Optimization of the fermentation medium for the mycelium production from Cordyceps militaris using Response Surface Methodology

By

CHANG REN YEH

Thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology, Assumption University

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ACKNOWLEDGEMENT

I am indebted to my thesis advisor **Dr. Churdchai Cheowtirakul**, dean in faculty for his understandings, dedications and help me to complete this thesis. Without his guidance and encouragements it would be impossible for me to complete the research. The valuable experiences and attitudes in conducting researches are the best thing I can receive and will always remember in my future career or studies.

I would also like to express my greatest appreciation to my dear teacher from the Graduate school of Biotechnology, Mrs. Nootrudee Siriboon, Dr. Viyada Kunathigan , and Dr. Wunwisa Kasaekoopt for their love, inspiration and knowledge bestowed upon me throughout the program, which helped me greatly in completing this study. Especially, A. Nootrudee Siriboon. Without her this thesis can be finished.

Finally, I would like to thank Dr. Apinya Assavanig as a committee to be kind and patient for this thesis founding. As well as USDA-ARS Plant Protection Research Unit

US Plant, Soil & Nutrition Laboratory provide the culture *Cordyceps militaris* (ARSEF 5007) and Mr. Richard A. Humber sent the culture, without the culture this research work won't be started. Thank you very much.

ABSTRACT

Fermentation of *Cordyceps militaris* for the production of mycelium was conducted using non-static rotational shaker at room temperature (~30°C) during June to July in 2008. Medium composition was screened by varying carbon sources from 5 types of compounds, nitrogen sources from 3 types of compounds, and 4 salts. It was found that molasses, yeast extract, $MnSO_4 \cdot H_2O$ and $MgSO_4 \cdot 7H_2O$ provided the highest mycelium production with significantly difference at $\alpha < 0.05$. To optimize the medium composition, two statistical methods were applied. Firstly, Fractional Factorial Design, FFD, was used to identify the highly influential factor from 5 factors from the screening including pH. The result indicated that molasses and pH had highly affected on the mycelium production with Pearson correlation of 0.433 and 0.528, respectively. Lastly, Response Surface Methodology was applied to optimize the selected factors from FFD and Central Composite Design, CCD, was used to interpret and demonstrate the coordinate that could elevate the mycelium production from the optimized medium. According to the research finding, the optimum fermentation medium for the mycelium production by *Cordyceps militaris* was 3.485% molasses (w/v), 2% yeast extract (w/v), 0.2 % of $MnSO_4 \cdot H_2O$, and 0.2 % of $MgSO_4 \cdot 7H_2O$; initial pH was 6.26 under the condition where the temperature was 30°C and fermentation time were10-day.

Keywords: mycelium, *Cordycep militaris*, medium optimization, response surface methodology

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CHAPTER I

INTRODUCTION

This chapter provides a brief outline of research "Optimization of the fermentation medium for the mycelium production from *Cordyceps militaris* using Response Surface Methodology" The chapter begins with the background of *Cordyceps* species, and then introduces the subject of *Cordyceps militaris* and recent studies on *Cordyceps militaris* and better research for better ideas. The scope of study was how to archive the goal of better mass production.

1.1 Background of *Cordyceps* species

Cordyceps is a rare medicinal mushroom, known in China for centuries. In general usage of the term "*Cordyceps*" usually refers specifically to the specific species *Cordyceps sinensis*, but there are also many other closely related species that come under the general term of *Cordyceps*. While *Cordyceps sinensis* is the most well known throughout the world, there are many other species in the genus *Cordyceps* in which modern science found as valuable medicinal properties as well (John, 2004).

1.1.1 The subject *Cordyceps militaris*

Cordyceps militaris (*Clavicipitaceae, Ascomycotina*), is also known as the Chinese caterpillar fungi that has potential to replace Dong-Chong-Xia-Cao (*Cordyceps sinensis*) as a Chinese herb for extracting Cordycepin. Cordycepin was first extracted from *Cordyceps militaris* (Mizuno, 1999) and then found to be present in *Cordyceps sinensis* (Huang, 2003) and *Cordyceps kyuhuensis* (Ling, 2002). During the past several years, the

polysaccharides and cordycepin produced by *Cordyceps* had been interested due to their various biological and pharmaceutical properties and activities (Bae, 1998). Adenosine and Cordycepin, in Cordyceps-related species, have usually been assumed to be bioactive ingredients in most reports (Hsu, 1999). Adenosine has a number of actions that give it as a possible cardio-protective and therapeutic agent for chronic heart failure (Kitakaze, 2000). Adenosine is also a local hormone with numerous tissue-specific biological functions. In the myocardium, adenosine is released in small amount at a constant basal rate during normoxia, and the effects of such interventions have been observed during ischemia and reperfusion (Sommerschid, 2000). Cordycepin (3'-deoxyadenosi, 3'-dA) is a nucleoside analogue which exhibits antileukemic activity against terminal deoxynucleotidyl transferase-positive (TdT+) leukemic cells and has been considered as a therapeutic agent for TdT+ leukemia (Kodama, 2000)

1.1.2 Recent studies on *Cordyceps militaris* and research idea

Most of the recent studies on *Cordyceps militaris* are about the extraction of adenosine, cordycepin (Wang, 2005; Wen, 2005), polysaccharides (Wang, 2005; Ruan, 2005), and also about cultivating of artificial fruity body by silk-warm or rice. As *Cordyceps militaris* gains the popularity of researcher's view, a research idea comes out that how to make mass production on those active substrates mentioned above. In this study, the purpose was to find the suitable fermentation condition for the production of mycelium of *C. militaris*. As the amount of adenosine, cordycepin, and polysaccharides depend on the quantity of mycelium.

1.2 Objective

This research aims to optimize the fermentation condition for the growth of mycelium in order to lower the production cost of *Cordyceps militaris* mycelium at industrial scale level. Response surface methodology (RSM) is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed values. By using RSM, the research hopes to answer the above research question and provided statistical data that would provide insight for further studies.

CHAPTER II

LITERATURE REVIEW

2.1 Name of *Cordyceps*

The ability to attack or invade insects has appeared frequently in fungi. Most insect pathogens are either from the *Entomopthorales*, a *Zygomycete* order, or from the Ascomycetes and related Deuteromcetes. Insects can be attacked by microorganism which often live in soil or within substrates during larva or pupa stages, or in the adult, often as aerial from. Most species are specialized to attack one stage of insect. Ascomycete fungi consist hundreds of obligate insect parasites. Many of these are tropic and therefore little investigated. The best known Ascomycete genus of insect parasites is *Cordyceps*, with several hundred species. It commonly infects larva or pupa, producing yeast-like cells in the haemocoel, and usually killing its host some weeks after initial infection. Continued hyphal growth within the body produces a kind of sclerotium which acts as a survival stage for the fungus. Finally, when conditions are favorable, an aerial fruit body emerges from the sclerotium, and bears perithecia and conidia. (Michael, 1994)

The name *Cordyceps* comes from the Latin words: *cord* and *ceps*, meaning "club" and "head". In historical and general usage the term "Cordyceps" usually refers specifically to the specific species *Cordyceps sinensis*, but there are also many other closely related species that come under the general term of *Cordyceps*. Although *Cordyceps sinensis* is the species of *Cordyceps* that well known throughout the world, but there are many other species in the genus *Cordyceps* in which modern science has found

valuable medicinal properties in as well. For example, Cordycepin was first extracted from *Cordyceps militaris* (Mizuno, 1999) and then found to be present in *Cordyceps sinensis* (Huang, 2003) and *Cordyceps kyuhuensis* (Ling, 2002).

2.1.1 General description

Cordyceps is a well-known parasitic genus. Most attack *Lepidoptera* and *Coleoptera*, but in the tropics some attack adult spiders. *Cordyceps militaris* produces orange-colored, stalked, club-shaped stromata, 10-40 mm tall, bearing numerous perithecia immersed in the swollen apex (as show in figure 1 below). These arise in the autumn from mummified larva, pupa or adults in the soil. Ascospores are released and, on infection, short, cylindrical hyphal bodies develop in the haemocoel of the insect. These Ascospores are increasing by budding and become distributed throughout the body. Death occurs after about 5-7 days. After death, the hyphae of *Cordyceps spp.* developed from the hyphal bodies and the dead insect becomes plugged with a dense mass of fungal tissue (as in Figure 2). At this stage it is virtually mummified. Its tissues are extremely resistant to decay due to the production of the antimicrobial nucleotide cordycepin by the fungus. This ensures that other saprotrophic micro-organisms in the soil do not plunder the reserves required to produce the talked ascocarps. (Ingold, 1993)

Figure 2.1 The orange-colored, stalked, club-shaped stromata of *Cordyceps militaris* (source: http://www.nifg.org.uk/cordyceps.htm)

Figure 2.2 The Fruity body on a mummified insect

(Source http:// www.dkimages.com/ discover/Home/ Plants/Fungi-Monera-Protista/ Mushrooms-and-other-Fungi/Truffles/ Clavicipitaceae/ Orange-Caterpillar-fungus/ Orange-Caterpillar-fungu-1.html)

2.1.2 Mycological information

Kingdom – Fungi

Phylum – *Ascomycota*

Class –*Sordariomycetes*

Order – *Hypocreales*

Family – *Clavicipitaceae*

Genus – *Cordyces*

Species – *Cordyceps militaris*

English name – Cordyceps mushroom, Chinese caterpillar fungus

Japanese names – sanagitake, サナギタケ

Chinese names – Bei-Dong-Chon-Xia-Cao, 北冬蟲夏草

2.1.3 History and traditional uses

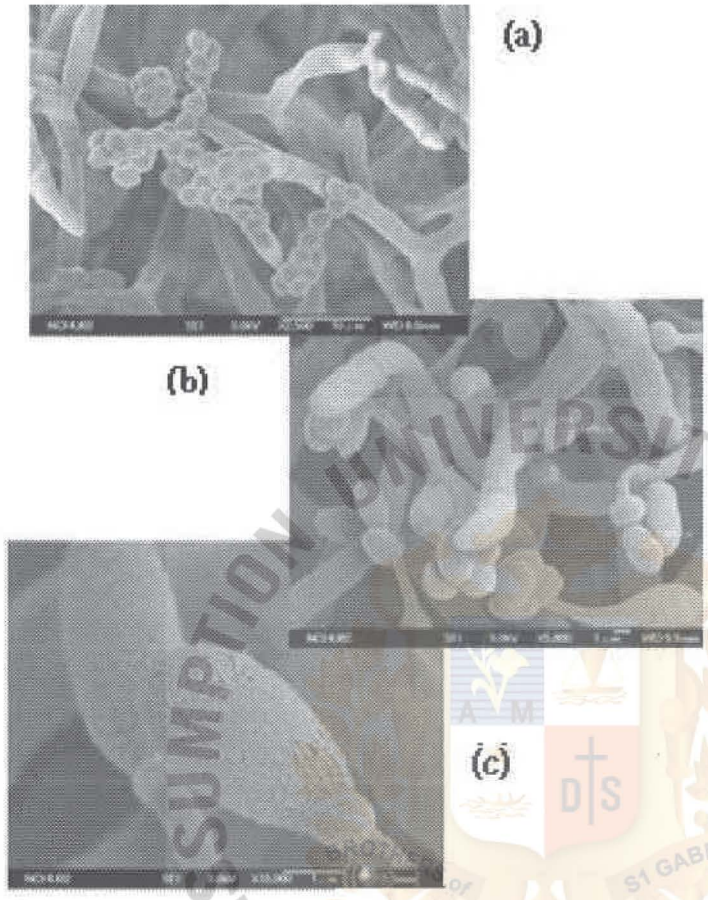
In traditional Chinese medicine, there are three most famous and upper class tonic herbs: ginseng, deer horn and *Cordyceps spp.* (Winter warm summer grass) . In several periods of ancient China, Cordyceps was used in the Emperor's palace only. In the compendium of medical herb 1596, compiled by Lǐ Shízhēn, Dong-Chon-Xia-Cao has the functions on strengthen the body, restore various functions or homeostasis leading to a balance, improve lung, improve kidney and other impressive functions.

Figure 2.3 Direct consumption on the *Cordyceps militaris* using water to extract active compound

(source: http://www.jin.ne.jp/fukuju/info-ex/2007/04/)

2.1.4 Habitat and area

The *Cordyceps militaris* is mainly found in China, Japan, and Taiwan. It lives in arm and pine of forest with the altitude of 2200~2400 m. Its fruit bodies occur from late July to late September, in the mild, moist and rainy habitat. Unlike *Cordyceps sinensis, Cordyceps militaris* are much wider spread thought out the world. The hosts infected by *C. militaris* are much wider compare with *C. sinensis.*



(a), conidiophores

(b) conidia

(c) electro-microscope

Figure 2.4 The conidia clusters (or chain) produced from carpophore of *Cordyceps militaris*

(Source: http://store.pchome.com.tw /isobio/HM/ view_sup_epaper.htm? sup_paper_no=000118921)

2.2 Active ingredient interactions

There is some evidence that alteration of the body's blood glucose metabolism in patients consuming *Cordyceps* often results in reduction of oral or injected antidiabetic medications. It is also posited that the naturally occurring antiretroviral compounds found

in *Cordyceps* (2'3' dideoxyadenosine , or *Cordycepins*) could result in increased effectiveness or decreased dosage requirements or patients undergoing concurrent therapy with other antiretroviral drugs. Caution should be exercised in these patients, especially considering the newer, more potent hybrid strains of *Cordyceps* being developed, and the targeted medicinal compounds they are being selectively cultivated for. Many of the antiretroviral drugs currently on the market have quite considerable toxicity, and it is hoped that the incorporation of *Cordyceps* into the treatment regimen of those patients undergoing such therapy might result in a reduction of some of these more toxic synthetic drugs, while sacrificing none of the efficacy. While no detrimental drug interactions have yet been noted in the scientific literature, caution should be advised, as both the fields of pharmaceutical discovery and *Cordyceps* cultivation are both rapidly expanding. With any substance of such considerable bioactivity as Cordyceps has proven to be, some drug interaction is always a possibility.

2.2.1 General nutritional components of *cordyceps*

Cordyceps contains a wide range of compounds considered nutritional. It contains all of the essential amino acids, vitamins B1, B2, B12, E, and K, a wide range of sugars including monosaccharide, disaccharide, and oligosaccharide and many different polysaccharides (some of amazing and unique complexity), proteins, sterols, nucleosides, and a wide range of trace elements (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr.)

2.2.2 Major bioactive constituents - Cordycepin

Cordycepin [3'-deoxyadenosine] and Cordycepic acid [d-mannitol] were the initial bioactive compounds first isolated from the *Cordyceps militaris* species (Mizuno, 1999). A study by (Chen and Chu,1996), announced the characterization of cordycepin [3' deoxyadenosine] and 2'-deoxyadenosine, using nuclear magnetic resonance (NMR) and infrared spectroscopy (IR) in an extract of *Cordyceps sinensis*. Other components found included various saccharides, and polysaccharides of varied and amazing complexities. Beta-glucans, Beta-manans, cross-linked beta-mannan polymers, and complex polysaccharides consisting of both 5 and 6 carbon sugars joined together in branch chains comprising both alpha and beta-bonds. Many nucleosides have been found in *Cordyceps*, including uridine, several distinct structures of deoxyuridines, adenosine, 2',3' dideocyadenosine, hydroxyethyladeosine, cordycepin [3'deoxadenosine], cordycepin triphophate, guanidine, deoxyguanidine, and a variety of other very unique altered and deoxygenated nucleosides that are found no where else in nature.

2.2.3 Major bioactive constituents - polysaccharides

In the fungal kingdom, and particularly in *Cordyceps,* the polysaccharides are perhaps the best known and understood of the medicinally active compounds (Wasser, 2002). A number of polysaccharides and other sugar derivatives such as cordycepic acid [d-mannitol] have been identified and their pharmacological activity has been reported. Research has shown these polysaccharides to be effective in regulating blood sugar to have anti-metastatic effect (Nakamura et al, 1999) and antitumor effect. (Bok et al, 1999)

2.3 Therapeutic applications

Table 2.1 Use of Cordyceps and its products in various medical treatments (rearranged from Huang, Y. et all, 1987)(reference from Wasser, S.P., 2002; Nakamura K, et all, 1999; Bok J.W. et all, 1999)

Animal study/clinical trial/treatment	Effectiveness
Improvement of physical performance	
- Ratio between ATP and inorganic phosphate	- Increased (p<0.001)
- Hypoxic environment	- Prolonged survival and more efficient use of oxygen (p<0.001)
Senescence	
- Intolerance to cold, fatigue, dizziness	- Improved (P<0.001)
- Tinnitus	- Improved (P<0.001)
- Hyposexuality	- Improved (P<0.05)
- Amnesia	- Improved (P<0.003)
- Scavenging activity of superoxide dismutase, SOD (reduction in oxygen-free radical)	- Significant increase in red blood cell SOD activity (P<0.001) even in different disease conditions
Reproductive functions	
- Sperm count	- Higher (P<0.05), increased, may promote spermatogenesis
- Malformed spermatozoa	- Reduced
- Survival rate of spermaozoa	- Increased
Endocrine system	- Attenuated adrenal and spleen antrophy – an increased capability to adopt a cold environment
Cardiovascular system and circulatory function	
- Super – ventricular or ventricular arrhythmia	- Complete/partial recovery of ECG, effective for tachyarrhythmia and bradyarrhythmia
- Ischaemic heart disease	- Clinical improvement in chest distress and palpitation
- Blood fibrinogen and viscosity	- Reduced (P<0.01)
- Contracted aorta	- Artery relaxation seen
- Dilation of arteries and increased blood supply	- Can dilate the coronary and cerebrovascular arteries
- Heart rate	- Effective in reducing heart rate
- Hyperlipidaemia	- Can control
- Atherosclerosis	- Acts against the formation
- Total cholesterol and triglycerides	- Reduced (P<0.001)
- Both LDL and vLDL cholesterol	- Decreased (P<0.001)

- HDL cholesterol - Blood glucose	- Increased (P<0.001) - Reduced (P<0.01)
Respiratory system - Intratrachael secretion - Cough latent period - Cough frequency - Chronic bronchitis, bronchial asthma	 - Increased (P<0.001) facilitating expectoration - Prolonged (P<0.001) - Decreased (P<0.001) - Significant clinical improvement
Kidney and renal function - Blood nitrogen urea - Serum ceratinine - Haemglobin - Kidney toxicity - Cellular sodium pump - Chronic interstitial oedema haemorhage fibrosis and tubular denaturation-necrosis - Morphological damages induced by cyclosporin	 - Reduced (P>0.005) - Reduced (P>0.005) - Increased (P>0.005) - Nephro-protective effect - More activated - Reduced - Prevented
Hepatic system (chronic hepatitis and related disease conditions) - Hepatitis – B, thymol turbidity test - Increased SGPT - Serum albumin	 - Returned to normal in one-third of the patients - Over half of the patients recovered to normal range - Increased
Cancer (anti-proliferative activity) - Spontaneous metastases of B16 melanoma - Drug resistant Lewis lung carcinoma (LLC) - Primary weight of tumors - In *vivo* anti-tumor activity - Patients with advanced cancer receiving conventional cancer therapies - Various types of tumor	 - Decreased liver weight (P<0.01) - Decreased liver weight (P<0.05), significantly fewer meta-static foci than control - Reduced by 20% in LLC and 47% in B16 M compared to control - LLC cells decreased by 96%, B16M cells decreased b 62% - Restoring of immune cell functions - WBC maintained at <3000 mm^{-3}, tumor size significantly reduced in half of the patients

2.3.1 Possible uses as agent in anticancer

Cordyceps militaris is a parasitic fungus and has been used in Chinese medicine to treat numerous illnesses so as an anticancer drug. Active compound of *C. militaris* was extracted with methanol, and then, methanol extract was further fractionated with hexane, ethyl acetate, butanol and water. Anticancer effects were examined. The cytotoxicity of *C. militaris* fractions on cancer cell lines and normal cell lines were tested and the anticancer molecule was purified. Tested cancer cell lines were B16F10 (melanoma), Hela (cervixcarcinoma), SKOV3 (adenocarcinoma), K562 (leukemia), HepG2 (hepatoblastoma), Du1459 (prostate carcinama), MKN-45 (stomach adenocarcinoma) and A549 (lung carcinoma) cells. Among the cell lines, K562 and Du1459 were highly inhibited by *C. militaris* butanol fraction. After different purification steps (silicagel column chromatography, Sephadex-LH column chromatography and HPLC), the study had given two different anticancer molecules from butanol fraction, one is cordycepin and the other is a new compound as F-1. F-1 is more active than cordycepin to prohibit cancer cell line. It has been proposed to name F-1 as 'militarin'. (Ho et al, 2004)

2.3.2 Side effects

Very few toxic side effects have been demonstrated with *Cordyceps* use, although a very small number of people may experience dry mouth, nausea or diarrhea. Many people find that when they first take *Cordyceps*, they will experience a feeling of mental clarity, sometimes bordering on the state induced in the early stages of LSD intoxication, where the colors all seem brighter and everything seems to stand out with crystal clarity. These effects usually clear up within a couple of days of *Cordyceps* used. There have been reported very occasional allergic reactions to Cordyceps, but this type of reaction is not common. There is little published data on the use of Cordyceps in pregnant or lactating women, or in very young children, so normal appropriate precautions should be taken with these types of patients.

2.3.3 Toxicity

Cordyceps has proven to be a very non-toxic herbal substance with the obviously wide-ranging physical effects on the body. While no human toxicity has been reported, animal models have found an LD 50 of 27 g/kg when injected in mice. Given by mouth to rabbits for 3 months at 80 grams/day, no abnormalities were seen from blood tests or in kidney or liver function. (Huang, et all, 1987). Cordyceps is thought to be a very safe substance with a minimal potential of toxicity.

2.4 Related species and artificial cultivation method

2.4.1 Cordyceps sinensis

Cordyceps generally refer to *Cordyceps sinensis*, It was found in the mountain in south of China. Formation of stroma of *Cordyceps sinensis* and its artificial cultivation method were not found in the other article.

2.4.2 Cordyceps militaris

The *Youtouga* larva was submerged into the suspended spore broth made from Cordyceps militaris for 30 seconds. The submerged *Youtouga* larva was then put on a plate box that was covered with sphagnum on both top and bottom. The plate box was closed with a cover. Then the box was placed in room temperature under sunlight. After 40 days, a colony was formed, and 85-90 days stroma was formed.

Without insect larva

0.6% dry yeast extract, 1.5% Probian, 0.2% peptone, 2.2% (w/v) agar powder dissolved in distilled water. After sterilized, 1.5% glucose solution, 0.01% Inosinic solution were added into the solution in 100ml Erlenmeyer flask. Stroma of *Cordyceps militaris* were incubated in 100ml Erlenmeyer flask in 18°C. Around 41 days conidiophores generated. After 80-95 days fruity body was formed.

2.4.3 Cordyceps formicarum

0.6% dry yeast extract, 0.15% gluten, 2.2% (w/v) agar powder dissolved in distilled water. After sterilized, 1.5% glucose solution, 0.01% Inosinic solution were added into the solution in 100ml Erlenmeyer flask. Stroma of *Cordyceps formicarum* was incubated in 100ml Erlenmeyer flask in 18°C. After 171 days fruity body was formed.

2.4.4 Cordyceps takaomontana

0.6% dry yeast extract, 0.15% gluten, 2.2% (w/v) agar powder dissolved in distilled water. After sterilized, 1.5% glucose solution, 0.01% Inosinic solution were added into the solution in 100ml Erlenmeyer flask. Stroma of *Cordyceps takaomontana* was incubated in 100ml Erlenmeyer flask in 18°C. After 120 days fruity body was formed.

(Original from https://www.jpo.go.jp/shiryou/s_sonota/hyoujun_gijutsu/kinoko/2-3-1.pdf)

It contains proteins, polysaccharides, D-Manitol, cordycepin, cordyceps acids, amino acid, eggosteral peroxide, cordyceptide A and trace elements.

Figure 2.5 Cordyceps mycelium powder from American store website

Figure 2.6 The Cordycepin capsule in Japan website shop

(source: http://www.gabataro.com/SHOP/101.html)

Figure 2.7 Glucasepin W from the crystal cellulose of *Cordyceps militaris*.

(Source: http://www.mmjp.or.jp /oze-syokukin/sonoiti/h-s1.htm)

Figure 2.8 The Cordyceps extract with chicken essential.
(source: http://www.ibnest.com/english/shopping.asp)

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

Cordyceps militaris (ARSEF 5007)

3.1.2 Equipments

Oven – Memmert, model 600

Glassware

Non-rotary flask shaker – IKA Labortechnik, K5501 digital

Microprocessor pH Meter – Hanna Instruments, pH211

3.1.3 Chemical and medium

PDB kit – Britania company

Peptone - Britania company

Yeast extract - Britania company

Milk powder

Glucose – UNIVAR from Asia Pacific Specialty Chemical Limited

Corn flour

Molasses

Rice flour

K_2HPO_4 - MERCK

$MgSO_47{\cdot}H_2O$- CARLO ERBA

$ZnSO_47{\cdot}H_2O$- Fluka

$MnSO_4 \cdot H_2O$- CARLO ERBA

3.2 Experiment Design

In order to obtain a suitable medium for mycelium production, a series of experimental design was conducted to investigate the possible effects of various medium components on the mycelium weight. First, the preferable nutrient of carbon sources, nitrogen sources, and essential elements for mycelium production were determined by varying one nutrient at one time while keeping the others constant using a 2-factor factorial design that each nutrient as one factor and level of nutrient in the medium as another factor. Second, a modified experimental design from Liu C.et.al (2007)), a factional factorial designs were used for identifying the highly influential nutrient(s) in the medium composition. Thirdly, 2 of the most influential factors were studied to optimize they level for the medium composition used Response Surface Method (RSM) with Central Composite Design (CCD) to interpret the result.

3.3 Preparation of the culture and medium

3.3.1 Preparing starter culture

C. militaris (ARSEF 5007) culture used in this experiment was obtained from USDA-ARS Plant Protection Research Unit, US Plant, Soil & Nutrition Laboratory. The culture was activated in PDA medium at 25°C for 8 days. The starter culture was prepared by punching out 1 x 1 x 1 cm^3 from the inoculated the PDA plate with a sterilized cylindrical cutter. (Adapted from Mina, 2006).

3.3.2 Preparing the filter paper

No.1 Filter paper was dried until gaining a constant weight in a previously sterilized oven at 40°C. The dried filter paper was weighed and recorded its weight before using in filtering the mycelium

3.3.3 Preparing Broth

The broth was prepared based on PDA formula. C source, N source, two essential elements were varied with kind of amount and pH value were adjusted to pH 6.. The broth was sterilized in an autoclave 121°C for 30 minutes.

3.4 Experimental procedure

3.4.1 Screening of the media components using one factor at a time

The purpose of the first step was to screen the medium components, carbon sources, nitrogen sources and essential elements, for the mycelium production. 1% and 2 % concentration of carbon sources - rice flour, glucose, sucrose, molasses and corn flour - and nitrogen sources - milk powder, yeast extract, and peptone - were studied. 0.1% and 0.2% concentration of essential elements (K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$) was added.

3.4.1.I Design to test the effect of different carbon sources (rice flour, glucose, sucrose, molasses and corn flour) on mycelium production.

Five different carbon sources, rice flour, glucose, sucrose, molasses and corn flour, each with two different levels, 1% and 2%, were added into the culture medium. The other components in the medium consisted of 1% malt extract (w/v), 1% yeast extract (w/v), 0.2% K_2HPO_4 (w/v), 0.2% $MgSO_4 \cdot 7H_2O$ (w/v) and pH value was adjusted to pH 6. The inoculums were prepared by punching out 1 cm^3 of starter culture and were added into the flask. The flasks were shook in a rotary shaker at 180 rpm at $32^{O}C$ for 10 days. The mycelia were pouring into a plastic tube and centrifuged at 5500 rpm for 5 minutes. The supernatant was discarded. The wet precipitate was filtered through the prepare filter paper. Then, the mycelium was dried in an oven at $60^{o}C$ for 2 days until a constant weight was obtained. The dried mycelium was weighed and recorded its weight. The C source which could give the highest amount of mycelium had been selected for further study.

3.4.1.II Design to test the effect of different nitrogen sources (milk powder, yeast extract, and peptone) on mycelium production.

Three nitrogen sources, milk powder, yeast extract, and peptone, each with two different levels, 1% and 2%, were added into culture medium the other components in the medium consisted of 1% malt extract (w/v), 2% Molasses (w/v) (selected from

experiment of screening carbon source), 0.2% K_2HPO_4 (w/v), 0.2% $MgSO_4 \cdot 7H_2O$ (w/v) and pH value was adjusted to pH 6. The procedure was done as the same as in Part 3.4.1.I. The N source which could give the highest amount of mycelium had been selected for further study.

3.4.1.III Design to test the effect of different essential elements on metabolism of mycelium production

Four different essential elements, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$, each with two different levels of concentration, 0.1% and 0.2%, are added into culture medium. The other components in the medium consist of 2% Molasses (w/v) (selected from experiment of screening carbon source), 2% yeast extract (w/v) (selected from experiment of screening nitrogen source) and pH value was adjusted to pH 6. The procedure was done as the same as in Part 3.4.1.I. The salt(s) which could give the highest amount of mycelium had been selected for further study.

3.4.2 Fractional Factorial Designs and analysis

The purpose of the first optimization step was to identify which component in the medium was the most influential of the production of mycelium. Fractional factorial design is very useful statistic tool to identify the important components and potential interactions between 2 factors or more factors in the reduced experiments. The number of experiments can be reduced by using only part of the fractional factorial designs without losing the information about the main effects. FFD (2_{IV}^{6-2}); was used and 16 treatments were obtained, as shown in Table 3.1. Four control runs were added to verify the most significant factor affected on the weight of the mycelium. Each run of experiment was conducted in duplicate. The variables were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i$$

Where x_i is the coded value of an independent variable, X_i is the real level of an independent variable, X_0 is the real level of an independent variable at the center point, and ΔX_i is the step change level.

The range and the levels of the variables, both coded values and natural values, investigated in this study are given in the following Table3.2. The weight of mycelium was considered as the dependent variable or response (Y_i).

Table 3.1 FFD for optimizing media of the production of mycelium by *Cordyceps militaris*.

Run	x_1	x_2	x_3	x_4	x_5	Net Weight (mg, Y_i)
1	+1	+1	-1	+1	-1	81.63
2	+1	+1	+1	+1	+1	112.03
3	+1	-1	-1	+1	+1	94.9
4	-1	+1	+1	+1	-1	62.2
5	+1	-1	+1	+1	-1	75.2
6	+1	-1	-1	-1	-1	74.08
7	-1	+1	-1	-1	-1	59.23
8	-1	-1	+1	-1	-1	78
9	+1	-1	+1	-1	+1	90.25
10	-1	+1	-1	+1	+1	98.58
11	-1	-1	+1	+1	+1	68.65
12	-1	+1	+1	-1	+1	72.7
13	-1	-1	-1	+1	-1	64.78
14	-1	-1	-1	-1	+1	72.83
15	+1	+1	+1	-1	-1	62.55
16	+1	+1	-1	-1	+1	97.825
17	0	0	0	0	0	96.9
18	0	0	0	0	0	103.7
19	0	0	0	0	0	94.13
20	0	0	0	0	0	97.68

$x_1 = (X_1-2)/1$, $x_2 = (X_2-2)/1$, $x_3 = (X_3-0.2)/0.05$, $x_4 = (X_4-0.2)/0.05$, $x_5 = (X_5-6)/0.5$

Table 3.2 Levels of variables used in the fractional factorial design for optimizing media for the production of mycelium

Independent variables	Levels		
	-1	0	+1
Molasses (w/v) (X_1)	1	2	3
yeast extract (w/v) (X_2)	1	2	3
$MgSO_4 \cdot 7H_2O$ (w/v) (X_3)	0.15	0.2	0.25
$MnSO_4 \cdot H_2O$ (w/v) (X_4)	0.15	0.2	0.25
pH (X5)	5.5	6.0	6.5

Adapted from Liu C. et. al (2007)

3.4.3 Optimization of key component concentrations using a Central Composite Design (CCD)

The Box-Wilson Central Composite Design, commonly called `a central composite design,' contains an imbedded factorial or fractional factorial design with center point that is augmented with a group of `star points' that allows an estimation of a curvature. If the distance from the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center of the design space to a star point is ± α with $|\alpha| > 1$. The precise value of α depends on certain properties desired for the design and on the number of factors involved in the experiment.

To maintain rotation ability of the star points (Figure 4.5), the value of α will depend on the number of experimental runs in the factorial portion of the CCD:

$$\alpha = [\text{number of factorial runs}]^{1/4}$$

The medium components that significantly affected mycelium production were optimized using a CCD design. The variables were coded according to the equation [1]:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad [1]$$

Where x_i was the coded variable of a nutrient factor (carbon source, nitrogen source, essential elements, pH), X_i was the natural variable of the nutrient factor. X_o was the value of the natural variable at the center point, and ΔX_i was the step change value. The variables and levels were shown in Table 4.1.

The statistical software has defined a full CCD design for 2 factors consisting of 13 combinations plus the replicates at the center point (4 star points and 5 replicates at the center point to estimate the experimental error and to investigate the suitability of the proposed model)as showed in Figure 4.5.

(Modified from internet source http://www.itl.nist.gov/div898/handbook/pri/section3/pri3361.htm)

3.5 Statistical Analysis

For the analysis of each experiment result, a several calculation had been conducted. 2-factor factorial design was analysis using Microsoft Excel version 2002 to perform analysis of variance (ANOVA) at $\alpha > 0.05$ and Duncan Multiple Range Tests (DMRT) was conducted when there was significant effect from ANOVA. Fractional factorial design (FFD) in 2^{k-p} was used to estimate the effect of the treatment obtained from a quarter reduction of 2^6 experiment and analyzed by SPSS version 14.0. In Central Composite Design, Data plot (Design Expert- trial version obtained from the site - http://www.statease.com/soft_ftp.htm) was used to analyze the result in the form of ANOVA for Response Surface Quadratic Model table, Contur plot and Response surface plot. The Contur plot and Response surface plot were obtained to visualize the effect of the factors.

CHAPTER IV

RESULT AND DISCUSSION

4.1 Screening of the media components

4.1.1 Effect of different carbon sources (rice flour, glucose, sucrose, molasses and corn flour) on mycelium production

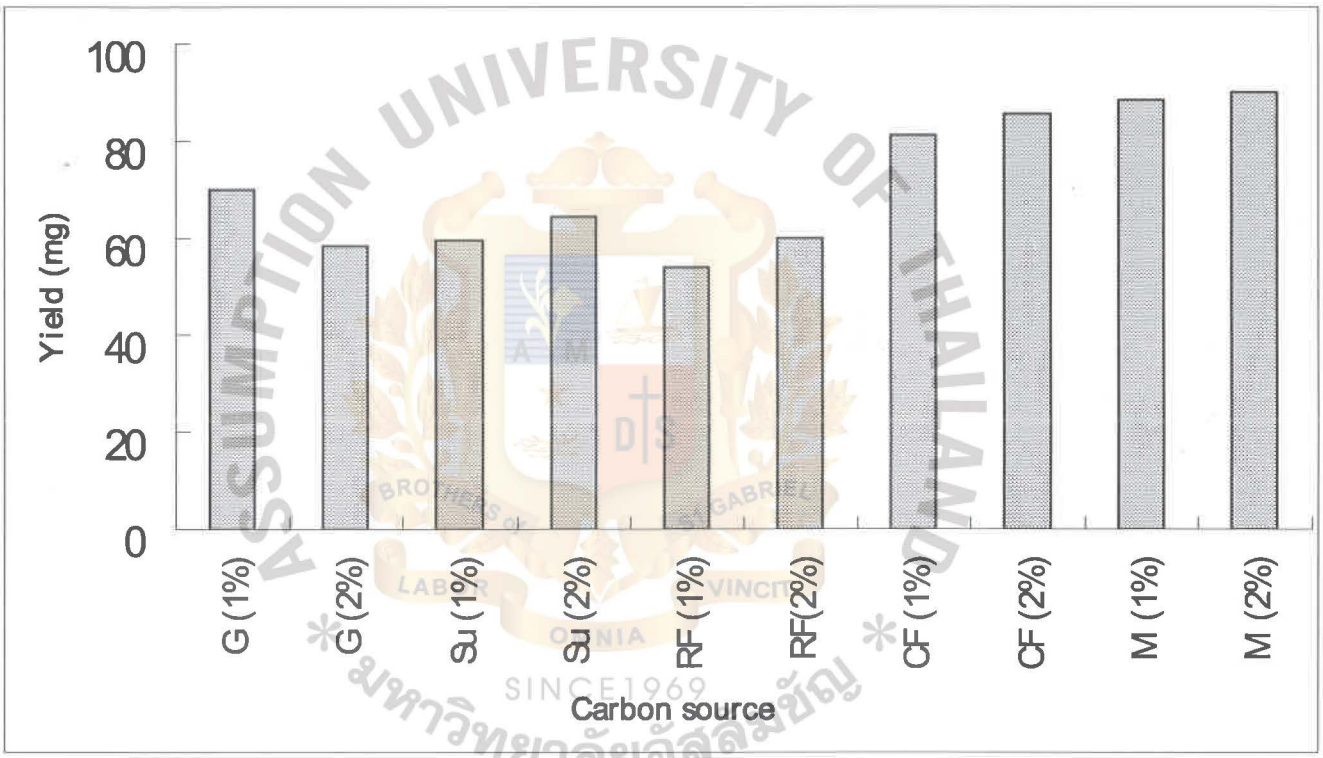


Figure 4.1 Effect of different carbon sources on mycelium production by *Cordyceps militaris*. The medium consisted of 1% malt extract (w/v), 1% yeast extract (w/v), 0.2% K_2HPO_4(w/v), and 0.2% $MgSO_4 \cdot 7H_2O$(w/v)) with initial pH6.0, culture time 10 days. G1 = glucose 1% (w/v), G2 = glucose 2% (w/v), S1 = Sucrose1% (w/v), S2 = Sucrose2% (w/v), R1 = Rice flour 1% (w/v), R2 = Rice flour 2% (w/v), C1 = Corn flour 1% (w/v), C2 = Corn flour 2% (w/v), M1 = molasses 1% (w/v), M2 = molasses 2% (w/v).

Carbon source is an important factor in microbial growth and it plays the main role in cell division. The effect of five carbon sources, rice flour –R, glucose - G, sucrose –S, molasses -M and corn flour –C, on the production of mycelium by *Cordyceps militaris* was summarized in Figure 4.1. Two concentrations of each carbon sources used in the media were studied. Statistic analysis indicated that the kind of C source was significantly affected the mycelium production while the concentration of C source and their interaction had no significantly effect (α > 0.05%). 2% of each carbon source was favorable to the mycelium production, but not in the 2 % of Glucose. Glucose, sucrose and rice flour had poor induction effect compared to corn flour and molasses. In figure 1, both 1% and 2% molasses gave high results in production of mycelium. The highest net weight of mycelium was observed when 2% molasses was used as carbon source. Both 2 % corn flour and 2 % molasses showed significant results for the growth of mycelium. In the DMRT, the corn flour and molasses were significantly different. So 2 % of molasses was chosen as carbon source for subsequent experiments on mycelium production.

4.1.2 Effect of different nitrogen sources (milk powder, yeast extract, and peptone) on mycelium production.

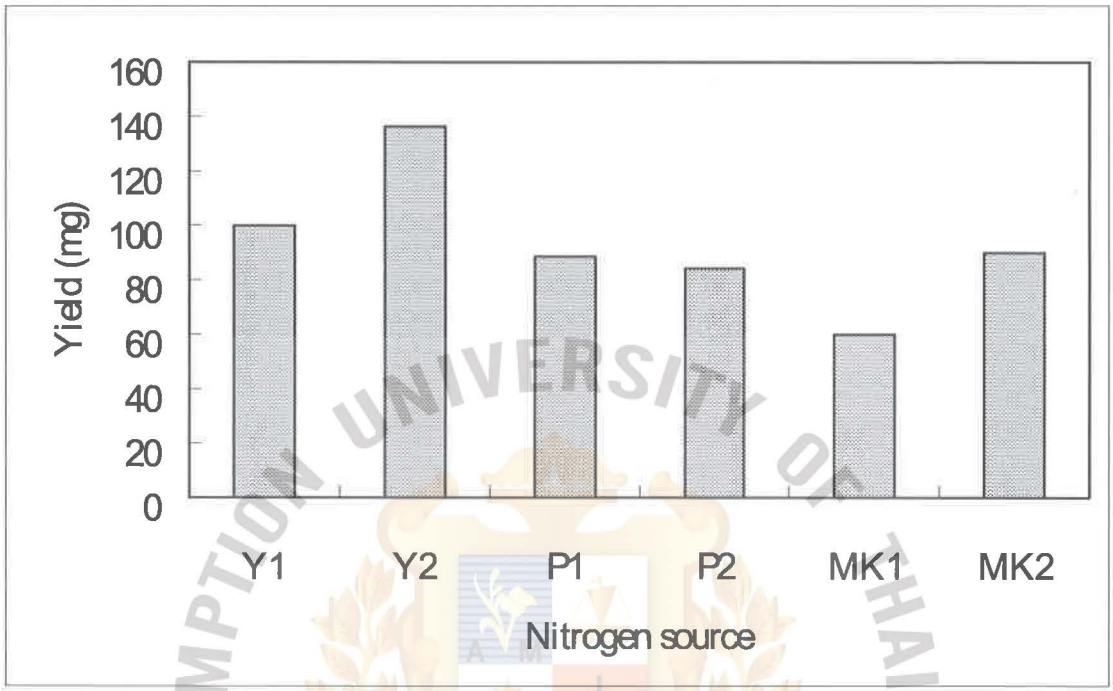


Figure 4.2 Effect of different nitrogen sources on mycelium production by *Cordyceps militaris*. The medium consist of 1% malt extract (w/v), 2% Molasses(w/v), 0.2% K_2HPO_4(w/v), and 0.2% $MgSO_4 \cdot 7H_2O$(w/v) with initial pH6.0, culture time 10 days. Y1 = yeast extract 1% (w/v), Y2 = yeast extract 2% (w/v), P1 = peptone 1% (w/v), P2 = peptone 2% (w/v), M1 = milk powder 1% (w/v), M2 = milk powder 2% (w/v).

In the experiment of screening nitrogen sources (milk powder - M, yeast extract - Y, and peptone –P) were used to provide nitrogen element for essential growth of an organism. 1% of milk powder gave the lowest result in the production of mycelium according to the net weight. Both 2 % and 1 % of peptone had poor induction effects in the production. 2 % of yeast extract gave the highest production of the weight mass as shown in Figure 4.2. Statistic analysis indicated that all type of nitrogen sources were non – significant. The level concentration of each nitrogen source and their interactions were also non – significant. The results were non significant, it could be due to the large deviation

between two different set of experiments or from different production period. However the yeast extract were cheap among these three types of nitrogen source. Moreover, 2% of yeast extract gained the highest yield of mycelium from the Figure 4.2. 2 % of yeast extract was selected to be used as nitrogen sources for further screening and optimizing the experiment.

4.1.3 Effect of different essential elements on mycelium production

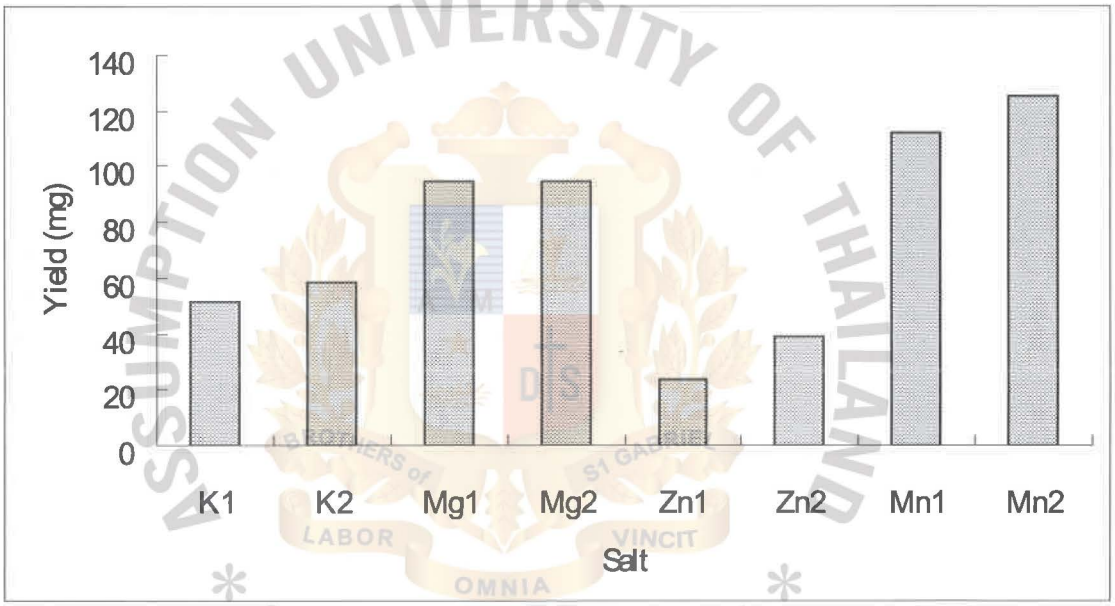


Figure 4.3 Effect of different essential elements on mycelium production by *Cordyceps militaris*. The medium consist of 2% Molasses (w/v), and 2% yeast extract (w/v), with initial pH6.0, culture time 10 days. Mg1 = 0.1% of $MgSO_4 \cdot 7H_2O$ (w/v), Mg2 = 0.2% of $MgSO_4 \cdot 7H_2O$ (w/v), Mn1 = 0.1 of $MnSO_4 \cdot H_2O$ (w/v), Mn2 = 0.2 % $MnSO_4 \cdot H_2O$ (w/v), K1 = 0.1% of K_2HPO (w/v), K2 = 0.2% of K_2HPO (w/v) , Zn1 = 0.1% of $ZnSO_4 \cdot 7H_2O$ (w/v), Zn2 = 0.2% of $ZnSO_4 \cdot 7H_2O$ (w/v).

In the growth of microorganism, carbon source and nitrogen source play the major role, and essential elements play the minor role. Although essential elements are minor in proportion of growth factor but they cannot be neglected. They provide several supports in cellular function. Many essential elements such as magnesium, calcium, potassium and numerous trace elements like manganese, and zinc are required in those cellular functions. The average weight of *C. militaris* mycelium yield from media containing different essential elements, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$, was illustrated in Figure 11. Statistic analysis indicated that the type of mineral elements had highly significant effect on the mycelium production which the concentration and their interaction were not. From figure 4.3, it demonstrated that Mn and Mg were preferred for *Cordyceps militaris* in the mycelium production. Both 0.1 % and 0.2 % of $MgSO_4 \cdot 7H_2O$ gave high production in a limited time. The result obtained from 0.1% and 0.2% of $MnSO_4 \cdot H_2O$ were 112.3 mg and 125.4 mg that were highest among the other essential elements. Poor growth of mycelium was observed for $ZnSO_4 \cdot 7H_2O$.

4.2 Fractional Factorial Designs and analysis

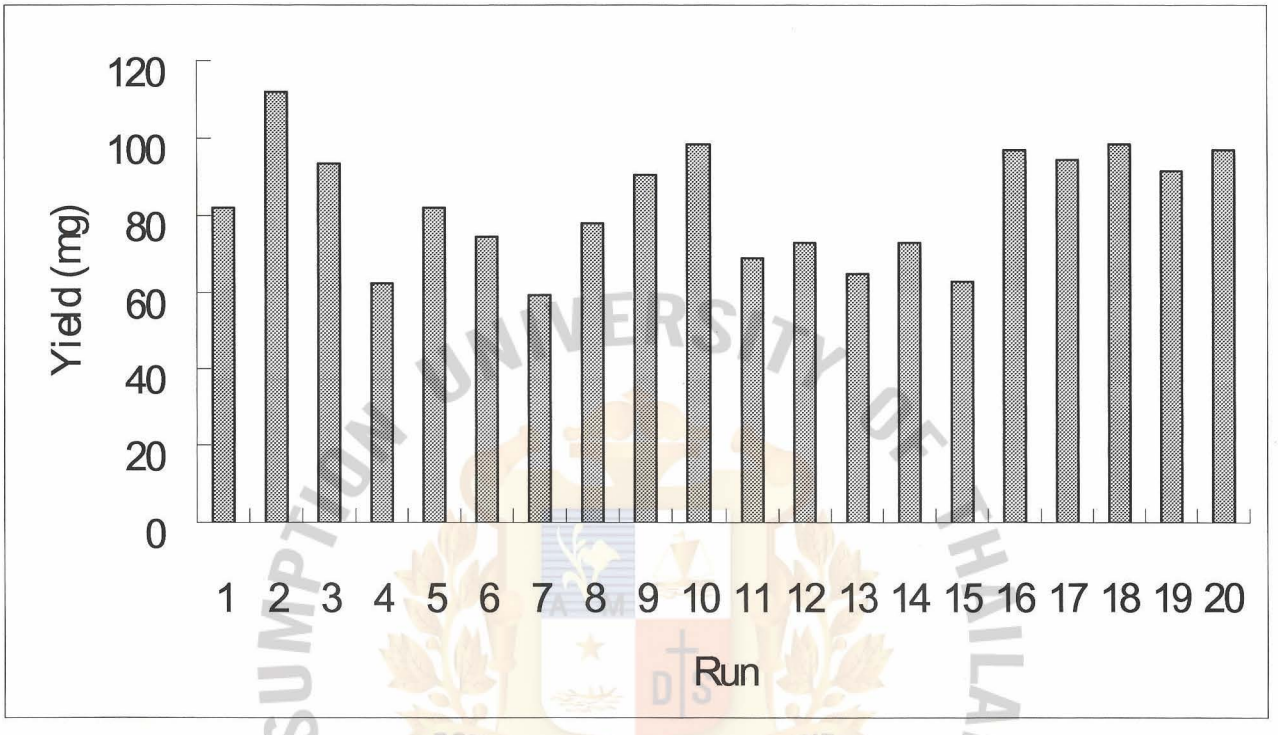


Figure 4.4 Result of fractional factorial design for optimizing media for the production of mycelium by *Cordyceps militaris*. The culture time is 10 days.

In fractional factorial design (FFD), 5 important factors (molasses, yeast extract, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, pH) were evaluated for their impact on the mycelium. Table 3.2 shows the levels of variables selected used in FFD while Table 3.1 presented the design and average results of the FFD from 20 runs. The result summary (part 2 Summary of Fractional Factorial Designs and analysis in Appendix) suggested that molasses (X_1) and pH (X_5) significantly affected the mycelium production. The result on

ANOVA also showed that molasses (X_1) and pH (X_5) were found to be significant at level of 95 %. The other three factors - yeast extract (X_2), $MnSO_4 \cdot H_2O$ (X_3), and $MgSO_4 \cdot 7H_2O$(X_4) were not significant at the probability of 95 %. The predicted regression equation (2) was determined and showed below where Y_i was a weight of mycelium, X_1 was concentration of Molasses, X_2 was concentration of yeast extract, X_3 was the concentration of $MgSO_4 \cdot 7H_2O$,X_4 the concentration of $MnSO_4 \cdot H_2O$, X_5 was pH value:

$$Y_i = 51.30 + 7.27\,X_1 + 1.45X_2 - 17.59X_3 + 69.10X_4 + 17.74\,X_5 \text{-------} (2)$$

The Pearson correlation table showed that molasses (X_1) and pH (X_5) had highly positive effects to the mycelium production. Based on the above equation, the concentration of molasses (X_1) could be adjusted to obtain higher amount of mycelium. Also, by increasing pH (X_5) could gain higher amount of mycelium. Therefore, in the Central Composite Design (CCD), molasses and pH were the main factors to find the optimum mycelium production.

4.3 Optimization of key component concentrations using a central composite design (CCD)

Box – Wilson CCD with 4 star points and 5 replicates at a center point was used for optimizing mycelium production for molasses (X_1) and pH (X_5). The statistical software (Design expert, version 7.1.5) defined a full CCD design for 2 factors consisting of 13 combinations plus the replicates at the center point (4 star points and 5 replicates at the center point to estimate the experimental error and to investigate the suitability of the proposed model); details are in Table 4.2. Figure 4.5 a brief idea of CCD design.

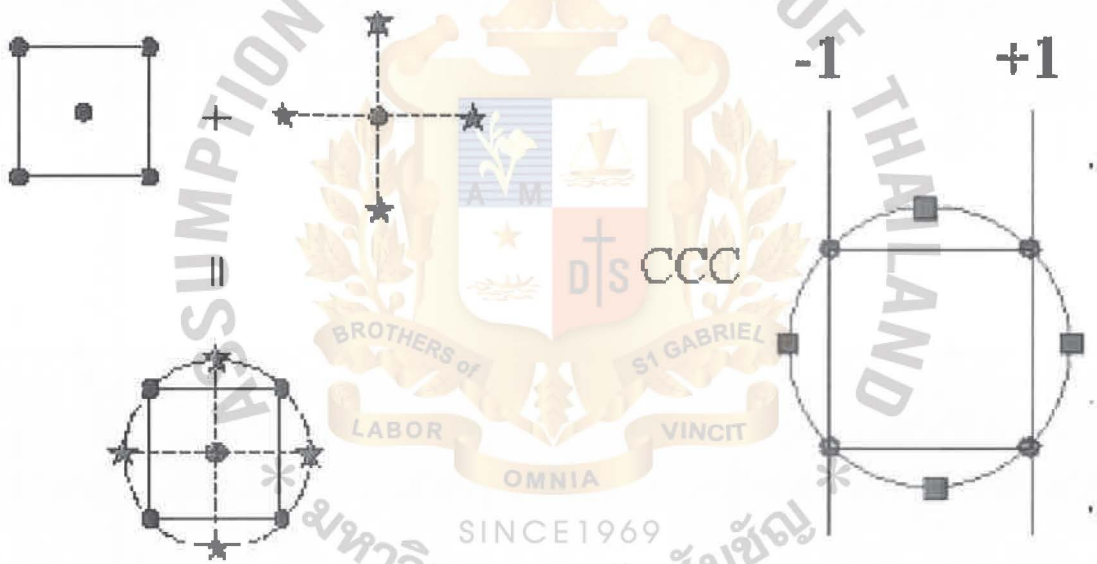


Figure 4.5 Generation of central composite design for two factors. (Modified from internet source http://www.itl.nist.gov/div898/handbook/pri/section3/pri3361.htm)

Table 4.1 shows the levels of selected variables used in CCD. A wide range of molasses (X_1), and pH (X_5) had been used. Table 4.2 presents the design and results of the CCD for 13 observations. Figures 4.6 and 4.7 demonstrate a contur-plot and response surface plot of the combine effect of the molasses and pH on the mycelium production. The result indicated that the linear effect of molasses was highly significant (ANOVA showed that molasses (X_1) had P-value 0.0016) to the yield, Y_{ii}. Referred to Figure 4.6, the Contur plot showed a shift toward increased X_i (Molasses). The 2nd-order polynomial equation (3) represented a model from Central Composite Design as the following equation:

$$Y_{ii} = 92.10 + 27.89\,X_1 + 5.54\,X_5 + 5.64\,X_1{*}X_5 - 17.25\,X_1^2 - 22.30\,X_5^2 \qquad (3)$$

The model showed that molasses, pH and their interaction had positive effects while the quadratic effects of molasses and pH were negative.

Table 4.1 Levels of variables used in central composite design for optimizing the production of mycelium by *Cordyceps militaris*.

	Levels				
Independent variables	-1.414	-1	0	1	1.414
Molasses (w/w, X_1)	0.233	0.75	2	3.25	3.768
pH (X_5)	4.68	5	6	7	7.42

Table 4.2 CCD result for optimizing media for the production of mycelium by *Cordyceps militaris*.

Observation	x1 **(molasses)**	x5 **(pH)**	Y_{ii} **(Net Weight mg)**
1	-1	-1	29.4
2	1	-1	85.1
3	-1	1	35.3
4	1	1	113.55
5	0	0	94.7
6	0	0	90.95
7	**-1.414**	0	12.75
8	**1.414**	0	75.825
9	0	**-1.414**	30.65
10	0	**1.414**	37.725
11	0	0	92.7
12	0	0	88.85
13	0	0	93.275

$X_1 = (X_1 - 2)/1.5$; $X_5 = (X_5 - 6.0)/1$

The 3-dimensional graph and its corresponding contur plot obtained from the calculated response surface plots are shown in Figures 14 and figure 15. It was shown in the response surface plot that the mycelium would reach the maximum at (X_1= 0.99, X_5 = 0.26), equivalent to 3.485 % of molasses (X_1) and 6.26 in pH (X_5). Statistical analysis indicated that the model was highly significant at P = 0.0016.

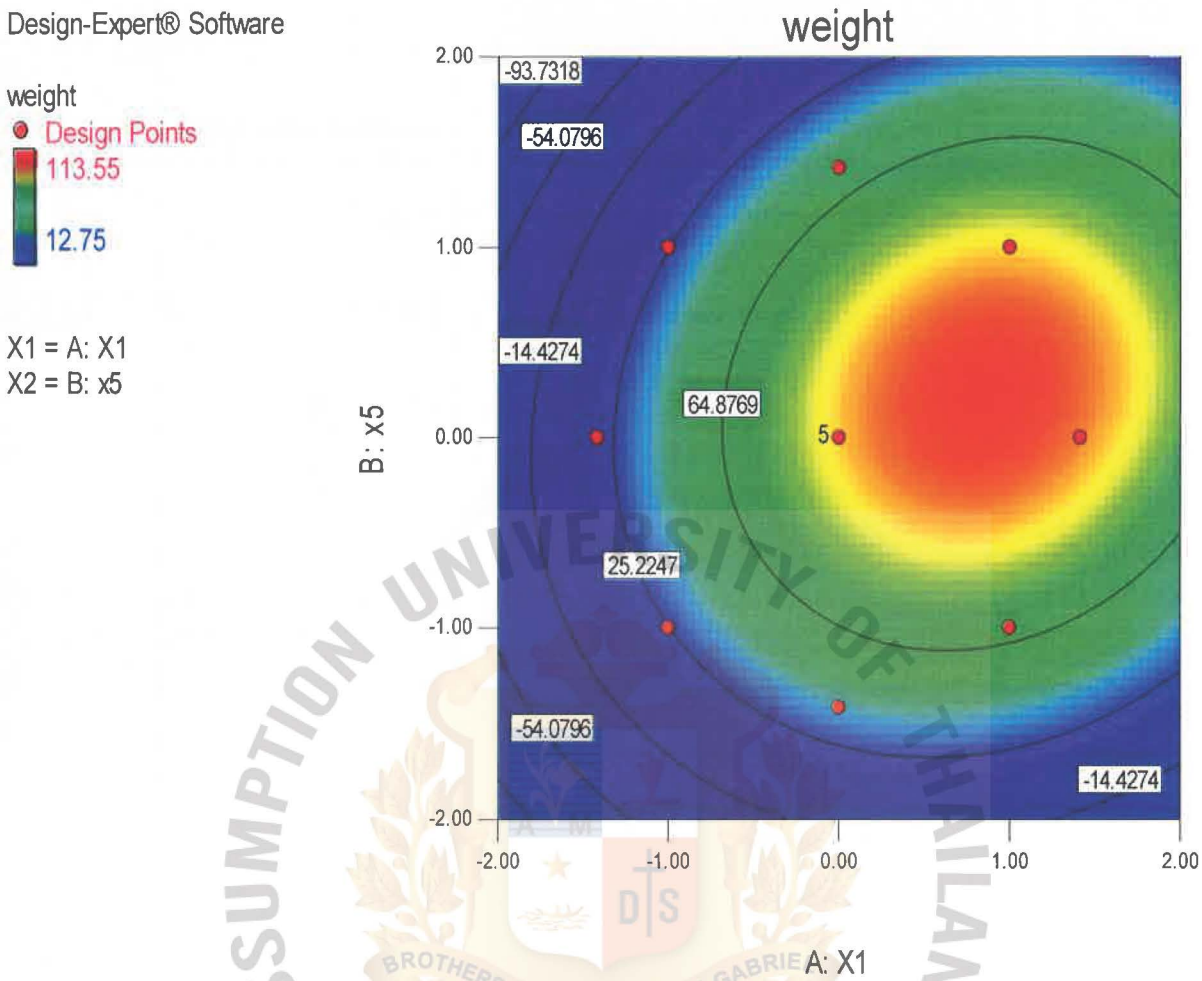


Figure 4.6 Contur plot of the combined effect of concentration of molasses and pH on the mycelium production

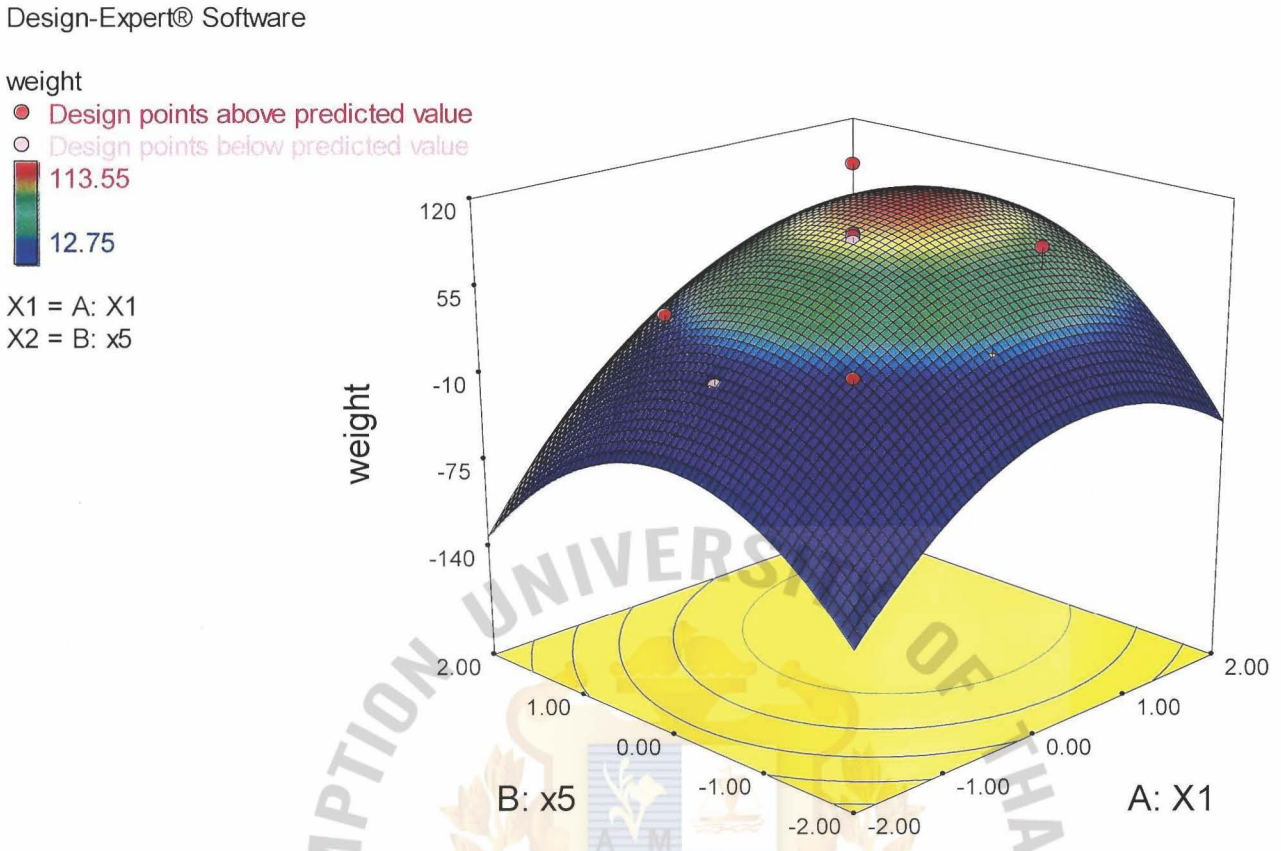


Figure 4.7 Response surface plot of the combined effect of concentration of molasses and pH on the mycelium production

CHAPTER V

CONCLUSION

This research aims to optimize the fermentation condition for the mycelium of growth, in order to lower the production cost of *Cordyceps militaris* mycelium at industrial scale level. Fermentation of *Cordyceps militaris* of production of mycelium was conducted using non-static rotational shaker at 180rpm at room temperature (~30°C) for 10 days. Molasses was selected as a carbon source, Yeast extract as nitrogen source, $MnSO_4 \cdot H_2O$, and $MgSO_4 \cdot 7H_2O$ as essential elements from screening using two-factor factorial design. By using 2_{IV}^{6-2} of FFD, 5 factors were investigated for their potential effects on the mycelium production. Molasses and pH were found to have high influence. Furthermore, CCD was used to observe the concentration of molasses and pH effect on mycelium yield using contur plot and response surface plot to illustrate where the optimum coordinate that could provide the maximum yield in the production. According to the research finding, the optimum fermentation medium for the mycelium production by *Cordyceps militaris* was 3.485% molasses (w/v), 2% yeast extract (w/v), 0.2 % of $MnSO_4 \cdot H_2O$, and 0.2 % of $MgSO_4 \cdot 7H_2O$; initial pH was 6.26 under the condition where the temperature was 30°C and 10-day fermentation time.

5.1 Suggestion

The research was conducted on net weight of mycelium yield which is not measured Cordycepin, polysaccharide or other active compounds that food microbiologist are interested in. Mostly, the *Cordyceps militaris* is found in the area of China, Japan, Korea, and Taiwan where the temperature is cooler compared with Thailand. The temperature measured during the experiment of this research was 30°C ± 2°C. It was suggested that the temperature should be controlled at 25°C to 26°C to achieve a faster growth rate (Yao, 1993). Therefore, a research conducted on an extraction of the active compounds from mycelium of *Cordyceps militaris* is required to provide the benefit for industry. It was also recommended to explore in greater detail of how mycelium and cordycepin were related. Moreover, due to the limitation of the small sample size, a larger-scale research is suggested to achieve a more conclusive result.

Appendix

Raw Data

Part I: Screening of the media components using one factor at a time

Carbon sources

Carbon source	Glucose (1%)	Glucose (2%)	Sucrose (1%)	Sucrose (2%)	Rice Flour (1%)	Rice Flour (2%)	Corn Flour (1%)	Corn Flour (2%)	Molasses (1%)	Molasses (2%)
1st	67.2	55.9	59.6	61.925	55.9	66.3	80.3	80.2	91	93.2
2nd	71.7	57.6	57.9	64.4	50.5	50.5	79.6	97.5	84.3	81.3
3rd	75.6	64.4	62.1	64.6	54.4	69.4	84.1	82.3	89.7	92
4th	66.2	55.5	58.1	66.3	55.1	54	81.2	82	88.5	94.3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	3307.947093	4	826.9867731	118.7571	2.2E-08	3.478049691
Columns	8.81792	1	8.81792	1.266273	0.28675	4.964602701
Interaction	229.5601675	4	57.39004188	8.241335	0.003303	3.478049691
Within	69.636825	10	6.9636825			
Total	3615.962005	19				

Duncan's multiple range test									
Range	9		df err	10					
s	0.64								
	2	3	4	5	6	7	8	9	
r0.05	3.15	3.3	3.37	3.43	3.46	3.47	3.47	3.47	
s.r0.05	2.02	2.11	2.16	2.20	2.22	2.22	2.22	2.22	
Arrange trt from low to high									
RiceFlour1p	Sucrose1p	Glucose2p	RiceFlour2	Sucrose1p	Glucose1p	CornFlour1	CornFlour2	Molass1p	Molass2p
53.9675	57.925	58.35	60.045	64.305	70.175	81.3	85.5	88.38	90.1875

Nitrogen sources

Nitrogen source	Yeast Extract (1%)	Yeast Extract (2%)	Peptone (1%)	Peptone (2%)	Milk powder (1%)	Milk powder (2%)
1st	95.2	147.1	89.7	89.2	62.9	94.7
2nd	103.7	122.6	90.8	75.4	62.7	86.8
3rd	94	140.6	86.7	82.6	62.6	90.8
4th	108.5	134.8	88.3	79.9	52.5	88.7

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	3998.814	2	1999.407	306.46165	9.11E-07	5.14325285
Columns	1246.441	1	1246.441	191.04981	8.92E-06	5.987377584
Interaction	972.1929	2	486.0965	74.507057	5.8E-05	5.14325285
Within	39.145	6	6.524167			
Total	6256.593	11				

Duncan's multiple range test						
Range	7		df err	6		
s	1.92					
	2	3	4	5	6	7
$r_{0.05}$	3.46	3.58	3.64	3.68	3.68	3.68
$s.r_{0.05}$	6.64	6.88	6.99	7.07	7.07	7.07

Arrange trt from low to high					
mk1	p2	p1	mk2	y1	y2
60.18	84.03	88.88	90.25	100.35	136.28

Essential elements

Essential elements	K_2HPO_4 (0.1%)	K_2HPO_4 (0.2%)	$MgSO_47H$ O (0.1%)	$MgSO_47H$ O (0.2%)	$ZnSO_47H_2$ O (0.1%)	$ZnSO_47H_2$ O (0.2%)	$MnSO_4H_2$ O (0.1%)	$MnSO_4H_2$ O (0.2%)
1st	49.1	68.5	87.8	89.4	23.0	42.5	103.1	105.5
2nd	57.2	54.7	103.7	93.8	26.1	33.6	123	136.3
3rd	50.3	57.6	97.2	100.9	24.8	43.2	106.8	125.8
4th	48.6	52.4	90.9	94.1	20.4	35.8	116.5	134

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	18565	3	6188.333	536.912	1.47E-09	4.066181
Columns	304.2844	1	304.2844	26.40032	0.000887	5.317655
Interaction	146.0339	3	48.67797	4.223397	0.045834	4.066181
Within	92.20629	8	11.52579			
Total	19107.52	15				

Duncan's multiple range test							
Range	7		df err	8			
s	**1.79**						
	2	3	4	5	6	7	
$r_{0.05}$	3.26	3.39	3.47	3.56	3.56	3.56	
$s.r_{0.05}$	**5.83**	**6.06**	**6.20**	**6.36**	**6.36**	**6.36**	
Arrange trt from low to high							
zn1	zn2	k1	k2	mg1	mg2	mn1	mn2
23.58	38.78	51.3	58.3	94.9	97.5	112.3375	125.39

Part II :Fractional Factorial Design Data

Correlations

		Yeild	X1	X2	X3	X4	X5
Pearson Correlation	Yeild	1.000	.433	.086	-.052	.206	.528
	X1	.433	1.000	.000	.000	.000	.000
	X2	.086	.000	1.000	.000	.000	.000
	X3	-.052	.000	.000	1.000	.000	.000
	X4	.206	.000	.000	.000	1.000	.000
	X5	.528	.000	.000	.000	.000	1.000
Sig. (1-tailed)	Yeild	.	.028	.359	.413	.192	.008
	X1	.028	.	.500	.500	.500	.500
	X2	.359	.500	.	.500	.500	.500
	X3	.413	.500	.500	.	.500	.500
	X4	.192	.500	.500	.500	.	.500
	X5	.008	.500	.500	.500	.500	.
N	Yeild	20	20	20	20	20	20
	X1	20	20	20	20	20	20
	X2	20	20	20	20	20	20
	X3	20	20	20	20	20	20
	X4	20	20	20	20	20	20
	X5	20	20	20	20	20	20

Model		Unstandardized Coefficients		Sig.
		B	Std. Error	Upper Bound
1	(Constant)	51.309	42.324	.245
	X1	7.270	3.113	.035
	X2	1.452	3.113	.648
	X3	-17.594	62.268	.782
	X4	69.094	62.268	.286
	X5	17.741	6.227	.013

Summary of Fractional Factorial Designs and analysis

Correlations

		Yeild	X1	X2	X3	X4	X5
Pearson Correlation	Yeild	1.000	.433	.086	-.052	.206	.528
	X1	.433	1.000	.000	.000	.000	.000
	X2	.086	.000	1.000	.000	.000	.000
	X3	-.052	.000	.000	1.000	.000	.000
	X4	.206	.000	.000	.000	1.000	.000
	X5	.528	.000	.000	.000	.000	1.000
Sig. (1-tailed)	Yeild	.	.028	.359	.413	.192	.008
	X1	.028	.	.500	.500	.500	.500
	X2	.359	.500	.	.500	.500	.500
	X3	.413	.500	.500	.	.500	.500
	X4	.192	.500	.500	.500	.	.500
	X5	.008	.500	.500	.500	.500	.
N	Yeild	20	20	20	20	20	20
	X1	20	20	20	20	20	20
	X2	20	20	20	20	20	20
	X3	20	20	20	20	20	20
	X4	20	20	20	20	20	20
	X5	20	20	20	20	20	20

Model		Unstandardized Coefficients		Sig.
		B	Std. Error	Upper Bound
1	(Constant)	51.309	42.324	.245
	X1	7.270	3.113	.035
	X2	1.452	3.113	.648
	X3	-17.594	62.268	.782
	X4	69.094	62.268	.286
	X5	17.741	6.227	.013

Part III: Summary of Central Composite Designs and analysis

Run	1	2	3	4	5	6	7	8	9	10	11	12	13
1st	28.2	88.4	35.2	109.4	91.9	96.9	14.4	75.4	23.2	39.5	93.8	87.5	92.7
2nd	32.6	82.2	35.8	123.2	94.5	88.9	9.0	77.2	33.4	34.9	91	91.1	93.7
3rd	28.9	83.6	37.4	108.2	98	89.6	19.5	72.1	31	32.9	92.4	88	92.2
4th	27.9	86.2	32.8	113.4	94.4	88.4	8.1	78.6	35	43.6	93.6	88.8	94.5

Response 1 weight

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	11514.39	5	2302.88	9.16	0.0056	significant
A-X1	*6224.58*	*1*	*6224.58*	*24.76*	*0.0016*	
B-x5	*245.93*	*1*	*245.93*	*0.98*	*0.3556*	
AB	*127.13*	*1*	*127.13*	*0.51*	*0.5000*	
A^2	*2070.90*	*1*	*2070.90*	*8.24*	*0.0240*	
B^2	*3460.57*	*1*	*3460.57*	*13.76*	*0.0076*	
Residual	1759.89	7	251.41			
Lack of Fit	*1739.50*	*3*	*579.83*	*113.77*	*0.0003*	*significant*
Pure Error	*20.39*	*4*	*5.10*			
Cor Total	13274.28	12				

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	92.10	1	7.09	75.33	108.86	
A-X1	27.89	1	5.61	14.64	41.15	1.00
B-x5	5.54	1	5.61	-7.71	18.80	1.00
AB	5.64	1	7.93	-13.11	24.38	1.00
A^2	-17.25	1	6.01	-31.47	-3.04	1.02
B^2	-22.30	1	6.01	-36.52	-8.09	1.02

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