OPTIMIZATION ON ADENOSINE PRODUCTION FROM *Cordyceps militaris* USING SUBMERGED FERMENTATION

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ABSTRACT

Cordyceps militaris, a medicinal mushroom, is widely used in traditional herbal medicine in East Asia. Adenosine is the main compound found in *C. militaris* which has many biological and pharmacological properties including anti–aging, skin regeneration, anti–wrinkle as well as and anti–hair fall. In this study, the production of adenosine from *C. militaris* was influenced by various factors including (1) fungal strains, (2) carbon source, (3) nitrogen source and (4) initial pH. To optimize the adenosine production, an experimental design based on the Taguchi method (Qualitek–4 software) was operated in this study. The experimental factors considered include (1) fungal strains (SH01, ATCC 34165 and a hybrid strain), (2) glucose concentration (20–60 g/L), (3) yeast extract concentration (5–20 g/L) and (4) initial pH (4.0–7.0). Nine treatments obtained from Taguchi–base experimental design were performed under 250–mL shaking flask containing 50 mL culture medium for 30 days in triplicate. It was found that the cultivation of *C. militaris* SH01 strain under optimal condition (40 g/L glucose, 20 g/L yeast extract and initial pH 4.0) showed the maximum adenosine production and productivity which were 8.662±0.269 mg/g and 2.495±0.077 mg/g.d, respectively, which was higher than expected result for 1.44 times. However, the

scalability in 5–L shaking flask shown the productivity of adenosine at 1.849±0.094 mg/g.d which was lower than in 250–mL shaking flask. Thus, these findings provide an effective strategy for enhancing adenosine production, and have potential to apply in large scale for healthcare, food and cosmetic purposes.

Keywords: Adenosine, *Cordyceps militaris*, Process Optimization, Submerged Fermentation, Taguchi Method

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CHAPTER 1

INTRODUCTION

Cordyceps militaris is an entomopathogenic fungus belonging to the class *Ascomycetes*. It is the most famous traditional Chinese medicinal mushroom and used in China, Japan, Korea and other oriental countries including Thailand (Schmidt et al., 2003; Shih et al., 2007; Tian et al., 2010). *Cordyceps militaris* can produce many kinds of bioactive compounds for example; exopolysaccharides, D–mannitol, cordycepin, adenosine and other nucleosides which can be used in medicinal and pharmacological purposes (Gu et al., 2007; Lim et al., 2012; Mao et al., 2005; Schmidt et al., 2003; Zhang et al., 2019). Adenosine has been known as a nucleoside which occurs naturally in our body and considered as a precursor of cordycepin (Xia et al., 2017). Adenosine was exhibited many interesting properties in medicinal, heal care product and food as well as cosmetic industry due to their bioactivities including anti–inflammatory, anticancer, and antioxidant, anti–aging, skin regeneration, anti–wrinkle as well as anti–hair fall properties (Kim et al., 2022; Marucci et al., 2022; Vuong Hoai et al., 2020; Xiao & Xiong, 2013). Besides, it can be used as a cardioprotective and therapeutic agent for chronic heart failure (Kitakaze & Hori, 2000), a neuromodulator and inhibitor of neurotransmitter releasing in the central nervous system (Gomes et al., 2011; Ribeiro, 1995).

Adenosine, a distinctive compound, is naturally present in the fruiting bodies of *Cordyceps militaris*, whether cultivated on lepidopteran larvae or artificial solid media (Lim et al., 2012; Oh et al., 2019). However, considering the extended growth period of up to 60 days required to produce these fruiting bodies, this process may not be viable for large–scale production. Numerous studies have focused on the cultivation of *C. militaris* in artificial media to boost the production of mycelium biomass, thereby satisfying the growing demand for its metabolites (i.e., adenosine and cordycepin). Among the various techniques, solid–state fermentation, and liquid fermentation

methods–including submerged cultivation, surface liquid cultivation, and two–stage cultivation–are the most widely adopted for adenosine and cordycepin extraction (Lim et al., 2012; Mao & Zhong, 2004; Masuda et al., 2007; Masuda et al., 2006; Sari et al., 2016; Schmidt et al., 2003; Shih et al., 2007; Wen et al., 2016; Wen et al., 2014). In term of the growth period, it takes around 60 days to yield the fruiting bodies. To meet the increasing demand of desired metabolites, submerged fermentation has been carried out and optimized to efficiently produce mycelial biomass, adenosine and other biological compounds in a smaller space and in less time with lower risk of contamination (Ke & Lee, 2019; Shih et al., 2007). Various *Cordyceps* species have been studied for maximizing adenosine production. Submerged cultivation of *Cordyceps sinensis* has been studied for optimum production and found the maximal adenosine yield of 0.714±31.74 mg/g of mycelium at the end of 14 days of growth period (Ghatnur et al., 2015). The submerged culture of *Cordyceps cicadae* resulted in more efficient adenosine productions (1.157±376 mg/g) than using solid–fermentation (Ke & Lee, 2019). The adenosine content of *Hirsutella sinensis* reached 1.76±11 mg/g when used rice bran as substrate for submerged fermentation (Ren et al., 2021). Nevertheless, there are several factors which effect on the production of biomass and their metabolites including screening the different strains, medium composition optimization, adding agents into the medium, initial pH and light irradiation (Das et al., 2008; Das et al., 2010; Dong et al., 2012; Fan et al., 2012; Mao et al., 2005; Masuda et al., 2011; Shih et al., 2007).

This study aims to investigate the process optimization to enhance the adenosine production of *Cordyceps militaris* using submerged fermentation. Moreover, the technique for optimizing fermentation conditions for adenosine production by Taguchi–based experimental design was applied for determination of the significant parameters. The optimal process under submerged fermentation from this research will be used for commercial scale–up in medical and pharmacological purposes.

1.1 Objectives

1.1.1 To determine the optimal cultivation parameters for adenosine production in *Cordyceps militaris*, using a Taguchi–based experimental design approach

1.1.2 To upscale the production of adenosine in *Cordyceps militaris*, guided by the conditions recommended from the Taguchi–based experimental design findings

1.2 Scope of Research

In this study, the optimization of the submerged fermentation process was explored to enhance the yield of adenosine from *Cordyceps militaris*. The determination of critical factors including the fungal strain, concentrations of carbon and nitrogen, and the initial pH was facilitated by a Taguchi–based experimental design to fine–tune the fermentation conditions. The production of metabolites, including adenosine, was subsequently extracted and quantified using HPLC analysis. Parameters such as dry cell weight, residual glucose, and pH were monitored throughout the cultivation process. The findings led to the establishment of an optimal submerged fermentation protocol, which was then applied to commercial scale–up for medical and pharmacological applications.

CHAPTER 2

LITERATURE REVIEW

2.1 *Cordyceps militaris*

Cordyceps is the largest and most diverse genus classified in the family Clavicipitaceae, which is a well–known ascomycetes parasitic fungus as endoparasitoids. It invades and grows within an insect larvae as a host, eventually killing the host and forming fruiting bodies on the host's surface (Masuda et al., 2007). *Cordyceps* species are diverse and most abundant in humid temperate and tropical forest, with a widely distribution in North America, Europe, East and Southeast Asian countries, particularly China, Japan, Nepal, Vietnam, Bhutan, Korea, including Thailand (Holliday & Cleaver, 2008; Olatunji et al., 2018). *Cordyceps militaris* is the second most studied member of the genus popularly and widely used as traditional Chinese medicinal mushroom in China, Japan and Korea and other oriental countries (Schmidt et al., 2003; Shih et al., 2007; Tian et al., 2010).

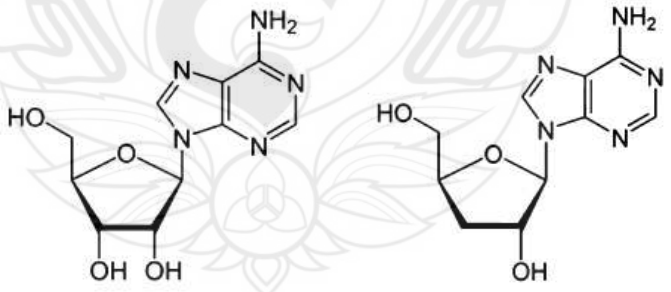
2.2 Bioactive Compounds and Their Biological Activities

In recent years, many active components and pharmacological effects of *C. militaris* have been studied. Both the mycelia and the fruiting body of *C. militaris* contain a variety of physiologically active compounds such as polysaccharide, cordycepin, cordycepic acids (D–mannitol), adenosine, carotenoids and other nucleoside (Gu et al., 2007; Lim et al., 2012; Schmidt et al., 2003; Zhang et al., 2019). Among these active compounds, adenosine and cordycepin are two majors of bioactive compounds which can be extracted from mycelia and the fruiting body of *C. militaris*.

Adenosine is a major nucleoside that is composed of adenine and d–ribose sugar which naturally occurring compound in the body (Figure 2.1A). It plays a crucial role

in energy metabolism and has been recognized as an energy transfer and signal transduction in cells for its ability to protect and prevent tissue damage with its bioactivities including anti–inflammatory, anticancer, and antioxidant characteristics (Vuong Hoai et al., 2020; Yoon et al., 2022). In addition, it can be used as a cardioprotective and therapeutic agent for chronic heart failure (Kitakaze & Hori, 2000), and a neuromodulator and inhibitor of neurotransmitter releasing in the central nervous system (Gomes et al., 2011; Ribeiro, 1995). In addition, adenosine widely used in cosmetic industry leading to anti–aging, skin regeneration, anti–wrinkle as well and anti–hair fall (Kim et al., 2022; Marucci et al., 2022). Moreover, the studies of Kim et al. (2021) shown that both adenosine and cordycepin can activate the adenosine A2B receptor in cultured human dermal fibroblasts, leading to increased intracellular cAMP levels and enhanced mitochondrial membrane potential (Kim et al., 2021)

Cordycepin (3′–Deoxyadenosine) is a derivative of the nucleoside adenosine, which absence of the hydroxy group at the 3′ position of its ribose ring (Figure 2.1B). It has been used as a biologically active metabolite with therapeutic potential including neuroprotection, lung and kidney protection, immunological stimulating, anti–cancer, antitumor, antiviral, anti–microbial and anti–inflammatory activities (Cui, 2015; De Silva et al., 2012; Nakamura et al., 2005; Pao et al., 2012; Schmidt et al., 2003; Tuli et al., 2014).



(A) (B)

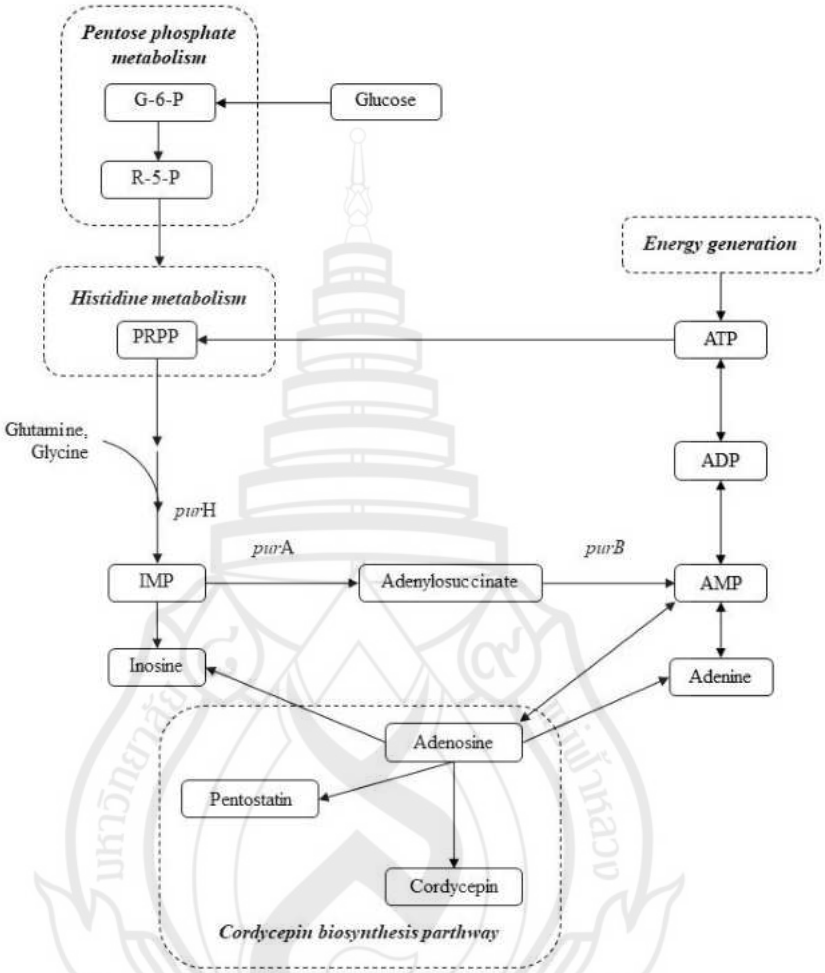
Figure 2.1 Chemical structures of (A) adenosine and (B) cordycepin

2.3 Biosynthetic Pathways of Adenosine

Adenosine is generally synthesized through several pathways. Firstly, adenine reacts with 1–phosphate ribose to yield phosphoric acid and adenosine. Secondly, S–adenosyl–homocysteine (SAH) undergoes hydrolysis to produce homocysteine and adenosine via the methionine pathway. Lastly, ATP or ADP is converted into AMP, which is subsequently hydrolyzed into adenosine by the enzyme 5'–nucleotidase. These processes collectively contribute to the synthesis of adenosine (Liu et al., 2019). Moreover, adenosine is one of the main compounds produced from *Cordyceps* sp. which is related to cordycepin synthesis. The intricate metabolic pathways of *C. militaris* are revealed by omics technologies (i.e., genomics, transcriptomics, proteomics and metabolomics) which also provide strong empirical support for the initial knowledge. Purine metabolism is one of the key factors that affect nucleoside production including IMP, AMP, adenine, adenosine as well as cordycepin (Figure 2.2). Glucose is first converted into glucose–6–phosphate (G–6–P), which is subsequently transformed into ribose–5–phosphate (R–5–P) through the pentose phosphate pathway. R–5–P then serves as substrate for the purine nucleotide pathway, which involves a series of changes from phosphoribosyl pyrophosphate (PRPP) to IMP and further to AMP, adenine and adenosine, and ultimately result in cordycepin (Chang et al., 2024; Wang et al., 2023; Zhang et al., 2016).

According to the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of *C. militaris*, the varieties enzymes required for constructed the biosynthetic pathways of adenosine and possible cordycepin biosynthesis pathway (Figure 2.3). It suggested that NT5E (5′–nucleotidase) is a key enzyme for the production of 3'–deoxyadenosine from 3'–dAMP. The crucial role of ADK, ADEK, and NT5E were revealed in phosphorylation and dephosphorylation in adenosine metabolic pathway, it is possible that these enzymes are also involves in phosphorylation and dephosphorylation in the cordycepin biosynthesis pathway (Lin et al., 2016; Xiang et al., 2014). The core of cordycepin biosynthetic pathway can be proposed as AMP was converted to ADP by adenosine kinase (ADEK). ADP was deoxidized to 3'–dADP (3'–deoxyadenosine 5'–diphosphate) by ribonucleotide reductase (NRDJ), then dephosphorylated into 3′–dAMP (3'–deoxyadenosine 5'–phosphate) by

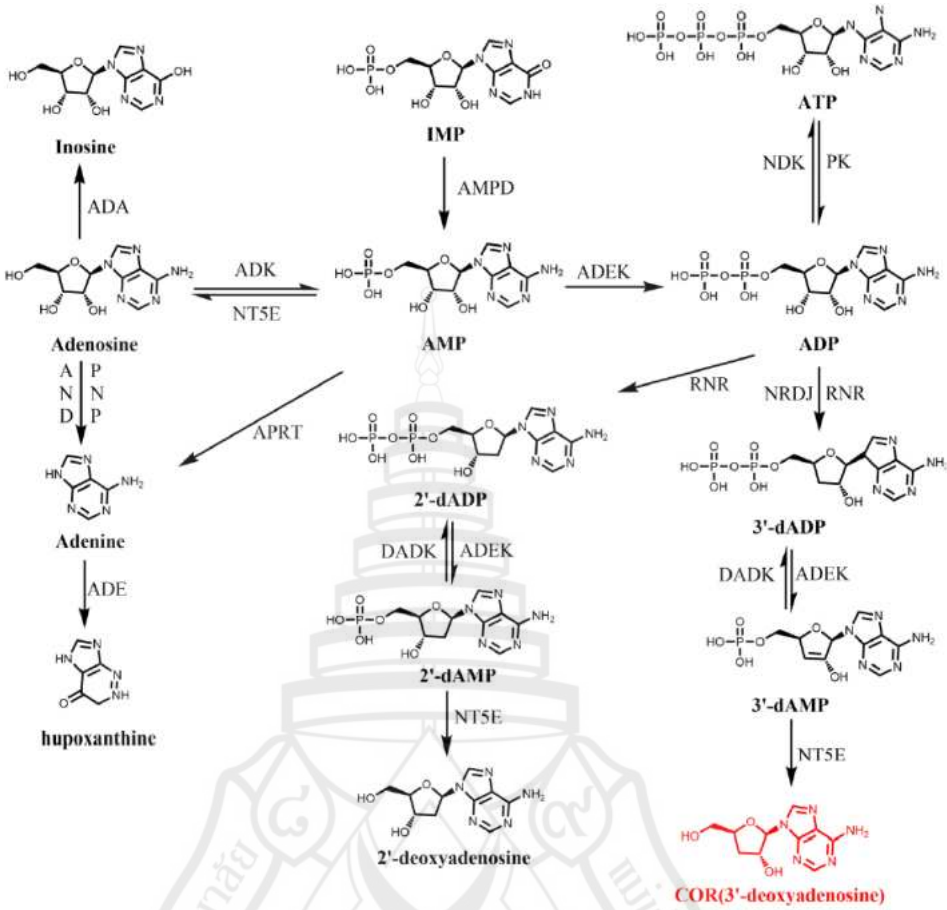
ADEK. NT5E catalyzes 3′–dAMP into the final product of cordycepin (Wang et al., 2022).



Note G–6–P: glucose–6–phosphate; R–5–P: ribose–5–phosphate; PRPP: phosphoribosyl pyrophosphate; IMP: inosine monophosphate; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate

Source Zhang et al. (2016) and Wang et al. (2023)

Figure 2.2 Putative pathway of adenosine and cordycepin synthesis via pentose phosphate pathway (PP) and purine nucleotide pathway

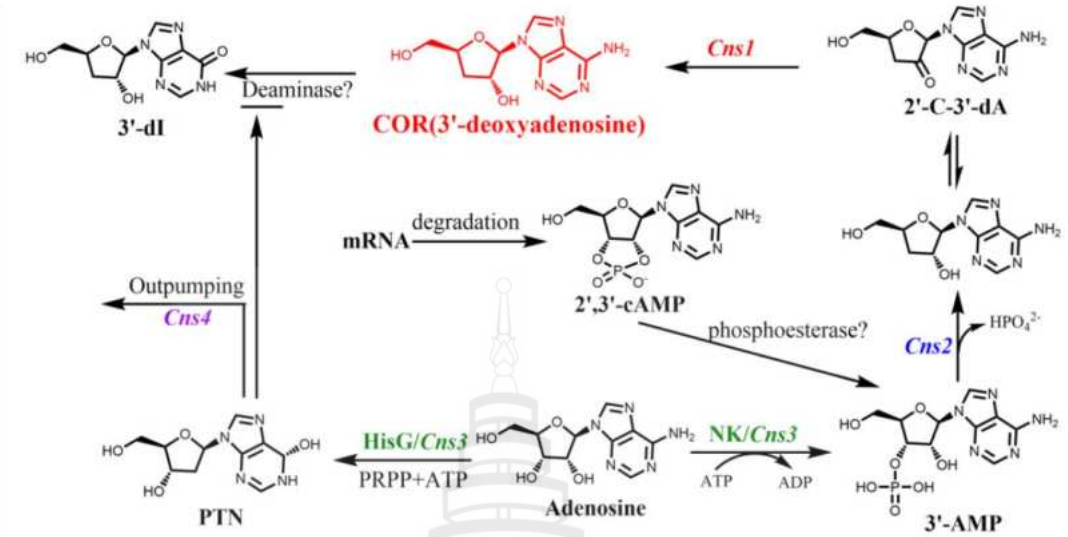


Note IMP: inosine monophosphate; AMP: adenosine monophosphate; ADA, adenosine deaminase; ADE, adenine deaminase; ADK: adenosine kinase; ADEK, adenylate kinase; ADN, adenosine nucleosidase; ADK, adenosine kinase; DADK, deoxyadenylate kinase; NDK, nucleosidediphosphate kinase; AMPD, AMP deaminase; dAMP: deoxyadenosine monophosphate; ADP: adenosine diphosphate; dADP: deoxyadenosine diphosphate; RNR: ribonucleotide reductases; APRT, adenine phosphoribosyltransferase; NT5E, 5'–nucleotidase; PNP, purine nucleoside phosphorylase; RNR, ribonucleotide triphosphate reductase

Source Wang et al. (2022)

Figure 2.3 The adenosine metabolism pathway and possible cordycepin biosynthesis pathway

Moreover, bioinformatics analysis, genetic and molecular biology were become to enhance the understanding of adenosine and cordycepin network. The study of Xia et al. (2017) elucidates a gene cluster that plays a pivotal role in metabolite biosynthesis. The cluster comprises four physically linked and highly conserved genes, designated as *Cns1* through *Cns4*. *Cns1* is characterized by an oxidoreductase/dehydrogenase domain, while *Cns2* harbors a metal–dependent phosphohydrolase domain and is part of the Histidine decarboxylase (HDc) family of proteins. *Cns3* features a nucleoside/nucleotide kinase (NK) at the N–terminal and a HisG domain at the C–terminal. *Cns4* functions as a putative ATP–binding cassette transporter (ABC transporter). *Cns1* and *Cns2* are integral to the biosynthesis of cordycepin, whereas *Cns3* is crucial for generating pentostatin, which inhibits the deamination of cordycepin to 3′–deoxyinosine by obstructing adenosine deaminase activity. *Cns4* serves as a self–regulatory detoxification pathway for cordycepin, moderating its production and safeguarding it from deamination by facilitating the conjugative synthesis of pentostatin (Figure 2.4) (Wang et al., 2022; Xia et al., 2017).



Note PRPP, phosphoribosyl pyrophosphate; 2′–C–3′–dA: 2′–carbonyl–3′–deoxyadenosine; 2′,3′–cAMP: 2′,3′–cyclic monophosphate; NK: an N–terminal nucleoside kinase; HisG: a C–terminal HisG family of ATP phosphoribosyltransferases; PTN: pentostatin; 3′–dI: 3′–deoxyinosine

Source Wang et al. (2022)

Figure 2.4 Delineation of adenosine and cordycepin biosynthesis pathway

2.4 Methods of Enhancement of Adenosine Production

The demand for adenosine and cordycepin from *Cordyceps militaris* is on the rise due to their recognized medical benefits. These bioactive compounds are used as functional components in health care products, foods, and cosmetics. In the relevant literature, various methods have been reported for producing *C. militaris* fruiting bodies and mycelia to enhance metabolite production, with a focus on commercial applications. Both solid–state fermentation (SSF) and liquid fermentation (LF) have gained prominence in the production of *C. militaris* fungal fruiting bodies and mycelium. These techniques aim to meet the increasing demand for adenosine and cordycepin.

2.4.1 Solid–State Fermentation

To enhance the demand of their metabolite, the various solid substrates will give different levels of adenosine and cordycepin. *C. militaris* cultured on millet showed a high level of adenosine, while a high cordycepin level obtained on soybean (Lim et al., 2012). In addition, the optimization of solid–state culture from Wen et al. (2014) suggested a 67.96% increase in fruiting body yield and a 63.17% increase of cordycepin, whereas adenosine content slightly increased leading to 2.67±0.04 mg/g at the later stage of culture. In the study of Adnan et al. (2017) on fermentation conditions such as pH, temperature, incubation time and solid substrates (wheat, oat and rice) were affected on cordycepin production. To maximize cordycepin production, the optimal combination of temperature, pH, and incubation time were 25 °C, 5.5, and 21 days, respectively. In their studied, it showed that the solid substrates, rice medium showed highest cordycepin production (814.60 mg/g) followed by oat and wheat medium (638.85 and 565.20 mg/g, respectively).

2.4.2 Liquid Fermentation

Adenosine and cordycepin content produced from solid state fermentation are mainly detected in the fruiting body. In contrast with liquid fermentation, their metabolite can be produced from mycelium but shorten the cultivation time for metabolites production. Liquid fermentation techniques, including submerged culture, surface liquid culture, and two–stage culture, have proven effective for obtaining adenosine and cordycepin from *Cordyceps* sp. Submerged fermentation is a method that allows fungi to grow in a liquid medium. This technique can yield higher quantities of the desired product due to optimal conditions, which include shaking or agitating the medium to provide dissolved oxygen and nutrients for microbial growth and metabolism (Mao & Zhong, 2004; Zhang & Zhong, 2013). This technique can be utilized for large–scale adenosine production, and the process can be easily scaled up to meet industrial demands. It has been demonstrated that *Cordyceps cicadae* produces higher levels of adenosine under submerged fermentation than solid fermentation, and in a shorter time frame (Ke & Lee, 2019). The production of biomass and adenosine in *Cordyceps* sp. in submerged fermentation is influenced by various factors such as pH, temperature, nutrient availability, and agitation (Fan et al., 2012; Hung et al., 2015; Mao et al., 2005;

Ren et al., 2021; Shih et al., 2007; Xie et al., 2009). Several *Cordyceps* species have been researched to improve the production of adenosine, a key bioactive compound. The submerged culture of *Cordyceps sinensis* produced the highest adenosine production with 0.714±31.74 mg/g of mycelium after 14 days of fermentation (Ghatnur et al., 2015). In addition, Ke and Lee (2019) compared the adenosine production of *Cordyceps cicadae* under submerged and solid–state fermentation. The submerged culture of *C. cicadae* produced a higher adenosine concentration of 1.157±376 mg/g, greater than the solid fermentation. Ren et al. (2021) investigated the use of rice bran as a substrate for the submerged fermentation of *Hirsutella sinensis*, a *Cordyceps*–like fungus. This approach significantly increased the adenosine concentration to 1.76±11 mg/g, demonstrating the potential of optimizing substrate selection to enhance adenosine synthesis. Surface/static fermentation, on the other hand, is a method for cultivating fungi in static conditions after seeding the inoculum. In this method, a mycelial sheet forms on the surface of the medium, and some mycelia settle at the bottom. This method has been the most studied and optimized for liquid fermentation. Research has been conducted on the effects of medium compositions, culture condition adjustments, and strain improvements to optimize biomass and metabolite enrichment under surface culture conditions. (Das et al., 2008; Ke & Lee, 2019; Kunhorm et al., 2019; Masuda et al., 2007; Masuda et al., 2006; Sari et al., 2016; Suparmin et al., 2017). A two–stage fermentation process, following the Box–Behnken experimental design, achieved maximum cordycepin production (2.2 g/L) by combining 8 days of shake–flask fermentation with 16 days of static culture at pH 6 with 45 g/L yeast extract (Shih et al., 2007). Under two–step fermentation, the biomass of *C. militaris* was rapidly obtained with sufficient nutrition and dissolved oxygen in shake stage. In the static stage, the conidiophore was developed and achieved the accumulation of cordycepin reached to 2.62 g/L (Tang et al., 2015).

2.5 The Factors Influencing the Adenosine Production

The various parameters, such as fungal strain carbon/nitrogen ratios, medium composition, LED irradiation (using different wavelengths), pH, temperature, and culture time have been studied to optimize adenosine production.

2.5.1 The Fungal Strain

The fungal strain is a critical factor that can significantly influence metabolite production including adenosine and cordycepin of *C. militaris*, a good strain would capable to be an effective producer of its metabolite (Das et al., 2010; Kontogiannatos et al., 2021). Moreover, adenosine and cordycepin production can vary among strains from different regions (Liu et al., 2017). Screening for high–producing strains, as well as genetic engineering and mutagenesis approaches, have demonstrated the potential to enhance adenosine yields. A mutant strain obtained through proton beam irradiation exhibited a remarkable 72% increase in cordycepin productivity compared to the control (Das et al., 2008). Notably, genetic engineering approaches have been successfully employed to enhance adenosine production in other organism like *Bacillus subtilis*, where adenosine production was increased from 7.40 to 14.39 g/L through upregulation of the *pur*A gene (Li et al., 2019). Similar strategies could be applied to *C. militaris* to improve adenosine yields.

2.5.2 The Medium Composition

Regarding medium composition, carbon and nitrogen were the most common nutritional sources in the media which are related to cell growth and metabolite biosynthesis. Carbon is the essential ingredient for fruiting body development under solid–state fermentation. Different carbon sources were optimized for *Isaria tenuipes* cultured, Luem Pua glutinous rice and Riceberry rice provided the highest yield of adenosine (7.54 and 7.52 mg/g, respectively) when used as carbon source (Woraphokanunt et al., 2021). Notably, glucose and sucrose are frequently employed as carbon sources to stimulate fruiting body formation (Patthanajuck & Bunnag, 2021). However, glucose is a monosaccharide that is easier to absorb than sucrose which directly interact with the central carbon metabolism as needed for biomass formation. Glucose medium showed the highest yield of fruiting body (5.5 g) as well as production of adenosine (0.957 mg/g) and cordycepin (3.2146 mg/g) (Li et al., 2020). In liquid cultivation, carbon i.e., glucose, sucrose and dextrose play a crucial role for mycelium formation. Mao et al. (2005) reported initial glucose concentration at 40 g/L was favorable to cordycepin production (15.1 mg/g) and cell growth was increased in parallel with an increase of initial glucose concentrations (25–55 g/L). Besides, the biomass rapidly

increased when cultured *C. militaris* in 4% of glucose concentration medium (Gu et al., 2007). However, the research did not directly report the effect of glucose on adenosine production in *Cordyceps militaris* liquid cultures. The combination of the carbon source with an appropriate nitrogen source, such as yeast extract or peptone, also plays a crucial role in maximizing adenosine yields.

Aside from the carbon source, the nitrogen source was also an important factor in biomass and accumulation of metabolites. Under solid–state fermentation, fresh pupa was the most effective nitrogen source for increasing fruiting body formation. However, skim milk powder was the greatest nitrogen source for producing adenosine (2.598 mg/g) (Li et al., 2020). Yeast extract and peptone had an effect in promoting the *Ganoderma lucidum* mycelium growth (Fang & Zhong, 2002). For *C. militaris*, yeast extract and peptone served as nitrogen source and obtained the maximal adenosine concentration at 1.266 mg/g and 1.093 mg/g respectively (Ke & Lee, 2019). In comparison with organic nitrogen sources and inorganic nitrogen sources, Shih et al. (2007) and Chang et al. (2024) suggested that the organic nitrogen sources were more favorable than inorganic nitrogen sources. Organic nitrogen sources especially yeast extract were examined for their effects on the productivity of adenosine (16.7 mg/L. d) and cordycepin (17.2 mg/L. d), which higher than peptone and corn steep powder, as well as inorganic nitrogen sources i.e., NH_4Cl and $NH_4H_2PO_4$ (Shih et al., 2007). Notably, peptone combined with corn steep liquor hydrolysate emerged as the optimal nitrogen source, which suggested dry mycelium weight and cordycepin was 10.72 g/L and 27.29 mg/L, respectively (Chang et al., 2024). In addition, the study of *C. militaris* (BH and DA strain) was obtained the highest biomass production in medium supplemented with 20 g/L of yeast extract. The suitable nitrogen source for enhancing adenosine production varied in different strains, which suggested the yeast extract (10 g/L) for BH strain and peptone (20 g/L) for DA strain (Patthanajuck & Bunnag, 2021).

Furthermore, the combination of carbon and nitrogen was interesting for enhancing the yield of mycelium and its metabolites. The optimal concentrations of glucose (86.2 g/L) and yeast extract (93.8 g/L) in surface liquid culture led to significantly higher cordycepin production (2.79 times higher) in the mutant strain compared to the wild strain (Das et al., 2010). Moreover, the central composite design

and response surface analysis revealed that a carbon–to–nitrogen ratio of 42.0 $g_{glucose}$/L and 15.8 $g_{peptone}$/L, respectively, resulted in optimal cordycepin content (345.4 mg/L) (Mao et al., 2005). The optimized composition of culture media demonstrate a clear trend of increasing cordycepin and adenosine content in the *Cordyceps militaris* was obtained 1.9% and 0.24%, respectively (Solakov et al., 2022). The response surface methodology (RSM) was employed to achieve adenosine concentration was 61.84 mg/L under the optimal medium with 10.33 g/L of yeast extract and 27.24 g/L of sucrose (Yang et al., 2014).

2.5.3 Initial pH

The initial pH of the medium plays a critical role in fungal growth, cellular membrane function, requirement of nutrient consumption for their growth and the metabolic reactions in *C. militaris* (Adnan et al., 2017; Yang et al., 2014). Moreover, acidic pH has been reported that it was more suitable for mycelial growth and metabolites production for many kinds of *ascomycetes* and *basidiomycetes*, including *Cordyceps* sp. (Hsieh et al., 2005; Park et al., 2001; Shih et al., 2007). From Wen et al. (2014) studied, pH 5.5–6.0 was suggested for maximizing fruiting body and cordycepin yield of 1.81 g and 7.40±0.01 mg/g, respectively (Wen et al., 2014). It is similar to the result for maximum cordycepin production (381 mg/L) by *C. militaris* in medium pH 5.5 (Adnan et al., 2017). The effect of adenosine production by *Cordyceps* sp. due to the initial pH in submerged fermentation has not been extensively studied. Nevertheless, the maximum adenosine production (109.7 mg/L) and productivity (21.08 mg/L.d) in the medium with initial pH was 7.0. Meanwhile, pH 4.0 gave the maximum cordycepin production and productivity which was 315.2 mg/L and 12.6 mg/L.d, respectively (Shih et al., 2007).

2.5.4 Other Factors

Others condition factors including cultivation time, temperature, additives and LED irradiation (using different wavelengths) on *Cordyceps* cultivation had been studied. The optimal process conditions of *C. sinensis* (temperature 28 °C, pH 7, and inoculum volume 10%) provided the best yields in both adenosine (0.745 mg/g) and cordycepin (0.714 mg/g), along with a reasonable biomass yield (4.53 g/L) (Ghatnur et al., 2015). Additional compounds, such as ferrous sulfate (1 g/L), increased cordycepin production

by 70% compared to the control, reaching 0.60 g/L (Fan et al., 2012). Selenium supplementation influenced adenosine accumulation, with the most rapid increase observed within the range between 0–5 ppm selenium, however it was not significant for cordycepin production (Dong et al., 2012). Despite media composition, the light also matters for *C. militaris* production, red light (620–630 nm) was optimal for mycelial growth and adenosine accumulation, while blue light favored cordycepin accumulation (Chiang et al., 2017; Dong et al., 2013; Dong et al., 2012; Jiaojiao et al., 2018).

CHAPTER 3

MATERIALS AND METHODOLOGY

The effect of factors influenced on adenosine production from *C. militaris* was examined to provide the optimal condition for maximizing of the adenosine yield. The methods for optimization of submerged fermentation and confirmation of the optimal condition in 250–mL and 5–L shaking flask were summarized in Figure 3.1.

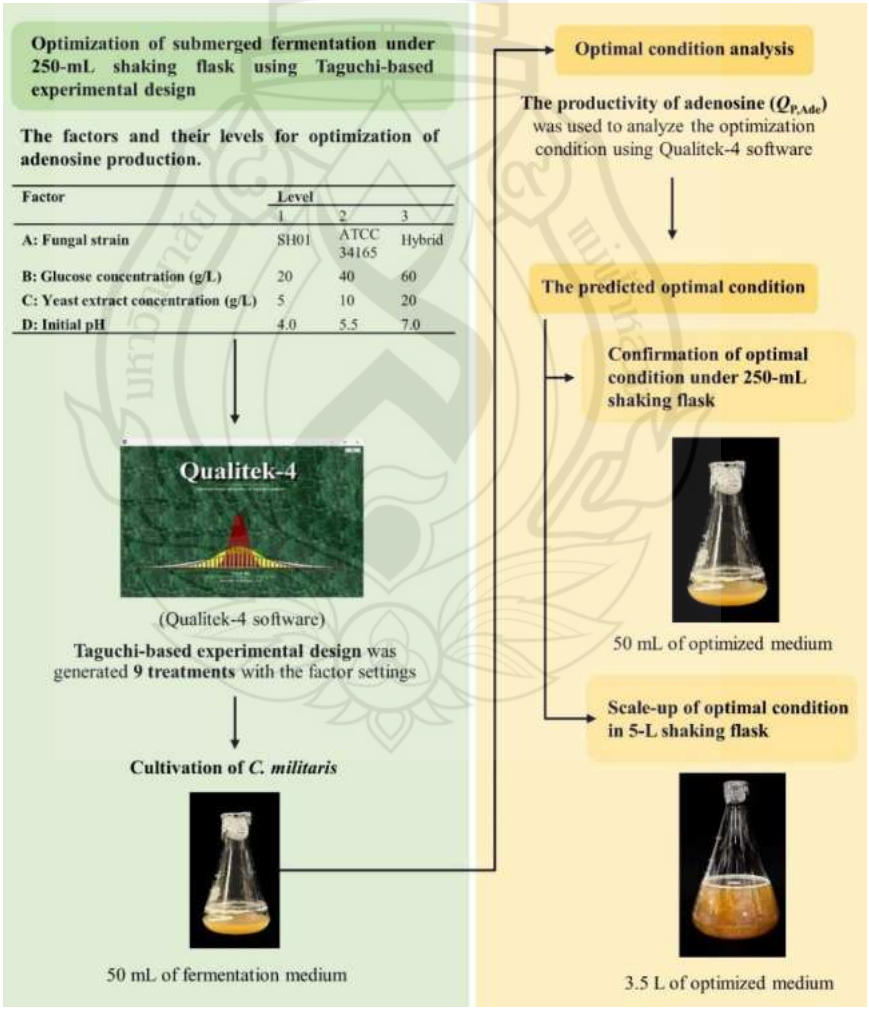


Figure 3.1 The methodological workflow for optimization on adenosine production

3.1 Maintenance and Culture of *Cordyceps militaris*

In this experiment, three strains of *C. militaris* with the potential to produce adenosine and cordycepin were investigated. These strains included: SH01 obtained from Shanghai, China, ATCC 34165 purchased from the American Type Culture Collection (ATCC) and the hybrid strain created by hybridizing SH01 and ATCC 34165. Three fungal strains were cultured on potato dextrose agar (PDA) at 20 °C for 21 days, the resulting fungal cultures were then stored at 4°C as stock. For seed culture preparation, the medium was modified based on the method of Shih et al. (2007) which was 40 g/L glucose, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The initial pH was adjusted to 7.0. Agar discs (5 mm in diameter) were punched out from PDA plates using a sterilized cork borer. Ten discs were inoculated into 200 mL of seed culture medium and incubated in darkness at 25 °C on a rotary shaker (150 rpm) for 5 days (Shih et al., 2007).

3.2 Optimization of Submerged Fermentation under 250–mL Shaking Flask using Taguchi–based Experimental Design

The various parameters including (1) fungal strain, (2) initial glucose concentration, (3) initial yeast extract concentration and (4) initial pH were selected to study the effect of fermentation condition. Beside glucose and yeast extract, the medium composition for the submerged fermentation consists of the following components: 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$. The optimization experiments were carried out in 250 mL shaking flask containing 50 mL culture medium. After inoculating with 10% (v/v) of the seed culture, the culture was incubated at 25 °C in a rotary shaker incubator at 110 rpm for 30 days, and samples were separately collected at various intervals every 3 days for analysis.

A Taguchi–based experimental design was used in this experiment. The experimental factors were: (A) fungal strain, (B) glucose concentration, (C) yeast extract concentration, and (D) initial pH. Each factor was assessed at three levels (Table 3.1). Qualitek–4 software (Nutek Inc., Bloomfield Hills, MI, USA) was used to design

the experiment and identify the optimal values of the variables. An orthogonal array of nine experiments with the factor settings shown in Table 3.2 was carried out. All experiments were performed in triplicate.

Table 3.1 The factors and their levels for optimization of adenosine production

Factor	Level		
	1	**2**	**3**
A: Fungal strain	SH01	ATCC 34165	Hybrid
B: Glucose concentration (g/L)	20	40	60
C: Yeast extract concentration (g/L)	5	10	20
D: Initial pH	4.0	5.5	7.0

Table 3.2 The factor levels for the production of adenosine by *Cordyceps militaris*

Treatment	Factor [a]			
	A	**B**	**C**	**D**
1	SH01	20	5	4.0
2	SH01	40	10	5.5
3	SH01	60	20	7.0
4	ATCC 34165	20	10	7.0
5	ATCC 34165	40	20	4.0
6	ATCC 34165	60	5	5.5
7	Hybrid	20	20	5.5
8	Hybrid	40	5	7.0
9	Hybrid	60	10	4.0

Note [a] A, fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); and D, initial pH.

3.3 Confirmation of Optimal Condition under 250–mL Shaking Flask

The productivity of adenosine ($Q_{P,Ade}$) was used to analyze the optimization condition using Qualitek–4 software (Nutek Inc., Bloomfield Hills, MI, USA). The optimization condition was carried out in 250–mL shaking flask containing 50 mL optimized medium. The cultured was incubated at 25 °C on a rotary shaker incubator at 110 rpm for 30 days. The samples were separately collected at various intervals every 3 days for analysis.

3.4 Scale–up of Optimal Condition in 5–L Shaking Flask

The optimal condition obtained from No. 3.3 was applied to a 5–L shaking flask containing 3.5 L of the optimal medium to evaluate scalability. Ten percent (v/v) of the seed culture was introduced to the optimal medium to reach a final volume at 3.5 L. The culture was then incubated at 25 °C with a rotary shaking at 150 rpm, without pH control, for a duration of 5 days. Each flask was harvested to represent the sampling. The content of adenosine and cordycepin, dry cell weight, residual glucose, and pH levels were examined in triplicate.

3.5 Determination of Mycelia Dry Weight, pH and Residual Glucose

A 50 mL of samples including mycelium and supernatant was collected every 3 days for 30 days from shake flasks. The samples were subjected for centrifugation at 8000 rpm for 15 min to separate mycelium and supernatant. Besides, the mycelium obtained from 5–L shaking flask was also harvested. The mycelium was rinsed with distilled water, and then collected by filtration through the 45 µm nylon membrane. The mycelium was freeze–dried prior measurement of dry cell weight and extraction to determine adenosine and cordycepin content. The weight of freeze–dried mycelium was examined and cell concentration (C_x)was calculated by following equation.

$$C_X\ (g/L) = \frac{W_2(g) - W_1(g)}{V(mL) \times 10^3}$$

where W_1 is the weight of container (g), W_2 is the weight of container and freeze–dried mycelium (g), V is volume of sample (mL).

The supernatant was subjected for pH measurement with a pH meter and residual glucose concentration using DNS method modified by Chuenprasert et al. (2021) (Miller, 1959). Briefly, to determine the residual glucose, 0.5 mL of supernatant were mixed with 0.5 mL of DNS solution. The reaction was boiled at 95 ºC for 5 min, then left until it cooled for 5 min. The sample was then measured at 540 nm using a microplate reader (Biotek Synergy HT Multi–Mode Microplate Reader, USA) (Chuenprasert et al., 2021). The calibration curve was generated with standard glucose (0.2–1.0 mg/mL) and the residual glucose concentration was estimated using the linear equation as follow:

$$Glucose\ concentration\ (g/L) = \frac{A_{540} \times dilution\ factor}{slope}$$

3.6 Extraction of Adenosine and Cordycepin

Adenosine and cordycepin were extracted and measured according to the method described by ChokeUmnuay and Owatworakit (2021). The crude extract was prepared from freeze–dried mycelium (50 mg) by extracting with 1 mL of dH_2O, and then was incubated at 60 °C for 3 hours. After centrifugation at 8000 rpm, 4 °C for 15 min, the supernatant was filtered through a 0.2 µm nylon syringe filter. The resulting solution (1 µL) was analyzed by a HPLC analysis.

3.7 HPLC Conditions for Metabolite Analysis

Analytical HPLC was carried out on a Waters ACQUITY Arc System (Waters Corporation, Milford, USA) consisting of a quaternary gradient pump, an autosampler,

and a 2998 Photodiode array (PDA) detector, connected to Empower 3 Software. A Kinetex C18 (4.6 × 250 mm, 5 μm, Phenomenax, Inc., Torrance, California, USA) was used. The isocratic elution method was adopted with a flow rate of 0.2 mL/min for separation of standards and samples using mobile phase consisting of methanol and 0.2% formic acid aqueous solution (95:5, V/V). The system operated at 30 °C and the detection wavelengths were set at 260 nm for adenosine and cordycepin.

Standards of adenosine and cordycepin (both from Sigma–Aldrich; USA) were prepared at 1 mg/mL. Working standard solutions were prepared by repeated dilution to give eight respective concentrations with RO water (100–0.78 μg/mL). The calibration curves were constructed by plotting the peak areas versus the concentrations of each standard. The linearity was demonstrated by a correlation coefficient (r^2) greater than 0.999.

3.8 Kinetic Parameters Estimation

For comparison of the experimental results, the volumetric rate of biomass production (Q_X) was calculated for the efficiency of the process for mycelium production, as follows:

$$Q_X = \frac{C_{X(a)} - C_{X(b)}}{t_{(a)} - t_{(b)}}$$

where Q_X is volumetric rate of cell production (g/L.d), C_X is cell concentration (g/L), t is time (d) and a, b represents the date of harvesting.

Yield of product ($Y_{P/X}$) was calculated as follows:

$$Y_{P/X} = \frac{C_P}{C_X}$$

where $Y_{P/X}$ is yield of product from freeze–dried mycelium (mg/g), C_P is concentration of product (i.e., adenosine and cordycepin) (g/L), C_X is cell concentration (g/L).

The productivity of adenosine (Q_P) was calculated as follows:

$$Q_{P,Ade} = \frac{Y_{Ade/X(a)} - Y_{Ade/X(b)}}{t_{(a)} - t_{(b)}}$$

where $Q_{P,Ade}$ is productivity of adenosine (mg/g.d), $Y_{Ade/X}$ is yield of adenosine from freeze–dried cell (mg/g), t is time (d) and a, b represents the date of harvesting.

3.9 Statistical Analysis

Experimental and statistical analysis was calculated using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) of data and graphics were generated by Qualitek–4 software (Nutek Inc., Bloomfield Hills, MI, USA) and Microsoft Excel 365, respectively. Data were presented as mean±standard derivative

CHAPTER 4

RESULTS AND DISCUSSION

4.1 The Effect of Factors Influence Mycelium Growth and Adenosine Production under Submerged Fermentation in 250–mL Shaking Flask

The effect of various factors on the condition specified in Table 3.1 under the submerged fermentation of *Cordyceps militaris* were examined. The fungal strain, the concentrations of both carbon source (glucose) and nitrogen source (yeast extract) and initial pH were investigated using Taguchi–based experimental design, which can help to identify and quantify the optimal values of the variables. After cultivation, the cell growth, residual glucose, pH value, adenosine and cordycepin production were determined. In all conditions, the cell concentration increased with time until day 3–6 as shown in Figure 4.1A. Treatment 3, provided the maximum cell concentration ($C_{X,\ max}$) and cell productivity ($Q_{X,max}$) were 30.982±0.386 g/L on day 6 and 6.309±0.480 g/L.d on day 3, respectively (Table 4.1). Consistence with the changes of residual glucose concentration during the fermentation in each treatment, since the carbon sources were slowly consumed by *C. militaris* (Gu et al., 2007). The initial glucose concentrations of each treatment were carried out at 20, 40 and 60 g/L according to Table 3.2, which gradually decreased with the mycelium growth (Figure 4.1B). The pH value during the cultivation process was decreased from 4.0–7.0 to 2.5–5.8 during exponential growth phase then increased on day 6 (pH 4.3–7.7) (Figure 4.1C). These suggested that mycelium grew due to the consumption of carbon sources, leading to the organic acid was produced and consequently reduced the pH value of media (Shih et al., 2007).

For the production of adenosine and cordycepin, the maximum yield of adenosine was observed in treatment 5, registering 4.080±0.415 mg/g on day 3.

Meanwhile, the peak cordycepin production was noted as 4.809±0.359 mg/g on day 21 of treatment 3 (Figure 4.2A&B, Table 4.1). The productivity (Q_P) is commonly used to indicate the efficiency of the process. The productivity of adenosine ($Q_{P,Ade}$) which was cultured under different conditions of submerged fermentation is shown in Table 4.2. The maximum $Q_{P,Ade}$ was found in the experimental treatment 5 (1.360±0.204 mg/g.d). Referring to Figure 4.2 and Table 4.2, the best condition was the *C. militaris* strain ATCC 34165 grew under glucose 40 g/L, yeast extract 20 g/L, and an initial pH of 4.0 (as seen in treatment 5).

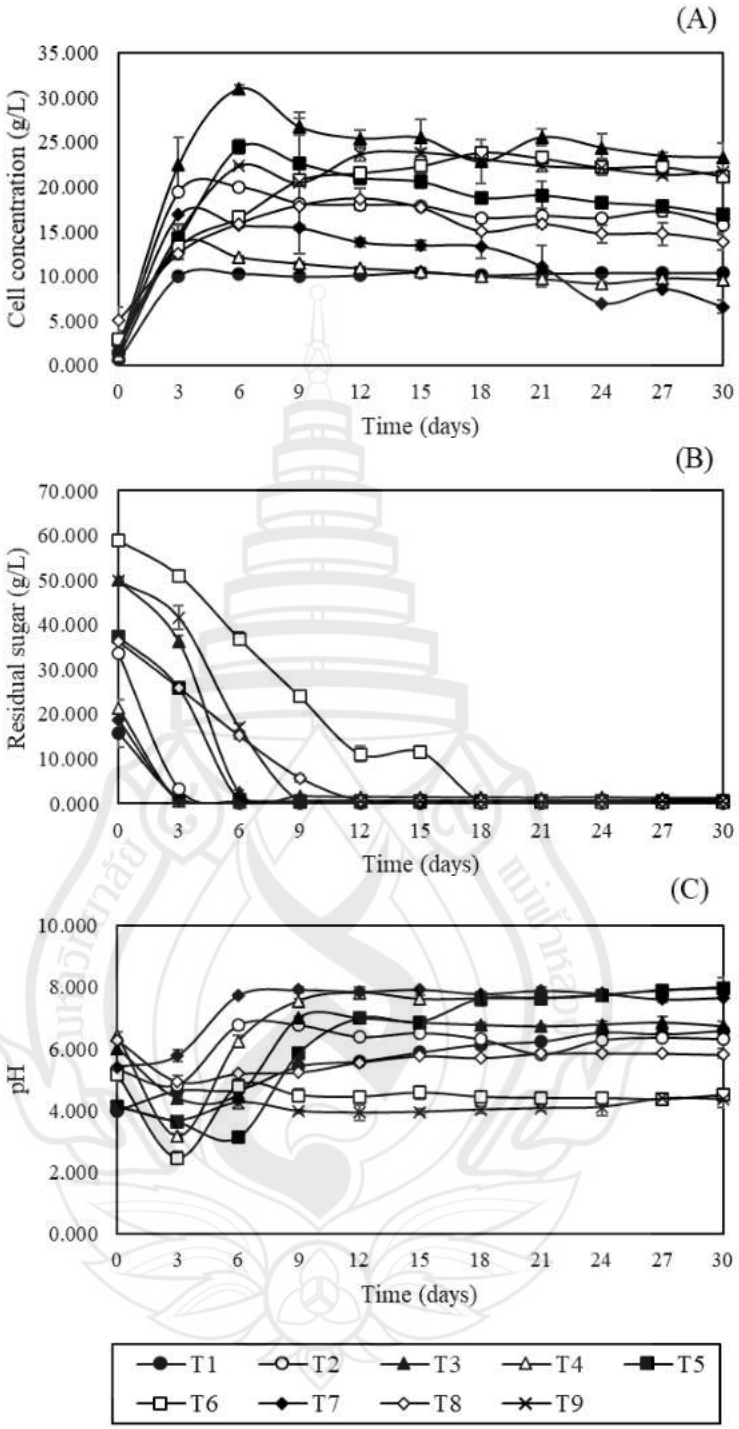


Figure 4.1 Changes in (A) cell concentration, (B) residual glucose and (C) pH by various conditions cultivated in 250–mL shaking flask

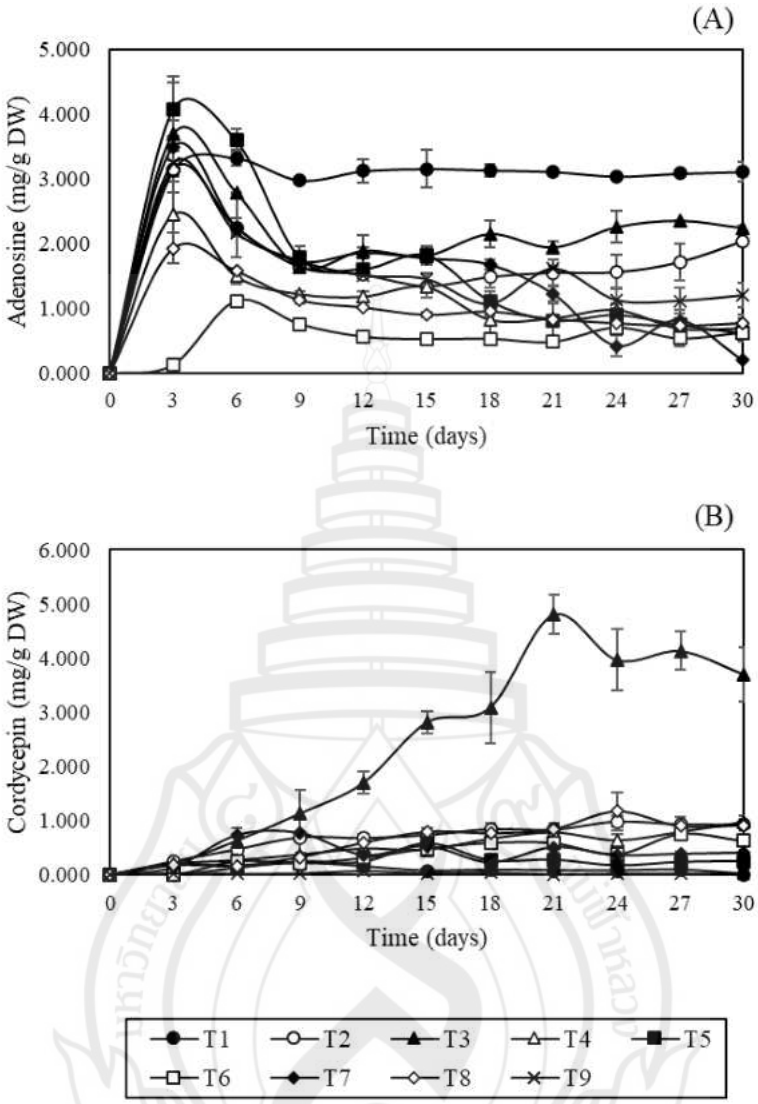


Figure 4.2 Yields of (A) adenosine and (B) cordycepin produced by various conditions cultivated in 250–mL shaking flask

Table 4.1 Maximum mycelium biomass, adenosine and cordycepin produced by various conditions

Treatment	Maximum cell concentration[a] ($C_{X,max}$) (g/L)	Maximum cell productivity[a] ($Q_{X,max}$) (g/L.d)	Maximum yield of adenosine[a] ($Y_{Ade/X,max}$) (mg/g)	Maximum yield of cordycepin[a] ($Y_{Cor/X,max}$) (mg/g)
1	10.408±0.031	3.088±0.039	3.324±0.123	0.224±0.017
2	19.980±0.383	5.970±0.080	3.161±0.127	0.986±0.105
3	30.982±0.386	6.309±0.480	3.694±0.890	4.809±0.359
4	13.522±0.225	4.154±0.110	2.459±0.507	0.992±0.092
5	24.499±0.833	3.807±0.187	4.080±0.415	0.587±0.108
6	23.895±0.532	3.435±0.489	1.118±0.030	0.764±0.115
7	16.866±0.122	5.084±0.003	3.490±0.410	0.772±0.039
8	18.695±1.149	2.505±0.301	1.931±0.237	1.179±0.346
9	23.871±0.421	4.316±0.378	3.247±0.189	0.085±0.060

Note [a] calculated based on maximum value

Table 4.2 The factor levels and productivity of adenosine for the different treatments

Treatment	Factor [a]				Productivity of adenosine ($Q_{P,Ade}$) (mg/g.d)
	A	B	C	D	
1	SH01	20	5	4.0	1.043±0.004
2	SH01	40	10	5.5	1.054±0.042
3	SH01	60	20	7.0	1.231±0.216
4	ATCC 34165	20	10	7.0	0.820±0.120
5	ATCC 34165	40	20	4.0	1.360±0.204
6	ATCC 34165	60	5	5.5	0.046±0.025
7	Hybrid	20	20	5.5	1.163±0.137
8	Hybrid	40	5	7.0	0.644±0.079
9	Hybrid	60	10	4.0	1.082±0.063

Note [a] A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

4.2 Optimal Condition Analysis under 250–mL Shaking Flask

The statistical analysis of the main effect of various factors and levels on the adenosine productivity is summarized in Table 4.3. The main effect of a factor was calculated as a percentage of its individual effect divided by the sum of the main effects of all the factors. The concentration of yeast extract (factor C; main effect = 40.071%) emerged as the most significant factor influencing adenosine production, followed by initial strain (factor D; main effect = 24.197%) and fungal strain (factor A; main effect = 21.879%). The glucose concentration (factor B; main effect = 13.853%) had less significant effect as presented in (Table 4.3). An analysis of variance (ANOVA) was performed to determine the contributions of individual factors to changes in the response, i.e., productivity of adenosine (Table 4.4). According to the ANOVA results

in Table 4.4, the concentration of the yeast extract (factor C) had the most significant effect on the adenosine productivity ($p < 0.01$).

Table 4.3 The percentage main effect of each factor on the productivity of adenosine

Level	Factor [a]			
	A	**B**	**C**	**D**
Productivity of adenosine ($Q_{P,Ade}$) (mg/g.d)				
1	1.109	1.008	0.577	1.161
2	0.741	1.019	0.985	0.754
3	0.963	0.786	1.251	0.898
Minimum value	0.741	0.789	0.577	0.754
Maximum value	1.109	1.019	1.251	1.161
Main effect [b]	0.368	0.233	0.674	0.407
% Main effect [c]	21.879	13.853	40.071	24.197

Note [a] A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

[b] Main effect = maximal value – minimal value.

[c] % Main effect = (100 × main effect)/total main effect. Total main effect is 1.682.

Table 4.4 Analysis of variance (ANOVA)[a] of factors affecting the productivity of adenosine

Factor [b]	DOF	Sum of Squares	Variance	F–Ratio	Pure Sum	Percent P (%)	Confidence (%)	Significant level
A	2	0.616	0.308	22.355	0.588	14.638	100.00	0.000
B	2	0.310	0.155	11.250	0.282	7.026	99.93	0.001
C	2	2.074	1.037	75.266	2.046	50.907	100.00	0.000
D	2	0.769	0.385	27.907	0.741	18.444	100.00	0.000
Other/Error	18	0.248	0.014			8.986		
Total	26	4.020	0.155			100.000		

$Y_{expected} = \overline{T} + (\overline{A}_{opt} - \overline{T}) + (\overline{B}_{opt} - \overline{T}) + (\overline{C}_{opt} - \overline{T}) + (\overline{D}_{opt} - \overline{T})$

Note $Y_{expected}$, the expected value; $\overline{T}$, the grand average of performance; $\overline{A}_{opt}$, maximum average effect of factor A; $\overline{B}_{opt}$, maximum average effect of factor B; $\overline{C}_{opt}$, maximum average effect of factor C; $\overline{D}_{opt}$, maximum average effect of factor D.

[a] ANOVA was for the experiments shown in Table 4.2.

[b] A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

* Significant at $p < 0.01$

From the optimization condition analysis, yeast extract concentration was the most significant factor effect on adenosine production (main effect = 40.071%). The nitrogen source significantly influences biomass and metabolite accumulation. Organic nitrogen sources exhibited greater effectiveness in promoting mycelium growth and metabolite production (Chang et al., 2024; Shih et al., 2007). Yeast extract was more favorable for mycelium growth, adenosine and cordycepin production than other organic nitrogen source i.e., peptone a corn steep powder, as well as inorganic nitrogen sources including NH_4Cl and $NH_4H_2PO_4$ (Shih et al., 2007). Previous studies suggested that the highest mycelium was achieved when using 10 g/L yeast extract, while the highest adenosine and cordycepin production occurred when 15 g/L yeast extract was used. Both strains of *C. militaris* (BH and DA strain) achieved the highest biomass production in medium supplemented with 20 g/L of yeast extract, while the suitable nitrogen source for adenosine production varied between strains. The highest adenosine content was obtained in medium supplemented with 10 g/L of yeast extract for the BH strain, whereas DA stain achieved in 20 g/L peptone (Patthanajuck & Bunnag, 2021). As the previous report has revealed the yeast extract composition including amino acids, lipid, vitamins, minerals and other soluble components with crucial of fungal nutritional sources (Zarei et al., 2016). Peptide and certain amino acid have been demonstrated for stimulating of adenosine via the metabolic pathway of purine and cordycepin (Chang et al., 2024). Therefore, the yeast extract might have a potential for adenosine production. The optimal yeast extract concentration was 20 g/L, with lower concentration (5 and 10 g/L) proving less desirable in the present work.

The second main effect of adenosine production was the initial pH (main effect = 24.197%). In this study, an initial pH of 4.0 proved optimal as displayed in Figure 4.3, whereas the initial pH at 5.5 and 7.0 was not suitable for adenosine production. In generally, the initial pH of the medium has a significant impact on fungal cell structure, cellular membrane function, nutritional consumption for fungal growth, and metabolic processes (Adnan et al., 2017; Yang et al., 2014). The research of *ascomycetes* and *basidiomycetes*, including *Cordyceps* sp. had been found that the initial pH value an acidic pH was more conductive to mycelium growth and metabolite production (Hsieh et al., 2005; Park et al., 2001; Shih et al., 2007). The result for maximum cordycepin production by *C. militaris* in medium pH 5.5 (Adnan et al., 2017), which similar to Wen

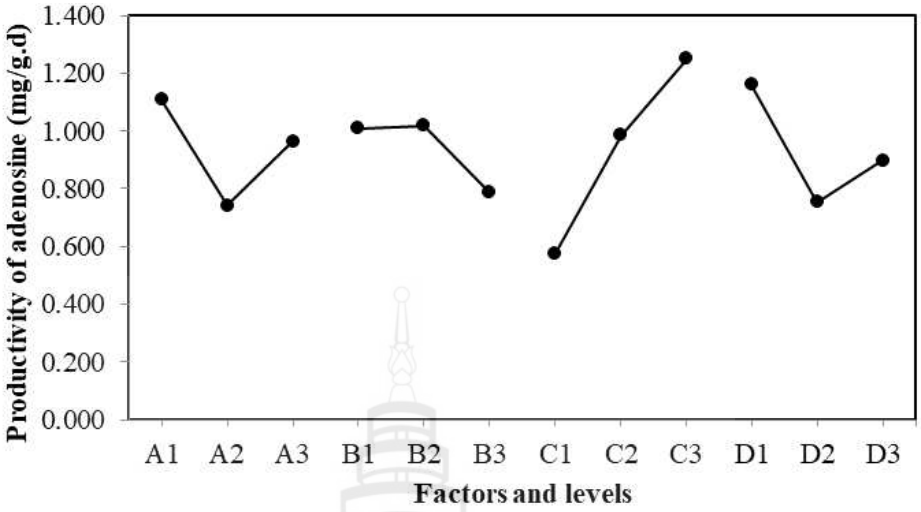
et al. (2014) studied. The finding suggested that at pH 5.5–6.0 of medium achieved the maximizing fruiting body and cordycepin yield (Wen et al., 2014). However, the study of initial pH influenced on the adenosine production had been scarcely explored. Shih et al. (2007) reported the maximum adenosine was shown in medium at initial pH 7.0, cordycepin was maximized at pH 4.0.

Besides, the fungal strain which is the third significantly effect on adenosine production (main effect = 21.879%). To achieve good productivity, one of the most critical factors is the strain used. A good strain, capable of being a robust producer of its metabolites, is necessary (Das et al., 2010; Kontogiannatos et al., 2021). Furthermore, adenosine and cordycepin synthesis might change between strains from various regions (Liu et al., 2017). Screening for high–producing strains, along with genetic engineering and mutagenesis approaches, have revealed the potential to applied for improving adenosine and cordycepin production in *C. militaris* (Das et al., 2008; Li et al., 2019). This study establishes *C. militaris* strain SH01 as the most suitable choice for adenosine production when using the optimal medium.

For the glucose concentration, it indicated the less effect than other factors on adenosine production. Carbon source is necessary for fungal growth, energy generation, and maintenance of cell. Fungi require a source of carbon to synthesize their biomass and energy. The kind and concentration of carbon sources can significantly impact fungal growth, metabolism, and the production of various bioactive compounds (Gu et al., 2007; Mao et al., 2005; Park et al., 2001; Woraphokanunt et al., 2021). Glucose and sucrose are common carbon sources used in fungal cultivation, especially glucose, which is easily metabolized and absorbed by fungi and directly interacted with the central carbon metabolism as needed for support biomass formation (Patthanajuck & Bunnag, 2021). The glucose medium was achieved the highest fruiting bodies yield as well as adenosine and cordycepin (Li et al., 2020). In addition, glucose also play a critical role for mycelium formation under liquid fermentation. The initial glucose concentration at 40 g/L was beneficial for cordycepin production, and cell growth increased corresponding with a rise in the initial glucose concentrations (25–55 g/L) (Mao et al., 2005). In the tested range of this work, the glucose concentration (20–60 g/L) had a relatively minor impact on adenosine production under *C. militaris*' submerged fermentation. However, an initial glucose concentration of 40 g/L was identified as

optimal. This aligns with Gu et al. (2007) studied, which observed a rapid increase in biomass when using 4% glucose. The studies did not directly report the effect of glucose on adenosine production in *Cordyceps militaris* liquid cultures. Nevertheless, glucose is the starting material change to ribose–5–phosphate (R–5–P) which is substrate for purine pathway to synthesize the certain nucleotide such as IMP and further to AMP, adenine and adenosine, and eventually result in cordycepin (Chang et al., 2024; Wang et al., 2023; Zhang et al., 2016). These metabolic pathways might suggest that glucose was beneficial for adenosine synthesis.

However, mycelial growth and adenosine production in *C. militaris* are related processes, but they are not directly proportional. Mycelial growth is favored by acidic conditions and continues over a longer period, while adenosine production is highest early on and is favored by neutral to alkaline conditions (Shih et al., 2007). Adenosine is produced during the mycelial growth phase, but its levels decrease as cordycepin production increases (Lin et al., 2022). The relationship between mycelial growth and adenosine production is complex and involves the interplay of various factors, varied among individual strains and suitable culture conditions. From these findings, *C. militaris* strain SH01 cultured under 40 g/L glucose, 20 g/L yeast extract, and initial pH 4.0 condition (Figure 4.3) was suggested as the predicted optimal condition for mycelium formation and adenosine production.



Note A, Fungal strain; B, glucose concentration (g/L); C, yeast extract concentration (g/L); D, initial pH.

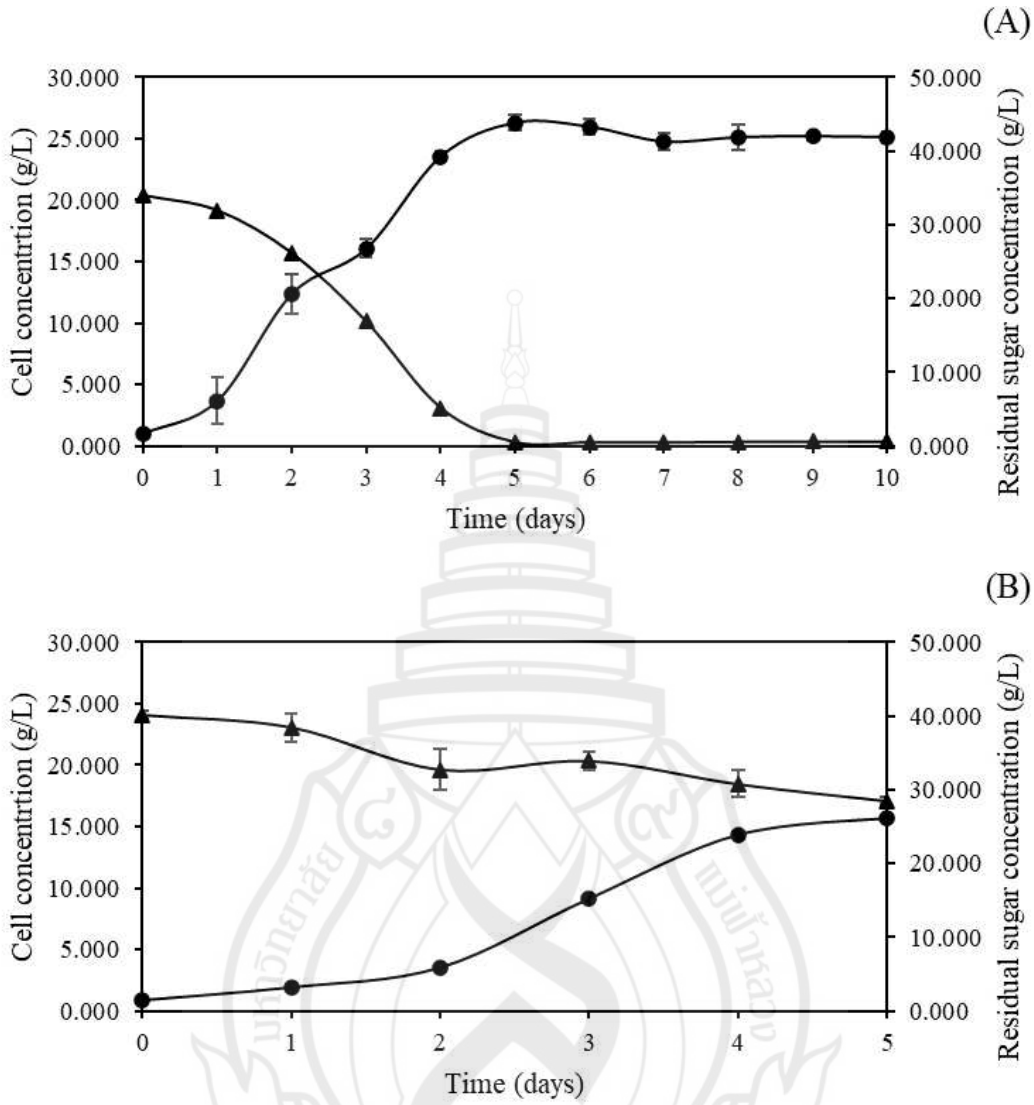
Figure 4.3 Productivity of adenosine for the various factors and levels

4.3 Confirmation of Optimal Condition under 250–mL and 5–L Shaking Flask

As depicted in Figure 4.3, the predicted optimal condition of the factors for maximizing the productivity of adenosine were as follows: A1 (A=SH01), B2 (B=40 g/L), C3 (C=20 g/L) and D3 (D=pH 4.0). Table 4.5 summarizes the maximum cell production ($C_{X,max}$) and cell productivity ($Q_{X,max}$) as well as yield of adenosine ($Y_{Ade/X,max}$) and cordycepin ($Y_{Cor/X,max}$) from cell, after confirming the optimal conditions as suggested by Taguchi–based experimental design. The result indicated that the kinetics of residual glucose concentration supported the conclusion, as the carbon sources were gradually consumed by *C. militaris*. The fungal grew for approximately 3 days, during which the carbon source was nearly depleted. The maximum cell production ($C_{X,max}$) (26.295±0.626 g/L) was achieved after five days of fermentation, followed by a stationary phase. During the log phase and the stationary phase, the residual glucose levels gradually decreased (Figure 4.4A). However, the maximum cell productivity ($Q_{X,max}$) of 5.703±0.734 g/L. d was obtained on the second day of fermentation.

Concurrently, adenosine production increased as mycelium grew (Ghatnur et al., 2015). The maximum yield of adenosine ($Y_{Ade/X,max}$) was obtained on the second day of fermentation (8.662±0.269 mg/g) but it declined after the third day. In contrast, the exponential phase of cordycepin production occurred around the fifth day, and the maximum yield of cordycepin was achieved on the seventh day (6.054±0.603 mg/g). However, the level of cordycepin increased when adenosine content was decreased (Figure 4.5A). This finding aligns with Xia et al. (2017), who established that adenosine is the direct precursor of cordycepin. Besides, adenosine is immediately transformed into pentostatin via the action of *cns*3, which inhibit the adenosine deaminase activity to block the deamination of cordycepin changed to non–toxic 3'–deoxyinosine. However, pentostatin levels should be investigated further for evidence of the adenosine transformation and the cordycepin accumulation.

For these values of the factors, the predicted the adenosine productivity was 1.727 mg/g.d. A validation experiment conducted at the optimal settings of the factors A; fungal strain was SH01, B; glucose concentration was 40 g/L, C; yeast extract concentration was 20 g/L and D; initial pH was 4.0 at a working volume of 250–mL shaking flask gave a measured productivity of 2.495±0.077 mg/g.d (Table 4.6). Thus, the measured data were increased 1.44 times of predicted result. The observed value of $Q_{P,Ade}$ of the validation experiment was significantly different ($p < 0.01$) from the experimental value seen in treatment 5 ($Q_{P,Ade}$ =1.360±0.204 mg/g.d; Table 4.2) at factor values of A; fungal strain was ATCC 34165, B; glucose concentration was 40 g/L, C; yeast extract concentration was 20 g/L, and D; initial pH was 4.0.



Note Cell concentration (●)

Residual glucose (▲)

Figure 4.4 The changes of cell concentration and residual glucose under optimal condition cultivated in (A) 250–mL and (B) 5–L shaking flask

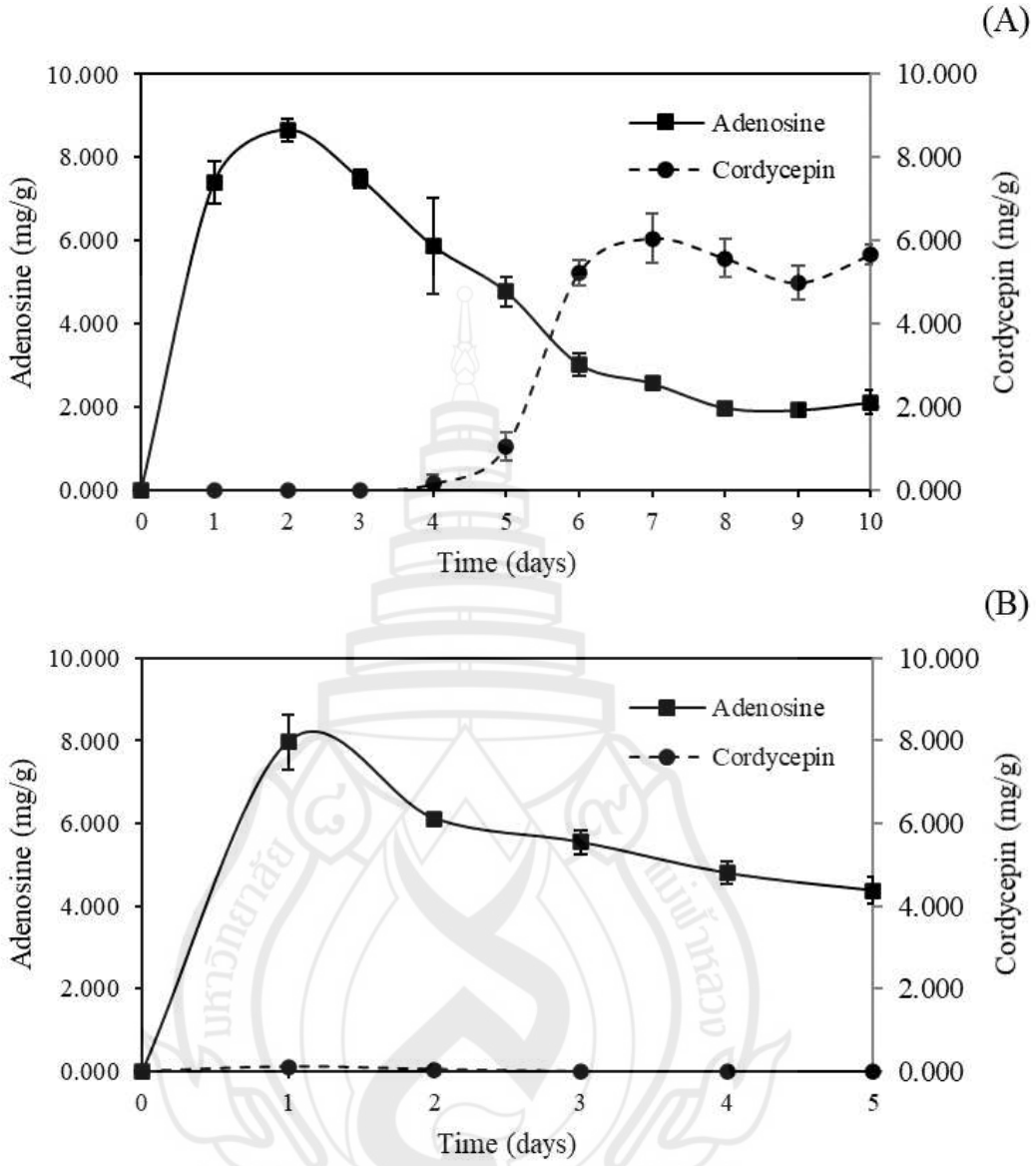


Figure 4.5 Yield of adenosine and cordycepin by optimal condition cultivated in (A) 250–mL and (B) 5–L shaking flask

The scalability of submerged fermentation conditions was assessed using a 5–L shaking flask with a working volume of 3.5 L. The *Cordyceps militaris* SH01 strain was cultivated under optimal conditions predicted from a Taguchi–based experimental design, specifically, 40 g/L glucose, 20 g/L yeast extract, and an initial pH of 4.0. During cultivation, the growth of *C. militaris* mycelium gradually increased, while residual glucose slightly decreased (Figure 4.4B). At the end of the process, the cell

concentration (C_X) reached 15.685 g/L, and the maximum productivity ($Q_{X,max}$) was 3.361 g/L.d at 4 days of fermentation (Table 4.5). The level of adenosine increased as the mycelium grew, whereas cordycepin yield was not produced during the first 5 days of fermentation (Figure 4.5B). The yield of adenosine ($Y_{Ade/X,max}$) was 7.967±0.674 mg/g obtained on the first day of fermentation, and then slightly decreased after the second day (Table 4.5). As a result of the 250–mL shaking flask experiment, cordycepin production began after 5 days, suggesting that cordycepin levels in the 3.5 L volume might also be produced after the fifth day of fermentation.

In the validation experiment conducted at the optimal condition in a 5–L shaking flask, the productivity of adenosine ($Q_{P,Ade}$) was 1.849±0.094 mg/g.d which was lower than that cultivated in the 250–mL flask (Table 4.6). However, the cell concentration and productivity of adenosine obtained under optimal conditions in the 5–L flask were less than those in the 250–mL shaking flask.

4.4 Limitation and Future Direction

For liquid culture, the working volume is an important consideration due to the role of dissolved oxygen (DO) in large–scale production (Kang et al., 2014; Wen et al., 2017). Oxygen affects cell growth, nutrient uptake, and metabolite formation in the fermentation of many microorganisms, such as *Bacillus brevis*, *Antrodia cinnamomea*, and *Ganoderma lucidum* (Narta et al., 2011; Shih et al., 2006; Zhang & Zhong, 2013). Higher levels of DO were beneficial to cell growth, but cordycepin production was higher when oxygen was reduced to 30% (Mao & Zhong, 2004). However, adenosine production peaked early in the growth cycle before declining as biomass increases (Ghatnur et al., 2015). Thus, this suggested that the dissolved oxygen required for cell growth and adenosine production.

For large–scale production, the bioreactor is more suitable strategy than shaking flask. Nevertheless, the stirred–tank bioreactor has been used to provide oxygen in the process. A lot of bubbles were formed, leading to the sticking of mycelium around bioreactor tank and resulting in a loss of mycelium yield in culture media. It means that it did not accurately represent the system's entire yield. Thus, the providing of dissolved

oxygen should be improved further by optimization of working volume or change the impeller type instead of the Rushton turbine, for example, the Intermig impeller.

Table 4.5 Mycelium biomass, adenosine and cordycepin produced by *C. militaris* under optimal conditions of submerged fermentation using the Taguchi–based experimental design

Kinetic parameters	Working volume (mL)	
	50	**3500**
Maximum cell production ($C_{X,max}$) (g/L)	26.295±0.626 (D5)	15.685±0.000 (D5)
Maximum cell productivity ($Q_{X,max}$) (g/L.d)	5.703±0.734 (D2)	3.361±0.000 (D4)
Maximum yield of adenosine from cell ($Y_{Ade/X,max}$) (mg/g)	8.662±0.269 (D2)	7.967±0.674 (D1)
Maximum yield of cordycepin from cell ($Y_{Cor/X,max}$) (mg/g)	6.054±0.603 (D7)	0.281±0.004 (D5)

Table 4.6 Productivity of adenosine under optimal conditions

Working volume (mL)	Optimal conditions [a]				Productivity of adenosine (Q_p) (mg/g.d)	
	A	B (g/L)	C (g/L)	D	Expected result	Measured value[c]
50	SH01	40	20	4.0	1.727 [b]	2.495±0.077
3500	SH01	40	20	4.0	1.727 [b]	1.849±0.094

Note [a] A, Fungal strain; B, Glucose concentration; C, Yeast extract concentration; D, Initial pH.

[b] Expected result was calculated as $Y_{expected} = \overline{T} + (\overline{A}_{opt} - \overline{T}) + (\overline{B}_{opt} - \overline{T}) + (\overline{C}_{opt} - \overline{T}) + (\overline{D}_{opt} - \overline{T})$. The grand average of performance, $\overline{T}$, was 0.938.

[c] Calculated based on yield of adenosine on day 3 of fermentation

CHAPTER 5

CONCLUSION

In this study, the effect of various factors including fungal strain, glucose concentration, yeast extract concentration and initial pH were studied in order to obtain a suitable condition for enhance adenosine production from *C. militaris*. Yeast extract concentration have more significantly affect than other factors. According to Taguchi method, the optimal condition for maximizing adenosine production from cultures *C. militaris* SH01 strain consists of 40 g/L glucose, 20 g/L yeast extract and initial pH 4.0 were identified at 50–mL working volume. The maximum adenosine productivity (2.495±0.077 mg/g.d) were obtained, which higher than higher than expected result 1.44 times. However, the scalability demonstrated in the 5–L shaking flask resulted in a lower adenosine productivity (1.849±0.094 mg/g.d) compared to the 250–mL shaking flask. Other factors, such as dissolved oxygen, warrant further investigation to enhance mycelium yield for large–scale production. Consequently, these findings could pave the way for improved adenosine production, potentially benefiting sectors such as healthcare, food, and cosmetics.

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APPENDICES

APPENDIX A

THE CHANGES IN PARAMETERS BY VARIOUS CONDITIONS CULTIVATED IN 250–ML SHAKING FLASK

Table A1 Mycelium biomass produced by various conditions

Time	Cell concentration (g/L)								
(day)	T1	T2	T3	T4	T5	T6	T7	T8	T9
0	0.741±0.052	1.553±0.415	1.910±0.627	1.059±0.187	2.809±1.037	3.012±0.531	1.614±0.113	5.051±1.526	1.887±0.310
3	10.006±0.170	19.461±0.232	21.061±2.137	13.522±0.225	14.231±1.595	13.316±1.323	16.866±0.122	12.566±0.651	14.835±0.884
6	10.267±0.245	19.980±0.383	30.982±0.386	12.143±0.187	24.499±0.833	16.626±0.200	15.726±0.008	15.875±0.354	22.357±0.091
9	9.948±0.133	18.165±0.119	26.721±0.946	11.425±0.232	22.671±0.622	20.799±0.019	15.420±0.240	17.921±0.191	25.055±0.270
12	10.089±0.030	18.000±0.483	25.444±0.958	10.907±0.198	21.112±0.821	21.607±0.479	13.819±0.479	18.695±1.149	23.678±0.734
15	10.408±0.031	17.879±0.163	25.511±2.105	10.538±0.391	20.635±0.812	22.389±0.135	13.478±0.509	17.671±0.120	23.871±0.421
18	10.080±0.057	16.532±0.420	22.847±2.052	10.025±0.341	18.833±0.458	23.895±0.531	13.368±1.409	15.003±1.545	23.167±0.750
21	10.254±0.054	16.753±0.456	25.583±0.921	9.722±0.291	19.103±1.501	23.255±0.572	11.105±2.326	15.805±0.399	22.370±0.598
24	10.350±0.215	16.491±0.266	24.435±1.521	9.164±0.204	18.316±0.151	22.236±0.950	6.976±0.006	14.735±0.982	22.066±0.199
27	10.352±0.198	17.273±0.565	23.501±0.283	9.791±0.167	17.896±0.577	22.300±0.534	8.603±0.245	14.764±1.250	21.360±0.294
30	10.362±0.054	15.683±0.704	23.348±1.557	9.597±0.333	16.877±0.647	21.233±0.369	6.579±0.714	13.846±0.881	21.713±0.981

Table A2 Residual glucose in various conditions

Time	Residual sugar concentration (g/L)								
(day)	T1	T2	T3	T4	T5	T6	T7	T8	T9
0	15.914±3.392	33.753±1.083	50.159±0.326	21.500±1.746	37.352±0.660	58.899±0.172	18.652±1.494	36.431±0.799	49.886±0.848
3	1.295±0.004	3.419±0.298	36.257±1.280	0.649±0.172	25.855±0.924	51.066±1.236	0.752±0.018	26.050±0.252	41.679±2.679
6	0.212±0.001	0.519±0.028	2.428±0.739	0.422±0.005	0.897±0.166	36.899±1.489	0.584±0.013	15.188±0.580	17.118±0.764
9	0.220±0.010	0.545±0.021	1.604±0.088	0.388±0.022	0.698±0.039	23.980±1.047	0.570±0.001	5.785±0.751	0.549±0.013
12	0.217±0.002	0.504±0.011	1.465±0.037	0.419±0.025	0.678±0.051	11.130±1.636	0.645±0.055	0.651±0.136	0.591±0.031
15	0.214±0.000	0.524±0.024	1.373±0.114	0.396±0.013	0.668±0.035	11.612±1.247	0.613±0.036	0.639±0.081	0.520±0.024
18	0.185±0.002	0.505±0.014	1.316±0.009	0.371±0.010	0.634±0.017	0.466±0.070	0.633±0.039	0.604±0.056	0.517±0.027
21	0.215±0.002	0.514±0.022	1.278±0.021	0.377±0.006	0.643±0.022	0.350±0.006	0.665±0.108	0.643±0.117	0.492±0.023
24	0.206±0.013	0.492±0.048	1.315±0.024	0.368±0.033	0.593±0.013	0.356±0.033	0.712±0.017	0.582±0.123	0.526±0.016
27	0.182±0.001	0.498±0.050	1.215±0.123	0.340±0020	0.601±0.024	0.398±0.032	0.834±0.016	0.685±0.080	0.559±0.016
30	0.199±0.022	0.516±0.011	1.159±0.046	0.358±0.008	0.589±0.040	0.468±0.039	0.839±0.024	0.731±0.117	0.545±0.027

Table A3 Yield of adenosine produced by various conditions

Time	Yield of adenosine (mg/g)								
(day)	T1	T2	T3	T4	T5	T6	T7	T8	T9
0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	3.128±0.013	3.161±0.127	3.694±0.890	2.459±0.507	4.080±0.451	0.137±0.090	3.490±0.410	1.931±0.237	3.247±0.189
6	3.324±0.123	2.257±0.150	2.794±0.008	1.502±0.127	3.601±0.182	1.118±0.030	2.257±0.455	1.581±0.038	2.175±0.027
9	2.984±0.022	1.647±0.054	1.650±0.057	1.227±0.032	1.789±0.184	0.762±0.039	1.729±0.112	1.135±0.057	1.740±0.055
12	3.125±0.172	1.530±0.008	1.896±0.231	1.188±0.070	1.614±0.058	0.568±0.011	1.860±0.083	1.021±0.026	1.515±0.044
15	3.162±0.288	1.350±0.184	1.818±0.072	1.336±0.050	1.827±0.137	0.531±0.001	1.772±0.032	0.906±0.032	1.460±0.082
18	3.139±0.092	1.491±0.176	2.155±0.206	0.832±0.211	1.121±0.003	0.537±0.077	1.679±0.082	0.955±0.048	1.074±0.189
21	3.117±0.016	1.555±0.207	1.947±0.100	0.852±0.006	0.816±0.261	0.489±0.010	1.232±0.120	0.841±0.013	1.617±0.042
24	3.043±0.011	1.564±0.268	2.259±0.243	0.983±0.088	0.903±0.040	0.707±0.021	0.426±0.165	0.775±0.058	1.135±0.181
27	3.088±0.011	1.721±0.283	2.361±0.002	0.693±0.013	0.749±0.008	0.542±0.120	0.821±0.155	0.736±0.050	1.122±0.193
30	3.116±0.143	2.043±0.081	2.249±0.028	0.697±0.212	0.619±0.131	0.632±0.068	0.202±0.028	0.778±0.080	1.209±0.188

Table A4 Yield of cordycepin produced by various conditions

Time	Yield of cordycepin (mg/g)								
(day)	T1	T2	T3	T4	T5	T6	T7	T8	T9
0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	0.195±0.051	0.248±0.008	0.210±0.081	0.206±0.096	N/A	N/A	0.136±0.021	0.178±0.003	0.034±0.012
6	0.165±0.012	0.470±0.099	0.629±0.124	0.271±0.088	0.131±0.023	0.256±0.100	0.730±0.146	0.168±0.028	0.022±0.011
9	0.224±0.017	0.694±0.034	1.135±0.441	0.380±0.277	0.248±0.156	0.222±0.113	0.772±0.039	0.327±0.062	0.029±0.010
12	0.159±0.070	0.680±0.042	1.702±0.198	0.492±0.113	0.240±0.034	0.316±0.027	0.365±0.010	0.596±0.150	0.085±0.060
15	0.078±0.005	0.737±0.029	2.815±0.197	0.471±0.094	0.587±0.108	0.494±0.134	0.533±0.056	0.800±0.078	0.036±0.022
18	0.095±0.034	0.846±0.119	3.094±0.660	0.676±0.090	0.271±0.079	0.597±0.115	0.231±0.058	0.774±0.105	0.051±0.022
21	0.085±0.043	0.835±0.113	4.809±0.359	0.796±0.127	0.283±0.088	0.592±0.216	0.511±0.106	0.849±0.082	0.008±0.003
24	0.081±0.031	0.986±0.105	3.971±0.559	0.649±0.116	0.177±0.040	0.385±0.079	0.365±0.045	1.179±0.346	0.026±0.009
27	0.097±0.003	0.934±0.146	4.137±0.355	0.806±0.092	0.243±0.076	0.764±0.115	0.387±0.063	0.903±0.072	0.023±0.011
30	0.018±0.003	0.927±0.032	3.708±0.497	0.992±0.092	0.253±0.075	0.631±0.200	0.410±0.050	0.923±0.097	0.029±0.007

Table A5 pH value during cultivation by various conditions

Time	pH								
(day)	T1	T2	T3	T4	T5	T6	T7	T8	T9
0	3.970±0.042	5.320±0.020	6.023±0.108	6.433±0.099	4.133±0.006	5.163±0.055	5.415±0.021	6.300±0.066	4.157±0.006
3	4.630±0.198	4.827±0.038	4.390±0.040	3.190±0.085	3.630±0.135	2.467±0.060	5.790±0.184	4.950±0.173	3.680±0.061
6	4.665±0.007	6.763±0.130	4.527±0.479	6.257±0.188	3.133±0.021	4.800±0.085	7.740±0.042	5.207±0.031	4.263±0.012
9	5.430±0.057	6.767±0.055	7.020±0.020	7.563±0.074	5.853±0.023	4.493±0.059	7.915±0.092	5.250±0.017	3.983±0.055
12	5.595±0.078	6.387±0.049	7.003±0.035	7.817±0.189	6.993±0.076	4.443±0.219	7.865±0.078	5.573±0.070	3.933±0.251
15	5.885±0.049	6.513±0.076	6.853±0.071	7.613±0.124	6.873±0.050	4.583±0.234	7.930±0.000	5.780±0.026	3.953±0.101
18	6.145±0.007	6.307±0.076	6.777±0.076	7.633±0.225	7.630±0.174	4.440±0.050	7.785±0.007	5.717±0.025	4.030±0.122
21	6.240±0.028	5.827±0.012	6.727±0.072	7.647±0.057	7.630±0.160	4.407±0.155	7.895±0.092	5.857±0.021	4.077±0.075
24	6.545±0.021	6.280±0.122	6.790±0.087	7.750±0.070	7.723±0.097	4.403±0.155	7.795±0.064	5.870±0.066	4.117±0.294
27	6.480±0.099	6.360±0.092	6.853±0.186	7.890±0.079	7.903±0.042	4.367±0.227	7.615±0.106	5.860±0.036	4.393±0.116
30	6.600±0.042	6.317±0.284	6.737±0.146	7.993±0.337	7.953±0.038	4.503±0.281	7.665±0.007	5.817±0.098	4.380±0.286

APPENDIX B

THE CHANGES IN PARAMETERS UNDER OPTIMAL CONDITION CULTIVATED IN 250–ML AND 5–L SHAKING FLASK

Table B1 Mycelium biomass, residual glucose and pH value during cultivation under optimal conditions

Time (day)	Cell concentration (g/L)		Residual glucose concentration (g/L)		pH	
	50 mL	3500 mL	50 mL	3500 mL	50 mL	3500 mL
0	0.961±0.180	0.884±0.000	34.017±0.818	40.110±0.572	4.317±0.006	4.077±0.012
1	3.673±1.877	1.933±0.000	31.961±2.612	38.455±1.897	4.390±0.010	4.123±0.015
2	12.368±1.573	3.533±0.000	26.171±0.928	32.756±2.795	4.540±0.010	4.097±0.025
3	16.043±0.749	9.137±0.000	16.891±0.229	33.925±1.219	4.580±0.020	4.030±0.000
4	23.546±0.443	14.328±0.000	5.195±0.401	30.824±1.827	4.373±0.116	4.083±0.006
5	26.295±0.626	15.685±0.000	0.532±0.078	28.546±0.462	4.580±0.302	4.027±0.006
6	25.969±0.645	N/A	0.527±0.055	N/A	5.463±0.177	N/A
7	24.755±0.693	N/A	0.490±0.034	N/A	6.517±0.093	N/A
8	25.117±1.018	N/A	0.568±0.021	N/A	5.653±0.015	N/A
9	25.192±0.272	N/A	0.607±0.042	N/A	5.643±0.032	N/A
10	25.124±0.419	N/A	0.627±0.019	N/A	5.617±0.012	N/A

Table B2 Yield of adenosine and cordycepin produced by optimal conditions

Time (day)	Yield of adenosine (mg/g)		Yield of cordycepin (mg/g)	
	50 mL	**3500 mL**	**50 mL**	**3500 mL**
0	N/A	N/A	N/A	N/A
1	7.395±0.514	6.292±0.011	N/A	0.065±0.065
2	8.662±0.269	4.687±0.392	N/A	0.073±0.025
3	7.486±0.231	2.961±0.025	N/A	0.110±0.002
4	5.873±1.143	2.523±0.014	0.158±0.193	0.237±0.010
5	4.775±0.364	2.036±0.010	1.032±0.341	0.281±0.004
6	3.032±0.271	N/A	5.216±0.302	N/A
7	2.567±0.134	N/A	6.054±0.603	N/A
8	1.978±0.111	N/A	5.579±0.453	N/A
9	1.919±0.004	N/A	4.986±0.420	N/A
10	2.108±0.293	N/A	5.651±0.237	N/A

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