)

(Dr. Mantana Jamklang)
Member

(Assoc. Prof. Dr. Chatchai Jothityangkoon)
Vice Rector for Academic Affairs
and Quality Assurance

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural
Technology

กฤษณะ ศรีพิไล: ผลของแหล่งไนโตรเจนจากพืชในอาหารเพาะเลี้ยงต่อการผลิตคอร์ไดเซปิน และวิถีการสังเคราะห์ทางชีวเคมีของถั่งเช่าสีทองในการเพาะเลี้ยงพื้นผิวของเหลว (EFFECT OF VEGETABLE MEDIUM NITROGEN SOURCES ON CORDYCEPIN PRODUCTION AND BIOSYNTHESIS PATHWAY OF *Cordyceps militaris* IN LIQUID SURFACE CULTURE) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร.ปริญญา น้อยสา, 74 หน้า.

คำสำคัญ: ถั่งเช่าสีทอง/แหล่งไนโตรเจนที่ปราศจากสัตว์/คอร์ไดเซปิน/เมล็ดผัก/การเพาะเลี้ยงพื้นผิวของเหลว

ถั่งเช่าสีทอง (*Cordyceps militaris*) เป็นเห็ดสมุนไพรที่นิยมบริโภคเพื่อสุขภาพในทวีปเอเชีย ในศตวรรษที่ 21 สารออกฤทธิ์ทางชีวภาพที่สำคัญชนิดหนึ่งที่ผลิตจากถั่งเช่าสีทองโดยเฉพาะคือ สารคอร์ไดเซปิน ถั่งเช่าสีทองได้รับการศึกษาอย่างกว้างขวางว่ามีศักยภาพสูงสำหรับกิจกรรมทางชีวภาพ เช่น สารต้านอนุมูลอิสระ, ต้านมะเร็ง, ต้านการอักเสบ, กระตุ้นภูมิคุ้มกัน, ต้านจุลชีพ ฯลฯ การศึกษานี้ได้ทำการศึกษาผลกระทบของสภาวะการเพาะเลี้ยง และสารสกัดจากเมล็ดผัก (VSEP) เป็นแหล่งเสริมของ ไนโตรเจนที่ปราศจากสัตว์สำหรับการผลิตคอร์ไดเซปินของถั่งเช่าสีทองในการเพาะเลี้ยงพื้นผิวของเหลว สภาวะการเพาะเลี้ยงถูกตรวจสอบเบื้องต้น พบว่าสภาวะการเพาะเลี้ยงที่เหมาะสมคือปริมาตรอาหารเพาะเลี้ยง 100 มิลลิลิตร ขนาดหัวเชื้อร้อยละ 10 และระยะเวลาการเพาะเลี้ยง 30 วัน การผลิตคอร์ไดเซปินสูงสุดถูกสังเกตภายใต้เงื่อนไขของการเสริมด้วยสารสกัดจากถั่วเหลือง (SBEP) และการเสริม SBEP 80 กรัมต่อลิตร จะเพิ่มการผลิตสารถั่งเช่าเป็น 2.52 กรัมต่อลิตร ซึ่งมากกว่ากลุ่มควบคุม (เปปโทน) Real-time PCR ถูกนำมาใช้เพื่อตรวจสอบระดับการถอดความ และผลลัพธ์แสดงให้เห็นว่าการเสริมด้วย SBEP 80 กรัมต่อลิตร ช่วยเพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับวิถีเมตาบอลิซึมของคาร์บอน เมแทบอลิซึมของกรดอะมิโน และยีนหลักสองยีนที่เกี่ยวข้องกับการสังเคราะห์ทางชีวภาพของคอร์ไดเซปินอย่างมีนัยสำคัญเมื่อเทียบกับเงื่อนไขการเสริมด้วยเปปโตน ภายใต้เงื่อนไขการเพาะเลี้ยงที่เหมาะสม แบบจำลองคาดการณ์การตอบสนองสูงสุดของการผลิตคอร์ไดเซปิน คือ 2.64 กรัมต่อลิตร ที่ปริมาตรอาหารเพาะเลี้ยง 147.5 มิลลิลิตร ขนาดหัวเชื้อร้อยละ 8.8 และเวลาเพาะเลี้ยง 40 วัน เงื่อนไขการเพาะเลี้ยงที่เหมาะสมนี้สามารถใช้เพื่อเพิ่มการผลิตคอร์ไดเซปินในถังหมักชีวภาพขนาดใหญ่ สามารถดำเนินการวิจัยเพิ่มเติมเพื่อประเมินความเป็นไปได้ทางเศรษฐกิจของกระบวนการนี้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา _____

KRITSANA SRIPILAI: EFFECT OF VEGETABLE MEDIUM NITROGEN SOURCES ON CORDYCEPIN PRODUCTION AND BIOSYNTHESIS PATHWAY OF *Cordyceps militaris* IN LIQUID SURFACE CULTURE. THESIS ADVISOR: ASSOC. PROF. PARINYA NOISA, Ph.D., 74 PP.

Keyword: *Cordyceps militaris*/ANIMAL-FREE NITROGEN SOURCES/ CORDYCEPIN/ VETGETABLE SEED/LIQUID SURFACE CULTURE

Cordyceps militaris is a medicinal mushroom that is popularly consumed for health benefits in Asia in the 21st century. One of the important bioactive compounds that are produced specifically from cordyceps is cordycepin. *C. militaris* is widely studied as having high potential for biological activities such as antioxidant, anticancer, anti-inflammatory, immunomodulatory, antimicrobial, etc. This study investigated the effects of culture conditions and vegetable seed extract powder (VSEP) as a supplementary source of animal-free nitrogen on the production of cordycepin by *C. militaris* in liquid surface culture. Cultivation conditions were initially examined. It was found that the optimum culture conditions were 100 ml of culture medium, 10% inoculum and the cultivation time of 30 days. The highest cordycepin production was observed under soybean extract powder (SBEP) conditions, and 80 g/L of SBEP supplementation increased cordycepin production to 2.52 g/L, which was greater than the control (peptone). Real-time PCR was used to examine the transcription levels, and the results showed that supplementing with SBEP 80 g/L significantly increased the expression of genes associated with the carbon metabolic pathway, amino acid metabolism, and two key genes involved in the cordycepin biosynthesis compared to peptone-supplemented culture. Under optimal culture conditions, the model predicted a maximum response of cordycepin production of 2.64 g/L at a working volume of 147.5 ml, an inoculum size of 8.8%, and a cultivation time of 40 days. This optimized culture condition could be used to increase cordycepin production in large-scale bioreactors. Additional research will be conducted to assess the economic viability of this process.

School of Biotechnology
Academic Year 2022

Student's Signature _____
Advisor's Signature _____

ACKNOWLEDGEMENT

The completion of this master's thesis was made possible by the assistance and support of several individuals. Without them, this research might not have been finished. Therefore, I would like to express my sincere gratitude and admiration for them.

First and foremost, I would like to express my sincere gratitude to my thesis advisor, Assoc. Prof. Dr. Parinya Noisa, for the invaluable opportunity to be his student. He gave me excellent assistance, consistent guidance, patience, constant encouragement throughout the course of this research, and other support that helped me successfully complete this study. In addition to teaching me to study, he taught me a variety of skills that I can use in my future work. It was a wonderful experience for me.

In addition, this work was supported by the funding for Graduate Research Grant in Agriculture and Agro-industry from the Agricultural Research Development Agency (ARDA; public organization) for Fiscal Year 2022 (HRD65050082) and the One Research One Grant (OROG) scholarship (Suranaree University of Technology, SUT).

I wish to thank all members of CBAI-Lab for all their friendship, teaching, guidance, assistance, and good encouragement all the time. Special thanks, I would like to be grateful to Suranaree University of Technology for a scholarship and laboratory facility support during my study.

Finally, I would like to thank my family for their support, encouragement, and faith throughout the period of this study. This work could not have been achieved without their support.

Kritsana Sripilai

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	=	Percent
°C	=	Degree Celsius
V	=	Volume
L	=	Liter
g	=	Gram
µg	=	Microgram
nm	=	Nanometer
µm	=	Micron
min	=	Minute
s	=	Second
mg	=	Milligram
ml	=	Milliliter
VSEP	=	Vegetable seed extract powder
SBEP	=	Soybean seed extract powder
HPLC	=	High pressure liquid chromatography
RSM	=	Response surface methodology
BBD	=	Box-Behnken design
rpm	=	Revolutions per minute
PDA	=	Potato dextrose agar
ubi	=	Polyubiquitin binding protein
GltS	=	Glutamate synthase

LIST OF ABBREVIATIONS (Continued)

NADP-GDH	=	NADP-specific glutamate dehydrogenase
AST	=	Aspartate transferase
cns1	=	Oxidoreductase domain-containing protein
NT5E	=	5′-nucleotidase
pfkA	=	6-phosphofructokinase
zwf	=	Glucose 6-phosphate dehydrogenase
aceA	=	Isocitrate lyase
PCR	=	Polymerase chain reaction
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
cDNA	=	Complementary DNA
Eq	=	Equation
Ct	=	Cycle threshold
MDW	=	Mycelial dry weight
$MgSO_4 \cdot 7H_2O$	=	Magnesium sulphate heptahydrate
KH_2PO_4	=	Potassium dihydrogenphosphate

CHAPTER 1
INTRODUCTION

1.1 Significance of the research

Currently, the use of herbal extracts for medicinal to health, which the market is vast. The demand for herbs is higher popular and experienced an extraordinary rise in the herbal products such as food supplement, functional drink and cosmetic (Williamson et al., 2020). At present, there are many kinds of herbs that are beneficial to health, which production of the different biological compound and biological activity could be designed and formulated of products for high efficiency the production to be sufficient for the industrial sector and reduce costs in the production process. In this study, the cultivation of *Cordyceps militaris* for cordycepin production was determined.

C. militaris is a medicinal mushroom that has high potential in pharmaceuticals for health and treatment in East Asia. It is an entomopathogenic fungus that belongs to the class Ascomycetes in the family Cordycipitaceae (Tuli et al., 2014; Dong et al., 2015). Cordyceps can produce bioactive compounds such as cordycepin, cordycepic acid, adenosine, cordymin, carotenoid, cordyxanthins, cordycedipeptide A, polysaccharides, ergosterol, mannitol, myriocin, fibrinolytic enzyme, fatty acids, and amino acids that are created intracellular of cordyceps (Dong et al., 2013; Liu et al., 2015). *C. militaris* is considered one of the most expensive medicinal mushrooms in China, and it is very successful for nutraceuticals and cosmeceuticals. The cultivation of *C. militaris* can be scaled up to a commercial level (Wang et al., 2021).

Cordycepin (3′-deoxyadenosine) is the nucleoside adenosine that lacks a hydroxyl group at the 3′position of ribose. The chemical formula of cordycepin is $C_{10}H_{13}N_5O_3$, the major biologically active metabolite of *C. militaris*, which is the most widely studied for medical value (Soltani et al., 2018). Overgaard-Hansen. (1964) revealed that the biosynthesis of purine is crucial for the cordycepin synthesis.

Cordycepin is biosynthesized from two precursors, including 2′-carbonyl-3′-deoxyadenosine (2′-C-3′-dA) from adenosine pathway and adenosine-3′-monophosphate (3′dAMP) from adenosine diphosphate (ADP) pathway. From adenosine, 2′-C-3′-dA is converted to cordycepin by oxidoreductase domain-containing protein (cns1)., and 3′dAMP is converted to cordycepin by 5′-nucleotidase (Lin et al., 2016). In ADP pathway, adenosine monophosphate (AMP) is converted to ADP, and it is converted to 3′dAMP by enzyme ribonucleotide reductase (RNR). However, RNR was reported to not produce 3′-dADP in *C. militaris* and may be mediated by other enzymes (Kato et al. 2017). According to Xia et al. (2017), *C. militaris* can produce pentostatin that inhibits adenosine deaminase (ADA) activity to help protect cordycepin from deamination, which is very beneficial in developing the cultivation of *C. militaris* for the pharmaceutical industry. Cordycepin has high potential for health effects, such as antioxidant (Jaiboonma et al., 2020), anti-cancer (Chaicharoenaudomrung et al., 2018), anti-inflammatory (Choi et al., 2014), immunomodulatory (Jung et al., 2019), and hypoglycemic activities (Yu et al., 2016).

Nowadays, there are many strategies for enhancing cordycepin production in herbal industrials, such as applying artificial culture in liquid surface culture and submerged fermentation to substitution solid culture (Kunhorm et al. 2019). The liquid surface culture gives rise to higher cordycepin production in a small space, which uses a shorter cultivation time, and contamination is less than solid culture (Das et al., 2009). In addition, culture medium is essential for cordyceps culture, and nitrogen sources play an important role because they are directly related to mycelial growth and metabolic biosynthesis (Mao et al. 2005). Nitrogen sources are commonly obtained from ionogenic ($NaNO_3$, NH_4NO_3, Urea, and $(NH4)_2SO_4$) and organic sources (beef extract, casein, peptone, and yeast extract). According to Kumar et al. (2018), organic nitrogen sources are able to stimulate enzyme production and cell proliferation better than inorganic nitrogen sources. Nitrogen sources may even be crucial for mycelial growth and the synthesis of cordycepin by *C. militaris* in liquid surface culture. Kang et al. (2014) investigated various nitrogen sources (peptone, yeast extract, beef extract, casein, NH_4Cl, NH_4NO_3, $NaNO_3$, and carbamine) in liquid static culture, and found that peptone was the best nitrogen source for cordycepin production of 0.8 g/L. According to Chen et al. (2020), glutamine, glycine, and aspartate are related to the serial

conversion in the pathway that produces cordycepin. Suparmin et al. (2017) evaluated cordycepin production in liquid surface culture. They found that the glutamate synthase (GltS) precursor gene is related to the cordycepin biosynthesis pathway. Glutamine is converted to glutamate by GltS. Glutamate can be used in nucleotide biosynthesis, and aspartate is an important precursor for cordycepin biosynthesis. However, nitrogen sources derived from animals or microorganisms were expensive (Kim et al., 2010; Zhang and Liu 2016). The experiment of Luo et al. (2020) used wheat bran with pupa powder as a nitrogen source to reward the peptone and they found that it increased production of cordycepin, whereas it could remarkably reduce cost. Although the utilization of nitrogen new sources can reduce costs, it has not been able to enable people who do not eat meat to consume *C. militaris* as a source of health maintenance because culture media is still used as a nitrogen source derived from meat for cultivating *C. militaris*. Therefore, alternative sources of nitrogen from plants in order to enable people who do not consume meat to consume *C. militaris* for health benefits are required and also for the general population as well. Moreover, the use of plant-based nitrogen sources can also reduce environmental problems from livestock.

Traditional peptones, the alternative animal-free nitrogen source is vegetable extract. The proteins derived from vegetable seeds are comparable to peptone and cost approximately 1/100 or less than that (Raghavendra et al., 2020). Vegetable seeds also contain carbohydrates, lipids, vitamins, minerals, and amino acids. Proteins in vegetable seeds are essential for enzyme activity and influence purine synthesis, which is an important metabolite for cordycepin production of *C. militaris* (Suribabu et al., 2014). Recently, peptone made from plants was commercially available for use in microbial cultivation. However, it was discovered that high prices also led to increased production costs. This study aimed to develop an animal-free nitrogen source of *C. militaris* in liquid surface culture by using vegetable seed as the supplemented nitrogen source. Development of the animal-free nitrogen source was applied to cordycepin production, and the molecular mechanisms of the carbon metabolism, amino acid metabolism, and cordycepin biosynthesis pathways were investigated.

1.2 Research objectives

1.2.1 To optimize the cultural conditions including working volume, inoculum size, and cultivation time on cordycepin production of *C. militaris* in liquid surface culture.

1.2.2 To develop an animal-free culture medium for *C. militaris* cultivation in liquid surface culture using a nitrogen source derived from vegetable seed extract powder (VSEP).

1.2.3 To investigate the molecular mechanisms of the genes involved in the three significant biosynthetic pathways, including cordycepin biosynthetic, carbon metabolic, and amino acid metabolism.

1.2.4 To optimize the cultural conditions, including working volume, inoculum size, and cultivation time, for cordycepin production in an animal-free culture condition.

1.3 Research hypothesis

1.3.1 VSEP could be used as a nitrogen source for *C. militaris* in liquid surface culture.

1.3.2 Cordycepin production could be increased after using a nitrogen source derived from VSEP.

1.3.3 VSEP as a nitrogen source could promote molecular mechanisms in the cordycepin biosynthetic pathway, carbon metabolic pathway, and amino acid metabolism.

1.3.4 The optimal culture conditions were obtained under an animal-free culture condition.

1.4 Scope of thesis

The aim of this research is to develop an animal-free culture medium using a vegetable seed-derived nitrogen source. This alternative nitrogen source may be used in place of the commercial nitrogen source for *C. militaris* in liquid surface culture to increase the production of cordycepin and molecular mechanisms. In the primary experiment, the cultural conditions, including working volume, inoculum size, and cultivation time, were optimized by Response Surface Methodology (RSM) with

a Box-Behnken Design for high cordycepin production. In the secondary experiment, vegetable seeds were investigated as nitrogen sources for cordycepin production, and the molecular mechanisms involved in the cordycepin biosynthetic pathway (*cns1* and *NT5E*), carbon metabolic pathway (*pfkA*, *zwf*, and *aceA*), and amino acid metabolism (*GltS*, *NADP-GDH*, and *AST*). The cultural conditions were investigated after obtaining the nitrogen source derived from vegetable seeds for cordycepin production.

1.5 Expected results

This study attempted to obtain the optimal culture conditions for maximum cordycepin production of *C. militaris* in liquid surface culture. In addition, to replace commercial peptone, VSEP may bring as an excellent supplementary source of animal-free nitrogen for cordycepin production, and mRNA expression were also up-regulated of carbon and, amino acid metabolism, and cordycepin biosynthesis. The animal-free nitrogen has also contributed to the low-cost cultivation. Moreover, it is also beneficial for non-meat consumers to use cordyceps for health purposes.

1.6 References

Chaicharoenaudomrung, N., Jaroonwitchawan, T. and Noisa, P. (2018). Cordycepin induces apoptotic cell death of human brain cancer through the modulation of autophagy. Toxicol. In Vitro 46: 113-121.

Chen, B. X., Wei, T., Xue, L. N., Zheng, Q. W., Ye, et al. (2020). Transcriptome analysis reveals the flexibility of cordycepin network in *Cordyceps militaris* activated by L-alanine addition. Front. Microbiol. 11: 577.

Choi, Y.H., Kim, G.Y. and Lee, H.H. (2014). Anti-inflammatory effects of cordycepin in lipopolysaccharide-stimulated RAW 264.7 macrophages through Toll-like receptor 4-mediated suppression of mitogen-activated protein kinases and NF-kappaB signaling pathways. Drug. Des. Devel. Ther. 8: 1941-1953.

Das, S. K., Masuda, M., Sakurai, A., and Sakakibara, M. (2009). Effects of additives on cordycepin production using a *Cordyceps militaris* mutant induced by ion beam irradiation. J. Biotechnol. 8(13): 3041-3047.

Dong, C., Guo, S., Wang, W. and Liu, X. (2015) Cordyceps industry in China. Mycology 6(2): 121–129.

Dong, J.Z., Wang, S.H., Ai, X.R., Yao, L., Sun, Z.W., Lei, C., Wang, Y. and Wang, Q. (2013) Composition and characterization of cordyxanthins from *Cordyceps militaris* fruit bodies. J. Funct. Foods 5: 1450-1455.

Jaiboonma, A., Kaokaen, P., Chaicharoenaudomrung, N., Kunhorm, P., Janebodin, K., Noisa, P. and Jitprasertwong, P. (2020) Cordycepin attenuates salivary hypofunction through the prevention of oxidative stress in human submandibular gland cells. Int. J. Med. Sci. 17: 1733-1743.

Jung, S.J., Jung, E.S., Choi, E.K., Sin, H.S., Ha, K.C. and Chae, S.W. (2019) Immunomodulatory effects of a mycelium extract of cordyceps (*Paecilomyces hepiali*; CBG-CS-2): a randomized and double-blind clinical trial. BMC Complement Altern. Med. 19: 77.

Kang, C., Wen, T. C., Kang, J. C., Meng, Z. B., Li, G. R., and Hyde, K. D. (2014). Optimization of large-scale culture conditions for the production of cordycepin with *Cordyceps militaris* by liquid static culture. Sci. World. J. 4: 1–15

Kato, T., Admad, S. and Park, E. (2017) Functional Analysis of Ribonucleotide Reductase from *Cordyceps militaris* Expressed in *Escherichia coli*. Appl. Biochem. Biotechnol. 182(4): 1307-1317.

Kim, S.W., van Heugten, E., Ji, F., Lee, C.H. and Mateo, R.D. (2010) Fermented soybean meal as a vegetable protein source for nursery pigs: I. Effects on growth performance of nursery pigs. J. Anim. Sci. 88: 214-224.

Kumar, C.G., Sripada, S. and Poornachandra, Y. (2018) Status and future prospects of fructooligosaccharides as nutraceuticals. In Role of Materials Science in Food Bioengineering 451-503.

Kunhorm, P., Chaicharoenaudomrung, N. and Noisa, P. (2019) Enrichment of cordycepin for cosmeceutical applications: culture systems and strategies. Appl. Microbiol. Biotechnol. 103: 1681-1691.

Lin, S., Liu, Z.Q., Xue, Y.P., Baker, P.J., Wu, H., Xu, F., Teng, Y., Brathwaite, M.E. and Zheng,

Y.G. (2016) Biosynthetic pathway analysis for improving the cordycepin and cordycepic acid production in *Hirsutella sinensis*. Appl. Biochem. Biotechnol. 179: 633-649.

Liu, Y., Wang, J., Wang, W., Zhang, H., Zhang, X. and Han, C. (2015) The chemical constituents and pharmacological actions of *Cordyceps sinensis*. Evid-based Complement Altern. Med. 2015: 575063.

Luo, Q.Y., Cao, H.F., Liu, S.K., Wu, M., Li, S.S., Zhang, Z.Q., Chen, A.J., Shen, G.H., Wu, H.J., Li, M.L., Liu, X.Y., Jiang, Y., Bi, J.F. and He, Z.Y. (2020) Novel liquid fermentation medium of *Cordyceps militaris* and optimization of hydrothermal reflux extraction of cordycepin. J. Asian Nat. Prod. Res. 22: 167-178.

Mao, X.B., Eksriwong, T., Chauvatcharin, S. and Zhong, J.J. (2005) Optimization of carbon source and carbon/nitrogen ratio for cordycepin production by submerged cultivation of medicinal mushroom *Cordyceps militaris*. Process Biochem. 40: 1667-1672.

Overgaard-Hansen, K. (1964) The inhibition of 5-phosphoribosyl-1-pyrophosphate formation by cordycepin triphosphate in extracts of Ehrlich ascites tumor cells. Biochim. Biophys. Acta. 80: 504-507.

Raghavendra, R.M.V., Raja, R.M., Vijay, K.C., Dilip, M., Abrar A.K., Surekha B., Frank M.N., Tiara C.L., Abigail A., and Verma, M.K. (2020) An indigenous substitute for peptone as the basic ingredient of culture media. Adv. Res. J. Multidiscip. Discov. 50: 01-05.

Soltani, M., Malek, R.A., Elmarzugi, N.A., Mahomoodally, M.F., Uy, D., et al. (2018). Cordycepin: A biotherapeutic molecule from medicinal mushroom. Biol. Macrofungi. 319-349.

Suparmin, A., Kato, T., Dohra, H., and Park, E. Y. (2017). Insight into cordycepin biosynthesis of *Cordyceps militaris*: Comparison between a liquid surface culture and a submerged culture through transcriptomic analysis. PLoS One 12(11): e0187052.

Suribabu, K., Lalitha G. T., and Hemalatha, K. P. J. (2014). Optimization of various Nitrogen sources for the production of **α**-Amylase using *Brevibacillus borstelensis* R1 by Submerged fermentation. Int. J. Curr. Microbiol. Appl. Sci. 3(4): 791-800.

Tuli, H.S., Sandhu, S.S. and Sharma, A.K. (2014) Pharmacological and therapeutic potential of cordyceps with special reference to cordycepin. 3 Biotech 4: 1-12.

Wang, Y., Yang, Z., Bao, D., Li, B., Yin, X., Wu, Y., Chen, H., Tang, G., Li, N. and Zou, G. (2021) Improving hypoxia adaption causes distinct effects on growth and bioactive compounds synthesis in an entomopathogenic fungus *Cordyceps militaris*. Front. Microbiol. 12: 698436.

Williamson, E.M., Liu, X., Izzo, A.A. (2020) Trends in use, pharmacology, and clinical applications of emerging herbal nutraceuticals. Br. J. Pharmacol. 177(6): 1227-1240.

Xia, Y., Luo, F., Shang, Y., Chen, P., Lu, Y. and Wang, C. (2017) Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. Cell Chem. Biol. 24(12): 1479-1489.

Yu, S.H., Dubey, N.K., Li, W.S., Liu, M.C., Chiang, H.S., Leu, S.J., Shieh, Y.H., Tsai, F.C. and Deng, W.P. (2016) *Cordyceps militaris* treatment preserves renal function in type 2 diabetic nephropathy mice. PloS One 11: e0166342.

Zhang, Q. and Liu, Y. (2016) The strategies for increasing cordycepin production of *Cordyceps militaris* by liquid fermentation. Fungal Genom. Biol. 6: 134. doi:10.4172/2165-8056.1000134

CHAPTER 2
LITERATURE REVIEW

2.1 Botanical background of *C. militaris*

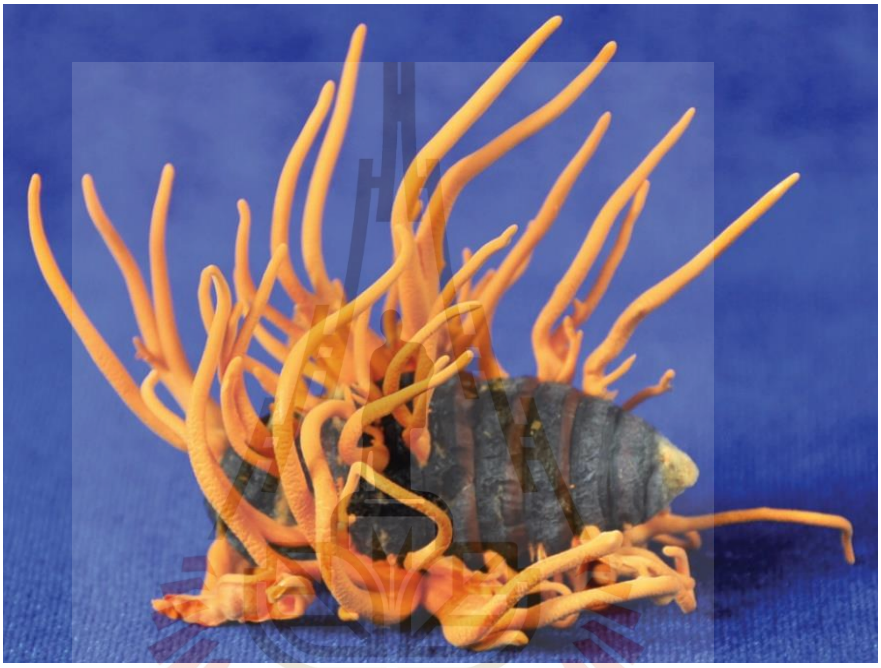


Figure 2.1 Morphology of *Cordyceps militaris* (Zheng et al., 2011).

C. militaris is a well-known Chinese medicinal mushroom used in pharmaceuticals for health and treatment. It is an entomopathogenic fungus that lives on the surface of arthropods Lepidopteron larvae and pupae during the winter and forms the fruiting body on pupae in the summer for reproduction (Tuli et al., 2014; Dong et al., 2015). Currently, 540 species are reported to be *Cordyceps*, which belongs to the class Ascomycetes, family Cordycipitaceae, and genus *Cordyceps*. *Cordyceps* is a medical herb that was discovered in Eastern Asian cultures (Wang et al., 2020). The species of *Cordyceps* are popular medical herbs, including *C. sinensis* (Ophiocordyceps sinensis) and *C. militaris* (Kunhorm et al., 2019). It biological and pharmacological properties have been continuously studied.

Classification of *C. militaris* was shown in Table 2.1 (Edited from Yang et al., 2020).

Table 2.1 Classification of *C. militaris*.

Classification	
Scientific name	*Cordyceps militaris*
Kingdom	Fungi
Phylum	Ascomycota
Class	Ascomycetes
Order	Hypocreales
Family	Cordycipitaceae
Genus	*Cordyceps*
Species	*militaris*

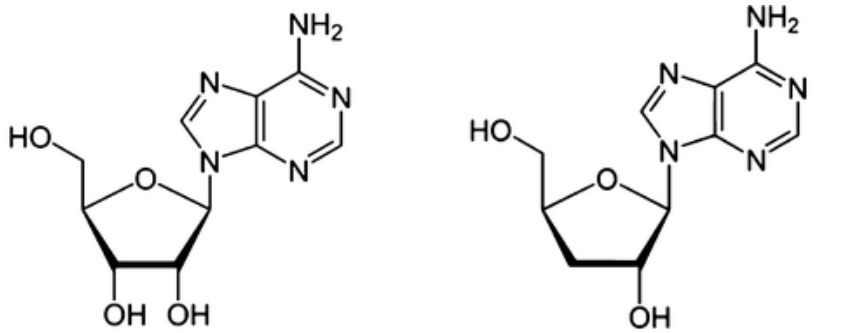
2.2 Chemistry of cordycepin

Cordycepin is a unique compound that is specifically produced by *Cordyceps* fungi, including *C. militaris*. It is a nucleoside analog of adenosine, with a lack of hydroxyl group at the 3′position of ribose (Tuli et al., 2013). Its chemical formula is $C_{10}H_{13}N_5O_3$. As mentioned, cordycepin is a water-insoluble organic compound and was first discovered by *C. militaris* in 1950 (Jedrejko et al., 2021). Although cordycepin can be naturally extracted from *Cordyceps* fungi, commercial uses of cordycepin are synthetic. Because the purification of natural cordycepin is difficult and the purity is low.

However, advances in extraction technology and cultivation techniques may make it possible to produce high purity cordycepin from Cordyceps fungi in the future. This would be beneficial for researchers and could potentially reduce the cost of cordycepin production for therapeutic use.

Table 2.2 Cordycepin molecular information.

Properties	Qualitative
Character (high purity at room temperature)	Crystal-like solid, colorless, odorless
Molecular weight	251.24 g·mol-1
Density	1.91 g/cm3
Melting Point	225 ℃
Boiling Point	627.2 ± 65.0 ℃ at 760 mmHg



(a) Adenosine ($C_{10}H_{13}N_5O_4$) **(b) Cordycepin ($C_{10}H_{13}N_5O_3$)**

Figure 2.2 The chemical structure of adenosine and cordycepin (Singpoonga et al. 2020).

2.3 Bioactive compound from *C. militaris*

C. militaris is known to contain a wide variety of bioactive compounds. In addition to cordycepin, *C. militaris* contains various bioactive compounds, such as adenosine, amino acids, cordycepic acid, ergosterol, mannitol, myriocin, fibrinolytic enzyme, lovastatin, carotenoid, cordyxanthins, and fatty acids (Das et al., 2010; Jedrejko et al., 2021; Liu et al., 2015). The nucleoside and polysaccharide are highly contained in *C. militaris* and have been studied for their biological activity in *vitro* and in *vivo*.

Research on the potential medicinal and nutritional benefits of *C. militaris* and its bioactive compounds is being conducted, and there is significant interest in their use in pharmaceutical industries. Some studies have suggested that *C. militaris* and its components may have anti-inflammatory, immunomodulatory, anti-tumor, and antioxidant effects, among others. Despite being intensively researched, the safe and efficacy of *C. militaris* as a medication and nutritional supplement are still under investigation.

Table 2.3 The list of two important bioactive compounds present in *C. militaris* and biological activity in pharmaceuticals.

Bioactive compound	Biological activity	References
Nucleosides (Cordycepin)	Anti-cancer	(Chaicharoenaudomrung et al., 2018)
	Anti-tumor and anti-metastatic	(Jin et al., 2018)
	Antioxidant	(Jaiboonma et al., 2020)
	Anti-inflammatory	(Choi et al., 2014)
	Anti-aging	(Zou et al., 2021)
	Hyperglycemia	(Ma et al., 2015; Yu et., 2016)
	Immunomodulatory	(Lee et al., 2020)
	Anti-microbial	(Jiang et al., 2013)
	Anti-fatigue	(Chai et al., 2022)
Polysaccharide	Antioxidant	(Miao et al., 2022)
	Anticancer	(Jing et al., 2014)
	Immunomodulatory	(Wang et al., 2012)
	Anti-inflammatory	(Lee et al., 2015)
	Anti-diabetic	(Zhu et al., 2016)
	Anti-hyperlipidemic	(Shang et al., 2020)

2.4 Biological activity of cordycepin

Cordycepin has been found to have many potential health benefits, including anti-cancer, anti-inflammatory, immunomodulatory, antioxidant, antimicrobial, anti-proliferative, and anti-metastatic effects. These effects are likely due to cordycepin's ability to influence many different signaling pathways in the body. Cordycepin has been shown to influence intracellular and extracellular signaling pathways in a variety of cells, tissues, and organ systems. It is associated with purine biosynthesis, DNA synthesis, and RNA synthesis, which are essential processes for cellular growth and replication (Tuli et al., 2016). Additionally, cordycepin has been found to inhibit the signal transduction of PI3K/mTOR/AKT and ERK signaling, which are pathways involved in cell growth and proliferation, and it is activated by the AMPK pathway, which is

involved in energy metabolism and cellular stress responses (Radhi et al., 2021). Overall, the ability of cordycepin to influence multiple signaling pathways and cellular processes may contribute to its diverse range of potential health benefits. However, more research is needed to fully understand the mechanisms behind these effects and to determine optimal dosages and methods of administration for therapeutic use. According to Brigham et al. (2013), the amount of cordyceps that should be consumed per day is 3 grams. Consumption of this magnitude falls within the safe limits for consumers.

2.4.1 Antioxidant

Research has shown that cordycepin extracted from *C. militaris* has antioxidant activity. In a study by He et al. (2013), cordycepin was found to have 2,2-diphenyl-1-picrylhydrazyl (DPPH) and •OH scavenging capacity comparable to that of vitamin C, indicating its potential as an antioxidant. Additionally, in a more recent study by Jaiboonma et al. (2020), cordycepin was investigated for its ability to prevent aging in human submandibular gland (HSG) cells by reducing ROS (reactive oxygen species) generation through upregulation of antioxidant genes such as Superoxide dismutase (SOD), Catalase (CAT), and Glutathione peroxidase (GPx). This suggests that cordycepin may have potential as an anti-aging agent through its antioxidant activity. It's important to note that while cordycepin has shown promising antioxidant activity in these studies, further research is needed to fully understand its mechanisms and potential as an antioxidant.

2.4.2 Anti-cancer

Cordycepin is a bioactive compound found in the fungus *C. militaris*. It has been extensively studied for its potential use as a chemotherapeutic agent due to its cytotoxicity and ability to induce apoptosis (programmed cell death) in cancer cells. Several studies have shown that cordycepin has promising anticancer effects, including the ability to inhibit cell proliferation, induce apoptosis, and suppress tumor growth in animal models. Cordycepin has also been found to sensitize cancer cells to chemotherapy and radiation therapy, potentially improving their effectiveness. While more research is needed to fully understand the mechanisms behind cordycepin's

anticancer effects and its potential as a natural drug for cancer patients, it is a promising area of study in the field of cancer research.

According to Chaicharoenaudomrung et al. (2018), they investigated the potential of cordycepin on human brain cancer cells, including SH-SY5Y and U251 cells. They found that cordycepin induced cytotoxicity in human brain cancer cells. Cordycepin can also induce oxidative stress and suppress antioxidant genes, including *GPX*, *SOD*, and *CAT*. Importantly, it can promote pro-apoptotic genes (*BAX*, *P53*, *Caspase-9*, and *Caspase-3*). Additionally, cordycepin shows autophagy activation by up-regulating *LC3I/II* gene. In addition, Schwenzer et al. (2021) found that the cordycepin-derived chemotherapy drug NUC-7738, which is an analogue of the natural nucleoside and bioactive component of the fungus *C. sinensis*, is 40 times more potent in killing cancer cells than the original compound. This research shows that cordycepin from *Cordyceps* is highly likely to be used as an anticancer drug for patients in the near future. In the near term, cordycepin from *Cordyceps* may be used as a therapeutic agent in cancer patients.

The study conducted by Zhou et al. on the effect of Jin Shui Bao (*Cordyceps*) on the immune system and quality of life in patients with advanced cancers. According to the study conducted by Zhou et al., which involved 36 patients with advanced cancer, Jin Shui Bao showed promising results. The researchers concluded that this product could restore cellular immunologic function and improve the quality of life for the patients involved in the study.

2.4.3 Anti-inflammation

Cordycepin has been found to have anti-inflammatory effects in various cell models, including macrophage cells, microglial cells, human OA chondrocytes, adipose tissue-derived mesenchymal stem cells, and cellosaurus cells. It has been shown to have protective effects against inflammatory injury in many diseases such as acute lung injury, asthma, rheumatoid arthritis, Parkinson's disease, hepatitis, atherosclerosis, and atopic dermatitis (Tan et al., 2020). Cordycepin has been found to inhibit the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as reduce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are important mediators of inflammation.

Additionally, cordycepin has been found to suppress the activation of nuclear factor-kappa B (NF-**κ**B), a transcription factor that plays a critical role in regulating the expression of pro-inflammatory genes (Phull et al., 2022). Overall, the anti-inflammatory properties of cordycepin make it a promising candidate for the treatment of various inflammatory diseases.

2.4.4 Anti-aging

Cordyceps is a type of fungus that has been traditionally used in traditional Chinese and Tibetan medicine for centuries. It has gained popularity in recent years due to its potential health benefits, including its potential effects on aging. While there is growing interest in the potential anti-aging properties of *Cordyceps*, it's important to note that many studies conducted on this topic are still in the preliminary stages, and further studies are needed to fully understand its effects on aging in humans. However, some studies have shown promising results and shed light on the potential mechanisms of action (Ji et al., 2009).

One area of interest is *Cordyceps* antioxidant properties. Oxidative stress, caused by an imbalance between free radicals and antioxidants in the body, is believed to play a role in aging and age-related diseases. *Cordyceps* contains various bioactive compounds that act as antioxidants and may help reduce oxidative stress, potentially slowing down the aging process. Another potential anti-aging mechanism of *Cordyceps* is its ability to modulate the immune system. As we age, our immune system tends to weaken, leading to increased susceptibility to infections and age-related diseases. *Cordyceps* has been found to possess immunomodulatory properties, meaning it can help regulate and enhance immune function, which may contribute to healthy aging.

Zou et al. (2021) investigated the effect of cordycepin on the restoration of damaged autophagy in aging cells. According to their research, Zou et al. discovered that cordycepin had an impact on cellular senescence and autophagy. They found that cordycepin promoted the activity of AMPK (adenosine monophosphate-activated protein kinase) and mTOR-p70S6K, which are signaling pathways involved in regulating cellular processes including autophagy and senescence. The study suggested that cordycepin's promotion of AMPK and inhibition of mTOR-p70S6K contributed to inhibiting premature cellular senescence. Cellular senescence refers to the state of

irreversible cell cycle arrest, and stress-induced senescence can occur due to various factors, including oxidative stress or DNA damage. Furthermore, the study highlighted the close relationship between stress-induced senescence and autophagy. Autophagy is a cellular process involved in the degradation and recycling of cellular components, including damaged proteins and organelles. Dysfunctional or impaired autophagy can contribute to cellular aging and the accumulation of cellular damage. They concluded that cordycepin has the potential to restore damaged autophagy in aging cells by modulating the AMPK and mTOR-p70S6K pathways, thereby inhibiting premature cellular senescence.

According to Chueaphromsri et al. (2023) investigated the effects of cordycepin, derived from *Cordyceps*, on anti-aging activities. The study found that cordycepin had several positive effects on maintaining the stemness of human mesenchymal stem cells (MSCs), which are a type of adult stem cells. It was observed that cordycepin enhanced the expression of SIRT1, a key anti-aging marker. Additionally, cordycepin contributed to delaying cellular senescence and aging of MSCs through multiple mechanisms. These mechanisms included enhancing autophagy, which is a cellular process that helps remove damaged components and maintain cellular health. Cordycepin also inhibited the activity of senescence-associated galactosidase, an enzyme associated with cellular aging. Furthermore, it helped maintain the proliferation rate of MSCs and increased telomere activity, which is associated with cellular longevity. These findings suggest that cordycepin from *Cordyceps* may have potential anti-aging properties and could play a role in maintaining the function and health of MSCs. However, it's important to note that further research is needed to validate these findings and explore the potential applications and safety of cordycepin in anti-aging therapies.

2.4.5 Immunomodulatory

Cordyceps has been traditionally believed to strengthen the immune system and improve overall health and vitality. It is often used in traditional medicine to support immune function and enhance the body's natural defenses against infections and diseases.

Ha et al. (2006) regarding the effects of *C. militaris* extract (CME) on macrophage and natural killer (NK) cell activity. According to their research, Ha et al. discovered that CME had several effects on immune cells. They found that CME enhanced nitric oxide (NO) generation by macrophages, which is an important immune response mediator. Additionally, CME activated macrophages and increased the expression of genes related to immune functions, such as IL-1 (Interleukin-1) and iNOS (inducible nitric oxide synthase). Furthermore, the study observed that CME increased NK cells and enhanced their cytotoxicity against YAC-1 cells, which are often used as target cells to assess NK cell activity. NK cells are a type of immune cell involved in recognizing and eliminating abnormal cells, including cancer cells. They concluded that CME could effectively modify the immune system and potentially be used as an herbal medication for immune system modulation and anti-cancer treatment.

Kang et al. (2015) reported on the effect of *C. militaris* extract (CME) on cell-mediated immunity in healthy Korean men. According to their research, Kang et al. extracted CME using ethanol and investigated its impact on immune function. They administered 1.5 g of CME per day to healthy volunteers over a period of 4 weeks. The study found that after the 4-week treatment (N = 39), levels of IL-2 (interleukin-2) and IFN-γ (interferon-gamma) were significantly increased compared to the placebo group (N = 40). IL-2 and IFN-γ are cytokines involved in promoting and regulating immune responses, particularly cell-mediated immunity. Furthermore, the study observed that CME increased NK (natural killer) cell activity and T cell proliferation. They suggested that CME could serve as a safe immunomodulator to enhance cell-mediated immunity.

2.4.6 Hyperglycemia

A study by Ma et al. (2015) investigated the effects of cordycepin from *C. militaris* in diabetic rats. According to their research, Ma et al. investigated the impact of cordycepin on diabetic rats. The rats were administered cordycepin at a concentration of 3,600 mg/day for 21 days. The study found that cordycepin treatment resulted in significant effects on blood glucose levels and hepatic glycogen. After the treatment period, there was a 47% reduction in blood glucose levels, indicating improved glucose control. Additionally, there was a 214% increase in hepatic glycogen, which suggests enhanced glycogen storage in the liver. These findings indicate that

cordycepin treatment may have beneficial effects in improving symptoms of metabolic syndrome associated with diabetes. Metabolic syndrome is a cluster of conditions, including high blood glucose levels, high blood pressure, abnormal cholesterol levels, and obesity, which collectively increase the risk of cardiovascular disease and diabetes. The study suggests that cordycepin may exert its effects by helping to control the absorption of glucose in the body. By reducing blood glucose levels and increasing hepatic glycogen storage, cordycepin may contribute to improved glucose metabolism and symptom management in diabetes.

Yu et al. (2016), investigated the effects of *C. militaris* powder (CMP) in rats with type 2 diabetic nephropathy. According to their research, Yu et al. investigated the impact of CMP on rats with type 2 diabetic nephropathy, a condition characterized by kidney damage due to diabetes. The study found that the administration of CMP resulted in several positive effects. One of the observed effects was the ability of CMP to lower blood glucose levels in the rats. This indicates an improvement in glucose control, which is beneficial for individuals with type 2 diabetes. Additionally, the study noted that CMP was able to restore kidney function in the rats with diabetic nephropathy. Diabetic nephropathy is a complication of diabetes that affects the kidneys and can lead to progressive kidney damage. The restoration of kidney function suggests a potential protective effect of *C. militaris* against diabetic nephropathy.

2.5 Biosynthesis of cordycepin

Cordycepin is an interesting molecule in *C. militaris* that has been studied extensively for its biosynthesis mechanism. Cordycepin is biosynthesized from two precursors, including 2′-carbonyl-3′-deoxyadenosine (2′-C-3′-dA) from adenosine pathway and adenosine-3′-monophosphate (3′dAMP) from adenosine diphosphate (ADP) pathway. Overgaard-Hansen (1964) has proposed that the biosynthesis of purine is crucial for the synthesis of cordycepin. Purine nucleotide was thus recognized as a crucial precursor for the synthesis of cordycepin in *C. militaris*. From adenosine, 2′-C-3′-dA is converted to cordycepin by oxidoreductase domain-containing protein (*cns1*), and 3′dAMP is converted to cordycepin by 5′-nucleotidase (NT5E) (Lin et al., 2016).

NT5E and cns1 are key enzymes in *C. militaris* that act as converters of intermediates to cordycepin. NT5E is involved in the conversion of AMP to adenosine,

which is reported to be an important precursor for cordycepin biosynthesis. According to Xia et al. (2017), the cns3 converts adenosine to 3′-dAMP, which is then dephosphorylated by the cns2 to 2′-C-3′-dA, which is then converted by the cns1 to cordycepin. In the future, studies on the mechanisms of cordycepin synthesis may be conducted to reveal more about them. Nitrogen sources have been reported to play an important role involving serial conversion in the cordycepin biosynthesis pathway (Chen et al., 2020).

Glucose is the starting material of cordycepin biosynthesis pathway and important precursor for the de novo purine nucleotide pathway (Zhang and Liu 2016). Glucose is converted to glucose-6-phosphate (G-6-P) and then can flux to ribose-5-phosphate (R-5-P) for substrate in de novo purine nucleotide pathway, and then R-5-P is metabolized to precursor for sequentially converted into phosphoribosyl pyrophosphate (PRPP), which can convert to inosine monophosphate (IMP). The de novo purine nucleotide related serial conversions of PRPP to IMP and nitrogen sources have been reported to have an important role in the purine pathway, in which glutamine, glutamate, glycine, and L-aspartate involve serial conversion as co-precursors in the cordycepin biosynthesis pathway (Oh et al., 2019; Chen et al., 2020). By PRPP is converted to ribosylamine-5-phosphat (Ribosylamine-5P) by amidophosphoribosyltransferase (*PurF*) which catalyzes of phosphoribosyl amine from PRPP and glutamate, then Ribosylamine-5P is converted to glycinamide ribonucleotide (GAR) by phosphoribosylamine--glycine ligase (*PurD*) which substrates of this enzyme are ATP, 5-phospho-D-ribosylamine and glycine, the products are ADP, phosphate, and N1-(5-phospho-D-ribosyl) glycinamide. GAR is converted to phosphoribosyl-N-formyl glycine amide (FGAR) by glycinamide ribonucleotide formyltransferase (*GAR FTase*), FGAR is converted to 5'-phosphoribosylformylglycinamidine (FGAM) by phosphoribosylformylglycinamidine synthase (*PurL*) that catalyzes the ATP-dependent conversion of formylglycinamide ribonucleotide (FGAR) and glutamine to products formylglycinamidine ribonucleotide (FGAM) and glutamate, FGAM is converted to aminoimidazole ribonucleotide (AIR) by phosphoribosylformylglycinamidine cyclo-ligase (*PurM*), AIR is converted to 5-aminoimidazole-4-carboxamide ribonucleotide (GAIR) by multifunctional protein ADE2 (*PAICS*), GAIR is converted to phosphoribosyl-aminoimidazole-succinocarboxamide (SAICAR) by phosphoribosyl-aminoimidazole-

succinocarboxamide synthase (*PurC*) that catalyze 5-amino-1-(5-phospho-D-ribosyl) imidazole-4-carboxylate, ATP and L-aspartate, SAICAR is converted to 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) by adenylosuccinate lyase (*PurB*), AIGAR is converted to 5-Formamidoimidazole-4-carboxamide ribotide (FAICAR) by bifunctional purine biosynthesis protein (*PurH*), which is subsequently converted to inosine monophosphate (IMP) by *PurH*, IMP is converted to adenylo-succinate by adenylosuccinate synthetase (*PurA*). Then it converted to adenosine monophosphate (AMP) by *PurB*, this final biosynthesis pathway is cordycepin (Lin et al., 2016). AMP is converted to adenosine diphosphate (ADP) by adenylate kinase (*AK*) and then ADP is converted to 3′-deoxyadenosine 5′-diphospate (3′-dADP) by ribonucleotide reductase (*RNR*), 3′-dADP is converted to 3′deoxyadenosine 5′-phosphate (3′-dAMP) by adenylate kinase (*AK*), 3′-dAMP is converted to cordycepin by 5′-nucleotidease (*NT5E*) from purine biosynthesis pathway (Kunhorm et al., 2019). In ADP pathway, adenosine monophosphate (AMP) is converted to ADP, and it is converted to 3′dAMP by enzyme ribonucleotide reductase (RNR). However, RNR was reported to not produce 3′-dADP in *C. militaris* and may be mediated by other enzymes (Kato et al., 2017). In *C. militaris*, the exact mechanism of cordycepin synthesis from the ADP precursor is still unclear.

Adenosine is a precursor for cordycepin, which adenosine is converted to adenosine-3′-monophosphate (3′-AMP) by *cns3*, and 3′-AMP is dephosphorylated to 2′-carbonyl-3′-deoxyadenosine (2′-C-3′-dA) by *cns2*, which is finally converted to cordycepin by *cns1* (Xia et al., 2017). From the biosynthesis of cordycepin, it can be concluded that the nitrogen source is extremely important in the production of cordycepin for *C. militaris* cultivation (Mao et al., 2005).

The 6-phosphofructokinase is a key enzyme that phosphorylates fructose 6-phosphate in glycolysis of *C. militaris* (Tang et al., 2018). The glucose-6-phosphate dehydrogenase is a key enzyme that catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate dehydrogenase in PPP, which is an important pathway for cordycepin biosynthetic and has high efficiency in *C. militaris* culture under hypoxia conditions (Suparmin et al., 2019). The isocitrate lyase is an essential enzyme in the glyoxylate cycle because it catalyzes the reversible formation of succinate and glyoxylate from isocitrate, which uses fatty acid substrates during growth. It also might trigger the catalysis of the initial reaction in the glyoxylate shunt to detoxify glyoxylate

during carbon consumption in *C. militaris* (Raethong et al., 2018). According to Zhang and Liu (2016), carbon sources serve as the initial building block for the de novo purine nucleotide pathway as well as the cordycepin biosynthesis.

The enzyme NADP-specific glutamate dehydrogenase (NADP-GDH) produces glutamate from **α**-ketoglutarate in *C. militaris* liquid surface culture. The product is then transferred into the TCA via the GABA pathway after GltS catalyzes the conversion of glutamate from **α**-ketoglutarate by glutamine, the reaction's nitrogen source. The AST can convert oxaloacetate to aspartate, and aspartate can also play an important intermediary role for SAICAR synthase (Suparmin et al. 2017). Hence, glutamate, glutamine, and aspartate are important precursors for serial conversion, and GltS, NADP-GDH, and AST are important enzymes to catalyze intermediation in amino acid metabolism to synthesize glutamate, glutamine, and aspartate as precursors in the cordycepin biosynthetic pathway. According to Lin et al. (2016), the de novo purine metabolism pathway necessitates some amino acids for catalyzed intermedia to cordycepin biosynthetic, such as glutamate, glutamine, glycine, and L-aspartate. The activation of amino acid conversion enhanced the synthesis of cordycepin substrate (Chen et al. 2020). The results might relate to the study of Kaushik et al. (2020), which found that the supplementation of amino acids (glycine, glutamine, and aspartate) in the culture medium enhances cordycepin production and significantly increases the expression of genes (*ADEK*, *RNR*, and *NT5E*) in the cordycepin biosynthetic pathway.

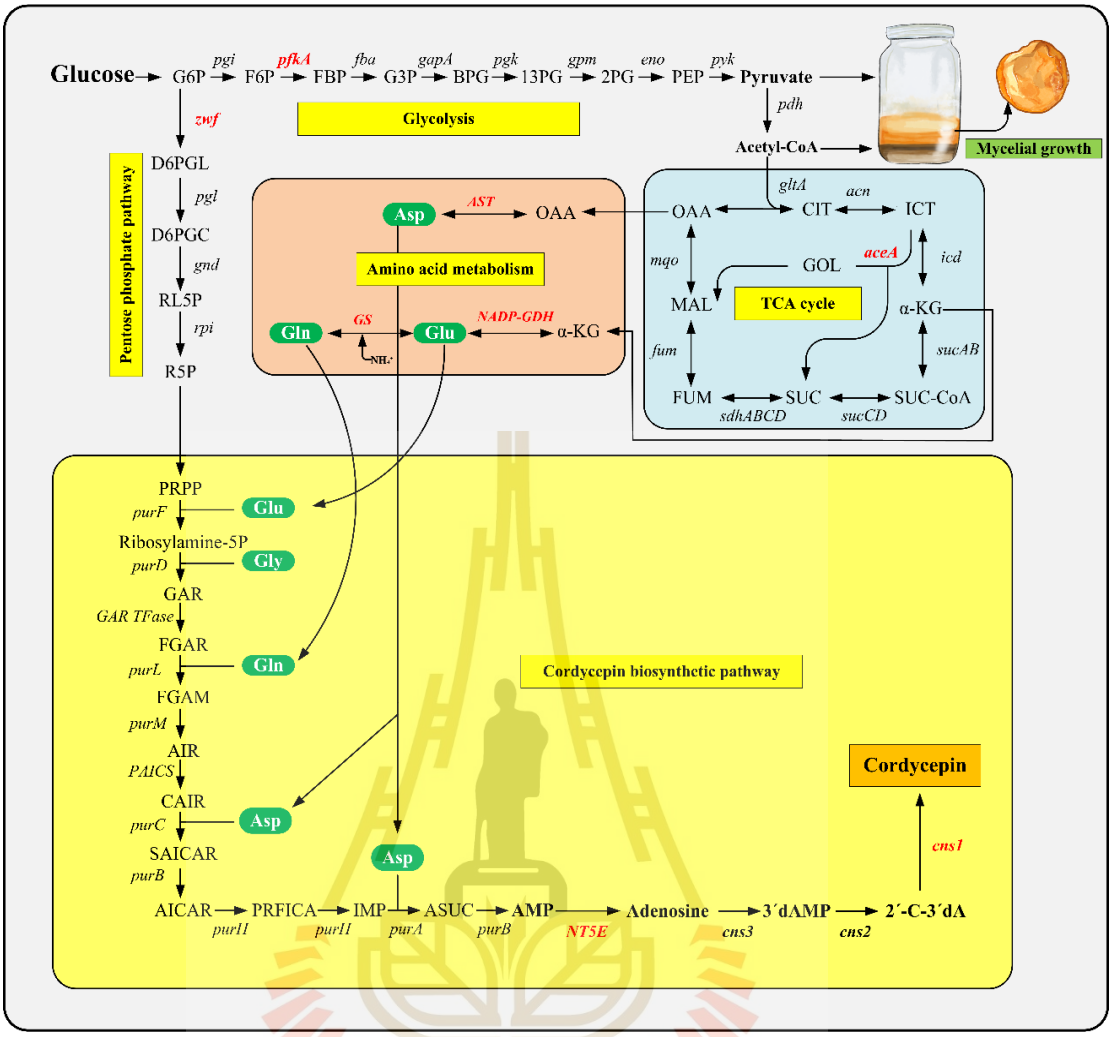


Figure 2.3 In liquid surface culture, *C. militaris* biosynthetic cordycepin by a de novo purine nucleotide pathway. There are descriptions of glycolysis, the pentose phosphate pathway, the TCA cycle, amino acid metabolism, and the cordycepin biosynthesis pathway.

2.6 Liquid surface culture

Liquid culture systems have been shown to be more efficient and effective for large-scale production of cordycepin. The solid culture method for cordycepin production has limitations in terms of its production capacity, cost, and time required for cultivation (Wen et al., 2014). Therefore, researchers have explored alternative methods to increase cordycepin production. One such method is liquid culture, which has shown promising results in terms of higher cordycepin yields and shorter cultivation times. Additionally, advances in biotechnology and genetic engineering have led to the

development of genetically modified strains of *C. militaris* with higher cordycepin production capacity.

Cordycepin production in liquid surface culture starts with the growth of aerial mycelium, which is supported by sufficient oxygen supply. The mycelium then goes through a period of differentiation, during which cordycepin is synthesized and secreted into the culture medium. The optimal cultivation conditions for cordycepin production in liquid culture vary depending on the strain of *C. militaris* and the specific medium used, but some general parameters include pH, temperature, and aeration rate (Suparmin et al., 2019). Oxygen is necessary for the growth and development of mycelium in the early stages of cultivation of *C. militaris*. During later stages, the mycelium synthesizes cordycepin, which is then secreted into the culture medium (Shih et al., 2007).

The liquid culture system for cordycepin production can be carried out using various methods, including liquid surface culture, submerged culture, and two-step (static/shaking) culture (Kunhorm et al., 2019). The liquid culture system for cordycepin production can be carried out using various methods, including liquid surface culture, submerged culture, and two-step (static/shaking) culture. Each method has its advantages and disadvantages, and the choice of method depends on factors such as the type of microorganism, the availability of equipment, and the desired yield of cordycepin. Liquid surface culture is a simple and cost-effective method that allows for the easy monitoring of the growth of mycelia and cordycepin production. Submerged culture involves growing the mycelium in a liquid medium, which provides better aeration and nutrient supply but requires more sophisticated equipment. Two-step culture involves growing the mycelium in a static culture followed by a period of shaking, which can increase the yield of cordycepin.

The study by Suparmin et al. (2017) found that the liquid surface culture of *C. militaris* resulted in a significant upregulation of genes associated with cordycepin biosynthesis, compared to submerged culture. This was attributed to the hypoxic conditions generated in the liquid surface culture, which were found to be optimal for cordycepin biosynthesis. Additionally, enzymes involved in purine nucleotide metabolism, such as PurA and PurC, were also upregulated in the liquid surface culture,

suggesting that this culture method can enhance the production of cordycepin in *C. militaris*.

In the experiment of Hung et al. (2009), they evaluated the effects of different strains of *C. militaris* on cordycepin production in liquid static culture. The strains BCC2816 and BCC2819 found to have the highest cordycepin production are 544.82 and 587.68 mg/L under temperature conditions at 25 °C, respectively. Additionally, Kang et al. (2014) optimized culture conditions for cordycepin production in *C. militaris* CGMCC2459. They found peptone (20 g/L); sucrose (24.7 g/L); $K_2HPO_4 \cdot 3H_2O$ (1.11 g/L); $MgSO_4 \cdot 7H_2O$ (0.90 g/L); VB_1 (10 mg/L); hypoxanthine (5.45 g/L); and L-alanine (12.23 g/L) to be the optimal culture media. And they cultured *C. militaris* in 700 mL of working volume in the 1000 mL glass jars under culture conditions at 25°C and cultivation time for 30 days and found the total content of cordycepin reached 1405.94 mg/bottle. Tang et al. (2018) investigated the different vegetable oils as the second carbon source in liquid static culture, and they found that adding 20 g/L of peanut oil enhanced cordycepin production to 5.29 g/L. It is important to note that the maximum cordycepin production reported by Das et al. (2009) is significantly higher than most other studies. While the liquid surface system is generally considered to be the best artificial method for *C. militaris* cultivation, the specific conditions used in this study may not be easily replicable and may require optimization for each individual production setup. It is also important to consider factors such as cost, scalability, and practicality when choosing a cultivation method for commercial production.

Table 2.4 Cordycepin production of *C. militaris* by liquid surface fermentation under different nitrogen source.

Strain	Nitrogen source	Culture conditions	Cordycepin production	References
C. militaris CGMCC2459	Peptone 20 g/L	Working volume 700 mL in 1000 mL glass jars, Temp 25°C for 35 days.	1405.94 mg/bottle	Kang et al., 2014
C. militaris NBRC 103752	Yeast extract 72.5 g/L	Working volume 100 mL in 500 mL Erlenmeyer flask, Temp 25°C for 15 days.	4.92 g/L	Suparmin et al., 2017
C. militaris NBRC103752-3	Yeast extract 72.5 g/L	Working volume 100 mL in 500 mL flask, Temp 25°C for 24 days.	312.15 g/L	Sari et al., 2016
C. militaris G81-3	Yeast extract 93.8 g/L	Working volume 150 mL, Temp 25°C for 20 days.	14.3 g/L	Masuda et al., 2014
C. militaris G81-3	Bacto yeast extract 45 g/L	Working volume 100 mL, Temp 25°C for 20 days.	3.1 g/L	Das et al., 2008
C. militaris CICC 14014	Tryptone 10 g/L, yeast extract 6 g/L	Working volume 100 mL, 250 mL flask, Temp 25°C for 20 days.	5.29 g/L	Tang et al., 2018
C. militaris SIP2	Peptone 20 g/L	Working volume 400 mL, 1,000 mL flask, Temp 25°C for 8 weeks.	2.835 g/L	Kunhorm et al., 2022

2.7 Nitrogen sources for cordycepin production

Nitrogen is a key element in amino acids, which are the building blocks of proteins and other important biomolecules. In microbial cultures, nitrogen sources are critical for cell growth and biosynthesis, as they provide the nitrogen required for the formation of new cells. Inorganic nitrogen sources, such as ammonium nitrate and urea, are often used in microbial cultures, as they are readily available and can be easily assimilated by microorganisms. Organic nitrogen sources, such as beef extract, casein, peptone, and yeast extract, can also be used and may provide additional nutrients that support microbial growth. The choice of nitrogen source can have a significant impact on the growth rate and productivity of microbial cultures, and selecting the appropriate nitrogen source is an important consideration in optimizing microbial culture conditions (Youcai and Tao 2021). Nitrogen is an essential nutrient for the growth and development of *C. militaris*, as well as for the biosynthesis of cordycepin. Nitrogen plays a crucial role in the production of purine nucleotides, which are the precursors for the synthesis of cordycepin. Therefore, the availability and type of nitrogen sources can affect the growth and cordycepin production of *C. militaris* (Vongsangnak et al., 2017).

Organic nitrogen sources are typically more effective at promoting cell growth and enzyme production compared to inorganic sources. This is because organic nitrogen sources, such as peptone and yeast extract, contain a variety of amino acids, peptides, and other growth factors that are readily assimilated by the mycelium. In contrast, inorganic nitrogen sources like ammonium nitrate and urea require additional energy and metabolic processes to convert into a form that can be used by the mycelium and may lead to decreased overall growth and enzyme production (Kumar et al., 2018). However, the optimal nitrogen source and concentration may vary depending on the specific strain of *C. militaris* being cultivated and the desired outcome of the production process.

Tuli et al. (2014) conducted a study to evaluate different nitrogen sources for cordycepin production in submerged fermentation of *C. militaris*. Among the tested

nitrogen sources, yeast extract was found to be the most suitable nitrogen source for cordycepin production, with a yield of 709 mg/L. In their study, Kang et al. (2014) optimized the cultivation conditions for *C. militaris* in a large-scale culture and found that the addition of peptone as a nitrogen source increased the production of cordycepin to 800 mg/L, which was higher than the production levels obtained using other nitrogen sources, such as yeast extract, beef extract, and malt extract. Suparmin et al. (2017) reported that in liquid surface culture, the up regulation of genes encoding glutamate synthase precursor was related to the cordycepin biosynthesis pathway. Glutamate, which is synthesized from α-ketoglutarate and glutamine, is an important nitrogen source that can be used in nucleotide biosynthesis, and aspartate, which can be synthesized from glutamate, is an important precursor for cordycepin biosynthesis. Peptone and yeast extract are rich in amino acids, which are essential for cell growth and metabolic processes. In particular, amino acids such as glutamate, glycine, and aspartic acid are important precursors for cordycepin biosynthesis (Lee et al., 2019).

Agricultural and food processing by-products such as soybean meal, corn steep liquor, and wheat bran are rich in organic nitrogen compounds and can be used as a low-cost and sustainable nitrogen source for microbial fermentation. These by-products not only reduce the cost of fermentation but also contribute to environmental sustainability by reducing waste. Furthermore, the use of these by-products can enhance the production of secondary metabolites such as cordycepin in *C. militaris* (Barrios-González, 2018).

2.8 Plant-base for nitrogen source

Amino acids such as glutamine, glycine, and aspartate are indeed important for the cordycepin biosynthesis pathway and purine synthesis. These amino acids play crucial roles in the metabolism of microorganisms, including *C. militaris*, and can impact the production of cordycepin (Kumar et al. 2019). Beans, peanuts, and seeds are indeed rich sources of essential nutrients, including amino acids, which play a crucial role in the growth and metabolism of microorganisms. Amino acids are the building blocks of

proteins and are essential for various cellular processes, including enzyme activity and the synthesis of important compounds like purines (Suribabu et al. 2014). Microorganisms, including fungi like *C. militaris*, require a balanced supply of amino acids to support their growth and various metabolic activities. The availability and composition of amino acids in the culture medium can have a significant impact on the growth, development, and production of desired metabolites, such as cordycepin. By incorporating bean, peanut, or seed extracts into the culture medium, you may be able to provide a rich source of amino acids and other nutrients that can support the growth and cordycepin production of *C. militaris*. Using vegetable seeds as a source of amino acids for cordycepin production in *C. militaris* offers potential advantages, as these amino acids are naturally synthesized by plants. The specific composition and concentration of amino acids in these extracts can vary depending on the type of seed or legume used. This aligns with the goal of using sustainable and plant-based nitrogen sources in fungal cultivation. It would be beneficial to explore the specific composition and concentration of amino acids in these seed extracts and their impact on cordycepin production. Further research and experimentation can help optimize the incorporation of vegetable seed extracts into the culture medium, enabling enhanced cordycepin production by *C. militaris*.

Song et al. (2018) investigated the effects of different nitrogen sources derived from seed meals on Pneumocandin B0 production by *G. lozoyensis* in submerged fermentation. They found that cotton seed powder was the most suitable nitrogen source for Pneumocandin B0 production, followed by cotton seed protein and corn meal. Soybean meal, on the other hand, was found to be less effective than yeast extract as a nitrogen source for Pneumocandin B0 production. This suggests that different types of seed meals may have varying effects on the production of secondary metabolites by microorganisms, including cordycepin production by *C. militaris*.

Li et al. (2021) focused on utilizing soybean curd residue waste materials as a solid medium for fermentation by *C. militaris* mycelium to produce crude polysaccharide. The study aimed to explore the potential of utilizing waste materials from soybean

curd residue as a sustainable and cost-effective substrate for *C. militaris* cultivation and polysaccharide production. The researchers investigated the biological activities of the crude polysaccharide produced in this process, including antioxidant, antitumor, and immunomodulatory activities. Polysaccharides derived from mushrooms, such as *C. militaris*, have gained attention due to their potential health benefits and therapeutic properties. They have been reported to possess various biological activities, including antioxidant effects, anti-tumor properties, and the ability to modulate the immune system.

By using soybean curd residue waste as a solid medium for *C. militaris* fermentation, Li et al. (2021). aimed to repurpose an agricultural waste product while simultaneously producing valuable crude polysaccharides. This approach aligns with the principles of sustainability and waste reduction. The results of their study could provide insights into the potential utilization of soybean curd residue waste as a substrate for *C. militaris* cultivation and the production of polysaccharides with bioactive properties. However, it's important to note that further research is usually required to evaluate the purification, characterization, and specific bioactivities of the crude polysaccharide product.

Vegetable seeds could potentially be a suitable nitrogen source for the cultivation of *C. militaris* in liquid surface culture to produce cordycepin.

Table 2.5 Amino acid composition of different legumes (g/100g).

Amino acid	Soybean	Mung bean	Black bean	Kidney bean	White bean	Sesame seed	Sunflower seed	Peanuts
Cysteine	1.33	0.5	-	1.0	0.56	2.06	1.48	1.55
Aspartic acid	11.70	8.53	9.58	8.4	7.62	9.88	10.33	10.68
Glutamic acid	16.54	12.54	14.15	10.2	8.42	16.54	13.98	16.92
Serine	5.49	3.85	3.57	3.1	2.65	6.62	4.04	4.79
Histidine	2.53	2.79	2.90	3.0	2.24	2.25	2.49	2.52
Glycine	4.18	3.22	3.25	3.6	2.99	2.06	4.13	4.32
Threonine	3.86	2.84	2.50	3.3	2.57	4.85	2.52	3.59
Methionine	1.26	1.25	1.29	1.0	0.99	1.87	1.22	1.03
Alanine	4.26	3.66	3.55	3.7	3.40	2.83	4.10	4.27
Arginine	7.23	6.44	6.43	6.1	5.16	7.45	6.97	6.84
Tyrosine	3.14	3.23	3.35	2.9	1.87	6.61	2.52	3.16
Tryptophan	1.28	0.64	0.76	-	0.57	1.25	-	0.94
Valine	4.80	4.63	4.55	5.1	4.32	5.54	4.39	3.89
Phenylalanine	4.94	5.80	5.67	5.2	3.85	6.34	5.05	4.19
Isoleucine	4.54	3.91	3.98	3.1	3.55	4.85	4.02	3.33
Leucine	7.78	7.40	7.41	7.9	5.64	7.57	6.70	6.85

Table 2.5 (continued).

Amino acid	Soybean	Mung bean	Black bean	Kidney bean	White bean	Sesame seed	Sunflower seed	Peanuts
Lysine	6.38	6.24	6.03	7.0	5.99	5.06	4.91	6.84
Proline	5.49	-	-	3.4	4.47	4.08	3.13	3.76
References	FAO 1992	Kudre et al., 2013	Audu et al., 2011	Sahasrabudhe et al., 1981	Fasuan et al., 2018	Akande et al., 2011	Leterme et al., 1990	16.92

Table 2.6 Proximate composition of different legume.

Type of plant	Crude protein (%)	Crude fat (%)	Crude Fiber (%)	Carbohydrate (%)	Ash (%)	Moisture (%)	References
Soybean	43.0	23.0	17.06	43	5.0	-	FAO 1992
Mung bean	26.30	1.40	12.56	68.39	3.91	-	Sahasakul et al., 2022
Kidney bean	23.00	1.38	20.93	70.48	5.13	-	
Black bean	29.17	1.72	21.83	58.12	3.06	8.27	Kotue et al., 2018
White bean	26.0	1.60	-	-	4.3	-	Sahasrabudhe et al., 1981
Sesame seed	22.65	56.9	4.3	8.8	3.2	3.15	Bwshaw et al., 2022
Sunflower seed	18.70	23.98	12.92	-	3.36	-	Akande et al., 2011
Peanuts	38.61	47.00	3.70	1.81	3.08	5.80	Atasie et al., 2009

2.9 References

Akande, K. E. (2011). Proximate and amino acid analyses of full-fat sunflower (Helianthus annuus L.) seed meal. Singapore J. Sci. Res. 1: 179-183.

Atasie, V. N., Akinhanmi, T. F., and Ojiodu, C. C. (2009). Proximate analysis and physico-chemical properties of groundnut (*Arachis hypogaea* L.). Pak. J. Nutr. 8(2): 194-197.

Barrios-González, J. (2018). Secondary metabolites production: physiological advantages in solid-state fermentation. Curr. Devel. Biotechnol. Bioeng. 257-283.

Beshaw, T., Demssie, K., Tefera, M., and Guadie, A. (2022). Determination of proximate composition, selected essential and heavy metals in sesame seeds (*Sesamum indicum* L.) from the Ethiopian markets and assessment of the associated health risks. Toxicol. Rep. 9: 1806-1812.

Brigham, A., Bryan, J. K., Ceurvels, J., et al. (2013). Cordyceps (*Cordyceps* spp.): An Evidence-Based Systematic Review by the Natural Standard Research Collaboration. Altern. Complement. Ther. 19(6): 326–336.

Chai, X., Pan, M., Wang, J., Feng, M., Wang, Y., et al. (2022). Cordycepin exhibits anti-fatigue effect via activating TIGAR/SIRT1/PGC-1α signaling pathway. Biochem. Biophys. Res. Commun. 637: 127-135.

Chaicharoenaudomrung, N., Jaroonwitchawan, T. and Noisa, P. (2018). Cordycepin induces apoptotic cell death of human brain cancer through the modulation of autophagy. Toxicol. In Vitro 46: 113-121.

Chen, B. X., Wei, T., Xue, L. N., Zheng, Q. W., Ye, et al. (2020). Transcriptome analysis reveals the flexibility of cordycepin network in *Cordyceps militaris* activated by L-alanine addition. Front. Microbiol. 11: 577.

Choi, Y. H., Kim, G. Y., and Lee, H. H. (2014). Anti-inflammatory effects of cordycepin in lipopolysaccharide-stimulated RAW 264.7 macrophages through Toll-like receptor 4-mediated suppression of mitogen-activated protein kinases and NF-κB signaling pathways. Drug Des. Devel. Ther. 8: 1941–1953.

Chueaphromsri, P., Kunhorm, P., Phonchai, R., Chaicharoenaudomrung, N., and Noisa, P. (2023). Cordycepin Enhances SIRT1 Expression and Maintains Stemness of Human Mesenchymal Stem Cells. In vivo 37(2): 596–610.

Das, S.K., Masuda, M., Hatashita, M., Sakurai, A., Sakakibara, M. (2008). A new approach for improving cordycepin productivity in surface liquid culture of *Cordyceps militaris* using high-energy ion beam irradiation. Lett. Appl. Microbiol. 47(6): 534-538.

Das, S.K., Masuda, M., Sakurai, A., and Sakakibara, M. (2009) Effects of additives on cordycepin production using a *Cordyceps militaris* mutant induced by ion beam irradiation. J. Biotechnol. 8(13): 3041-3047.

Das, S. K., Masuda, M., Sakurai, A., and Sakakibara, M. (2010). Medicinal uses of the mushroom *Cordyceps militaris*: current state and prospects. Fitoterapia 81(8): 961-968.

Dong, C., Guo, S., Wang, W., and Liu, X. (2015). Cordyceps industry in China. Mycology 6(2):121-129.

Fasuan, T. O., Gbadamosi, S. O., and Omobuwajo, T. O. (2018). Characterization of protein isolate from Sesamum indicum seed: In vitro protein digestibility, amino acid profile, and some functional properties. Food Sci. Nutr. 6(6): 1715–1723.

Ha, J. W., Yoo, H. S., Shin, J. W., et al. (2006). Effects of *Cordyceps militaris* extract on tumor immunity. Korean J. Oriental Med. 27(4): 12-29.

He, Y. T., Zhang, X. L., Xie, Y. M., Xu, Y. X., and Li, J. R. (2013). Extraction and antioxidant property in vitro of cordycepin in artificially cultivated *Cordyceps militaris*. Adv. Mat. Res. 750: 1593-1596.

Hung, L.T., Keawsompong, S., Hanh, V.T., Sivichai, S. and Hywel-Jones, N.L. (2009). Effect of temperature on cordycepin production in *Cordyceps militaris*. Thai J. Agric. Sci. 42(4): 219-225.

Jaiboonma, A., Kaokaen, P., Chaicharoenaudomrung, N., Kunhorm, P., Janebodin, K., Noisa, P., Jitprasertwong, P. (2020). Cordycepin attenuates salivary hypofunction through the prevention of oxidative stress in human submandibular gland cells. Int. J. Med. Sci. 17(12): 1733-1743.

Jedrejko K.J., Lazur, J., and Muszynska B. (2021). *Cordyceps militaris*: An Overview of Its Chemical Constituents in Relation to Biological Activity. Foods. 10(11): 2634.

Ji, D. B., Ye, J., Li, C. L., Wang, Y. H., Zhao, J., and Cai, S. Q. (2009). Antiaging effect of *Cordyceps sinensis* extract. Phytother. Res. 23(1): 116–122.

Jing, Y., Cui, X., Chen, Z., Huang, L., Song, L., Liu, T., et al. (2014). Elucidation and biological activities of a new polysaccharide from cultured *Cordyceps militaris*. Carbohydr. Polym. 102: 288-296.

Jiang, Q., Lou, Z., Wang, H., and Chen, C. (2019). Antimicrobial effect and proposed action mechanism of cordycepin against *Escherichia coli* and *Bacillus subtilis*. J. Microbiol. 57(4): 288–297.

Jin, Y., Meng, X., Qiu, Z., Su, Y., Yu, P., and Qu, P. (2018). Anti-tumor and anti-metastatic roles of cordycepin, one bioactive compound of *Cordyceps militaris*. Saudi J. Biol. Sci. 25(5): 991-995.

Kang, C., Wen, T. C., Kang, J. C., Meng, Z. B., Li, G. R., and Hyde, K. D. (2014). Optimization of large-scale culture conditions for the production of cordycepin with *Cordyceps militaris* by liquid static culture. Sci. World J. 2014.

Kang, H. J., Baik, H. W., Kim, S. J., Lee, S. G., et al. (2015). *Cordyceps militaris* enhances cell-mediated immunity in healthy Korean men. J. Med. food 18(10): 1164–1172.

Kato, T., Admad, S. and Park, E. (2017). Functional analysis of ribonucleotide reductase from *Cordyceps militaris* expressed in *Escherichia coli*. Appl. Biochem. Biotechnol. 182(4): 1307-1317.

Kim, S. W., Van Heugten, E., Ji, F., Lee, C. H., and Mateo, R. D. (2010). Fermented soybean meal as a vegetable protein source for nursery pigs: I. Effects on growth performance of nursery pigs. J. Anim. Sci. 88(1): 214-224.

Kotue, T. C., Jayamurthy, P., Nisha, P., Pieme, A. C., Kansci, G., Fokou, E., and Ashok, P. (2018). Proximate analysis and minerals of black bean seeds (*Phaseolus vulgaris* L.) used to manage sickle cell disease in west region of cameroon. People 6: 7.

Kudre, T. G., Benjakul, S., and Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. J. Sci. Food Agric. 93(10): 2429-2436.

Kumar, C.G., Sripada, S., and Poornachandra, Y. (2018). Status and Future Prospects of Fructooligosaccharides as Nutraceuticals. Role of materials science in food bioengineering: 451-503.

Kumar, V., Sharma, A., Kaur, R., Thukral, A. K., Bhardwaj, R., and Ahmad, P. (2017). Differential distribution of amino acids in plants. Amino Acids 49: 821-869.

Kumar, V., Sharma, A., Kohli, S.K., Yadav, P., Bali, S., Bakshi, P., Parihar, R.D., Yuan, H., Yan, D., He, Y., Wang, J., Yang, Y., Bhardwaj, R., Thukral, A.K. and Zheng, B. (2019) Amino acids distribution in economical important plants: a review. Biotechnol. Res. Innov. 3(2): 197-207.

Kunhorm, P., Chaicharoenaudomrung, N. and Noisa, P. (2019). Enrichment of cordycepin for cosmeceutical applications: culture systems and strategies. Appl. Microbiol. Biotechnol. 103: 1681-1691.

Lee, C.T., Huang, K.S., Shaw J.F., et al. (2020). Trends in the immunomodulatory effects of *Cordyceps militaris*: total extracts, polysaccharides and cordycepin. Front. Pharmacol. 11: 575704.

Lee, J. S., Kwon, D. S., Lee, K. R., Park, J. M., Ha, S. J., & Hong, E. K. (2015). Mechanism of macrophage activation induced by polysaccharide from *Cordyceps militaris* culture broth. Carbohydr. Polym. 120: 29-37.

Lee, S. K., et al. (2019). Improved cordycepin production by *Cordyceps militaris* KYL05 using casein hydrolysate in submerged conditions. Biomolecules 9(9): 461.

Leterme, P., Monmart, T., and Baudart, E. (1990). Amino acid composition of pea (*Pisum sativum)* proteins and protein profile of pea flour. J. Sci. Food Agric. 53: 107–110

Li, Y., Ban, L., Meng, S., Huang, L., Sun, N., Yang, H., Wang, Y. and Wang, L. (2021). Bioactivities of crude polysaccharide extracted from fermented soybean curd residue by *Cordyceps militaris*. Biotechnol. Biotechnol. Equip. 35(1): 334-345.

Lin, S., Liu, Z.Q., Xue, Y.P., Baker, P.J., Wu, H., Xu, F., et al. (2016). Biosynthetic pathway analysis for improving the cordycepin and cordycepic acid production in *Hirsutella sinensis*. Appl. Biochem. Biotechnol. 179(4): 633-649.

Liu, Y., Wang, J., Wang, W., Zhang, H., Zhang, X. and Han, C. (2015). The chemical constituents and pharmacological actions of *Cordyceps sinensis*. Evid-based Complement Altern. Med. 2015: 575063.

Ma, L., Zhang, S., and Du, M. (2015). Cordycepin from *Cordyceps militaris* prevents hyperglycemia in alloxan-induced diabetic mice. Nutr. Res. 35(5): 431-439.

Masuda, M., Das, S. K., Hatashita, M., Fujihara, S., and Sakurai, A. (2014). Efficient production of cordycepin by the *Cordyceps militaris* mutant G81-3 for practical use. Proc. Biochem. 49(2), 181-187.

Mao, X.B., Eksriwong, T., Chauvatcharin, S. and Zhong, J.J. (2005). Optimization of carbon source and carbon/nitrogen ratio for cordycepin production by submerged cultivation of medicinal mushroom *Cordyceps militaris*. Proce. Biochem. 40: 1667-1672.

Miao, M., Yu, W. Q., Li, Y., Sun, Y. L., and Guo, S. D. (2022). Structural elucidation and activities of *Cordyceps militaris*-derived polysaccharides: a review. Front. Nutr. 9: 898674.

Oh, J., Yoon, D.H., Shrestha, B., Choi, H.K., and Sung, G.H. (2019). Metabolomic profiling reveals enrichment of cordycepin in senescence process of *Cordyceps militaris* fruit bodies. J. Microbiol. 57(1): 54-63.

Overgaard-Hansen, K. (1964). The inhibition of 5-phosphoribosyl-1-pyrophosphate formation by cordycepin triphosphate in extracts of Ehrlich ascites tumor cells. Biochim. Biophys. Acta. 80: 504-507.

Phull, A. R., Ahmed, M., and Park, H. J. (2022). *Cordyceps militaris* as a bio functional food source: pharmacological potential, anti-inflammatory actions and related molecular mechanisms. Microorganisms 10(2): 405.

Radhi, M., Ashraf, S., Lawrence, S., Tranholm, A. A., Wellham, P. A. D., et al. (2021). A systematic review of the biological effects of cordycepin. Molecules (Basel, Switzerland). 26(19): 5886.

Raethong, N., Laoteng, K., and Vongsangnak, W. (2018). Uncovering global metabolic response to cordycepin production in *Cordyceps militaris* through transcriptome and genome-scale network-driven analysis. Sci. Rep. 8(1): 1-13.

Sahasakul, Y., Aursalung, A., Thangsiri, S., et al. (2022). Nutritional compositions, phenolic contents, and antioxidant potentials of ten original lineage beans in Thailand. Foods 11(14): 2062.

Sahasrabudhe, M. R., Quinn, J. R., Paton, D., Youngs, C. G., and Skura, B. J. (1981). Chemical composition of white bean (*Phaseolus vulgaris* L.) and functional characteristics of its air-classified protein and starch fractions. J. Food Sci. 46(4): 1079-1081.

Sari, N., Suparmin, A., Kato, T. et al. (2016). Improved cordycepin production in a liquid surface culture of *Cordyceps militaris* isolated from wild strain. Biotechnol. Bioproc. Eng. 21: 595–600.

Schwenzer, H., De Zan, E., Elshani, M., van Stiphout, R., Kudsy, M., et al. (2021). The Novel Nucleoside Analogue ProTide NUC-7738 Overcomes Cancer Resistance Mechanisms In Vitro and in a First-In-Human Phase I Clinical Trial. Clin. Cancer Res. 27(23): 6500–6513.

Shang, X. L., Pan, L. C., Tang, Y., Luo, Y., Zhu, Z. Y., Sun, H. Q., Meng, M., and Zhang, Y. M. (2020). 1H NMR-based metabonomics of the hypoglycemic effect of polysaccharides from *Cordyceps militaris* on streptozotocin-induced diabetes in mice. Nat. Prod. Res. 34(10): 1366–1372.

Shao, L. W., Huang, L. H., Yan, S., Jin, J. D., and Ren, S. Y. (2016). Cordycepin induces apoptosis in human liver cancer HepG2 cells through extrinsic and intrinsic signaling pathways. Oncol. Lett. 12(2): 995–1000.

Singpoonga, N., Rittiron, R., Seang-On, B., Chaiprasart, P., Bantadjan, Y. (2020). Determination of adenosine and cordycepin concentrations in *Cordyceps militaris* fruiting bodies using near-infrared spectroscopy. ACS Omega 5(42): 27235-27244.

Song, P., Yuan, K., Ji, X.J., Ren, L.J., Zhang, S., Wen, J.P. and Huang, H. (2018). Effects of cotton seed powder as the seed medium nitrogen source on the morphology and pneumocandin B_0 yield of *Glarea lozoyensis*. Front. Microbiol. 9: 2352.

Suparmin, A., Kato, T., Dohra, H., and Park, E. Y. (2017). Insight into cordycepin biosynthesis of *Cordyceps militaris*: Comparison between a liquid surface culture and a submerged culture through transcriptomic analysis. PLoS One 12(11): e0187052.

Suparmin, A., Kato, T., Takemoto, H. and Park, E.Y. (2019). Metabolic comparison of aerial and submerged mycelia formed in the liquid surface culture of *Cordyceps militaris*. MicrobiologyOpen 8: e00836.

Suribabu, K., Lalitha G.T., and Hemalatha K.P.J. (2014) Optimization of various nitrogen sources for the production of α-Amylase using *Brevibacillus borstelensis* R1 by submerged fermentation. Int. J. Curr. Microbiol. App. Sci. 3(4):791-800.

Tan, L., Song, X., Ren, Y., Wang, M., Guo, C., Guo, D., Gu, Y., Li, Y., Cao, Z., and Deng, Y. (2020). Anti-inflammatory effects of cordycepin: A review. Phytother. Res. 35(3): 1284-1297.

Tang, J., Qian, Z. and Wu, H. (2018). Enhancing cordycepin production in liquid static cultivation of *Cordyceps militaris* by adding vegetable oils as the secondary carbon source. Bioresour. Technol. 268: 60-67.

Tuli, H. S., Sandhu, S. S., and Sharma, A. K. (2014). Pharmacological and therapeutic potential of cordyceps with special reference to cordycepin. 3 Biotech 4: 1-12.

Tuli, H. S., Sharma, A. K., and Sandhu, S. S. (2014). Optimization of fermentation conditions for cordycepin production using *Cordyceps militaris* 3936. J. Biol. Chem. Sci. 1: 35-47.

Tuli, H.S., Sharma, A.K., Sandhu, S.S., and Kashyap, D. (2013). Cordycepin: A bioactive metabolite with therapeutic potential. Life Sci. 93(23): 863-869.

Vongsangnak, W., Raethong, N., Mujchariyakul, W., Nguyen, N. N., Leong, H. W., and Laoteng, K. (2017). Genome-scale metabolic network of *Cordyceps militaris* useful for comparative analysis of entomopathogenic fungi. Gene 626: 132-139.

Wang, M., Meng, X. Y., Le Yang, R., Qin, T., Wang, X. Y., et al. (2012). *Cordyceps militaris* polysaccharides can enhance the immunity and antioxidation activity in immunosuppressed mice. Carbohydr. Polym. 89(2): 461-466.

Wang, Y., Wang, R., Wang, Y., Li, Y., Yang, R. H., Gong, M., et al. (2020). Diverse function and regulation of CmSnf1 in entomopathogenic fungus *Cordyceps militaris*. Fungal Genet. Biol. 142: 103415.

Wen, T.C., Li, G.R., Kang, J.C., Kang, C., and Hyde, K.D. (2014) Optimization of solid-state fermentation for fruiting body growth and cordycepin production by *Cordyceps militaris.* Chiang Mai J. Sci. 41(4): 858-872.

Xia, Y., Luo, F., Shang, Y., Chen, P., Lu, Y. and Wang, C. (2017). Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. Cell Chem. Biol. 24(12): 1479-1489.

Yang, L., Li, G., Chai, Z., Gong, Q., and Guo, J. (2020). Synthesis of cordycepin: Current scenario and future perspectives. Fungal Genet. Biol. 143:103431.

Youcai, Z., and Tao, Z. (2021). Chapter 5 - combined anaerobic fermentation biohydrogen and biomethane production for sewage sludge and food waste. In Z. Youcai, and Z. Tao (Eds.). Biohydrogen production and hybrid process development: 311–444.

Yu, R., Song, L., Zhao, Y., Bin, W., Wang, L., et al. (2004). Isolation and biological properties of polysaccharide CPS-1 from cultured *Cordyceps militaris*. Fitoterapia 75(5):465-72.

Yu, S. H., Dubey, N. K., Li, W. S., Liu, M. C., et al. (2016). *Cordyceps militaris* treatment preserves renal function in type 2 diabetic nephropathy mice. PLoS One 11(11): e0166342.

Zhang, Q. and Liu, Y. (2016). The strategies for increasing cordycepin production of *Cordyceps militaris* by liquid fermentation. Fungal Genom. Biol.

Zheng, P., Xia, Y., Xiao, G., Xiong, C., Hu, X., Zhang, S., et al. (2012). Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. Genome Biol. 12: 1-22.

Zhu, Z. Y., Guo, M. Z., Liu, F., Luo, Y., Chen, L., Meng, M., Wang, X. T., and Zhang, Y. M. (2016). Preparation and inhibition on α-d-glucosidase of low molecular weight polysaccharide from *Cordyceps militaris*. Int. J. Biol. Macromol. 93(Pt A): 27–33.

Zuo, S. Q., Li, C., Liu, Y. L., Tan, Y. H., Wan, X., Xu, T., et al. (2021). Cordycepin inhibits cell senescence by ameliorating lysosomal dysfunction and inducing autophagy through the AMPK and mTOR–p70S6K pathway. FEBS Open bio. 11(10): 2705-2714.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

The *C. militaris* stain CM001 was purchased from Mushroom House at Phra Samut Chedi (Samut Prakan provinc, Thailand). For stock culture, *C. militaris* was stored on potato dextrose agar medium plate at 4°C. To grow *C. militaris* the fungal stock was subcultured and incubated at 25°C for 30 days.

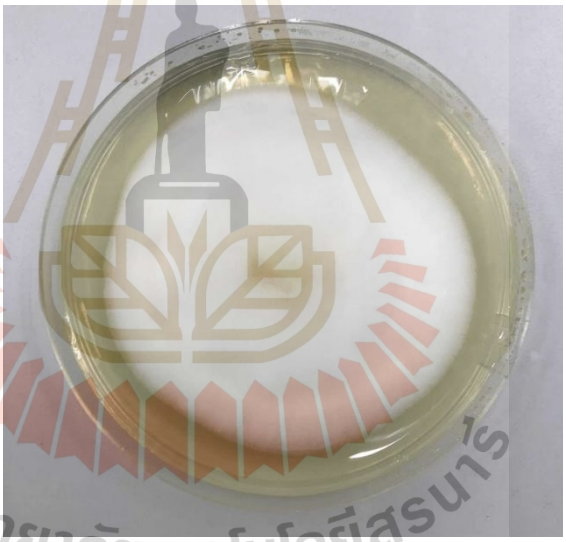


Figure 3.1 The *C. militaris* grown on PDA.

3.1.2 Vegetable seed as the nitrogen sources

Various vegetable seeds were obtained from department stores, including soybeans (Thai food industry (1964) CO., LTD, Bangkok, Thailand), mung beans, black beans, kidney beans, white beans, sesame seeds, peanuts (Thanya Farm Co., Ltd, Nonthabury, Thailand) and sunflower seeds.

3.2 Methods

3.2.1 Seed culture preparation and medium for *C. militaris*

The *C. militaris* incubated PDA was isolated to achieve conidia suspension. The supernatant was centrifuged at 10,000 rpm for 10 min. The final concentration of *C. militaris* used was 10^5 conidia/ml. Then, the conidia suspension was inoculated into 1,000 ml Erlenmeyer flask containing 600 ml of base seed culture media containing the following components; 24.7 g/L sucrose; 20 g/L peptone; 1.11 g/L KH_2PO_4; 0.9 g/L $MgSO_4 \cdot 7H_2O$; 10 mg/L vitamin B_1; and the pH was not adjusted and incubated on a rotary shaker incubator at 110 rpm for 7 days at 25°C (Kang et al., 2014). The seed culture preparation was inoculated into fresh culture medium for each experiment.

Figure 3.2 Seed culture of *C. militaris* after conidia inoculation at days 7.

3.2.2 Effect of cultural conditions (inoculum size, working volume, and cultivation time) of *C. militaris* in liquid surface culture

To obtain the optimum cultural conditions, each factor was performed to a completely randomized design (CRD). The levels of factors are as follows: the inoculum sizes were 5, 10, 15, and 20% v/v; the working volumes were 100, 150, 200,

and 300 ml; and the cultivation times were 10, 20, 30, and 40 days. There replicates have been performed for each treatment.

In the first experiment, a 480-ml jar with 100 ml of basal medium and 30 days of cultivation time were used to examine the inoculum size for the production of cordycepin and MDW. After obtaining the optimal inoculum size, the different working volumes were examined for cordycepin production. Finally, various cultivation times were investigated for cordycepin production by following the optimal inoculum size and working volume following the previous experiment.

3.2.3 Preparation of vegetable seed extract powder (VSEP)

Various vegetable seeds including SB: soybeans (Thai food industry (1964) CO., LTD, Bangkok, Thailand), MB: mung beans, BB: black beans, KB: kidney beans, WB: white beans, SS: sesame seeds, PN: peanuts (Thanya Farm Co., Ltd, Nonthabury, Thailand) and SF: sunflower seeds were obtained from department store. They were soaked in sterile DI water for 24 h, and then crushed to make them smaller. It was then boiled at 90°C for 15 min. The vegetable seeds were dried in a hot-air oven at 60°C for 3 days. The vegetable seeds were powdered using a powder grinder.

3.2.4 Effect of animal-free nitrogen source in liquid surface culture of *C. militaris*

To obtain the best animal-free nitrogen source derived from the vegetable seed, various VSEP conditions were supplemented at 20 g/L in a base culture medium free of nitrogen sources for the evaluation of cordycepin production and MDW.

After obtaining the best animal-free nitrogen source, the concentration was examined for high cordycepin production and MDW. The different concentrations, including 20–90 g/L, were supplemented into the nitrogen-free culture medium. The supplemented and non-supplemented peptone groups were used as positive control and negative control, respectively. The cultivation time is 30 days. Proximate composition was measured for the best VSEP by carrying out the experiment in accordance with the AOAC procedure (AOAC 1990). The Kjeldahl method was performed to measure protein content. The fat content was analyzed by the Soxtec 2050 Extraction Unit (Foss). Moisture and ash content were determined using a

Thermogravimetric Analyzer (TGA-701, LECO) and drying at 105 and 600 °C in a hot air oven, respectively. The fiber content was determined using Fibretherm-Automated Fibre Analysis (FT12, Gerhardt). Carbohydrate percentage was determined by calculation, following the formular below.

%Carbohydrate = 100% – (% moisture + % crude protein + % ash + % crude fat) Eq.(1)

3.2.5 Effect of animal-free nitrogen source on the transcriptional levels of *C. militaris* in liquid surface culture

Molecular mechanisms of the cordycepin production pathway, the carbon metabolic pathway, and amino acid metabolism were investigated through mycelium. The mycelium cultured in non-supplemented, peptone-supplemented, and animal-free nitrogen sources were collected after cultivation at 10, 20, and 30 days. The RNA was isolated, which was then converted to cDNA. The cDNA was investigated for gene expression involved in three significant pathways.

3.2.5.1 RNA isolation and cDNA synthesis

The mycelium fresh was collected to extract RNA using the NucleoSpin® RNA plant (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. The RNA concentration was measured using a microplate reader (BMG Labtech, Ortenbreg), and the ratio of absorbance at 260 nm to 280 nm was used to analyze RNA quality. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the ReverTra Ace® qPCR Master Mix kit (Toyobo., CO., LTD., Japan) following to the manufacturer's instructions. The cDNA was stored at -20°C for further use in quantitative real-time PCR.

3.2.5.2 Quantitative real-time PCR (qRT-PCR)

A quantitative real-time PCR (qRT-PCR) was used to determine the expression of key genes involved in carbon metabolism and cordycepin biosynthesis pathways. qRT-PCR was carried out on the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA). PCR reactions are prepared using SYBR® Green Master Mix (Thermo Fisher Scientific, Massachusetts, USA) with specific

quantitative real-time PCR primers in Table 3.2. Polyubiquitin binding protein (ubi) as the house-keeping gene was used as an internal control gene (Kunhorm et al. 2022). The thermal cycling conditions were as following follows: 95℃ for 1 min of denaturation, followed by 40 cycles of 30 s at 95℃, 30 s at 60℃, and 1 min at 72℃. The melting curve analysis was performed by heating at 95℃ for 15 s, 60℃ for 1 min, and 95℃ for 15 s. The biological replicates with two technical replicates were analyzed. The values of each gene expression were quantified by normalization with the ubi gene. The standard ΔCt method was calculated the values in the graph for relative fold change of non-supplemented as a negative control (Chen et al. 2011).

Table 3.1 Sequences of the primers used for the Real-Time PCR analysis.

Specific methabotic pathway	**Gene name**	**Primer**	**Sequence (5′- 3′)**
Housekeeping gene	Polyubiquitin binding protein (*ubi*)	Forward	GGTACATGGGCTACGGCTAC
		Reverse	TCCGACACAAACTCGTCCAG
Cordycepin biosynthesis pathway	Oxidoreductase domain-containing protein (*cns1*)	Forward	TCACGACCGCCGCACAATCC
		Reverse	CCAAGCCTGCTGGCACGGAG
	5′-nucleotidase (*NT5E*)	Forward	TGGACCTCACCATTCTGCAC
		Reverse	AGCTAGGCCGAAAAATCCCA
Carbon metabolic pathway	6-phosphofructokinase (*pfkA*)	Forward	GCTCTGAGCGCCTCTGTTAT
		Reverse	GCGCATCTTGAGCCATTCAG
	Glucose 6-phosphate dehydrogenase (*zwf*)	Forward	CAAGAAGTGCTGCTACCCCA
		Reverse	CTAGCCAGGTCCTTGCCAAA
	Isocitrate lyase (*aceA*)	Forward	CTCGCCAACGTTGACTACCT
		Reverse	CAATGTGAATACCGGCAGCG
Amino acid metabolism	Glutamate synthase (*GltS*)	Forward	TCGAGCACAACACAAACCTG
		Reverse	AGCCACTTCCTTGGTCGCA
	NADP-specific glutamate dehydrogenase (*NADP-GDH*)	Forward	GGCACCTCTTCCCATCACTC
		Reverse	TCTGGAGGATCTGGGTAGGC
	Aspartate transferase (*AST*)	Forward	TTGCCATTCTCCAGCGTTCA
		Reverse	GTCGATGATACGACCCGACA

3.2.6 Optimization of cultural conditions on cordycepin production by Response Surface Methodology (RSM) with the Box-Behnken Design (BBD)

After obtaining the best supplemental source of animal-free nitrogen for high cordycepin production of *C. militaris* in liquid surface culture, the cultural conditions were optimized. A statistical approach using BBD was conducted, which was a response to cordycepin production in the liquid surface culture. The three factors were significant variables for this study, including working volume (ml, X_1), inoculum size (%, X_2), and cultivation time (days, X_3), with each factor at three different levels, as shown in Table 3.3. The total BBD matrix of the experiment of 15 trials was given by a set of points at the midpoint of each edge of a multidimensional cube and three replications of the center points. All experiments were performed in triplicate. The value of cordycepin production was taken as response (Y), and experimental results were fitted with a multiple regression analysis, which was explained by a second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{i<j}^{4} \beta_{ij} x_i x_{ij}, \qquad \text{Eq.(2)}$$

Where Y is the predicted response (cordycepin production in this study, g/L, β_0, β_i, β_{ii} and β_{ij} constant coefficients, and X_i and X_j are the coded independent variables or factors. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was analyzed by F-test. The significance of the regression coefficients was tested by the t-test. Design-Expert Version 12 software package (StatEase Inc., Minneapolis, USA) was used for designing experiments as well as for regression and graphical analysis of the experimental data obtained.

Table 3.2 The result of Box-Behnken design (BBD) with experimental and predicted values of cordycepin production and experimental coded and level of the independent variables.

RUN	Independent variables (Coded)			Independent variables (actual)			Dependent variables
	X_1	X_2	X_3	X_1	X_2	X_3	*Y*: Cordycepin (g/L)
1	0	-1	-1	200	5	20	1.11
2	-1	0	-1	100	10	20	1.72
3	1	0	-1	300	10	20	1.55
4	0	1	-1	200	15	20	1.29
5	-1	-1	0	100	5	40	2.55
6	1	-1	0	300	5	40	2.05
7	0	0	0	200	10	40	2.49
8	0	0	0	200	10	40	2.37
9	0	0	0	200	10	40	2.48
10	-1	1	0	100	15	40	2.70
11	1	1	0	300	15	40	2.02
12	0	-1	1	200	5	60	0.26
13	1	0	1	300	10	60	0.18
14	-1	0	1	100	10	60	0.88
15	0	1	1	200	15	60	0.30

Independent variables (coded)	Levels		
	-1	0	1
X_1: Working volume (ml)	100	200	300
X_2: Inoculum size (%)	5	10	15
X_3: Cultivation time (days)	20	40	60

3.2.7 Analyses

3.2.7.1 HPLC analysis

A high-performance liquid chromatography (HPLC) is used to measure the concentration of cordycepin in a culture medium. The first step in the method involves filtering the culture medium through a cellulose acetate filter paper with a pore size of 0.22 µm. Then, the filtrate was proceeded for HPLC analysis. The HPLC system used in this method is an Agilent 1100 series, and the column used is a Zobax stable bound C18 with dimensions of 4.6 x 250 mm and a 3.5 µm particle size. The mobile phase used in the chromatography is a gradient of DI water (A) and methanol (B), as specified in Table 3.1. The flow rate of the mobile phase is 0.5 ml per min, and the column temperature is maintained at 30°C. Finally, the concentration of cordycepin in the culture medium is determined using a Diode Array Detector (DAD) set to a wavelength of 260 nm, and the result is reported in units of g/L.

Table 3.3 Shows the condition of the HPLC used in cordycepin analysis.

Time	Mobile phase A (DI type I)	Mobile phase B (Methanol)
0	85	15
5	85	15
10	80	20
15	60	40
20	40	60
25	20	80
30	0	100

3.2.7.2 MDW analysis

Mycelial dry weight (MDW) was determined by drying it in a hot air oven at 60°C for 3 days. A 4 digital weighing balance (Sartorius, Göttingen, Germany) was used to weigh the mycelial.

3.2.8 Statistical analysis

Data was presented as the means of triplicate experiments, and the error bars indicated the corresponding standard deviation (SD). All analyses were performed using the IBM SPSS Statistics version 26 software packet (IBM Corp., Armonk, N.Y., USA). The statistical significance was calculated by one-way or two-way analysis of variance (ANOVA) followed by Turkey's test ($p > 0.05$) to assess the difference between treatment groups and their respective controls.

3.3 References

AOAC (1990) Official methods of analysis, 15th ed.; AOAC International: Washington, DC, USA.

Chen, D., Pan, X., Xiao, P., Farwell, M.A. and Zhang, B. (2011) Evaluation and identification of reliable reference genes for pharmacogenomics, toxicogenomics, and small RNA expression analysis. J. Cell. Physiol. 226(10): 2469-2477.

Kang, C., Wen, T. C., Kang, J. C., Meng, Z. B., Li, G. R., and Hyde, K. D. (2014). Optimization of large-scale culture conditions for the production of cordycepin with *Cordyceps militaris* by liquid static culture. Sci. World. J. 4:1–15.

Kunhorm, P., Chueaphromsri, P., Chaicharoenaudomrung, N., and Noisa, P. (2022). Enhancement of cordycepin production from *Cordyceps militaris* culture by epigenetic modification. Biotechnol. Lett. 44(4): 581-593.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Effect of cultural conditions (inoculum size, working volume, and cultivation time) on cordycepin production and MDW

The optimal culture conditions, including inoculum size, working volume, and cultivation time, were evaluated for high cordycepin production and MDW in the liquid surface culture of *C. militaris*. The results found that 10% (v/v), 100 mL, and 30 days were required, respectively. The effect of inoculum size (5, 10, 15, and 20% v/v) is present in Figure 4.1A, where the maximum cordycepin production and MDW at 10% (v/v) inoculum were 2.83 ± 0.07 g/L and 1.83 ± 0.07 g, respectively, and were significantly higher than other conditions. The effect of working volume (100, 150, 200, 250, and 300 ml) is shown in Figure 4.2B. The production of cordycepin was maximized with 100 mL of culture medium that reached 2.96 ± 0.16 g/L. However, a high MDW of about 2.34 ± 0.05 g was obtained in 300 mL of culture. The results of cultivation times (10, 20, 30, and 40 days) were displayed in Figure 4.1C. The highest cordycepin production and MDW were obtained 30 days after seed culture, which reached 2.82 ± 0.12 g/L and 1.83 ± 0.07 g, respectively. At 40 days, the production of cordycepin significantly decreased as time passed.

Liquid culture was applied for hyperproduction of cordycepin, and submerged fermentation could enhance cordycepin production by about 15 – 30% (Xian et al., 2004). Working volume is related to dissolved oxygen concentration, and hypoxia conditions are essential for cordycepin biosynthesis (Suparmin et al., 2017). The low working volume was suggested for higher cordycepin production (Masuda et al., 2006). Inoculum size is significant for *C. militaris* cultivation, and 10% (v/v) was suggested for higher cordycepin production in the fermentation condition (Tuli et al., 2014). The cultivation time affected the production capacity of cordycepin and was related to glucose consumption (Das et al., 2010). *C. militaris* cell growth began with glucose

consumption, followed by cordycepin production, and ended cordycepin production when all the glucose and nitrogen were consumed (Masuda et al., 2007). Proteins in soybean are essential for enzyme activity and influence purine synthesis, which is an important metabolite for cordycepin production (Suribabu et al., 2014).

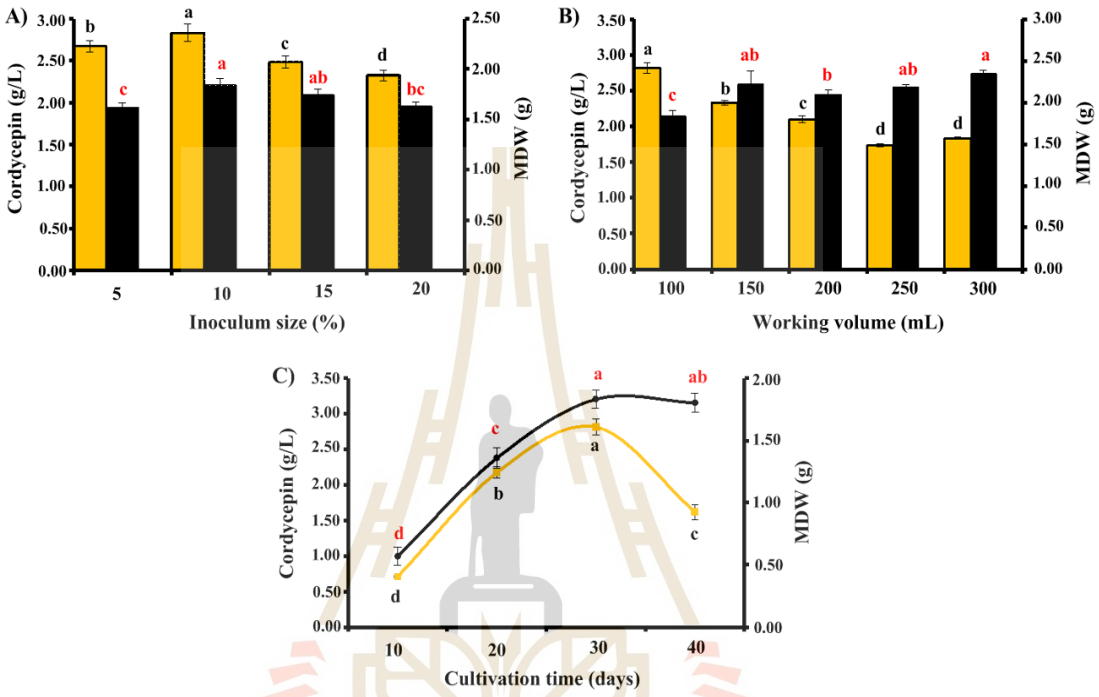


Figure 4.1 Effect of various conditions including inoculum size (A), working volume (B), and cultivation time (C) on cordycepin production and MDW of *C. militaris* in liquid surface culture. Bar charts were expressed as mean ± SD from three independent experiments; different superscripts represent significant ($p > 0.05$). Cordycepin; MDW; Cordycepin; MDW.

4.2 Effect of VSEP as the nitrogen source on cordycepin production

To obtain the best VSEP for use as the supplemented nitrogen source in *C. militaris* culture for high cordycepin production. For this experiment, the VSEP and peptone conditions were supplemented at a concentration of 20 g/L. The various VSEP were supplemented to a nitrogen source-free culture, with the peptone-supplemented nitrogen source serving as a positive control and the non-supplemented nitrogen source serving as a negative control. Cultivation time is for 30 days. As shown in Figure 4.2, our results showed that supplementation with soybean seed extract powder (SBEP)

significantly increased the cordycepin production by 0.36 g/L which was the highest value among these conditions. However, MDW was not significantly different.

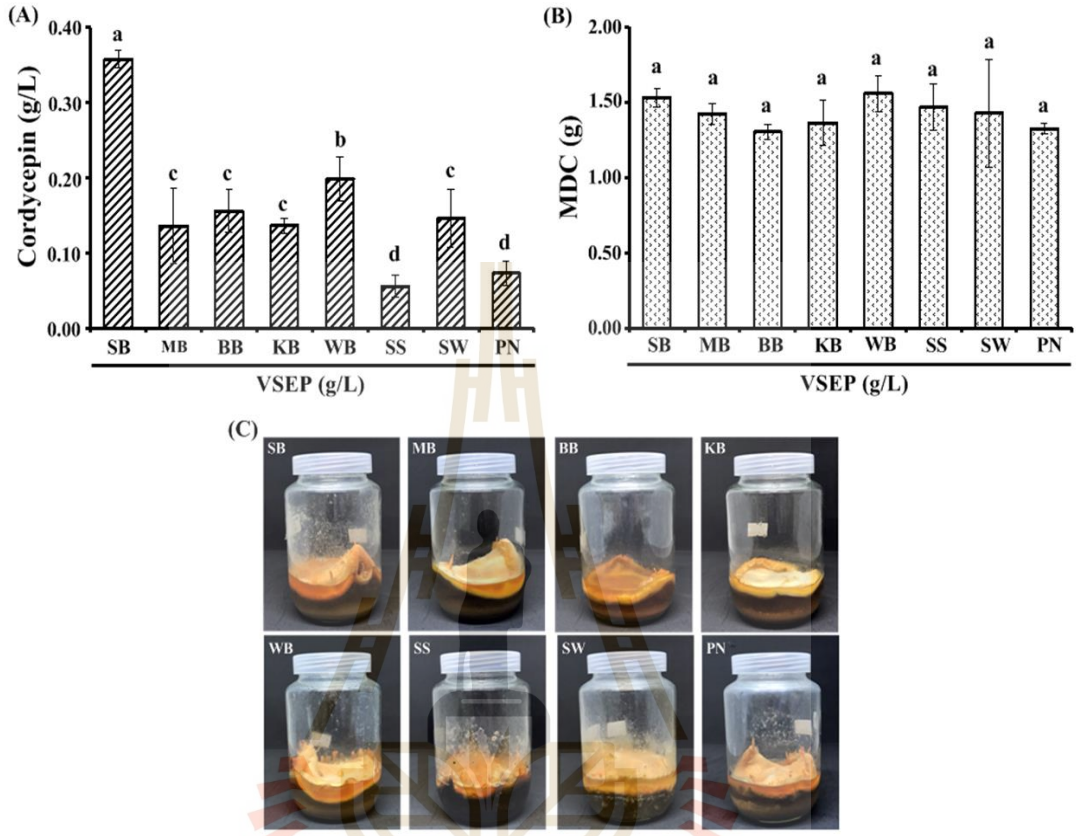


Figure 4.2 The effect of different VSEP nitrogen sources on (a) production of cordycepin and (b) MDW of *C. militaris* in liquid surface culture. (c) The morphology of *C. militaris* under different VSEP nitrogen sources at 20 g/L for 30 days. The value was represented as the mean ± SD (n = 3), where columns with different superscripts represent significant ($p > 0.05$). SB: Soybeans; MB: Mung beans; BB: Black beans; KB: Kidney beans; WB: White beans; SS: Sesame seeds; SF: Sunflower seeds; PN: Peanuts.

Prevously, Xiao et al. (2007) optimized molasses plus soybean meal hydrolysis medium for ehnahed actonin production from *Bacillus subtilis* CICC 10025 in fermentation, molasses and soybean meal hydrolysis were demonstrated to be more productive than pure sucrose and yeast extract plus peptone, respectively. According to Shariati et al. (2019) reported that soybean powder is the most effective nitrogen source on carotenoid production of *Blakeslea trispora* culture. Possibly, vegetable seed

might be used as a supplementary nitrogen source for high cordycepin production. More importantly, it may also replace peptone, which is a commercial nitrogen source for the cultivation of *C. militaris*. In this study, we found that SBEP supplementation as an animal-free nitrogen source has a high potential for *C. militaris* in liquid surface culture for cordycepin production.

Vegetable seed extract is a meat-free alternative to traditional peptones, and the nitrogen source is involved in de novo purine synthesis, which is an important pathway for cordycepin synthesis (Vongsangnak et al., 2017). Carbohydrates, lipids, vitamins, minerals, and amino acids are all found in vegetable seeds that are essential for enzyme activity and influence purine synthesis (Suribabu et al., 2014). Moreover, de novo purine biosynthesis is considered an important metabolic pathway for cordycepin production. The amino acid is a protein constituent which contains several amino acids required for cordycepin biosynthesis pathways, such as glutamine, glycine, and aspartate. These amino acids can be synthesized in plants (Kumar et al., 2019). Vegetable seeds, including soybean, mung bean, peanut, sesame, sunflower, black bean, kidney bean, and white bean, are an economic crop in the world, and they have a high protein content (Kumar et al., 2017). In a previous stulozoyensis and (2018) they investigated the effects of different seed medium nitrogen sources, including soybean meal, cotton seed powder, cotton seed protein, corn meal, soy peptone, tryptone, and yeast extract on pneumocandin B_0 production of G. lozoyensis, and found that cotton seed powder had higher pneumocandin B_0 production than yeast extract, and soybean meal was lower than yeast extract. In addition of protein in plant, the plant oil and fatty acids are stimulated on the exo-biopolymer production of *C. militaris* culture (Park et al., 2002).

4.3 Effect of various concentration of SBEP on cordycepin production

To obtain an optimal concentration of SBEP for high cordycepin production, The various concentrations, including 20 – 90 g/L, were supplemented into the culture medium free of nitrogen source. Cultivation time is for 30 days. The supplemented and non-supplemented peptone groups were positive control and negative control, respectively. The results showed that supplementing with SBEP at 80 g/L and 90 g/L significantly increased cordycepin production more than supplementing with peptone

up to 2.52 g/L and 2.58 g/L, respectively. The MDW production was increased up to 2.33 g and 2.39 g, respectively, as displayed in Figure 4.3A-B. However, this study focused on the optimal concentration of SBEP-supplemented on cordycepin production. Although the 90 g/L supplementation produced the highest cordycepin production, there was not significantly different from the 80 g/L supplementation. Therefore, a supplementation of 80 g/L concentration was selected in this study. It is also used in smaller amounts and has the same efficiency in the production of substances.

This finding is relevant to another study, in which Li et al. (2021) reported that using the soybean curd residue on *C. militaris* culture could produce edible mushrooms and have high medical efficiency. Nitrogen sources are considered important nutritional sources and have been used in large quantities for the production of cordycepin. Moreover, using commercial nitrogen sources is costly. SBEP is considered a premium product for replacing peptone as a nitrogen source in the cordycepin production of *C. militaris* in liquid surface culture. More importantly, SBEP is approximately 113.92 times cheaper than using peptone as a nitrogen source. The nutritional content of SBEP includes 37.0% protein, 20.9% fatty acids, 4.1% ash, 7.0% fiber, and 35.2% carbohydrate, as demonstrated in Table 4.1. In this study, it was found that supplementing with 80 g/L of SBEP as the nitrogen source also reduced costs by 3.51% when compared to peptone-supplemented at 20 g/L and increased cordycepin by up to 1.11 times. A previous study (Kunhom et al., 2022) used the strategy of epigenetic modification to enhance cordycepin production. However, such strategies have used chemicals to enhance cordycepin production, which may also increase the production cost. This study used the SBEP supplementation strategy of nutritional composition in culture medium, which may act as a precursor substance to the cordycepin biosynthetic pathway. The results found increased cordycepin production by a similar amount to the previous study. Therefore, SBEP supplementation demonstrably enhances the efficacy of cordycepin production, which is comparable to the use of epigenetic compounds and is also less expensive.

Table 4.1 Proximate composition from soybean seed extract powder (SBEP)

Composition	SBEP (%)
Protein	37.0
Fat	20.9
Ash	4.1
Moisture	2.8
Fiber	7.0
Carbohydrate	35.2

4.3.1 Time course of *C. militaris* in animal-free culture medium

After supplementation with 80 g/L of SBEP, the time course of cordycepin production was also examined. As shown in Figure 4.3D, the highest cordycepin production was discovered at 40 days, progressively declining to 45–60 days after inoculation.

Following the degradation of the primary carbon source in the culture medium, cordycepin may serve as a secondary source of carbon. The length of cultivation had an impact on cordycepin production capacity and was connected to glucose intake (Das et al., 2009). Consumption of glucose and production of cordycepin started cell growth, which ceased when all the available glucose in the culture medium was used (Masuda et al., 2007).

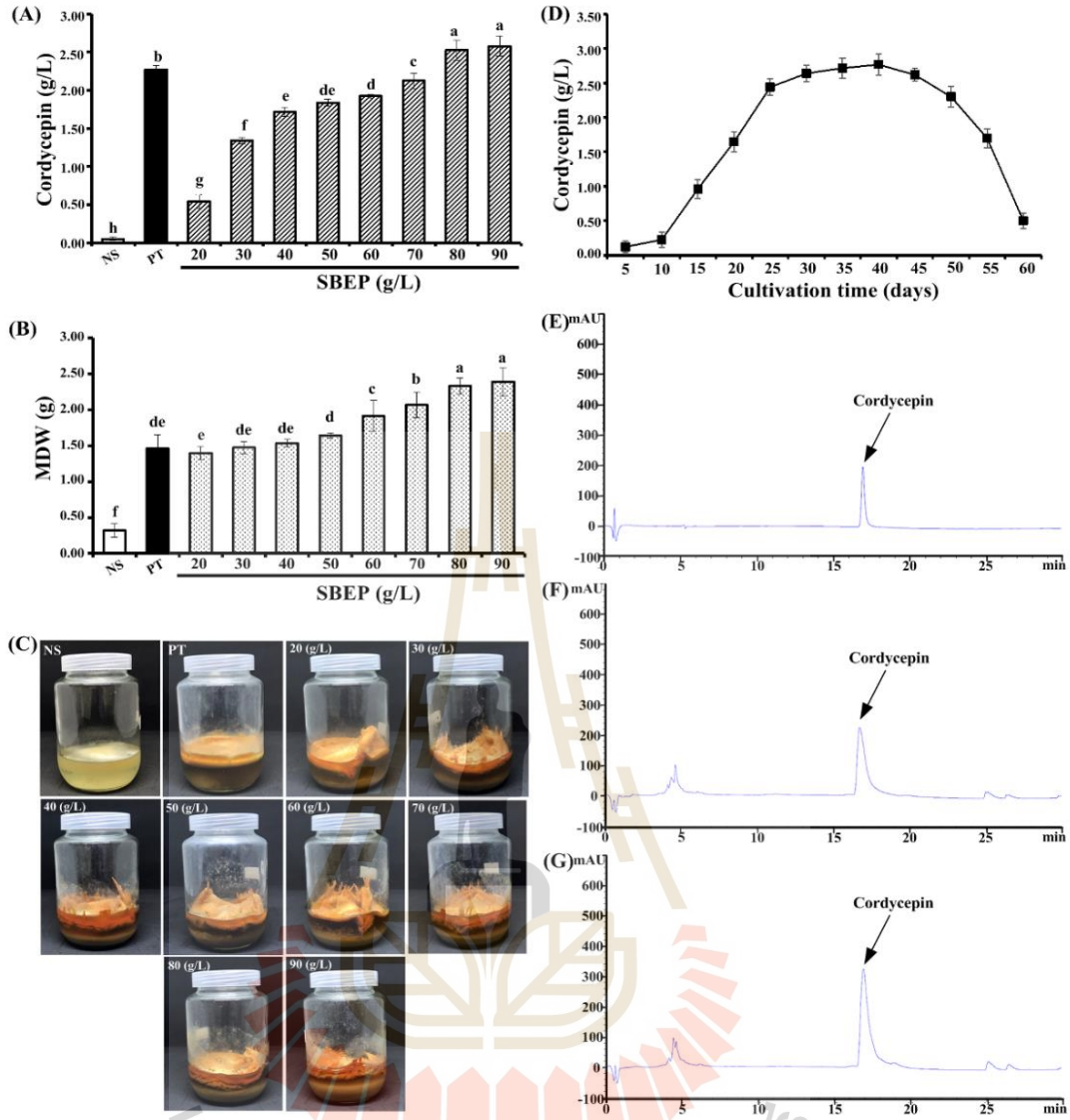


Figure 4.3 The various concentration of soybean seed extract powder (SBEP) on (A) cordycepin production and (B) MDW of *C. militaris* in liquid surface culture. (C) The morphology of *C. militaris* under different concentration of SBEP nitrogen sources in liquid surface culture for 30 days. (D) Time courses of ■ cordycepin production after using SBEP-supplemented. (E) HPLC chromatogram of cordycepin standard, (F) peptone-supplemented as a positive control, and (G) SBEP-supplemented at 80 g/L. The value was represented as the mean ± SD (n = 3), where columns with different superscripts represent significant ($p > 0.05$). NS: non-supplemented; PT: peptone-supplemented.

4.3.2 Comparison of animal-free nitrogen source between SBEP and soy protein isolated on cordycepin production

After obtained 80 g/L of SBEP as the animal-free nitrogen source for *C. militaris* by liquid surface culture. This experiment compares SBEP with soy protein isolated on cordycepin production. The experiment included five conditions: 1) a control with no added nitrogen source, 2) peptone as a nitrogen source, 3) SBEP at a concentration of 80 g/L, and 4) different concentrations of soy protein isolate ranging from 20-80 g/L. The experiment was carried out at a temperature of 25°C for a total of 40 days, under light conditions.

The results showed that the addition of soy protein isolate at different doses resulted in lower cordycepin production compared to 80 g/L of SBEP, which is an animal-free nitrogen source for the cultivation of *C. militaris* to produce cordycepin, as shown in Figure 4.4. These results suggest that SBEP may be a more effective nitrogen source than soy protein isolate for cordycepin production in liquid surface culture with *C. militaris*. The fact that SBEP is an animal-free nitrogen source also has important implications for the sustainability of using *C. militaris* as a source of cordycepin.

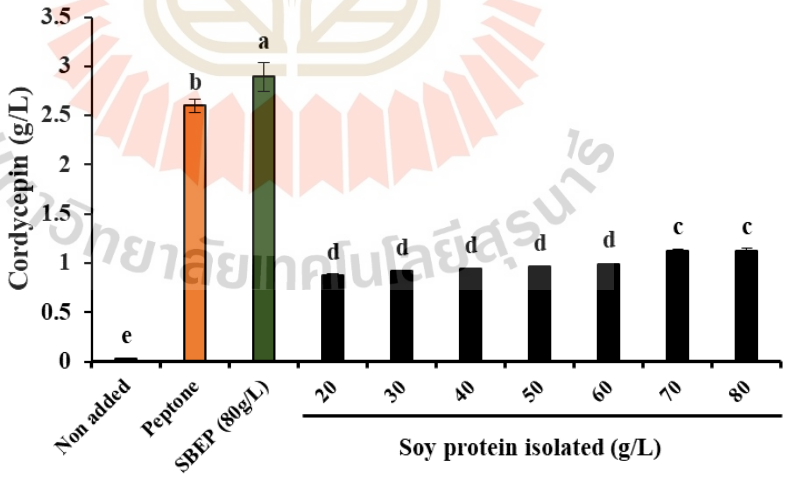


Figure 4.4 Comparison of SBEP and soy protein isolated as the animal-free nitrogen source on cordycepin production by *C. militaris* in liquid surface culture. The value was represented as the mean ± SD (n = 3), where columns with different superscripts represent significant ($p > 0.05$).

4.4 Effect of SBEP on the transcriptional levels of cordycepin biosynthesis pathway

The expression of genes involved in the cordycepin biosynthetic pathway was examined in this study. The transcription levels of two important genes including *cns1* and *NT5E* act to catalyze the formation of intermediates in the cordycepin biosynthetic pathways. The comparison of supplemented nitrogen sources (peptone and 80 g/L SBEP) with a non-supplemented nitrogen source. The result is shown in Figures 4.5. The results found that supplementation of nitrogen sources significantly increased the expression of two major downstream genes (*cns1* and *NT5E*) in the cordycepin biosynthesis pathway of *C. militaris* when compared non-supplemented nitrogen source conditions. However, supplementation of SBEP did not significantly increase the expression of *cns1* and *NT5E* genes at 10 and 20 days when compared with peptone supplemented. In addition, the expression of three genes was lower at 30 days.

The important genes involved in cordycepin biosynthesis have been identified, which has assisted us understand the molecular mechanism of downstream signaling. Overgaard-Hansen (1964) has proposed that the biosynthesis of purine is crucial for the synthesis of cordycepin. Purine nucleotide was thus recognized as a crucial precursor for the synthesis of cordycepin in *C. militaris*. Nitrogen sources have been reported to play an important role involving serial conversion in the cordycepin biosynthesis pathway (Chen *et al*. 2020). Cordycepin is an interesting molecule in *C. militaris* that has been studied extensively for its biosynthesis mechanism. *NT5E* and *cns1* are key enzymes in *C. militaris* that act as converters of intermediates to cordycepin. NT5E is involved in the conversion of AMP to adenosine, which is reported to be an important precursor for cordycepin biosynthesis. According to Xia et al. (2017), the *cns3* converts adenosine to 3′-dAMP, which is then dephosphorylated by the *cns2* to 2′-C-3′-dA, which is then converted by the cns1 to cordycepin. In the future, further studies on the mechanisms of cordycepin synthesis may indeed be conducted to gain a deeper understanding of this compound. Cordycepin, also known as 3'-deoxyadenosine, is a nucleoside analogue that is found naturally in *Cordyceps* fungi and has been of great interest due to its potential medicinal properties. By exploring the synthesis mechanisms of cordycepin, researchers can unravel the specific enzymatic reactions,

genetic pathways, and regulatory factors involved in its production. This knowledge can provide insights into the biosynthesis of cordycepin and potentially enable researchers to manipulate or optimize its production.

It is possible that as time passes, the genes that convert the intermediary to cordycepin become inactivated due to high intracellular cordycepin levels and are toxic to the cells. Genes are thus highly activated in 10 to 20 days to induce intracellular cordycepin accumulation, and their synthesis is inhibited in 30 days to reduce cell toxicity. According to Tang et al. (2018), cordycepin is excreted extracellularly into the culture medium to increase cordycepin accumulation and reduce toxicities. Cordycepin accumulation is presented in Figure 4.3D. Therefore, the experimental results indicated that the supplementation source of SBEP nitrogen could be used for peptone substitution as the nitrogen source for cultivation of *C. militaris* by liquid surface to produce cordycepin, and that it might also be applied at the industrial level.

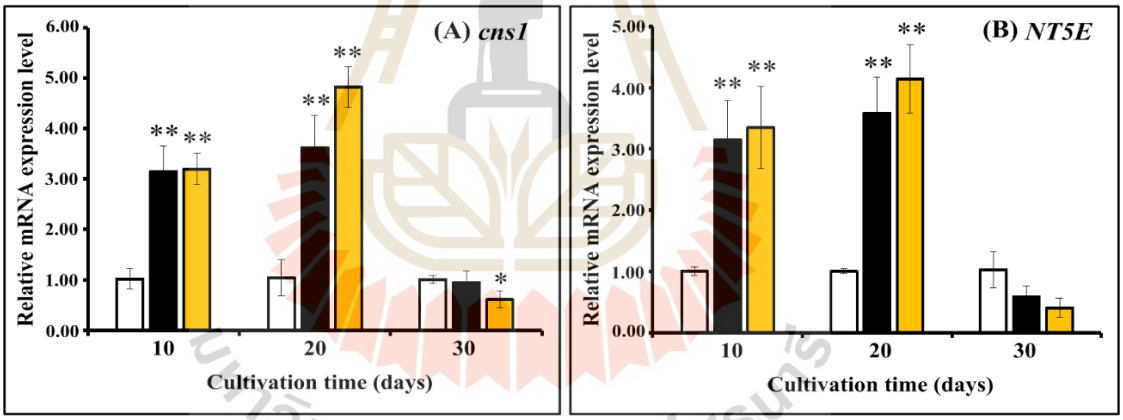


Figure 4.5 Transcriptional levels of *C. militaris* by liquid surface culture under different nitrogen sources. (A-B) the mRNA expression of major downstream genes in cordycepin biosynthesis pathway. The analysis was repeated in independent experiments with series of different cultures. The value was represented as the mean ± SD (n = 3). *$p < 0.05$, and ** $p < 0.01$ indicate statistically significant differences in the SBEP and peptone groups when compared to the non-added as the negative control, respectively, and # $p < 0.05$, and ## $p < 0.01$ indicate statistically significant differences in the SBEP when compared to peptone. ☐ Non-supplemented; ■ Peptone- supplemented; ■ SBEP-supplemented.

4.5 Effect of SBEP on the transcriptional levels of carbon metabolic pathway

The key enzymes in carbon metabolism are presented in Figure 4.4. In this study, specific genes in the carbon metabolic pathway for evaluation included *pfkA*; 6-phosphofructokinase in glycolysis, *zwf*; glucose-6-phosphate dehydrogenase in the PPP, and *aceA*; isocitrate lyase in the glyoxylate cycle. As result is demonstrated in Figure 4.6. Compared to a non-supplemented nitrogen source, SBEP-supplementation significantly increased the expression of three genes (*pfkA*, *zwf*, and *aceA*) involved in the carbon metabolism at 10, 20, and 30 days. When compared with peptone-supplemented as the positive control, SBEP-supplementation significantly increased the expression of *pfkA* at 10 days and *aceA* at 20 and 30 days.

Due to the SBEP contain carbohydrate (35.2%) and fatty acids (20.9%), which are considered the supplement of carbon sources. The 6-phosphofructokinase is a key enzyme that phosphorylates fructose 6-phosphate in glycolysis of *C. militaris* (Tang et al., 2018). The glucose-6-phosphate dehydrogenase is a key enzyme that catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate dehydrogenase in PPP, which is an important pathway for cordycepin biosynthetic and has high efficiency in *C. militaris* culture under hypoxia conditions (Suparmin et al., 2019). The isocitrate lyase is an essential enzyme in the glyoxylate cycle because it catalyzes the reversible formation of succinate and glyoxylate from isocitrate, which uses fatty acid substrates during growth. It also might trigger the catalysis of the initial reaction in the glyoxylate shunt to detoxify glyoxylate during carbon consumption in *C. militaris* (Raethong et al., 2018). According to Zhang and Liu (2016), carbon sources serve as the initial building block for the de novo purine nucleotide pathway as well as the cordycepin biosynthesis. Due to the SBEP contain carbohydrates and fatty acids in Table 4.1, which are strong secondary carbon sources that assist *C. militaris* produce increased cordycepin in liquid surface culture. However, the supplementation of SBEP as a source of animal-free nitrogen may increase fatty acid synthesis even more because there are sources of carbon, both sucrose and fatty acids. In support of this, the result of up regulated *aecA* was related to the report by Raethong et al. (2018) that fatty acid synthesis during carbon utilization may increase the toxicity from increased glyoxylate

accumulation, and *aceA* expression may mediate the increased toxicity of glyoxylate. It is a glyoxylate that is formed by a glyoxylate shunt. Additionally, it might also increase carbon absorption. Tang et al. (2018) confirmed that adding vegetable oils can enhance cordycepin production and enzyme activity in the carbon metabolism of *C. militaris* in liquid surface culture, and gene expression is also up regulated of key cordycepin biosynthetic. Similarly, Park et al. (2002) found that the addition of vegetable oils can increase the production of exo-biopolymer and mycelial biomass, and the type of fatty acid that markedly stimulated the biopolymer production was oleic acid and palmitic acid of *C. militaris* in fermentation culture. Therefore, the carbohydrase and fatty acid from the SBEP might be used as carbon sources in *C. militaris* by liquid surface culture as a substrate in culture media for the production of cordycepin and cell growth.

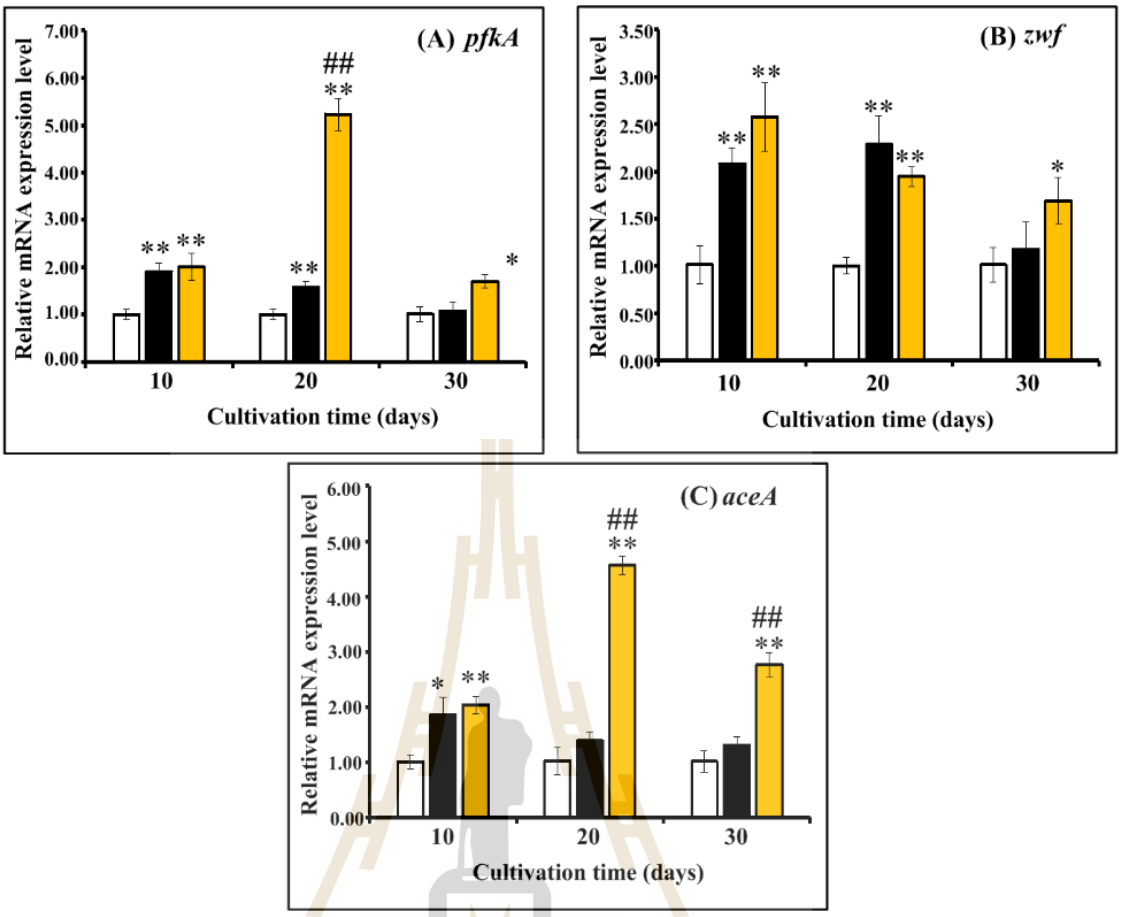


Figure 4.6 The investigation of carbon metabolism of *C. militaris* by liquid surface culture under different nitrogen sources. The analysis was repeated in independent experiments with series of different cultures. The value was represented as the mean ± SD (n = 3). *$p < 0.05$, and ** $p < 0.01$ indicate statistically significant differences in the SBEP and peptone groups when compared to the non-added as the negative control, respectively, and # $p < 0.05$, and ## $p < 0.01$ indicate statistically significant differences in the SBEP when compared to peptone. ☐ Non-supplemented; ■ Peptone-supplemented; ■ SBEP-supplemented.

4.6 Effect of SBEP on the transcriptional levels of amino acid metabolism

In addition to examining the transcription levels in cordycepin biosynthetic pathway and carbon metabolism. In this study, the transcription level in the amino acid metabolism is related to cordycepin production was also investigated. This experiment, the expression of amino acid-producing genes including *GltS*, *NADP-GDH*, and *AST* necessary to catalyze the precursor for cordycepin synthesis in *C. militaris* was examined. These results are displayed in Figure 4.7. At 10 days, the addition of both nitrogen sources found that the *AST* gene was significantly down-regulated, and the SBEP-supplemented significantly down-regulated the *NADP-GDH* gene when compared to peptone-supplemented as the positive control. However, the supplement of both nitrogen sources (peptone and SBEP-supplemented) significantly up-regulated the three genes in amino acid metabolism when compared with non-supplemented as a negative control at 20 and 30 days, and SBEP-supplemented was not significant when compared with peptone-supplemented as the positive control.

The enzyme NADP-specific glutamate dehydrogenase (NADP-GDH) produces glutamate from α-ketoglutarate in *C. militaris* liquid surface culture. The product is then transferred into the TCA via the GABA pathway after GltS catalyzes the conversion of glutamate from α-ketoglutarate by glutamine, the reaction's nitrogen source. The AST can convert oxaloacetate to aspartate, and aspartate can also play an important intermediary role for SAICAR synthase (Suparmin et al., 2017). Hence, glutamate, glutamine, and aspartate are important precursors for serial conversion, and GltS, NADP-GDH, and AST are important enzymes to catalyze intermediation in amino acid metabolism to synthesize glutamate, glutamine, and aspartate as precursors in the cordycepin biosynthetic pathway. According to Lin et al. (2016), the de novo purine metabolism pathway necessitates some amino acids for catalyzed intermedia to cordycepin biosynthetic, such as glutamate, glutamine, glycine, and L-aspartate. The activation of amino acid conversion enhanced the synthesis of cordycepin substrate (Chen et al., 2020). The results might relate to the study of Kaushik et al. (2020), which found that the supplementation of amino acids (glycine, glutamine, and aspartate) in the culture medium enhances cordycepin production and significantly increases the expression of genes (*ADEK*, *RNR*, and *NT5E*) in the cordycepin biosynthetic pathway. These results indicate the SBEP-supplemented in the culture medium of *C. militaris* in liquid surface culture contains an adequate precursor for synthesis of amino acids for

conversion of intermedia in the cordycepin biosynthesis pathway. According to Samanta *et al*. (2016), they reported the amino acid extract from soybean seed that they contained with 1.58% of aspartic acid, 3.28% of glutamic acid, and 0.51% of glycine, which might be amino acids in addition to their precursors that were derived from SBEP-supplemented culture medium. It also might be involved in another precursor for the conversion of an intermediate in cordycepin biosynthesis and amino acid metabolism.

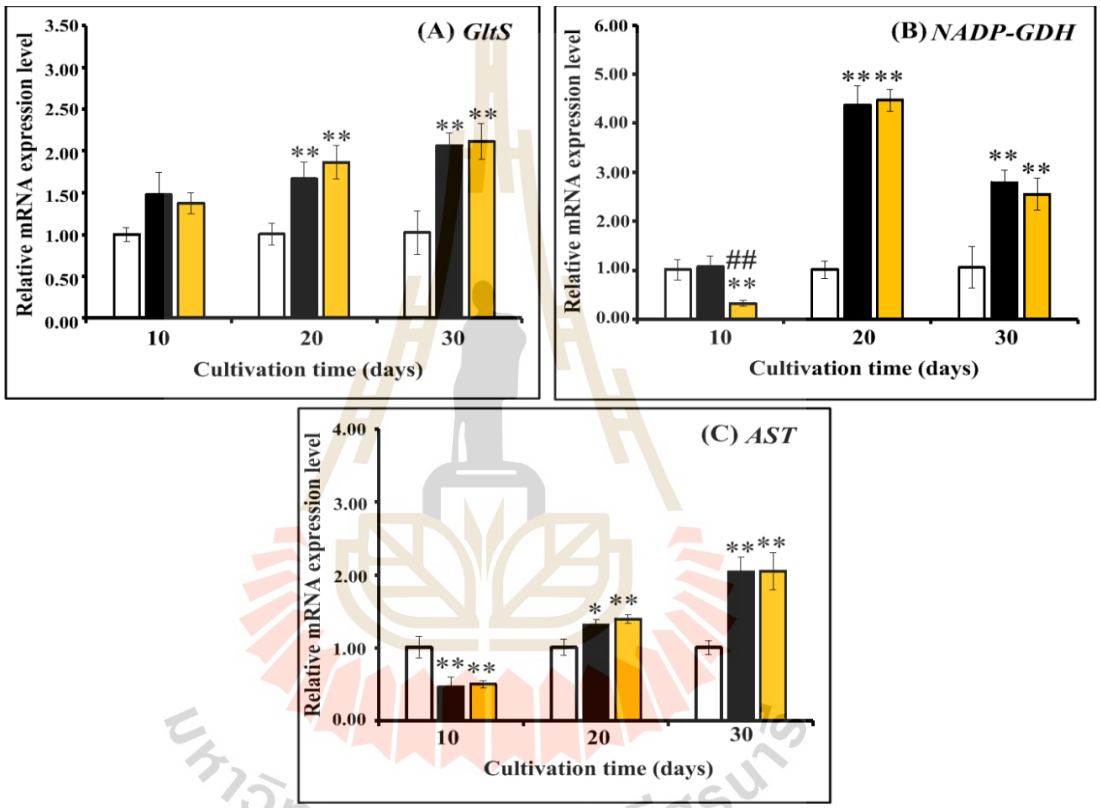


Figure 4.7 The investigation of amino acid metabolism of *C. militaris* by liquid surface culture under different nitrogen sources. The analysis was repeated in independent experiments with series of different cultures. The value was represented as the mean ± SD (n = 3). *$p < 0.05$, and ** $p < 0.01$ indicate statistically significant differences in the SBEP and peptone groups when compared to the non-added as the negative control, respectively, and # $p < 0.05$, and ## $p < 0.01$ indicate statistically significant differences in the SBEP when compared to peptone. □ Non-supplemented; ■ Peptone-supplemented; ■ SBEP-supplemented.

4.7 Optimization of various conditions using RSM with a BBD on cordycepin production of *C. militaris* in liquid surface culture

After obtaining SBEP 80 g/L as the supplementary source of animal-free nitrogen, the cultural conditions were investigated for the cordycepin production of *C. militaris* in liquid surface culture. The RSM analysis was conducted to obtain the high cordycepin production by using the above-mentioned results, as is shown in Table 3.3. The BBD was used to optimize the level of significant variables, including working volume, inoculum size, and cultivation time, and the effect of interactions on the value of cordycepin production. Table 4.2 presents the experimental design and the actual values of 15 BBD experiments are listed. As a function to describe the relationship between variables and responses, the second-order polynomial equation provided a mathematical model. The regression equation was described as follows:

$$Y = 2.45 - 0.42X_1 + 0.09X_2 - 0.44X_3 + 0.05\,X_1X_2 - 0.06\,X_1X_3 - 0.04X_2X_3 - 0.02X_1^2 - 0.25X_2^2 - 1.47X_3^2 \quad \text{Eq.(3)}$$

Where Y presents the value of cordycepin production (g/L), X_1 is a working volume (ml), X_2 is an inoculum size (%), and X_3 is a cultivation time (days). As shown in Table 4.3, the response surface model terms were displayed as a result of the ANOVA analysis, and the regression model was statistically significant at a 95% confidence level ($p < 0.05$). The model showed a high regression coefficient (R^2) of 0.9867, indicating that 98.67% of the variability in response could be explained by the data in the model. The F-value of the model was 41.28 and P-value was 0.0004, which indicated that the experimental data fitted well with a quadratic model.

Table 4.2 Regression and ANOVA for response surface quadratic polynomial model of Box Behnken design (BBD) experiments.

Source	Sum of Squares	df	Mean Square	F-value	*p*-value Prob>*F*
Model	11.11	9	1.23	41.28	0.0004**
X_1	1.41	1	1.41	47.1	0.001**
X_2	0.0678	1	0.0678	2.27	0.1926
X_3	1.57	1	1.57	52.67	0.0008**
X_1X_2	0.0113	1	0.0113	0.3793	0.5649
X_1X_3	0.0141	1	0.0141	0.4717	0.5227
X_2X_3	0.0049	1	0.0049	0.1652	0.7012
X_1^2	0.0019	1	0.0019	0.0643	0.8099
X_2^2	0.2216	1	0.2216	7.41	0.0417
X_3^2	7.93	1	7.93	265.1	< 0.0001**
Residual	0.1495	5	0.0299		
Lack of Fit	0.1403	3	0.0468	10.22	0.0905
Pure Error	0.0092	2	0.0046		
Cor Total	11.26	14			
R^2=0.9867					
Adj-R^2 = 0.9628					
CV=11.36%					

** significant at 1% level; * significant at 5% level.

Table 4.3 Fatty acid composition of soybean oil.

Fatty acid	Symbol	Weight percentage
Lauric	12:0	4.5
Myristic	14:0	4.5
Palmitic	16:0	11.6
Stearic	18:0	2.5
Oleic	18:1	21.1
Linoleic	18:2	52.4
Linolenic	18:3	7.1

Source: FAO (1992)

Figure 4.8 shows the three-dimensional response surface plot from Eq. 3. These were used to optimize the levels of all variables with respect to the cordycepin production value. From the response surface and contour plots, the value of the cordycepin production was found to be the highest in the tested range. The model predicted a maximum response of cordycepin production of 2.64 g/L at a level of working volume of 147.5 ml, an inoculum size of 8.8% v/v, and a cultivation time of 40.0 days as optimal culture conditions. To validate the predicted results of the model, this experiment was conducted using optimized cultural conditions, and found to have a cordycepin production of 2.62 g/L. This validate confirmed the model for stimulating and predicting the value of cordycepin production.

As previously reported, cordycepin is synthesized intracellularly, and it can excrete extracellularly in liquid medium culture (Frederiksen et al., 1965). Liquid culture was reported for hyperproduction of cordycepin, and submerged fermentation could enhance cordycepin production by about 15–30% (Xian and Jian 2004). Previously, Suparmin et al. (2019) were suggested to be optimal for cordycepin production in hypoxia conditions. As a result, this study was carried out in liquid surface culture for the cordycepin production of *C. militates*. In general, complex bioprocesses were regarded as the important stages ranging from seed culture preparation and fermentation to downstream processes that could also lead to failure on an industrial scale (Zou et al., 2011). The working volume, inoculum size, and cultivation time are generally investigated to obtain the optimal conditions for cordycepin production in *C. militaris* culture. The working volume of liquid culture medium is proportional to dissolved oxygen (DO) concentration, and low DO levels in the medium can result in increased cordycepin production by lowering effective energy intake and decreasing fluid shear stress (Tang et al., 2018). According to Masuda et al. (2006) studied medium depths at 1.8–5.3 cm of *C. militaris* in liquid surface culture, the lower liquid medium depth could enhance cordycepin production when at the end of cultivation time, and biomass production is proportional to the liquid medium depth. Inoculum size is a crucial biological parameter for the initial ratio of fungi that relates to working volume by liquid culture, and there may be significant for cordycepin production of *C. militaris* culture, and 10% v/v is suggested for higher cordycepin production, and high inoculum size promotes biomass production in the liquid culture (Tang et al., 2014; Tuli et al.,

2014; Ghatnur et al., 2015). Cultivation time is using utilization that is related to the secondary synthesis. Cordycepin production of *C. militaris* also has a relationship with the carbon source if the carbon source is used up in the culture medium, thus cordycepin might be a secondary carbon source and production of cordycepin in the culture medium is low (Tang et al., 2018).

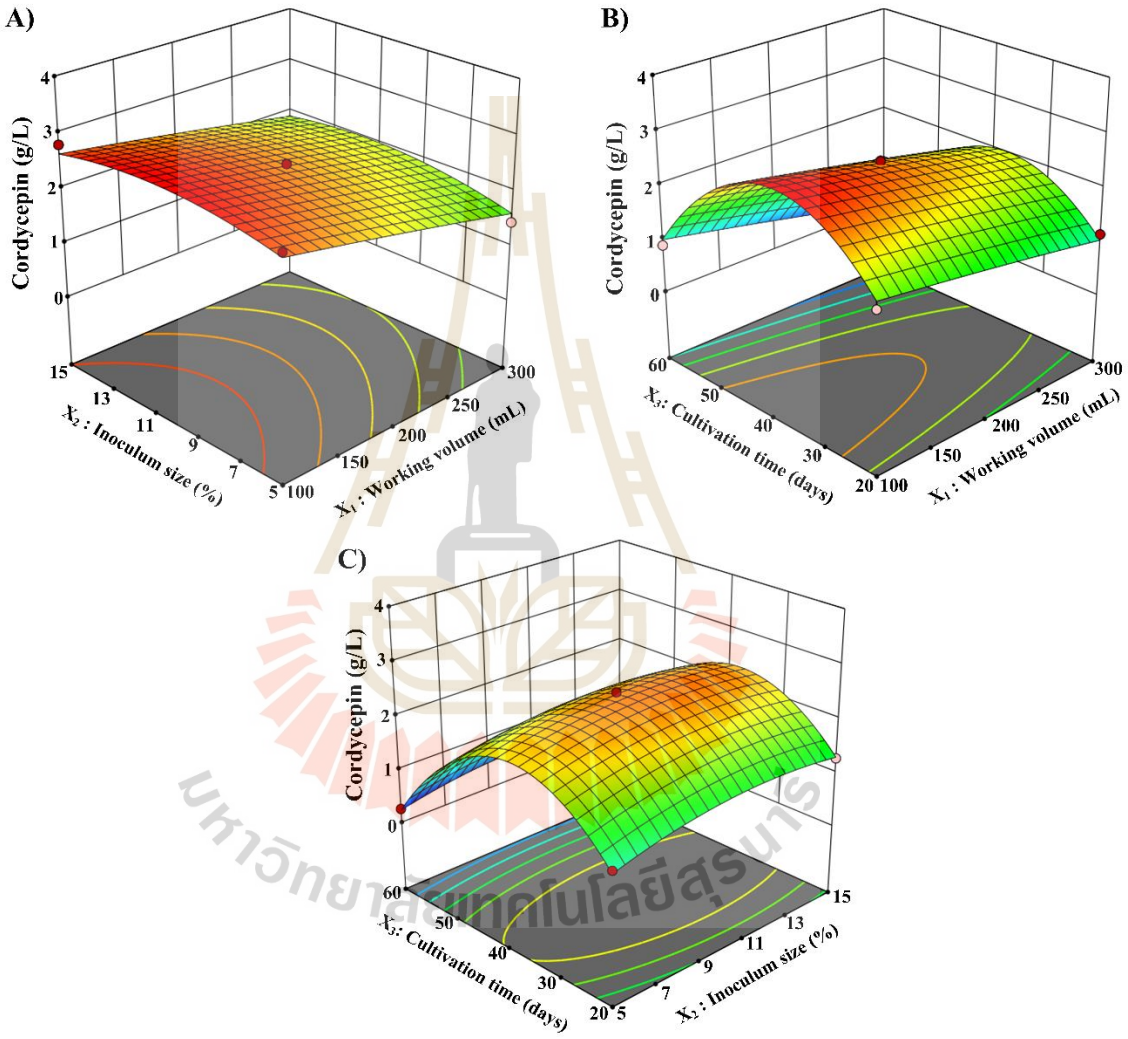


Figure 4.8 Three-dimensional response surface for cordycepin production (*Y*) by *C. militaris* in liquid surface culture. (A) Response surface plots for the value of *Y* showing the interaction between working volume and inoculum size; (B) response surface plots for the value of *Y* showing the interaction between working volume and cultivation time; and (C) response surface plots for the value of *Y* showing the interaction between of inoculum size and cultivation time.

4.8 References

Chen, B. X., Wei, T., Xue, L. N., Zheng, Q. W., Ye, et al. (2020). Transcriptome analysis reveals the flexibility of cordycepin network in Cordyceps militaris activated by L-alanine addition. Front. Microbiol. 11: 577.

Das, S. K., Masuda, M., Sakurai, A., and Sakakibara, M. (2009). Effects of additives on cordycepin production using a Cordyceps militaris mutant induced by ion beam irradiation. J. Biotechnol. 8(13): 3041-3047.

FAO (1992). Technology of production of edible flours and protein products from soybeans. (Electronic) Retrieved from http://www.fao.org/docrep/t0532e/t0532e02.htm (20 May 2013).

Frederiksen, S., Malling, H. and Klenow, H. (1965). Isolation of 3′-deoxyadenosine (cordycepin) from the liquid medium of Cordyceps militaris (L. ex Fr.) Link. Biochim. Biophys. Acta. 95: 189-193.

Ghatnur, S.M., Parvatam, G. and Balaraman, M. (2015). Culture conditions for production of biomass, adenosine, and cordycepin from Cordyceps sinensis CS1197: Optimization by desirability function method. Pharmacogn. Mag. 11: S448-S456.

Kaushik, V., Singh, A., Arya, A., Sindhu, S.C., Sindhu, A. and Singh, A. (2020). Enhanced production of cordycepin in Ophiocordyceps sinensis using growth supplements under submerged conditions. Biotechnol. Rep. 28: e00557.

Kumar, V., Sharma, A., Kaur, R., Thukral, A. K., Bhardwaj, R., and Ahmad, P. (2017). Differential distribution of amino acids in plants. Amino Acids 49: 821-869.

Kumar, V., Sharma, A., Kohli, S.K., Yadav, P., Bali, S., Bakshi, P., Parihar, R.D., Yuan, H., Yan, D., He, Y., Wang, J., Yang, Y., Bhardwaj, R., Thukral, A.K. and Zheng, B. (2019) Amino acids distribution in economical important plants: a review. Biotechnol. Res. Innov. 3(2): 197-207.

Kunhorm, P., Chueaphromsri, P., Chaicharoenaudomrung, N., and Noisa, P. (2022). Enhancement of cordycepin production from Cordyceps militaris culture by epigenetic modification. Biotechnol. Lett. 44(4): 581-593.

Li, Y., Ban, L., Meng, S., Huang, L., Sun, N., Yang, H., Wang, Y. and Wang, L. (2021). Bioactivities of crude polysaccharide extracted from fermented soybean curd residue by Cordyceps militaris. Biotechnol. Biotechnol. Equip. 35(1): 334-345.

Lin, S., Liu, Z.Q., Xue, Y.P., Baker, P.J., Wu, H., Xu, F., Teng, Y., Brathwaite, M.E. and Zheng, Y.G. (2016). Biosynthetic pathway analysis for improving the cordycepin and cordycepic acid production in Hirsutella sinensis. Appl. Biochem. Biotechnol. 179, 633-649.

Masuda, M., Urabe, E., Honda, H., Sakurai, A., and Sakakibara, M. (2007). Enhanced production of cordycepin by surface culture using the medicinal mushroom Cordyceps militaris. Enzyme Microb. Technol. 40(5): 1199-1205.

Masuda, M., Urabe, E., Sakurai, A., and Sakakibara, M. (2006). Production of cordycepin by surface culture using the medicinal mushroom Cordyceps militaris. Enzyme Microb. Technol. 39(4): 641-646.

Overgaard-Hansen, K. (1964). The inhibition of 5-phosphoribosyl-1-pyrophosphate formation by cordycepin triphosphate in extracts of Ehrlich ascites tumor cells. Biochim. Biophys. Acta. 80: 504-507.

Park, J.P., Kim, S.W., Hwang, H.J., Cho, Y.J. and Yun, J.W. (2002). Stimulatory effect of plant

oils and fatty acids on the exo-biopolymer production in Cordyceps militaris. Enzyme Microb. Technol. 31: 250-255.

Raethong, N., Laoteng, K., and Vongsangnak, W. (2018). Uncovering global metabolic response to cordycepin production in Cordyceps militaris through transcriptome and genome-scale network-driven analysis. Sci. Rep. 8(1): 1-13.

Samanta, A. K., Kar, T. R., Mukhopadhyay, A., Shome, D., and Konar, A. (2016). Eco-friendly

salt-free reactive dyeing of cotton (muslin) fabric after cationization with amino acid from soya. Text Res. J. 86(20): 2179-2192.

Shariati, S., Zare, D. and Mirdamadi, S. (2019). Screening of carbon and nitrogen sources using mixture analysis designs for carotenoid production by Blakeslea trispora. Food Sci. Biotechnol. 28: 469-479.

Song, P., Yuan, K., Ji, X.J., Ren, L.J., Zhang, S., Wen, J.P. and Huang, H. (2018). Effects of cotton seed powder as the seed medium nitrogen source on the morphology and pneumocandin B_0 yield of Glarea lozoyensis. Front. Microbiol. 9: 2352.

Suparmin, A., Kato, T., Dohra, H., and Park, E. Y. (2017). Insight into cordycepin biosynthesis of Cordyceps militaris: Comparison between a liquid surface culture and a submerged culture through transcriptomic analysis. PLoS One 12(11): e0187052.

Suparmin, A., Kato, T., Takemoto, H. and Park, E.Y. (2019). Metabolic comparison of aerial and submerged mycelia formed in the liquid surface culture of Cordyceps militaris. MicrobiologyOpen 8: e00836.

Suribabu, K., Lalitha G. T., and Hemalatha, K. P. J. (2014). Optimization of various Nitrogen sources for the production of α-Amylase using Brevibacillus borstelensis R1 by Submerged fermentation. Int. J. Curr. Microbiol. Appl. Sci. 3(4): 791-800.

Tang, J., Liu, Y. and Li, Z. (2014). Optimization of fermentation conditions and purification of cordycepin from Cordyceps militaris. Prep. Biochem. Biotechnol. 44: 90-106.

Tang, J., Qian, Z. and Wu, H. (2018). Enhancing cordycepin production in liquid static cultivation of Cordyceps militaris by adding vegetable oils as the secondary carbon source. Bioresour. Technol. 268: 60-67.

Tuli, H. S., Sharma, A. K., and Sandhu, S.S. (2014). Optimization of fermentation conditions for cordycepin production using Cordyceps militaris 3936. J. Biol. Chem. Sci. 1: 35-47.

Vongsangnak, W., Raethong, N., Mujchariyakul, W., Nguyen, N.N., Leong, H.W. and Laoteng, K. (2017). Genome-scale metabolic network of Cordyceps militaris useful for comparative analysis of entomopathogenic fungi. Gene 626: 132-139.

Xia, Y., Luo, F., Shang, Y., Chen, P., Lu, Y. and Wang, C. (2017). Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. Cell Chem. Biol. 24(12): 1479-1489.

Xian, B. M., and Jian, J. Z. (2004). Hyperproduction of cordycepin by two-stage dissolved oxygen control in submerged cultivation of medicinal mushroom Cordyceps militaris in bioreactors. Biotechnol. Prog. 20(5): 1408–1413.

Xiao, Z.J., Liu, P.H., Qin, J.Y. and Xu, P. (2007). Statistical optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate. Appl. Microbiol. Biotechnol. 74: 61-68.

Zhang, Q. and Liu, Y. (2016). The strategies for increasing cordycepin production of Cordyceps militaris by liquid fermentation. Fungal Genom. Biol.

Zou, X., Li, W.J., Zeng, W., Chu, J., Zhuang, Y.P. and Zhang, S.L. (2011). An assessment of seed quality on erythromycin production by recombinant *Saccharopolyspora erythraea* strain. Bioresour. Technol. 102: 3360-3365.

CHAPTER 5
CONCLUSION

The soybean extract powder (SBEP) was the best nitrogen source for the animal-free culture medium of *C. militaris*. A strategy of supplementing 80 g/L SBEP as the source of animal-free nitrogen could replace commercial peptone for *C. militaris* in the liquid surface culture. It could enhance cordycepin production while keeping production costs low. The maximum concentration of cordycepin was 2.52 g/L. Additionally, the molecular mechanisms of the carbon metabolic pathway and amino acid metabolism were also upregulated. Moreover, the two key genes in the cordycepin biosynthesis pathway (*cns1* and *NT5E*), carbon metabolism (*pfkA*, *zwf*, and *aceA*), and amino acid metabolism (*GltS*, *NADP-GDH*, and *AST*) were upregulated. Under the optimal culture conditions, the model predicted a maximum response of cordycepin production of 2.64 g/L at a working volume of 147.5 ml, an inoculum size of 8.8% v/v, and a cultivation time of 40.0 days. Importantly, this strategy could reduce the cost by 3.51% and increase the production of cordycepin by up to 1.11 times when compared to peptone. The results from this study confirm that vegan people could use cordyceps for health effects. Furthermore, the SBEP could be used to produce cordycepin on a large scale for commercialization by liquid surface fermentation.

VITAE

Mr. Kritsana Sripilai was born on October 31, 1997 in Chaiyaphum Province, Thailand. In 2015, he studied in School of Crop Production Technology, Institute of Agricultural Technology from Suranaree University of Technology, Thailand. He received Bachelor's Degree in B.Sc. (Crop Production Technology) in 2018. After graduation, he has been employed under the position of research assistant by Cell Based Assay and Innovation laboratory at Suranaree University of Technology.

In 2020, he studied at the School of Biotechnology, the Institute of Agricultural Technology, and Suranaree University of Technology. He received a One Research One Grant (OROG) scholarship to financially support his study for a degree in Master Science from Assoc. Prof. Dr. Parinya Noisa, Suranaree University of Technology, Thailand. In 2022, he received a Graduate Research Grant in Agriculture and Agro-industry from the Agricultural Research Development Agency (ARDA; public organization) for Fiscal Year 2022. The research topic is "EFFECT OF VEGETABLE MEDIUM NITROGEN SOURCES ON CORDYCEPIN PRODUCTION AND BIOSYNTHESIS PATHWAY OF *Cordyceps militaris* IN LIQUID SURFACE CULTURE". During his master's study, he obtained an opportunity to present her research work under the title "Animal-Free Formulation as a Nitrogen Source for *Cordyceps militaris* Culture" which was accepted to be published in The Annual Meeting at the Thai Society for Biotechnology International Conference Online (TSB 2022). He has also published one research article (Sripilai et al., 2023) as follow:

Sripilai, K., Chaicharoenaudomrung, N., Phonchai, R., Chueaphromsri, P., Kunhorm, P., and Noisa, P. (2023). Development of an animal-free nitrogen source for the liquid surface culture of *Cordyceps militaris*. Lett. Appl. Microbiol. 76(5). https://doi.org/10.1093/lambio/ovad053