Proximate Analysis of Formulated *Macrobrachium rosenbergii* Larvae Feed Using Egg Custard, *Moringa oleifera*, *Curcuma longa* (Turmeric) and Egg Shells

By

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A thesis submitted in fulfillment of the requirements for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

_____

Student

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Date:

I certify that the report of this final year project entitled "Proximate Analysis of Formulated *Macrobrachium rosenbergii* Larvae Feed Using Egg Custard, *Moringa oleifera*, *Curcuma longa* (Turmeric) and Egg Shells by Balqis Nur A'rasyi Binti Mohmad Noor, matric number F15A0274 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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Supervisor

Name:

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TABLE OF CONTENT

CHAPTER 3 METHODOLOGY

CHAPTER 4 RESULT AND DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION AND SYMBOLS

Abbreviation/Symbols	Full Name
%	Percentage
2D	Two Dimensional
3D	Three Dimensional
Adeq Precision	Adequate Precision
Adj R-squared	Adjusted R-squared
ANOVA	Analysis of Variance
BBD	Box-Behnken Design
℃	Degree Celsius
C.V.	Correlation of variance
CCD	Central Composite Design
CF	Crude fiber
CP	Crude protein
DF	Desirable function
DM	Dry matter
DoF	Department of Fisheries Malaysia
EC	Egg custard
EE	Ether extract
FAO	Food and Agriculture Organization of The United Nations
FIAT	Fakulti Industri Asas Tani
FPV	Fakulti Perubatan Veterinar
FYP	Final Year Project
g	Gram
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
kcal/g	Kilocalorie per gram

mg/kg	Milligram per kilogram
mL	Milliliter
mm	Millimeter
NaOH	Sodium hydroxide
NFE	Nitrogen free extract
Pred R-squared	Predicted R-squared
R	Response
R^2	Correlation coefficient
RSM	Response Surface Methodology
Std.	Standard
Std. Dev	Standard Deviation
UMK	Universiti Malaysia Kelantan

LIST OF EQUATIONS

NO.

Proximate analysis of formulated *Macrobrachium rosenbergii* larvae feed using Egg Custard, *Moringa oleifera, Curcuma longa* (Turmeric) and Egg shells

ABSTRACT

Macrobrachium rosenbergii or also known as Giant Freshwater Prawn is crustacean species that can be found in Malaysia. The decreasing number of this crustacean species in the water streams make it one of luxurious food in the market. Some researchers have done the research on alternative feed of *M. rosenbergii* larvae in order to lower the feeding cost and improve the growth rate. Nevertheless, the ingredients used in the feed still needed some cost to be invested. This project was proposed to analyze the nutrition value of *M. rosenbergii* larvae formulated feed by using 40 % of egg custard, 3 % of egg shells, 1 % of *Curcuma longa* (Turmeric) and 17.5% of *Moringa oleifera*. There were 13 different feed formulations by using Response Surface Methodology (RSM) in Design Expert Software version 10 which *M. oleifera* and *C. longa* were the variables coded while egg custard and egg shells were the based feed of the formulation. Proximate analysis has been conducted to observe the nutrition composition of the formulated feed and compared with the nutrient requirement of *M. rosenbergii* larvae which were protein, lipid, mineral and carbohydrates requirement. The result by Design Expert Software version 10 revealed that *M. oleifera* and *C. longa* gave the significant effect to protein and mineral requirement but not to lipid and carbohydrate requirement. The software also suggested the optimize formulation that near to the nutrient requirement of *M. rosenbergii* was Formulation 9, suggested percentage of 17.10 % *M. oleifera* and 1 % *C. longa* with 0.738 desirability.

Keywords: *Macrobrachium rosenbergii, Egg custard, Curcuma longa, Moringa oleifera, Egg shell, Response Surface Methodology (RSM)*

Analisis proksimat bagi makanan larva *Macrobrachium rosenbergii* yang diformulasikan menggunakan kastad telur, *Moringa oleifera*, *Curcuma longa* (kunyit) dan kulit telur

ABSTRAK

Macrobrachium rosenbergii atau juga dikenali sebagai udang galah adalah spesies krustasea yang boleh didapati di Malaysia. Penurunan bilangan spesies krustasea ini di aliran sungai menjadikannya salah satu makanan mewah di pasaran. Sesetengah penyelidik telah menjalankan penyelidikan mengenai makanan alternatif larva *M. rosenbergii* untuk mengurangkan kos makan dan meningkatkan kadar pertumbuhan. Walau bagaimanapun, ramuan yang digunakan dalam rumusan masih memerlukan sedikit kos untuk dilaburkan. Projek ini dicadangkan untuk menganalisis nilai nutrien rumusan makanan larva *M. rosenbergii* larva dengan menggunakan 40 % kastad telur, 3 % kulit telur, 1 % *Curcuma longa* (kunyit) dan 17.5 % *Moringa oleifera*. Terdapat 13 formulasi rumusan yang berbeza dengan menggunakan kaedah gerak balas permukaan (RSM) dalam Perisian Pakar Reka Bentuk versi 10 di mana *M. oleifera* dan *C. longa* adalah pemboleh ubah yang dikodkan manakala kastad telur dan kulit telur adalah makanan asas bagi perumusan. Analisis proksimat telah dijalankan bagi memerhatikan komposisi nutrisi makanan yang dirumus dan dibandingkan dengan keperluan nutrien larva *M. rosenbergii* iaitu keperluan protein, lemak, mineral dan karbohidrat. Hasil oleh Perisian Reka Bentuk versi 10 mendedahkan bahawa *M. oleifera* dan *C. longa* memberi kesan yang signifikan kepada keperluan protein dan mineral tetapi tidak kepada keperluan lemak dan karbohidrat. Perisian juga mencadangkan pengoptimuman rumusan yang hampir dengan keperluan nutrien *M. rosenbergii* adalah Perumusan 9, peratusan yang disarankan adalah 17.10 % *M. oleifera* dan 1 % *C. longa* dengan 0.738 kebolehinginan.

Kata kunci: *Macrobrachium rosenbergii, kastad telur, Curcuma longa, Moringa oleifera, kulit telur, Kaedah gerak balas permukaan (RSM)*

CHAPTER 1

INTRODUCTION

1.1 Research Background

Adult stage of *Macrobrachium rosenbergii* is known to have high disease resistance towards disease such as vibriosis (Khasani, Lusiastuti, Zairin & Alimuddin, 2018). It can be found in the river but in a less quantity because of the decreasing number of the species. The decreasing in number may because of the slow growth rate of the species or because of high mortality rate during its larval stages. The hatchery management is closely related to the early mortality of *M. rosenbergii* larvae such as the use of green water system, feed preparation and sanitary procedures (Roslim & Mohd Daud, 2012). Department of Fisheries Malaysia, DOF (2011) reported that the declining in *M. rosenbergii* production is due to decreasing supply of quality brood stock, low productivity and culture technology and also a high dependence on imported food for larval stages.

Herbs, plant sources and chemical substance was being used in the feed formulation to observe the nutrition value in the feed. Herbs especially *Curcuma longa* or commonly known as turmeric have been widely used as an antimicrobial agent and as

a home remedy for its natural source. It is used in the feed for its antimicrobial agent properties. *Moringa oleifera*, which is a type of legumes is high in protein and being used as a protein source in the feed. Chemical substance which is calcium carbonate was widely used in aquaculture farming and egg shells was used as a source of calcium carbonate in the formulated feed. The application of *C. longa*, *M. oleifera* and egg shells in *M. rosenbergii* larvae feed may help to be an alternative low-cost feed with the locally availability of the source.

1.2 Problem statement

Larvae stage of aquaculture species especially *Macrobrachium rosenbergii* larvae stage is a sensitive stage that need more attention during nursery period. The larvae need a best environment condition to survive as they need optimum condition for water quality with a good management for feed. Feed management is the most important in aquaculture farming as feed took over than 50 % of the production cost in aquaculture (FAO, 2009). Common feed used to feed *M. rosenbergii* larvae is live brine shrimp nauplii (BSN) or also known as *Artemia*. However, due to the high cost of *Artemia*, the common practice in the nursery is to feed the larvae with alternative feed which is egg custard to reduce the feed cost.

In normal practices, farmer will give egg custard in the morning while *Artemia* is given in the evening. A new formulated feed must need the optimum nutrient requirement for *M. rosenbergii* larvae to promote the growth rate and to reduce the mortality rate of the larvae. Thus, this study investigates the potential application of *Moringa oleifera*,

Curcuma longa and egg shell as a suitable ingredient to partially replace the protein content in the egg custard formulation.

1.3 Hypothesis

H_0 = Nutrition composition of *Macrobrachium rosenbergii* formulated larvae feed is not significant to the nutrient requirement with the amount of *Moringa oleifera, Curcuma longa* (Turmeric) and Egg shell applied.

H_1 = Nutrition composition of *Macrobrachium rosenbergii* formulated larvae feed is significant to the nutrient requirement with the amount of *Moringa oleifera, Curcuma longa* (Turmeric) and Egg shell applied.

1.4 Objectives

The objectives of the present study were:

a) To formulate *Macrobrachium rosenbergii* larvae feed using *Moringa oleifera, Curcuma longa* and egg shells
b) To determine the nutrient composition of the new egg custard formulation

1.5 Scope of study

This study focuses on the nutrition of alternative feed for *Macrobrachium rosenbergii* larvae which include feed formulation, proximate analysis and data analysis. The proximate analysis was conducted on the new egg custard formulated using *Moringa oleifera*, *Curcuma longa* and egg shells. The feed formulation has been conducted by optimizing by using Response Surface Methodology (RSM) in Design Expert Software version 10. Proximate analysis result revealed the basic nutrient composition in the feed such as moisture, crude protein, crude fiber, ether extract, ash and nitrogen free extract. The data have been analyzed by using Design Expert Software version 10 to know either the amount of ingredients applied has significant effect to the nutrient composition of the formulated feed compare to nutrient requirement of *M. rosenbergii* larvae.

1.6 Significance of study

Feed formulation is important in order to formulate an optimum feed with an optimum nutrient requirement based on the stage of the aquaculture species. The feed formulation involved egg custard mix, *Moringa oleifera* as the protein source, *Curcuma longa* as the additive (antimicrobial source) while egg shells as the source of calcium.

The proximate analysis is important to reveal the nutrients composition in the feed. The proximate analysis provides the information of the amount of water (moisture), ash (mineral), crude protein, crude fiber, crude fat and nitrogen free extract in the feed.

Data analysis is important to know the significance of data that have been obtained. The data is a prove to know either the study provide the fundamental scientific evidence or not.

1.7 Limitation of study

Feeding trial has not been done in this research because of some limitations and affected the actual result of this research finding. The feeding trial may result in more scientific and significant potential of applying *Moringa oleifera*, *Curcuma longa* and egg shells in egg custard formulated feed in order to promotes the growth rate and reduce the mortality of *Macrobrachium rosenbergii* larvae.

CHAPTER 2

LITERATURE REVIEW

2.1 General Background of *Macrobrachium rosenbergii*

Macrobrachium rosenbergii De Man (Giant Freshwater prawn) or locally known as udang galah in Malaysia is a crustacean animal that inhabit in freshwater environment. It belongs to the genus *Macrobrachium*, Bate (1868), which is the largest genus of the family *Palaemonidae* (FAO, 2002). *Macrobrachium rosenbergii* is a special crustacean which it lives in marine water during mating and larvae stage and then move to brackish water to grow into juveniles. Adult *M. rosenbergii* can be found widely distributed in most of the tropical and subtropical areas of the Indo-Pacific Region, including East Pakistan, India, Ceylon, Burma, Thailand, Malaysia, Indonesia, Philippines, Cambodia and Vietnam (Ling, 1969).

Male of *M. rosenbergii* can reach the total length of 320 mm and females could reach up to 250 mm (FAO, 1976). The size of the male is usually bigger and longer compare to female that usually slightly big at the body compartment. The body color usually greenish to brownish grey and sometimes blue while the antennae is often blue or orange in color. The eggs of *M. rosenbergii* change the color from orange to brownish-

grey in color when they are about to hatch. There are four general stages for *M. rosenbergii* life cycle which are eggs, larva, juveniles and adult (growth-out) (FAO, 1993).

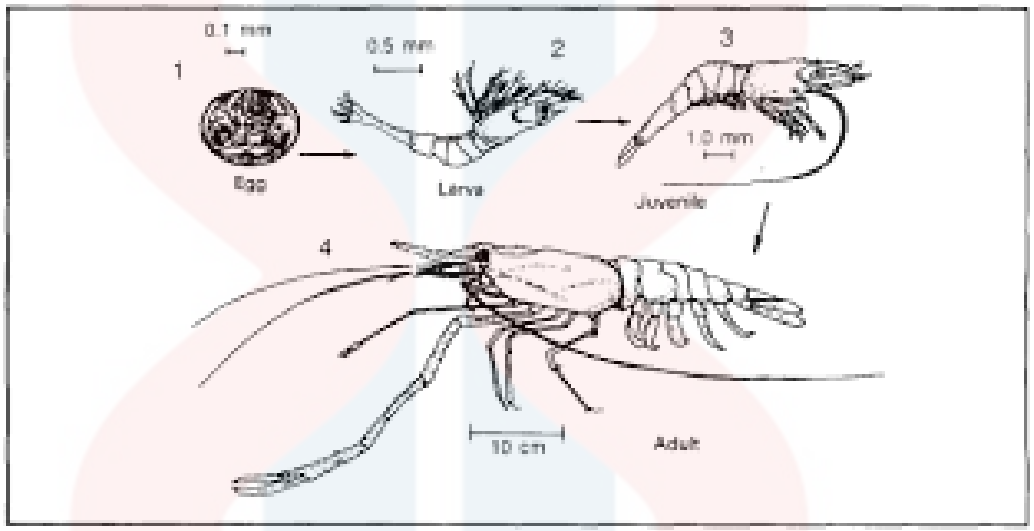

Figure 2.1: The life cycle of *Macrobrachium rosenbergii*

Source: (Chowdhury et al., 1993)

Figure 2.2: Adult *Macrobrachium rosenbergii*

2.1.1 *Macrobrachium rosenbergii* production in Malaysia

The demand for *Macrobrachium rosenbergii* in Malaysia is currently decreasing as the price is getting higher. This is because the production of the prawn from the river is getting low because of the illegal catching by the fishermen. As the activities cannot be prevented, Department of Fisheries Malaysia (DoF) take the initiatives to restocking *M. rosenbergii* larvae into the selected river.

The production trends for cultured giant freshwater prawn in Malaysia had fell 218 % from 627 million tonnes in 2013 to 197 million tonnes in 2006 even though Malaysia pioneered the breeding of this species in the late 50's, (Banu & Christianus, 2016). The fall in this species production may due to declining supply of good quality brood stock, low productivity, low development of culture technology and a high dependence on imported food for larval stages.

The aquaculture sector has been expanding in Malaysia for decades because of the natural habitat resources available such as rivers, lakes, ponds, estuaries and coastal area. Global production of *M. rosenbergii* output was first recognized and recorded in FAO statistics in 1970 (Banu & Christianus, 2016). Based on the report by New and Nair (2012), global production of crustaceans species, prawn is about 229,419 tonnes and 552 tonnes came from Malaysia.

Data from Department of Fisheries Malaysia, DoF (2017) stated that there are 427,015.43 tonnes of aquaculture production by state and culture system in year 2017. The data from DoF also stated that giant freshwater prawn, *M. rosenbergii* state the production of 293.74 tonnes from 102,596.83 tonnes aquaculture production from freshwater culture system in year 2017.

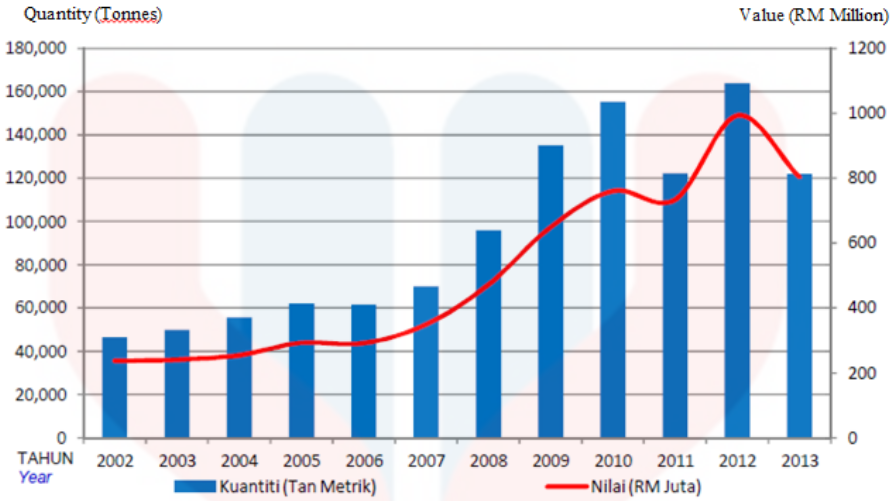

Figure 2.3: The production and value of freshwater fish from all freshwater aquaculture system in Malaysia

Source: (Anon, 2014)

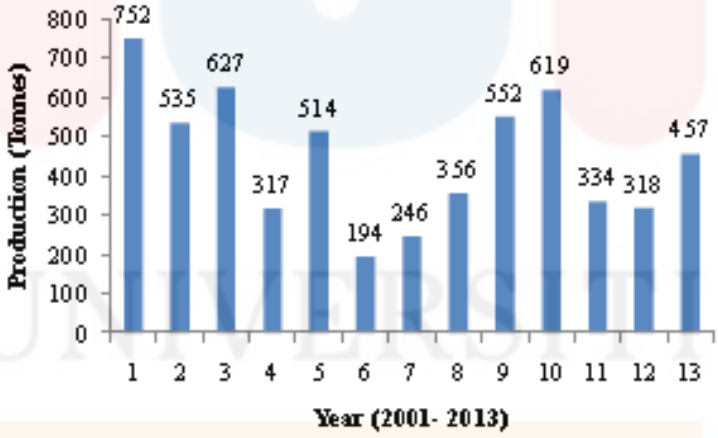

Figure 2.4: The production of giant freshwater prawn in Malaysia

Source: (Anon, 2008-2014)

2.1.2 Factors leads to low production of *Macrobrachium rosenbergii*

The decreasing number of the production year by year may because of several problems which included lack of quality brood stock, low quality of feed, shortage of water supply, low water quality and disease. Paper by Nair and Salin (2012) stated that, the declining in production also may affected by the poor seed quality of brood stock, pond water quality issue and increasing cost of production on feed account, labour and mandatory requirements.

Disease being a major factor that causes the low production of *Macrobrachium rosenbergii* larvae in hatcheries. The mortality of the larvae costs a high lost. Disease carrier, pathogens such as *Aeromonas* sp. and *Vibrio* sp. may contribute to the mortality of *M. rosenbergii* larvae. This is stated in the paper by Phatarpekar, Kenkre, Sreepada, Desai and Achuthankutty (2002), in which there is no major mortalities and abnormalities in larvae in the present study that may be attributed by the absence of *Aeromonas* sp. and *Vibrio* sp.

As reported by Nagarajan and Chandrasekar (2002) and New (2005), there are also the other problems encountered in rearing fresh water prawn included white muscle or idiopathic muscle necrosis, ciliate infestations, antenna and tail rot, soft shell, hard shell and low dissolved oxygen.

The disease also can easily be infected if the environmental farming is not suitable to the prawn. A study by Cheng, Liu, Hsu, and Chen (2002) concludes that low concentration of dissolve oxygen (DO) leads to immune system depression in *M. rosenbergii* and increase the susceptibility towards *Enterococcus* infection. Changes in water environment such as temperature, pH, DO and salinity, exposure towards ammonia,

heavy metals or other pollutants also could reduce the immune system abilities towards pathogen such as phenoloxidase activity, phagocytic activity, clearance efficiency and production of superoxide anions (Cheng & Chen, 2002).

2.2 Nutrition of *Macrobrachium rosenbergii*

The nutrient requirement for *Macrobrachium rosenbergii* is almost the same for all the life cycle stages. All the nutrients needed are included carbohydrates, protein and amino acids, lipids and fatty acids, vitamin and minerals. The nutrients can be got from the natural sources of the feed in the water streams or through the commercial and formulated feed.

FAO had summarized the suggested nutrient requirement by Mitra, Chattopadhyay and Mukhopadhyay (2005) in Table 2.1.

Table 2.1: The summary of nutrient requirements of giant freshwater prawn, *Macrobrachium rosenbergii*

Nutrients	Growth stages	Requirement
Protein (%)	Brood stock	38 – 40
	Juveniles (2^{nd} 4^{th} month)	35 – 37
	Adult (5^{th} 6^{th} month)	28 - 30
Carbohydrates (%)	For all stages	25 - 35
Lipids including phospholipids (%)	For all stages	3 - 7
Highly unsaturated fatty acids (%)		> 0.08

Cholesterol (%)	For all stages	0.5 – 0.6
Vitamin – C (mg/kg)	Growth out	100
Calcium/Phosphorus		1.5 – 2.0 : 1
Zn (mg/kg)		90
Other minerals		Quantitative requirements not yet known
Energy	Brood stock	3.7 – 4.0 kcal/g feed
	Other stages	2.9 – 3.2 kcal/g feed

Source: (Mitra et al., 2005)

2.2.1 *Macrobrachium rosenbergii* feed

Macrobrachium rosenbergii larvae which live in the water streams and pond usually feed for natural sources such as zooplankton (Mitra et al., 2005). In hatchery, feed that usually used in *M. rosenbergii* feeding system are brine shrimp nauplii (BSN), *Artemia* nauplii and prepared egg custard feed (EC) (New, 2002). Newly hatched *M. rosenbergii* larvae do not feed until the first moult which is occurs within 24 hours after hatching (Moller, 1978). Larvae are omnivorous animal that mainly feed on zooplankton (FAO: Natural food and feeding habits, 2018). The prawn larvae usually started to eat from day 2 of life in which brine shrimp nauplii, *Artemia* usually used to feed the larvae, (New, 2002).

There are many previous studies suggested that live *Artemia* as an excellent feed for *M. rosenbergii* larvae (Murai & Andrews, 1978). *Artemia* is fed to *M. rosenbergii* larvae for five times a day started on day 2 until day 4 of life and was prepared based on

the volume of water in the tank, not based on the number of *M. rosenbergii* larvae (New, 2002). Formulated feed such as egg custard can be given to *M. rosenbergii* larvae started on day 6. Formulated feed was given to the larvae in order to cut the cost of rearing as the cost of *Artemia* is high. The other alternative feed that commonly given to *M. rosenbergii* are included animal protein sources such as poultry by product meal, oyster meal, mussel meat meal, squid meal, shrimp meal, fishmeal and earthworm meal (Murai & Andrews, 1978; Mukhopadhyay, Rangacharyulu, Mitra, & Jana, 2003; Mitra et al., 2005; Nik Sin & Shapawi, 2017).

2.3 General background of Egg custard

Egg custard is the most common alternative feed used to feed *Macrobrachium rosenbergii* larvae. The ingredient needed to prepare the egg custard are 60 % egg and 40 % powdered milk (Ali, 2005). General egg custard recipe contains 34.59 % of protein make it suitable as alternative feed to replace the frequent used of *Artemia* in daily feeding schedule. Table 2.2 shows the proximate analysis of general egg custard recipe observed by (Ali, 2005).

On the other hand, some of researcher used to improve the general egg custard recipe in order to increase the nutrient composition of the alternative feed and achieve the objective of improving survival rate of *M. rosenbergii* larvae. A research by Nik Sin and Shapawi (2017) on innovative egg custard formulation revealed that innovative formulated feed with suitable feeding regime can successfully decrease the rearing period and increase the survival rate of *M. rosenbergii* larvae.

Table 2.2: Proximate analysis of general egg custard recipe

Food Value	**Percentage (%)**
Moisture	37.13
Protein	34.59
Lipid	13.78
Ash	6.02
Carbohydrate	8.48

Source: (Ali, 2005)

2.4 General background of *Moringa oleifera*

Moringa oleifera or also known as horseradish tree in English is a plant that natively in northern India, Pakistan and Nepal (Parrotta, 2009). It belongs to the genus Moringaceae with 14 known species (Olagbemide & Philip, 2014). *M. oleifera* is a miracle tree that has variety of purposes such as for medical. It has a potential used as antioxidant, anticancer, antibacterial, anti-inflammatory and antimicrobial agent (Anwar, Latif, Ashraf, & Hassan Gilani, 2006; Gopalakrishnan, Doriya, & Kumar, 2016).

M. oleifera is a multi-nutrient rich plant as it rich in minerals, vitamins and other nutrient composition. *M. oleifera* is an excellent food source to be used in human nutrition or for balanced diet development for animal nutrition due to the chemical composition values (Amabye, 2016).

Table 2.3: Proximate analysis of *Moringa oleifera* leaf powder

Element	**Value**
Moisture (%)	3.34 ±1.36
Protein (%)	10.71 ± 0.81
Lipid (%)	10.31 ± 1.2
Ash (%)	7.29 ± 0.84
Carbohydrates (%)	57.61 ± 2.19
Energy value (Kcal/100 g)	366.2 ± 4.23

N.B Values are mean ±SD, analyzed individually in triplicate, and are expressed as g/100 g leaf powder

Source: (Amabye, 2016)

2.5 General background of *Curcuma longa* (Turmeric)

Curcuma longa or known as Turmeric is herbs plant that belongs to family Zingiberaceae. It is a spice that have yellow in color and have aromatic smell. It has been used in culinary as spice and as a home remedy for their benefits. Some of the benefits of *C. longa* are as anti-oxidant, anti-inflammation, anti-microbial as it inhibits the growth and infection of pathogens such as bacteria and fungi (Chainani-Wu, 2003).

In aquaculture, *C. longa* have been used into the feed as the feed additives. The application of *C. longa* is to improve the feed intake and assimilation in *Macrobrachium rosenbergii* (Salini & Thomas, 2017). Moreover, *C. longa* also helps in improving immune system of *M. rosenbergii* and increase the survival rate of shrimp that challenged with pathogen (Alambra et al., 2012).

Table 2.4: Proximate analysis of *Curcuma longa* (Turmeric)

Parameter	**Composition (%)**
Moisture Content	8.92 ± 0.02
Dry Matter	91.00 ± 0.01
Ash Content	2.85 ± 0.02
Crude Fibre	4.60 ± 0.01
Crude Protein	9.40 ± 0.02
Fat	6.85 ± 0.00
Carbohydrate	67.38 ± 0.01

Values are means ± standard deviation of three determinations

Source: (Ahamefula, Onwuka, & Chibuzo, 2014)

2.6 General background of Egg shells

Egg shells is usually derived from the hard shell of the eggs. In the normal use, people usually used poultry egg shells to produce lime as egg shells (chicken egg shells) is high in mineral content especially calcium carbonate, which is about 73.54 % (Al-awwal & Ali, 2015).

Calcium carbonate is commonly lime that can be easily found as one of the earth elements. Calcium carbonate derives as $CaCO_3$. When calcium carbonate is heated, the chemical compound will change the form into calcium oxide and calcium dioxide. Calcium carbonate have been used widely in aquaculture farming as it neutralises the soil pH of the soil. Dolomite is a chemical substance that contain calcium and magnesium have been widely used to stabilize the pH of water and soil, and trigger crustaceans to develop new skin after moulting. Agricultural lime such as dolomite has no negative

effect to the prawn and it is safe to be used as source of calcium for proper growth and development (Blessing, Ibitoru, Ebinimi and Gabriel, 2014).

Table 2.5: Proximate analysis of chicken egg shells

Composition	**Percentage of weight (%)**
Moisture	0.5 ± 0.030
Ash	43.5 ± 0.032
Crude fibre	3.0 ± 0.300
Crude protein	1.35 ± 0.400
Carbohydrate	51.7 ± 0.440

Source: (Al-awwal & Ali, 2015)

2.7 Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is a collection of mathematical and statistical model that being used to building, developing, optimizing and improving an empirical model. Box and Wilson are the persons who introduced RSM in 1951 (Khairul Anwar & Mohamed Afizal, 2015). In RSM, the performance that being observed called as response while the input called as independent variables (Carley, Kamneva, & Reminga, 2004). There were many types of designs in RSM such as Central Composite Design (CCD), Box Behnken Design (BBD) and Optimal (Custom) Design (Kraber, 2014).

RSM is used to select and construct an appropriate model that can provide a significant and reliable information towards response which denoted as y. It is also used to determine a suitable model that best fits to the data and finding an optimal solutions in

order to produce an optimum value of response towards variables (Khuri, 2017). Moreover, RSM also generated polynomial equation regression to observe the effect of factors towards response whereas the significance of the best fitted model was determined by analysis of variance, ANOVA (Khairul Anwar & Mohamed Afizal, 2015).

CHAPTER 3

METHODOLOGY

3.1 Experimental Setup

The ingredients which are *Moringa oleifera*, *Curcuma longa* and egg shells were got from the local market, calcium carbonate powder from crushed egg shells while ingredients for egg custard which are milk power were from nearby store. The other equipment used were got from the Food Laboratory, FIAT's laboratory and FPV laboratory. The research was conducted in Food Laboratory for cooking, Animal Science Laboratory for sample preparation and FPV laboratory for proximate analysis.

3.2 Materials

The raw materials that being used were milk powder, eggs, *Moringa oleifera* leaves, *Curcuma longa* rhizomes and egg shells.

3.3 List of chemical reagents

The chemical reagents that have been used during proximate analysis were sulphuric acid (H_2SO_4), hydrochloric acid (HCl), sodium hydroxide (NaOH), petroleum ether, Kjeldahl tablet, boric acid, methyl red indicator and bromocresol green indicator.

3.4 Preparation of raw materials

The raw materials which are *Moringa oleifera* and *Curcuma longa* were got from the local market, dried for 24 hours, 105 ℃ in air-forced oven and being processed into powder. The egg shells were cleaned through running water, air dried before drying process in the air-forced oven for 24 hours, 105 ℃ and being crushed into powder. The egg custard was prepared by mixing the eggs and milk powder by using electrical mixer (until well combined) and being steamed on the water bath for 45 minutes to be a cake. The cake then was let to be cooled in the chiller for 24 hours and sieved into powder, 0.01 mm.

3.5 Design experimental by using Response Surface Methodology (RSM)

The new egg custard formulation for *Macrobrachium rosenbergii* larvae was designed by using Response Surface Methodology (RSM) with the Central Composite Design (CCD) model. There were two variables have been entered which were *Moringa oleifera* and *Curcuma longa* in a percentage unit. The lower level (-1) and high level (+1) of variables have been entered to get the suggested feed formulation. The suggested

percentage of *M. oleifera* was referred to the percentage of poultry by-product (PBM) in egg custard formulation by Nik Sin and Shapawi (2017) while percentage of *C. longa* was referred to a paper by Poongodi, Saravana Bhavan, Muralisankar and Radhakrishnan (2012).

Table 3.1: Experimental design by using Central Composite Design (CCD)

Variables	**Name**	**Unit**	**Low Level (-1)**	**High Level (+1)**
A	*Curcuma longa*	%	0	1.00
B	*Moringa oleifera*	%	0	17.10

There were 13 runs were shown for two variables that have been entered with different suggested percentage of variables for each formulation. The suggested 13 runs of the formulations were shown in the Table 3.2.

Table 3.2: Generated experimental runs by Central Composite Design (CCD)

Std.	**Run**	**A**	**B**
		***Curcuma longa* (%)**	***Moringa oleifera* (%)**
8	1	0.50	20.64
1	2	0.00	0.00
6	3	1.21	8.55
12	4	0.50	8.55
9	5	0.50	8.55
13	6	0.50	8.55
5	7	-0.21	8.55

11	8	0.50	8.55
4	9	1.00	17.10
2	10	1.00	0.00
7	11	0.50	-3.54
10	12	0.50	8.55
3	13	0.00	17.10

3.6 Proximate analysis

Proximate analysis was conducted to know the nutrient composition in the raw materials which are *Moringa oleifera, Curcuma longa* and egg custard. The proximate analysis was also done for the formulated feed. The analysis that have been conducted are moisture analysis, crude protein analysis (CP), ether extract/crude fat analysis (EE), crude fiber analysis (CF), ash analysis and nitrogen free extract analysis (NFE). The analysis was analyzed by using AOAC (1990) procedures.

3.6.1 Moisture Analysis (Dry matter content)

Moisture analysis is done to know Dry Matter content of the feed. The moisture content was done by determined by force air drying oven. The empty container selected to hold the sample was weighed and the was recorded (W1). Next, approximately 2 g of sample was weight (W2). The sample was placed in the container. The sample was dried in force air oven at 110 ℃ at 24 hours. The dried sample with container were weighted and recorded immediately after dried (W3). The weight of the dried sample (W3 - W1)

was divided by the weight of the wet sample (W2). Then, it was multiplied by 100 to get a percentage.

$$\text{DM } (\%) = \frac{W3 - W1}{W2} \times 100 \quad (3.1)$$

Where,

DM = Dry matter

W1 = Weight of empty container (g)

W2 = Weight of sample (g)

W3 = Weight of dried sample (g)

3.6.2 Crude protein analysis (CP)

Crude protein analysis is done to know the amount of crude protein in the feed. The crude protein was analyzed by using Kjeldahl method. Kjeldahl method was divided into 3 methods which are digestion, distillation and titration.

The first method is Kjeldahl digestion method. Approximately 1 g of sample was prepared with 12 mL of sulphuric acid and 2 pieces of Kjeldahl tablet in a Kjeldahl digestion tube and then was put into the digester system to be digested for about 1 hour 30 minutes. After the digestion, the sample was cooled down in the fume chamber for at least 1 hour 30 minutes before proceeding to the next step which was distillation method.

The second method is Kjeldahl distillation. The distillation system was let to be warm up for about 10 minutes. Then, the digestion tube and the conical flask (filled with 30 mL of receiver containing 4 % of boric acid, 1 mL of bromocresol green, 0.7 mL of methyl red and 100 mL of distilled water) was correlated to the distillation unit. After the distillation was completed, the sample then proceed for the third method.

The third method is Kjeldahl titration. The sample in the conical flask was titrated with 0.1 N of hydrochloric acid (HCl) which was added drop by drop until the receiver solution turns colour from green to greyish pink. The amount of crude protein determined according to Bhuiyan, Bhuyan, Anika, Sikder and Zamal (2016) :

$$\% \text{ Nitrogen} = \frac{\text{value of HCl} \times 0.1 \times 0.014}{\text{weight of sample}} \times 100 \quad (3.2)$$

$$\% \text{ Crude Protein} = \% \text{ Nitrogen} \times 6.25 \quad (3.3)$$

3.6.3 Ether extract/crude fat analysis (EE)

The ether extract analysis was determined by using Soxtec Extraction system. The aluminum cups were pre-heated in the air-forced drying oven for 30 minutes at 103 ℃ and were let to be cool in the desiccator for 20 minutes. The aluminum cups then were weighed, and the initial weight were denoted as W1. Approximately 1 g of the sample was weighed in a fine form into the thimble and denoted as W2. A layer of de-fatted cotton was placed on top of the sample and act as absorber. The thimbles were placed in the extraction unit by attaching them to the magnet. Extracting solvent, 80 mL of petroleum ether was added in the aluminum cup and were inserted to the extraction unit. The sample undergoes the following process which were, immersion, rinsing and

recovery. At the end, the aluminum cups contain extracted ether were dried in the oven at 105 °C for 30 minutes and then cooling it in the desiccator at room temperature for 20 minutes. The final weight of the aluminum cups was weighed and recorded as W3.

The amount of Ether Extract was calculated by:

$$\text{EE}\ (\%) = \frac{\text{W3}-\text{W1}}{\text{W2}} \times 100 \qquad (3.4)$$

Where,

EE = Ether extract

W1= Weight of empty aluminum cups (g)

W2 = Weight of sample (g)

W3 = Weight of aluminum cups with residue after extraction (g)

3.6.4 Crude fiber analysis (CF)

Crude fiber analysis was conducted by using fiber bag system. Firstly, fiber bags were prepared and dried into air-forced drying oven for 1 hour at 105 ℃ and then cooled in the desiccator for 30 minutes. Fiber bags then were weight and denoted as m_1. Approximately 1 g of sample was weighed to obtain m_2 value and was put into the fiber bag. Then, fiber bags with the glass spacers were inserted into the carousel. The samples then undergo de-fatting by washing it in the petroleum ether for at least three times and let dried for 2 minutes.

Next step involves two washing phases. In washing phase 1, the sample along with fiber bags were boiled in 360 mL of 0.13 N sulphuric acid solution (H_2SO_4) for 30 minutes after the solvent started to boil. Then, the sample with fiber bag undergo removal of acids by washing the samples with fiber bag in the hot water for three times and proceed for washing phase 2. In washing phase 2, the sample along with fiber bags were boiled in 300 mL of 0.13 N sodium hydroxide solution (NaOH) for 30 minutes after the solvent started to boil. Then, the sample with fiber bag undergo removal of alkali by washing the samples with fiber bag in the hot water for three times. After the washing procedure, the samples with fiber bags were removed from the carousel and dried in the air-forced drying oven for 4 hours at 105 ℃ and were placed in the desiccator for 30 minutes. The fiber bags then were weighed with the incinerating crucible and denoted as m_3. An incinerating crucible with empty fiber bags as blank and an empty incinerating crucible were weighed and denoted as m_5 and m_6 respectively. The fiber bags were heated in the furnace for at least 4 hours at 600 ℃ followed by cooling in the desiccator for 30 minutes. The crucibles containing burned samples (ash) were weighed and denoted as m_4. The crude fiber amount was determined based on the following formula:

$$\text{CF}\ (\%) = \frac{(m_3 - m_1 - m_4 - m_5)}{m_2} \times 100 \qquad (3.5)$$

Where,

CF = Crude fiber

m_1 = Weight of fiber bag (g)

m_2 = Weight of initial sample weight (g)

m_3 = Weight of incinerating crucible and dried fiber bag after digestion (g)

m_4 = Incinerating crucible and ash (g)

m_5 = Blank value of the empty fiber bag (g)

$$\text{Blank value} = (m_7 - m_6) \quad (3.6)$$

Where,

m_6 = Incinerating crucible (g)

m_7 = Incinerating crucible and ash of the empty fiber bag (g)

3.6.5 Ash analysis

Based on the method in AOAC (1990), an empty porcelain crucible and 2 g of sample was weighed and denoted as W1 and W2 respectively. The samples then were incinerated in the furnace at 600 ℃ for at least 4 hours. Then, the crucibles were transferred directly to desiccator to be cooled and weighted immediately and denoted as W3.

$$\text{Ash (\%)} = \frac{W3 - W1}{W2} \times 100 \quad (3.7)$$

Where,

W1 = Weight of empty crucible (g)

W2 = Weight of sample (g)

W3 = Weight of crucible and ash (g)

3.6.6 Nitrogen Free Extract (NFE) Analysis

Nitrogen Free Extract is analyzed by using a formula which is

$$\% \text{ NFE} = \text{DM} - (\% \text{ EE} + \% \text{ CP} + \% \text{ ash} + \% \text{ CF}) \quad (3.8)$$

Where,

NFE = Nitrogen free extract

DM = Dry matter

EE = Ether extract or crude lipid

CP = Crude protein

CF = Crude fiber

3.7 Required nutrient percentage

The required nutrient percentage of protein, lipid, mineral and carbohydrate were calculated as the equation below:

$$\text{Required nutrient (\%)} = \frac{\text{Obtained value from proximate analysis}}{\text{Nutrient requirement reference}} \times 100 \quad (3.9)$$

3.8 Feed formulation

The feed formulation had involved egg custard, *Moringa oleifera* powder, *Curcuma longa* powder and egg shell powder. The feed formulation has been formulated by using Response Surface Methodology (RSM) in Design Expert Software version 10. There were 13 runs of formulations from the software in which the amount of suggested *M. oleifera* and *C. longa* were added with 40 % of egg custard and 3 % egg shells powder as the based feed. The formulated feed undergoes proximate analysis to reveal the nutrition composition in each formulation. The formulation table of new egg custard formulation was presented in Table 3.3 while the calculation for feed formulation to achieve 100 % was shown below:

$$\text{Parameter amount} = \frac{\text{Percentages of parameter}}{\text{Total percentages of } Moringa\ oleifera + Curcuma\ longa} \times 57 \quad (3.10)$$

$$\text{Final percentage of parameter} = \frac{\text{Parameter}}{100} \times 30 \text{ g} \quad (3.11)$$

Table 3.3: Percentage of ingredients for each formulation (100 %)

FORMULATION	**INGREDIENTS**			
	Egg custard %	**Egg shell %**	***Moringa oleifera* %**	***Curcuma longa* %**
1	40	3	55.6518	1.3482

2	97	3	0	0
3	40	3	49.9334	7.0670
4	40	3	53.8508	3.1492
5	40	3	53.8508	3.1492
6	40	3	53.8508	3.1492
7	40	3	57	0
8	40	3	53.8505	3.1492
9	40	3	53.8505	3.1492
10	40	3	0	57
11	40	3	57	0
12	40	3	53.8505	3.1492
13	40	3	57	0

Table 3.4: Formulation of egg custard with egg shell, *Moringa oleifera* and *Curcuma longa* / ± 30 g

FORMULATION	**INGREDIENTS**			
	Egg custard/g	**Egg shell/g**	***Moringa oleifera*/g**	***Curcuma longa*/g**
1	12	0.90	16.70	0.40
2	29.10	0.90	0	0
3	12	0.90	14.98	2.12
4	12	0.90	16.16	0.95
5	12	0.90	16.16	0.95
6	12	0.90	16.16	0.95
7	12	0.90	17.10	0

8	12	0.90	16.16	0.95
9	12	0.90	16.16	0.95
10	12	0.90	0	17.10
11	12	0.90	17.10	0
12	12	0.90	16.16	0.95
13	12	0.90	17.10	0

3.9 Optimization studies and data analysis

The optimization studies were performed by using Design Expert Software version 10 by comparing the actual experimental results with the predicted data that have been obtained in the software. There were four (4) experimental responses (R) have been analyze which were Protein Requirement (%), Lipid Requirement (%), Mineral requirement (%) and Carbohydrate Requirement (%) which were labelled as R1, R2, R3 and R4 respectively. All the responses were undergo a series of evaluation which were analysis of variance (ANOVA), development of polynomial regression model equation, diagnostic plot for predicted value versus actual values and diagnostic plot for normal probability plots of residual. The experimental data was observed and analysed by using interaction plot, 2D contour plot as well as 3D surface plot generated by Design Expert Software version 10.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Proximate analysis studies

In this study, the effect of amount of *Moringa oleifera* and *Curcuma longa* in egg custard and egg shell mixture towards the nutrient composition in the feed was observed by using statistical experimental design (Design Expert Software version 10) with different percentage of parameters. Response Surface Methodology (RSM) was used in this study by using Central Composite Design (CCD) model. The model requires three levels for each factor which are low, middle and high. However, there were only two factors being coded in this experiment which are low and high. The factors coded represented percentage of *M. oleifera* and *C. longa* in which the effect of percentage of each factor towards the nutrient composition in formulated feed was investigated. In this experimental design, two level was coded which are - 1 and + 1. Table 4.1 shows the experimental factors (parameter) with the level coded in the design.

Four (4) responses were coded to determine the effect of *M. oleifera* and *C. longa* percentage in the formulated feed to required nutrient requirement of *Macrobrachium rosenbergii* which were protein requirement, lipid requirement, mineral requirement and

carbohydrate requirement. Table 4.2 shows the proximate analysis data of 13 different formulation of egg custard added with 3 % egg shells powder, *M. oleifera* powder and *C. longa* powder.

Based on the data in Table 4.2, formulation 1 stated the highest dry matter content, 95.6587 % with the lowest moisture content, 4.3413 %. The lowest was in formulation 10 with 91.2998 % of dry content and the highest moisture content, 8.9002 %. Next, formulation 2 stated the highest crude protein content with 31.0819 % and the lowest was formulation 10 with 17.8731 %. Formulation 8 stated the highest ether extract content which was 6.1833 % while formulation 10 stated the lowest ether extract amount, 2.5776 %. The highest crude fiber content was observed in formulation 5 with 7.1081 % while the lowest value was formulation 2 with 0.2583 %. The highest ash content value was observed in formulation 1 which was 13.3625 % and the lowest value was observed in formulation 2 which was 8.6405 %. NFE value stated the highest value which was 8.9002 % in formulation 10 and the lowest value 4.3413 % in formulation 1.

To conclude the result from Table 4.2, the most repeated formulation was formulation 10 in which the nutrient content such as crude protein, crude fat and ash was 17.8731 %, 2.5776 % and 8.6905 % respectively, differ to egg custard poultry by product (PBM) formulation by Nik Sin & Shapawi (2017), protein content was 55.63 %, fat content was 13.41 % and ash content was 8.23 %. The huge differ in crude protein and crude fat content value between formulation 10 and egg custard PBM may because of the high protein and fat value contain in PBM meal which about 78.89 % and 15.32 % respectively (Nik Sin & Shapawi, 2017).

Table 4.1: Experimental factors with level coded

Factor	Name	Unit	Actual Factors		Coded Factors	
A	*Curcuma longa*	%	0	1	- 1	+ 1
B	*Moringa oleifera*	%	0	17.10	- 1	+ 1

Table 4.2: Nutrient composition of 13 runs of formulated feed

Formulation	Dry Matter (%)	Crude Protein (%)	Crude Fat (%)	Crude Fiber (%)	Ash (Mineral) (%)	Nitrogen Free Extract (%)	Moisture (%)
1	95.6587	26.6419	4.0421	4.7245	13.3625	46.8877	4.3413
2	94.6910	31.0819	5.2228	0.2583	8.6405	49.4875	5.3090
3	93.8917	26.2250	5.5699	6.1700	11.1465	44.7803	6.1083
4	93.9089	27.0450	5.5820	6.3160	11.2707	43.6952	6.0911
5	93.9509	27.1813	5.9081	7.1081	11.0434	42.7100	6.0491
6	94.0212	26.9838	5.6902	6.4060	11.1951	43.7461	5.9788
7	93.9811	27.3438	5.5869	5.7791	11.5144	43.7569	6.0189
8	93.9829	27.1400	6.1833	5.8327	10.9909	43.8360	6.0171
9	93.6234	26.6044	5.4030	5.5583	10.9983	45.0594	6.3766
10	91.2998	17.8731	2.5776	3.5739	8.6905	58.5847	8.9002
11	93.5140	27.5075	6.0079	6.0711	11.4371	42.4904	6.4860

12	93.4909	27.3225	5.3788	5.9264	10.9864	43.8768	6.5091
13	93.4851	27.7113	5.5975	6.0726	12.1619	41.9	6.5149

4.2 Development of Regression Model Equation for Response 1 (Protein requirement)

Central Composite Design (CCD) which is a standard response surface methodology was selected to investigate the relationship between variables and parameters which were percentage of *Moringa oleifera* and *Curcuma longa* towards percentage of nutrient requirement of *Macrobrachium rosenbergii* larvae which were protein requirement, lipid requirement, mineral requirement and carbohydrates requirement. Table 4.3 shows a model of summary statistic generated by the software, Design Expert Software version 10 in which it suggested 2FI (2 Factorial Interaction) was the best model that fit the experimental response 1, protein requirement (%). The model summary statistics was focus on the model maximizing the "Adjusted R-squared" and the "Predicted R-Squared".

Table 4.3: Model summary statistics of Protein Requirement % (R1)

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	6.46	0.3358	0.2030	-0.4540	912.78	
2FI	4.57	0.7004	0.6005	-0.1785	739.77	Suggested
Quadratic	5.02	0.7190	0.5182	-0.9948	1252.23	
Cubic	1.78	0.9747	0.9393	-0.5765	989.67	Aliased

Table 4.4 shows the standard deviation and 2FI model for R^2 for response 1. To judge the adequacy and consistency of the experiment model, the coefficient of determination (R^2), the adjusted determination coefficient (adjusted R^2) and coefficient of variation (CV) were used (Pishgar-Komleh, Keyhani, Mostofi-Sarkari, & Jafari, 2012).

From the data, the relative correlation coefficient (R^2) was 0.7004. which was lower than 1.00. Low value of correlation coefficient indicates less relevance of the dependent variables in the model (Salehi, Noaparast, & Shafaei, 2016). For response 1, it indicates that the model only can explained 70.04 % of the response variability.

However, there were huge difference between the predicted R^2 value which was -0.1785 and adjusted R^2 which was 0.6005. The difference was may due to the possible problem or large effect in the model or data. Adequate precision was used to measures the signal to noise ratio and compares the range of the predicted values to the average prediction error in which the ratio that is greater than 4 is desirable and indicates the adequacy of model discrimination (Mourabet, Rhilassi, Boujaady, Bennani-Ziatni, & Taita, 2017). In this study, the adequate precision was 9.884 which was larger than 4 and indicates the adequate signal.

Table 4.4: The standard deviation and 2FI model for R^2 for Protein Requirement % (R1)

Std. Dev.	4.57	**R-Squared**	0.7004
Mean	66.67	**Adj R-Squared**	0.6005
C.V. %	6.86	**Pred R-Squared**	-0.1785
PRESS	739.77	**Adeq Precision**	9.884

Moreover, RSM also generated an empirical polynomial regression model of coded factors represented in Equation 4.1 in which it reflected the interaction and significance of variables towards response 1, protein requirement (%).

The coefficient represented in the equation, with one factor was referred to the effect of the individual factor while coefficient with two factors referred to the interaction effect between the factor. By comparing the coefficient in the equation, the significance of the factor (*C. longa* and *M. oleifera*) towards the responses can be determined. The positive sign indicates the positive effect to the responses whereas negative sign indicates negative effect to the responses (Behera, Meena, Chakraborty, & Meikap, 2018).

In this study, factor B, referred to *M. oleifera* gave a positive effect towards the percentage of nutrient requirement while factor A, *C. longa* was in contrast. However, factor AB, in which the interaction effect of *C. longa* and *M. oleifera*, was the biggest positive influencer in protein requirement percentage.

Equation 4.1:

Protein requirement, Y (%) = + 66.67 - 4.97A + 1.29B + 7.56AB (4.1)

4.3 Statistical Analysis for Response 1 (Protein Requirement)

Analysis of variance (ANOVA) was used in this study to further analyze the accuracy and significance of the experimental data. The F-test was used to check the statistical significance of the model whereas the coefficient of R^2 determined the accuracy of the fitted polynomial model and evaluated by the probability value (P-value) lower than 0.05 or at 95 % of confidence interval (Kim, Kim, Cho, & Hong, 2003; Behera, Meena, Chakraborty, & Meikap, 2018). Value F and p determined the significance of the coefficient term whereas the corresponding coefficient is denoted as significant when the F value is larger and the p value is smaller (Amini et al., 2008; Bai, Saren, & Huo, 2015). Table 4.5 showed the ANOVA table for surface 2 Factorial Interaction (2FI) model for response 1, protein requirement (%).

From the ANOVA table, F value of the model was 7.01 and p-value was 0.0099, which denoted the model as significant. There was only 0.99 % chance that the F-value could be large due to noise. From the table, the result showed that the coefficient A, B and interaction of AB were considered as significant in 2FI model for response 1, protein requirement. The largest F value stated on factor A (*C. longa*) at 9.45 showed that it was the most important factor contribute to the percentage of protein requirement.

Table 4.5: ANOVA table for surface 2FI model for Protein Requirement % (R1)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	439.66	3	146.55	7.01	0.0099	Significant
A-*Curcuma longa*	197.46	1	197.46	9.45	0.0133	
B-*Moringa oleifera*	13.37	1	13.37	0.64	0.4444	
AB	228.84	1	228.84	10.95	0.0091	

4.4 Predicted Values versus Actual Values for Response 1 (Protein Requirement)

There were predicted value generated by CCD model of Design Expert Software version 10 and actual value that have been obtained during the experimental studies in RSM data. Table 4.6 showed predicted values and actual values. The highest percentage of protein requirement was run 2 (formulation 2), with 77.70 % and was slightly different to the predicted value, 77.90 % of protein requirement.

On the other hand, the data also plotted a normal probability plot of residuals to determined either the error terms were distributed normally. Figure 4.1 shows the normal probability plot of residuals for response 1, protein requirement (%). Based on the figure, almost all the data point distributed near to the straight line indicates that there was only intangible difference of residuals and the data were distributed normally. Besides, Figure 4.2 shows the diagnostic plot of actual and predicted values of response 1, protein

requirement (%). From the figure, some of the data point were not lie near to the straight line but still indicates that the data were normally distributed.

Table 4.6: Predicted values versus actual values for Protein Requirement (R1)

Standard Order	Actual Value	Predicted Value	Residual	Internally Studentized Residual	Externally Studentized Residual	Run Order
1	77.70	77.90	-0.20	-0.067	-0.063	2
2	44.68	52.84	-8.16	-2.744	** -6.40	10
3	69.28	65.36	3.92	1.317	1.382	13
4	66.51	70.55	-4.04	-1.360	-1.438	9
5	68.36	73.69	-5.33	-1.422	-1.522	7
6	65.56	59.64	5.92	1.579	1.751	3
7	68.77	64.84	3.93	1.048	1.055	11
8	66.60	68.49	-1.89	-0.504	-0.482	1
9	67.95	66.67	1.29	0.293	0.278	5
10	68.31	66.67	1.64	0.374	0.355	12
11	67.85	66.67	1.18	0.270	0.255	8
12	67.61	66.67	0.95	0.216	0.204	4
13	67.46	66.67	0.79	0.181	0.171	6

** Case(s) with |External Stud. Residuals| > 3.86

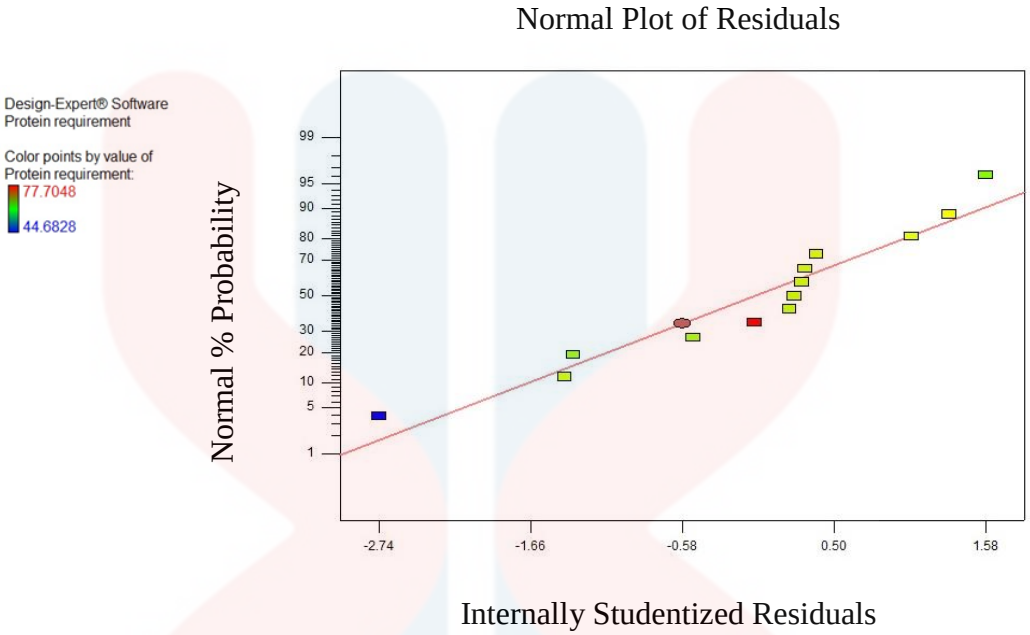

Figure 4.1: Normal plot of residuals of R1, Protein Requirement (%)

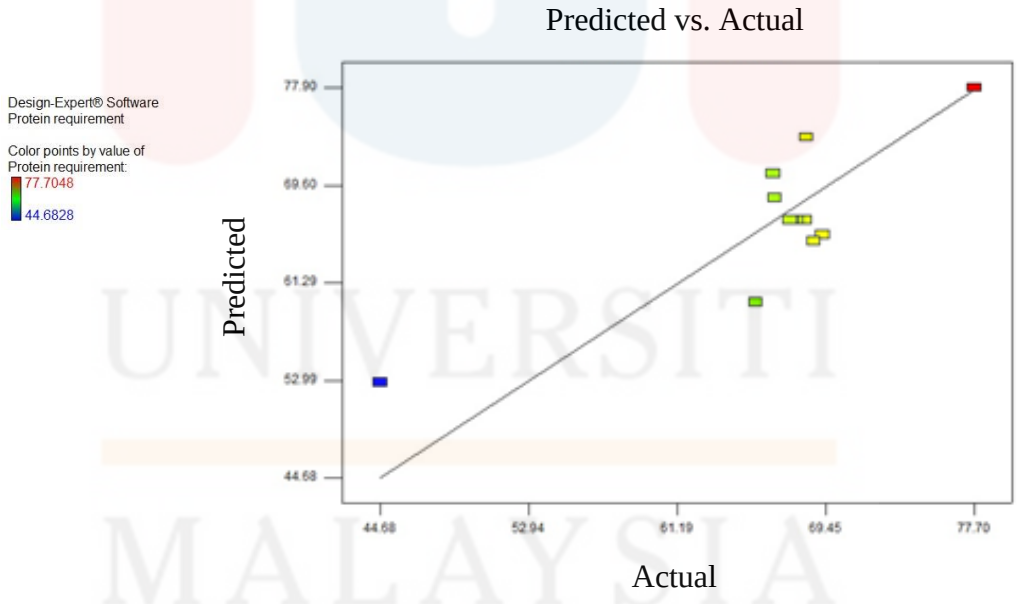

Figure 4.2: The diagnostic plot of actual versus predicted values of R1, Protein Requirement (%)

4.5 Effect of *Moringa oleifera* and *Curcuma longa* towards Response 1 (Protein Requirement)

In order to examine the effect of potential relationship between the variables, a two-dimension (2D) contour plot and a three-dimensional (3D) response surface graph was obtained. The optimum level of variables also can be identified to achieve the optimum level of protein requirement percentage by using the 2D and 3D plots. Figure 4.3 (a) shows the 2D contour plot while Figure 4.3 (b) shows the 3D plot of interaction effect of *Moringa oleifera* and *Curcuma longa* in the feed formulation towards Protein Requirement (%).

From both figures, the graph showed that percentage of protein requirement increase at 73.7276 % with the percentage of *C. longa* was in between 0.00 to 0.25 whereas percentage of *M. oleifera* was in between 4.28 to 8.55. The increment of protein requirement percentage is may because of the percentage of protein source in *M. oleifera* which about 31.5 % (Mbailao, Mianpereum, & Albert, 2014).

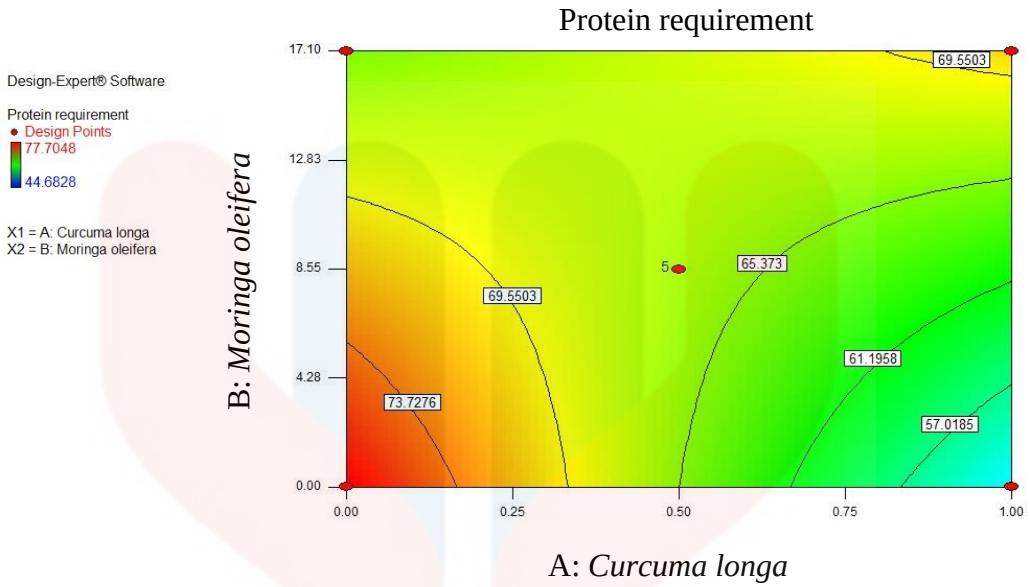

Figure 4.3(a): 2D contour plot of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R1, Protein Requirement (%)

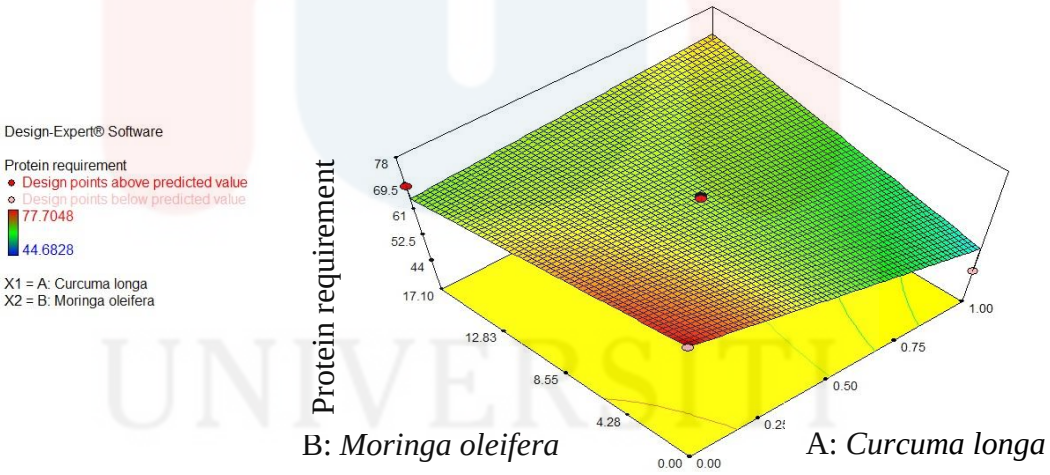

Figure 4.3(b): 3D response surface graph of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R1, Protein Requirement (%)

4.6 Development of Regression Model Equation for Response 2 (Lipid Requirement)

In order to determine the most fit model, Design Expert Software version 10 selected the highest order polynomial where the additional terms were significant, and the model was not aliased. Nevertheless, for response 2, lipid requirement (%), there was no suggested model that fit this response, thus 2FI model was selected to analyse the data. Table 4.7 shows the model of summary statistic generated by Design Expert Software version 10.

Table 4.7: The model of summary statistics of Lipid Requirement % (R2)

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	13.08	0.0940	-0.0872	-0.8662	3525.20	
2FI	12.72	0.2288	-0.0283	-1.8169	5320.87	
Quadratic	12.61	0.4106	-0.0105	-3.0011	7557.91	
Cubic	6.12	0.9007	0.7618	-3.2099	7952.23	Aliased

Table 4.8 shows the standard deviation and 2FI model for R^2 for response 2. From the data, showed that the relative coefficient (R^2) was 0.2288 which was lower than 1.00 and indicated that the dependent data in the model was less relevant. There was only 22.88 % of response variability can be explained by the model. Moreover, the was also a big difference between the predicted R^2 and adjusted R^2 which was -1.8169 and -0.0283 respectively. A negative predicted R^2 explained that the overall mean was a better

predictor of the response than the current model. High value of adequate precision which was 3.582 also indicates an inadequate signal.

Table 4.8: The standard deviation and 2FI model for R^2 for Lipid Requirement % (R2)

Std. Dev.	12.72	**R-Squared**	0.2288
Mean	68.86	**Adj R-Squared**	-0.0283
C.V. %	18.48	**Pred R-Squared**	-1.8169
PRESS	5320.87	**Adeq Precision**	3.582

The empirical polynomial regression model of coded factors for response 2 was represented in Equation 4.2 to determine the relationship and interaction between variables towards response. From the equation, the positive effect influencer for response 2 was factor B (*M. oleifera*) but the interaction factor AB (*C. longa* and *M. oleifera*) gave the biggest positive effect to the lipid requirement percentage in the formulated feed.

Equation 4.2:

$$\text{Lipid requirement, Y (\%)} = + 68.86 - 4.66A + 0.68B + 7.98AB \quad (4.2)$$

4.7 Statistical Analysis for Response 2 (Lipid Requirement)

To further analyze the data, analysis of variance (ANOVA) was being used in this study and reprented in Table 4.9. From the table, the F value and p-value of the model was 0.89 and 0.4827 respectively. The model was not significant due to p-value was bigger than 0.0500. There was also 48.27 % chance for F value to be this large due to noise.

Table 4.9: ANOVA table for surface 2FI model for Lipid Requirement % (R2)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	432.11	3	144.04	0.89	0.4827	not significant
A-*Curcuma longa*	173.80	1	173.80	1.07	0.3271	
B-*Moringa oleifera*	3.74	1	3.74	0.023	0.8826	
AB	254.57	1	254.57	1.57	0.2414	

4.8 Predicted Values versus Actual Values for Response 2 (Lipid Requirement)

Table 4.10 shows the predicted and actual values for response 2, lipid requirement (%). The highest percentage of lipid requirement was 80.51 % that referred to run 8 or

formulation 8. There was a huge different between the actual values and predicted values as predicted values recorded only 68.86 % of lipid requirement by formulation 8.

Besides, there was also probability plot of residuals plotted in Figure 4.4 while Figure 4.5 shows the diagnostic plot of actual and predicted values for response 2. From Figure 4.4, the data points were distributed near to the straight line which indicates that the data were normally distributed. On the other hand, from Figure 4.5, the data were distributed in group and two data points were outliers. The data were normally distributed except for the two outliers.

Table 4.10: Predicted values versus actual values for Lipid Requirement (R2)

Standard Order	**Actual Value**	**Predicted Value**	**Residual**	**Internally Studentized Residual**	**Externally Studentized Residual**	**Run Order**
1	68.01	80.82	-12.81	-1.548	-1.704	2
2	33.56	55.54	-21.98	-2.656	** -5.38	10
3	72.88	66.23	6.66	0.804	0.787	13
4	70.35	72.86	-2.51	-0.303	-0.287	9
5	72.75	75.45	-2.71	-0.259	-0.245	7
6	72.52	62.27	10.26	0.983	0.980	3
7	78.23	67.89	10.33	0.990	0.989	11
8	52.63	69.83	-17.20	-1.647	-1.858	1
9	76.93	68.86	8.07	0.660	0.638	5
10	70.04	68.86	1.18	0.096	0.091	12
11	80.51	68.86	11.65	0.953	0.948	8
12	72.68	68.86	3.82	0.313	0.296	4
13	74.09	68.86	5.23	0.428	0.408	6

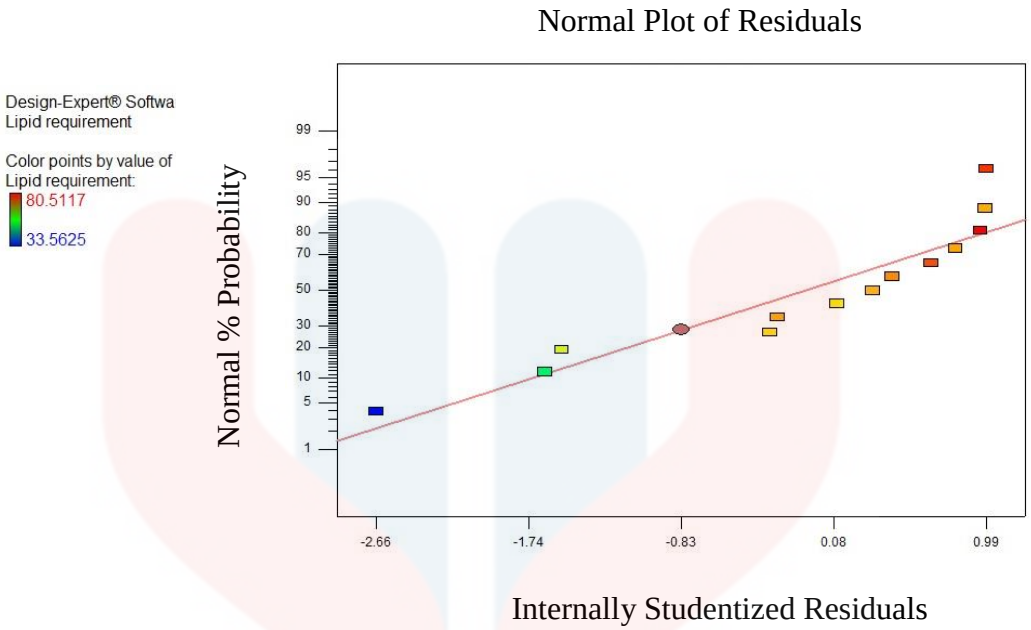

Figure 4.4: Normal plot of residuals of R2, Lipid Requirement (%)

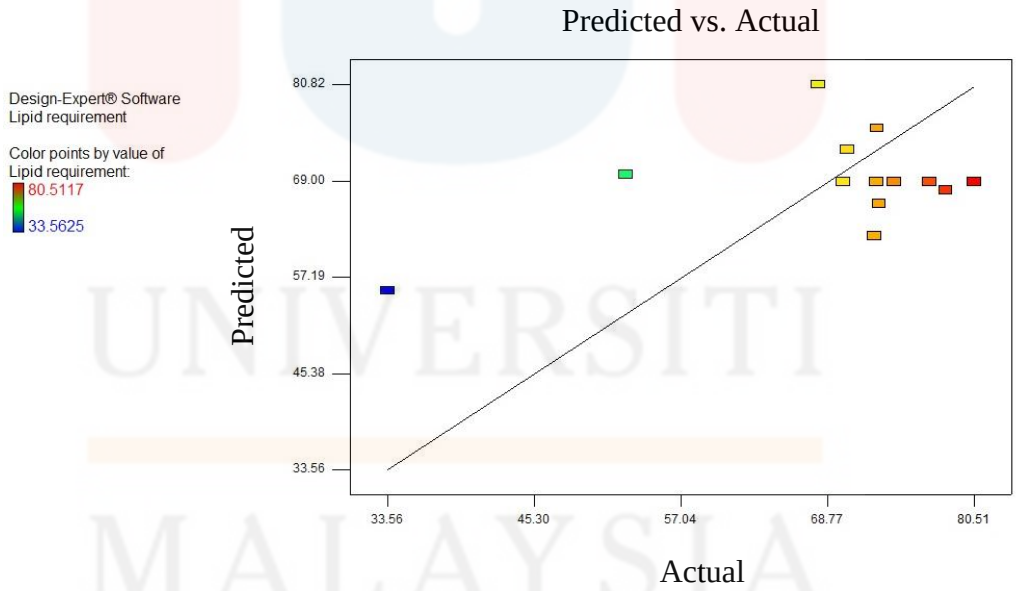

Figure 4.5: The diagnostic plot of actual versus predicted values of R2, Lipid Requirement (%)

4.9 Effect of *Moringa oleifera* and *Curcuma longa* towards Response 2 (Lipid Requirement)

The effect of *Moringa oleifera* and *Curcuma longa* towards lipid requirement percentage in the feed was determined by observing the 2D contour plot and 3D response surface graph as shown in Figure 4.6 (a) and Figure 4.6 (b). From both figures, it showed that lipid requirement in the feed formulation increase up to 76.6023% with the percentage of factor A (*C. longa*) was in between 0.00 to 0.25 while factor B (*M. oleifera*) was in between 4.28 and 8.55. Finding in previous research on *C. longa* crude lipid content was 6.85 % while *M. oleifera* was 2.5 % (Ahamefula et al., 2014; Mbailao et al., 2014).

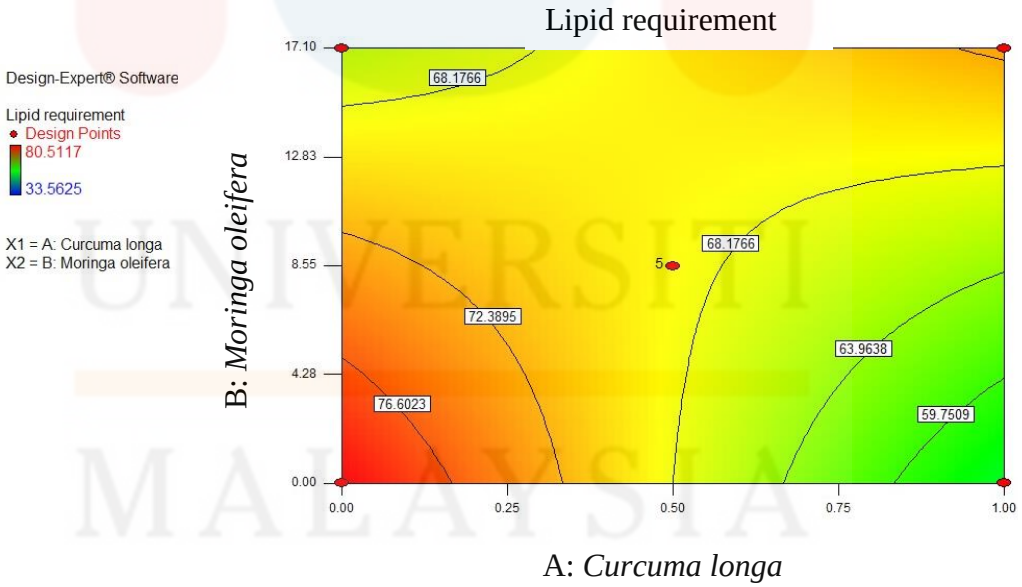

Figure 4.6 (a): 2D contour plot of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R2, Lipid Requirement (%)

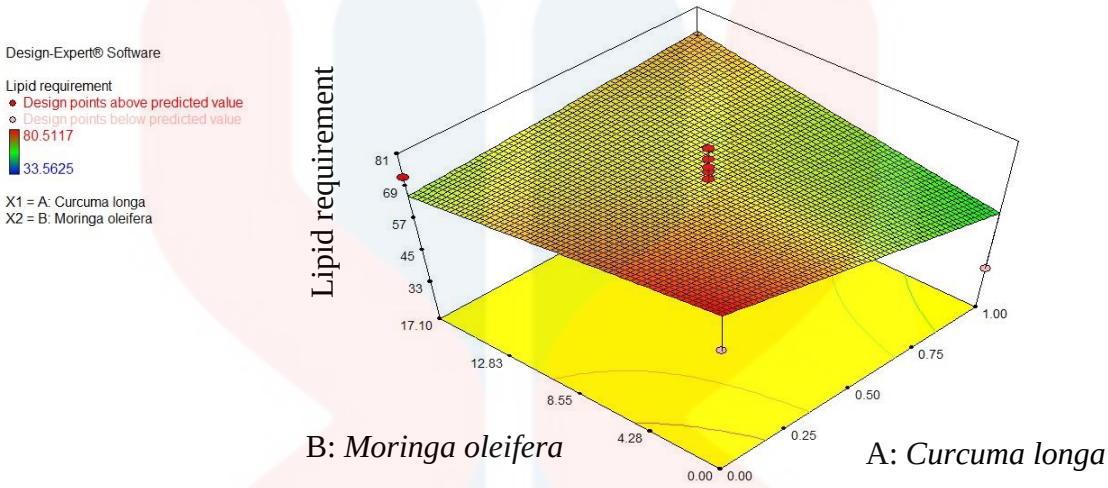

Figure 4.6 (b): 3D response surface graph of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R2, Lipid Requirement (%)

4.10 Development of Regression Model Equation for Response 3 (Mineral Requirement)

Table 4.11 shows the summary statistics of response 3, mineral requirement (%). From the table, the software suggested Linear model as the best fit. To respect the suggested model, Linear model was being used for response 3, mineral requirement (%). The aliased model which was Cubic model cannot be used as the model was far away from the best fit.

Table 4.11: Model summary statistics of Mineral Requirement % (R3)

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	31.50	0.5148	0.4178	-0.0662	21805.34	Suggested
2FI	32.51	0.5348	0.3797	-0.7458	35704.77	
Quadratic	34.24	0.5987	0.3120	-1.8340	57959.38	
Cubic	36.93	0.6666	0.1998	-20.1150	4.318E+005	Aliased

Table 4.12 shows the standard deviation and linear model for R^2 for response 3. The table shows that R^2 value was 0.5148, lower than 1.00 and indicates that there was less relevance of dependent variable in the model. For response 3, it indicates that the model only can explained 51.48 % of the response variability. There was also huge difference between predicted R^2 and adjusted R^2, - 0.0662 and 0.4178 respectively with adequate precision of 6.660 indicates the adequacy of model discrimination.

Table 4.12: The standard deviation and linear model for R^2 for Mineral Requirement % (R3)

Std. Dev.	31.50	**R-Squared**	0.5148
Mean	367.77	**Adj R-Squared**	0.4178
C.V. %	8.57	**Pred R-Squared**	-0.0662
PRESS	21805.34	**Adeq Precision**	6.660

The empirical polynomial regression model of response 3, mineral requirement (%) was represented in Equation 4.3. From the equation, individual factor B referred to *Moringa oleifera* have a positive effect to the mineral requirement percentage whereas individual factor A (*Curcuma longa*) gave the negative effect,

Equation 4.3:

Mineral requirement, Y (%) = + 367.77 - 6.81A + 35.63B (4.3)

4.11 Statistical Analysis for Response 3 (Mineral Requirement)

The analysis of variance (ANOVA) for linear model of response 3, mineral requirement was represented in Table 4.13. From the table, showed that the F value was 5.30 and p-value was 0.0269 and indicates it was significant as p-value low than 0.0500. There was only 2.69 % chance for F value to be this large due to noise. The F value of factor B (*Moringa oleifera*) was the largest, 10.24 which indicates that the factor was the most important contribute to the percentage of mineral requirement.

Table 4.13: ANOVA table for linear model for Mineral Requirement % (R3)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	10528.33	2	5264.16	5.30	0.0269	significant
A-Curcuma longa	370.75	1	370.75	0.37	0.5547	

B-Moringa oleifera	10157.57	1	10157.57	10.24	0.0095

4.12 Predicted Values versus Actual Values for Response 3 (Mineral Requirement)

The predicted and actual values of mineral requirement percentage was tabulated in Table 4.14. From the data, run order 5 or formulation 5 stated the highest percentage, 445.40 % and slightly different to the predicted value which was 418.16.

The normal probability diagnostic plot of residuals and the diagnostic plot of actual versus predicted value was shown in Figure 4.7 and Figure 4.8 respectively. From Figure 4.7, the data points lie near to the straight line and indicates that all the data were normally distributed. From Figure 4.8, some of the data points lie near the straight line and four data points were outliers. However, it still indicates that the data were normally distributed.

Table 4.14: Predicted values versus actual values for Mineral Requirement % (R3)

Standard Order	Actual Value	Predicted Value	Residual	Internally Studentized Residual	Externally Studentized Residual	Run Order
1	288.01	338.95	-50.94	-1.971	-2.391	2
2	289.67	325.33	-35.66	-1.380	-1.455	10
3	405.38	410.21	-4.83	-0.187	-0.178	13
4	366.60	396.60	-30.00	-1.161	-1.184	9
5	383.80	377.40	6.40	0.248	0.236	3

6	371.54	358.14	13.39	0.518	0.498	11
7	381.23	317.38	63.85	2.470	3.754	1
8	445.40	418.16	27.24	1.054	1.061	5
9	368.10	367.77	0.33	0.011	0.010	12
10	366.20	367.77	-1.57	-0.052	-0.049	8
11	366.35	367.77	-1.42	-0.047	-0.044	4
12	375.68	367.77	7.91	0.261	0.249	6
13	373.06	367.77	5.29	0.175	0.166	3

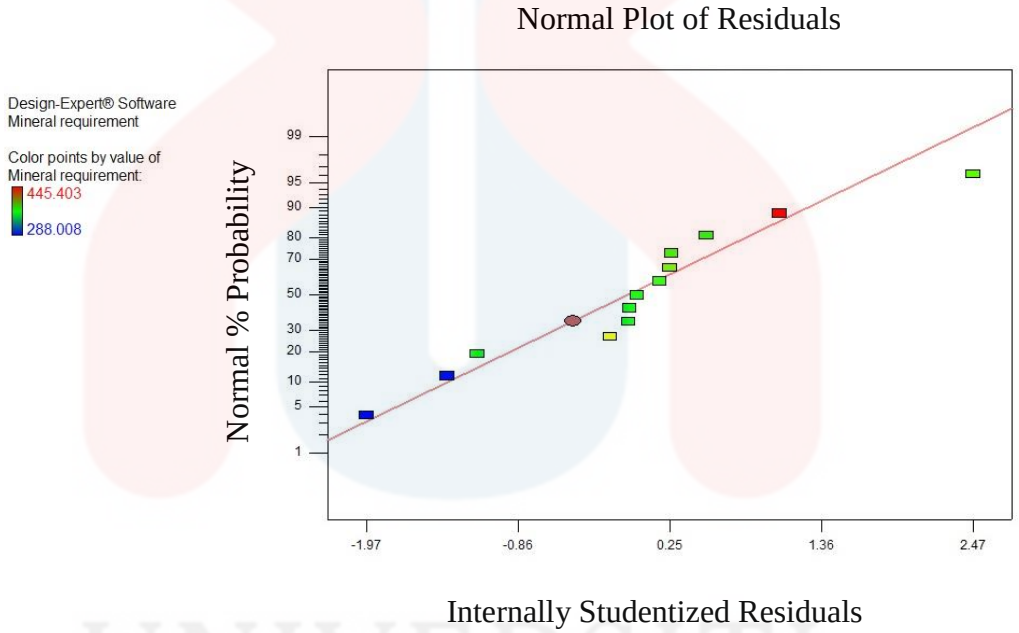

Figure 4.7: Normal plot of residuals of R3, Mineral Requirement (%)

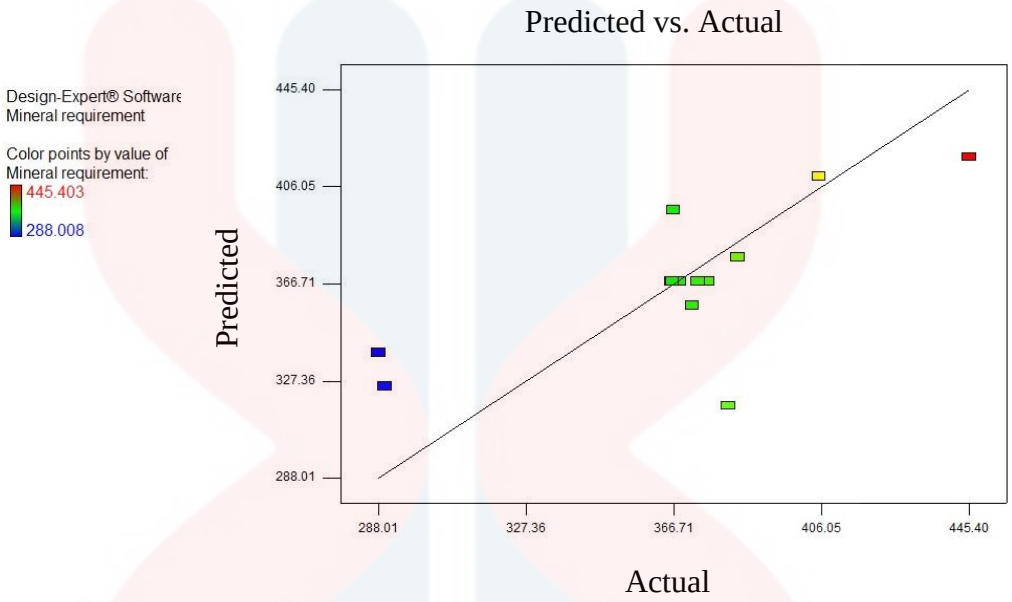

Figure 4.8: The diagnostic plot of actual versus predicted values of R3, Mineral Requirement (%)

4.13 Effect of *Moringa oleifera* and *Curcuma longa* towards Response 3 (Mineral Requirement)

The effect of *Moringa oleifera* and *Curcuma longa* towards mineral requirement percentage was observed in the 2D contour plot and 3D response surface graph as shown in Figure 4.9 (a) and Figure 4.9 (b). From the graph, showed that there was only individual interaction that influence the increasing percentage of mineral requirement for linear model. The mineral requirement increases up to 396.065 % in which it exceeded the 100 % of requirement for *M. rosenbergii* larvae if factor B (*M. oleifera*) percentage was in

between 12.83 to 17.10. The increasing in mineral value may because of the mineral content in *M. oleifera* which about 6.96 % (Mbailao et al., 2014).

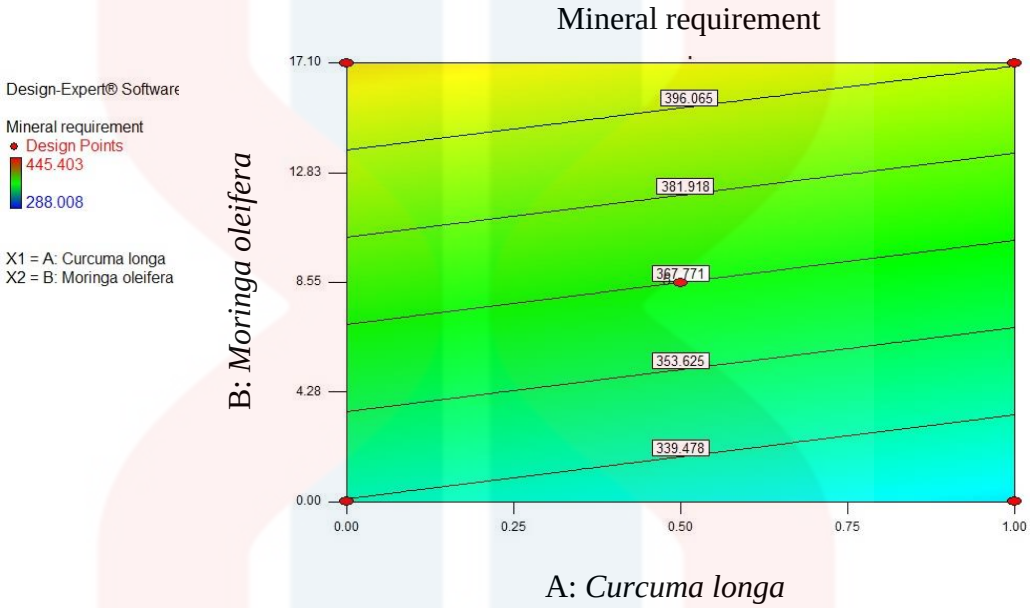

Figure 4.9 (a): 2D contour plot of interaction of *Moringa oleifera* and *Curcuma longa* towards R3, Protein Requirement (%)

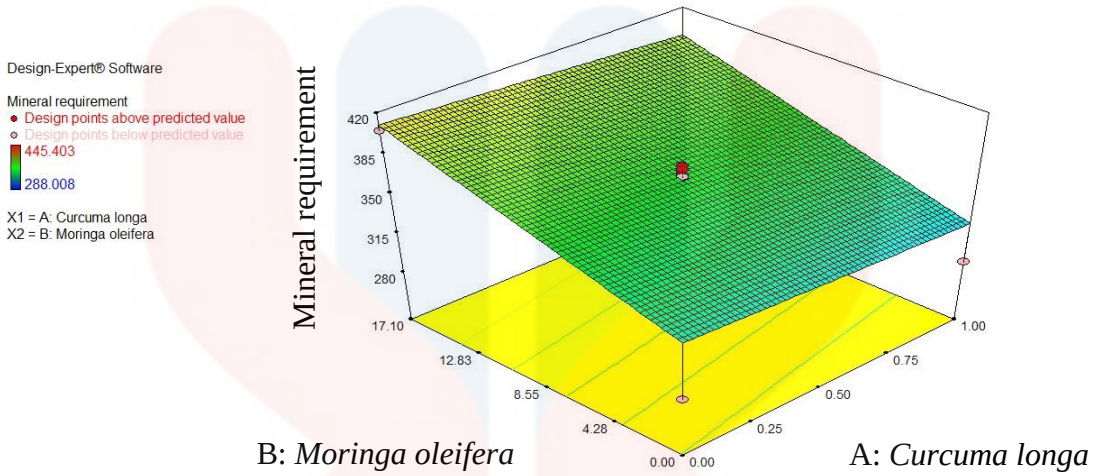

Figure 4.9 (b): 3D response surface graph of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R3, Mineral Requirement (%)

4.14 Development of Regression Model Equation for Response 4 (Carbohydrate Requirement)

Table 4.15 shows the model summary statistics of Carbohydrate Requirement % (R4). The software was suggested linear model as the best fit while cubic model was aliased as it far away to fit the response. Linear model was used for this response as to respect the suggested model by Design Expert Software version 10.

Table 4.15: Model summary statistics of Carbohydrate Requirement % (R4)

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	12.23	0.2186	0.0623	- 0.6432	3142.80	Suggested
2FI	12.57	0.2562	0.0082	- 1.9715	5683.08	
Quadratic	13.03	0.3787	-0.0650	- 3.3953	8406.32	
Cubic	7.83	0.8396	0.6151	- 9.0101	19144.86	Aliased

The standard deviation and linear model for R^2 for response 4 was shown in Table 4.16. From the data, the R^2 value was 0.2186 and indicates less relevance dependent variables in this model. It also showed that only 21.86 % of the response variability can be explained by the model. There were also a big different between predicted R^2 value and adjusted R^2 value, -0.6432 and 0.0623 respectively with adequate precision of 3.478. A negative R^2 value means that the overall mean was a better predictor to the response than the current model.

Table 4.16: The standard deviation and linear model for R^2 for Carbohydrate Requirement % (R4)

Std. Dev.	12.23	**R-Squared**	0.2186
Mean	129.85	**Adj R-Squared**	0.0623
C.V. %	9.42	**Pred R-Squared**	-0.6432
PRESS	3142.80	**Adeq Precision**	3.478

The empirical polynomial regression model of coded factors for response 4 was shown in Equation 4.4. From the equation, it explained that the biggest positive influencer was individual factor A, *Curcuma longa*. In contrast, individual factor B, *Moringa oleifera* gave the negative influence on the response.

Equation 4.4:

$$\text{Carbohydrate requirement, Y (\%)} = +129.85 + 4.89A - 5.32B \quad (4.4)$$

4.15 Statistical Analysis for Response 4 (Carbohydrate Requirement)

The ANOVA table for respnse 4, carbohydrate requirement was represented in Table 4.17. From the table, the F value was 1.40 and p-value was 0.2914. Due to the p-value was higher than 0.05, thus indicates that the model was not significant due to relative noise. The F value was bigger for factor B, *Moringa oleifera* denoted it as the most important factor that contribute to the percentage of carbohydrate requirement in the feed formulation.

Table 4.17: ANOVA table for linear model for Carbohydrate Requirement % (R4)

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	417.99	2	209.00	1.40	0.2914	not significant
A-*Curcuma longa*	191.63	1	191.63	1.28	0.2839	

B-*Moringa oleifera*	226.36	1	226.36	1.51	0.2466

4.16 Predicted Values versus Actual Values for Response 4 (Carbohydrate Requirement)

The predicted and actual values for response 4, carbohydrate requirement percentage, was tabulated in Table 4.18. From the table, the highest percentage of carbohydrate requirement was 167.38 % for run order 10 or referred to formulation 10. The predicted value was slightly different as it lower than the actual value, 140.06 %.

Figure 4.10 shows the normal plot of residuals while Figure 4.11 shows the diagnostic plot of actual and predicted value of response 4. From figure 4.10, all the data point was distributed near to the straight line except for one outlier indicate that the data was normally distributed. From Figure 4.11, the data points were distributed not far from the straight line except for one outlier. This also indicates that the data was normally distributed.

Table 4.18: Predicted values versus actual values for Carbohydrate Requirement % (R4)

Standard Order	**Actual Value**	**Predicted Value**	**Residual**	**Internally Studentized Residual**	**Externally Studentized Residual**	**Run Order**
1	141.39	130.27	11.12	1.109	1.123	2
2	167.38	140.06	27.32	2.724	** 5.09	10

3	119.71	119.63	0.079	0.008	0.008	13
4	128.74	129.42	-0.68	-0.068	-0.065	9
5	125.02	122.93	2.09	0.209	0.198	7
6	127.94	136.77	-8.83	-0.880	-0.869	3
7	121.40	137.37	-15.97	-1.592	-1.748	11
8	133.96	122.33	11.64	1.160	1.183	1
9	122.03	129.85	-7.82	-0.666	-0.646	5
10	125.36	129.85	-4.49	-0.382	-0.365	12
11	125.25	129.85	-4.60	-0.392	-0.375	8
12	124.84	129.85	-5.01	-0.426	-0.408	4
13	124.99	129.85	-4.86	-0.414	-0.396	6

** Case(s) with |External Stud. Residuals| > 5.08

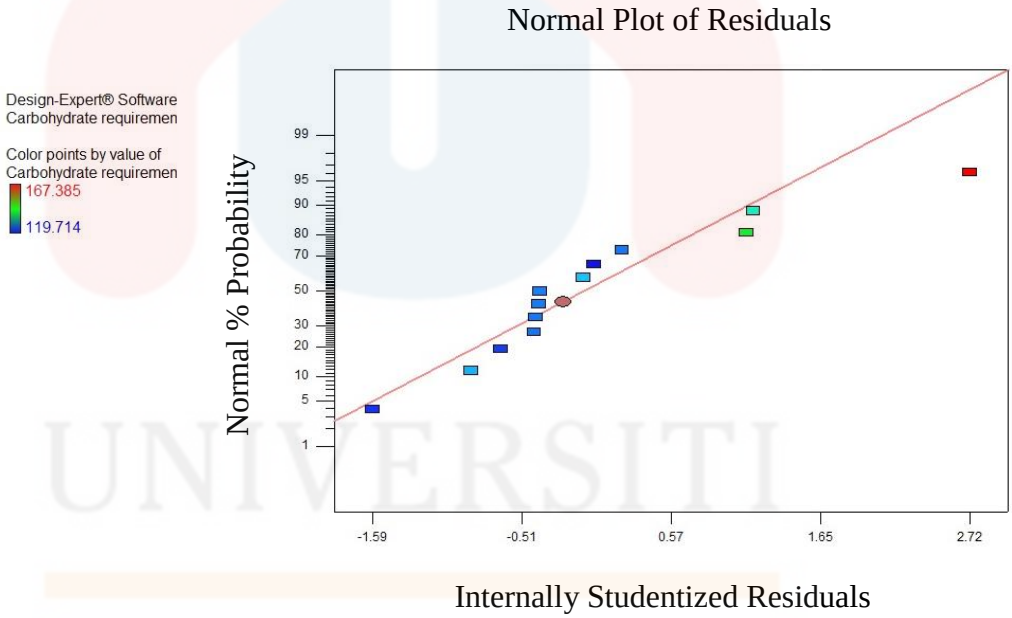

Figure 4.10: Normal plot of residuals of R4, Carbohydrate Requirement (%)

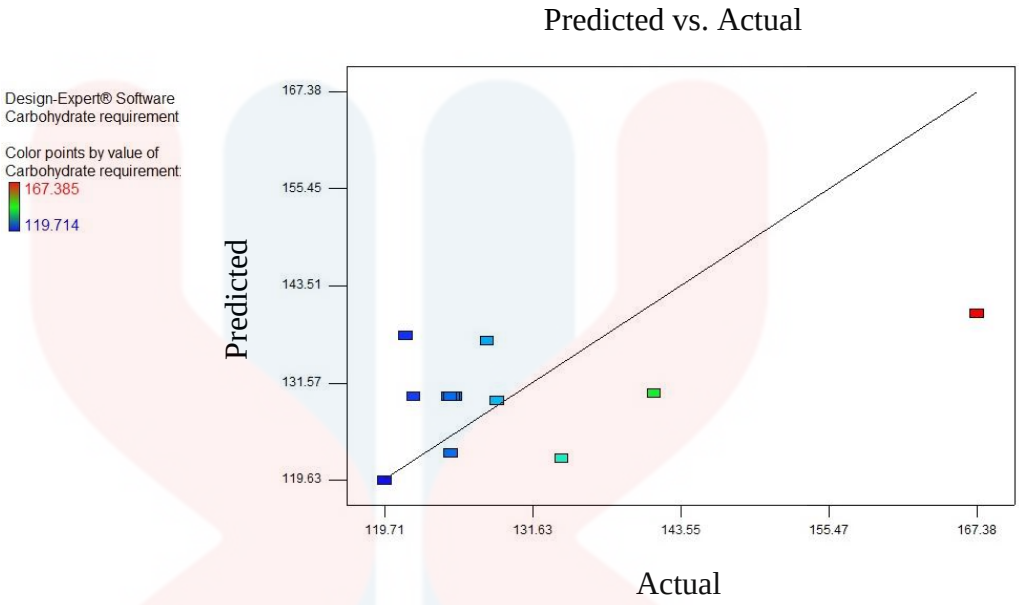

Figure 4.11: The diagnostic plot of actual versus predicted values of R4, Carbohydrate Requirement (%)

4.17 Effect of *Moringa oleifera* and *Curcuma longa* towards Response 4 (Carbohydrate Requirement)

2D contour plot and 3D response surface graph was obtained to determine the effect of *Moringa oleifera* and *Curcuma longa* towards response 4 as shown in Figure 4.12 (a) and Figure 4.12 (b) respectively. From both figures, observed that the percentage of carbohydrate requirement in the formulated feed increase by 136.658 % with the increasing percentage of individual factor A, *C. longa* in which it has exceed the 100 % of carbohydrate requirement needed by *Macrobrachium rosenbergii* larvae. This was may due to higher carbohydrate content in *C. longa* which was 67.38 % (Ahamefula et al., 2014).

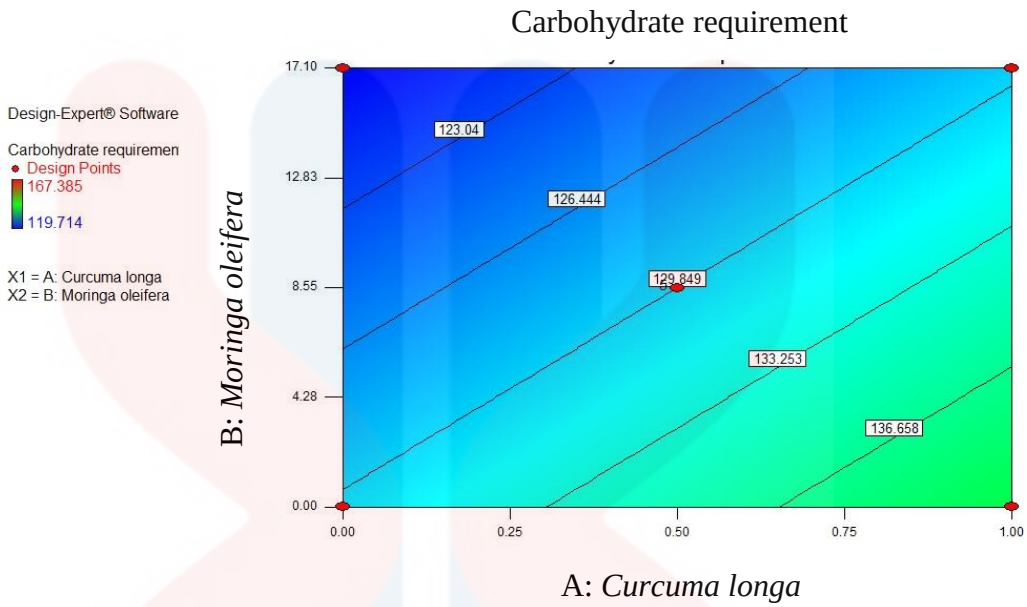

Figure 4.12 (a): 2D contour plot of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R4, Carbohydrate Requirement (%)

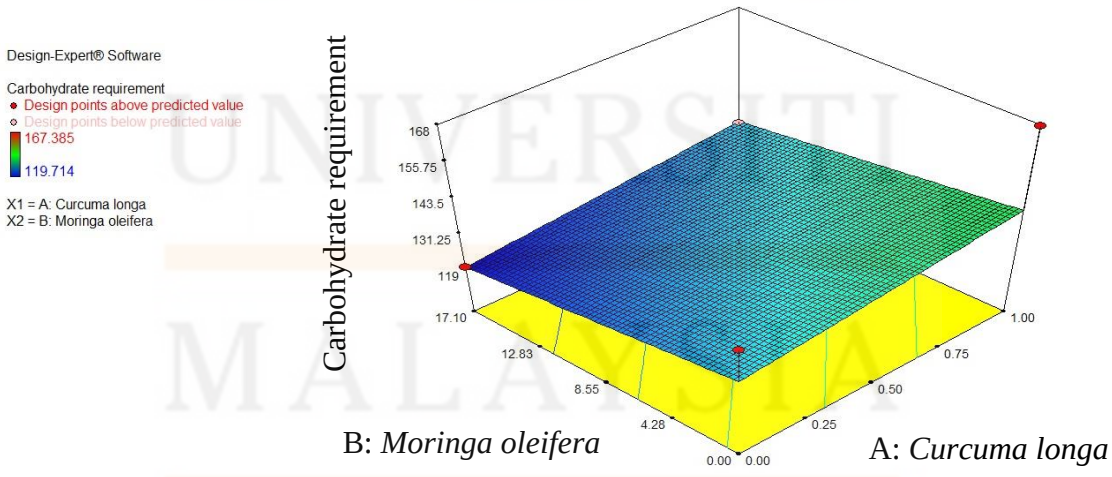

Figure 4.12 (b): 3D response surface graph of interaction effect of *Moringa oleifera* and *Curcuma longa* towards R4, Carbohydrate Requirement (%).

4.18 Numerical Optimization of Desirability Function for Factors and Parameters

The Design Expert software version 10 generated a desirability function (DF) in order to optimize the factors to obtain the optimize percentage of all parameters (responses). It aimed to optimize the multiple response processes and the most widely used in science and engineering (Mang et al., 2015). The range of DF values are in between zero (least value) to one (the most desirable value). The factors with the highest desirability value are considered as the most optimal parameter conditions (Manohar, Joseph, Selvaraj, & Sivakumar, 2013).

In this study, Design Expert Software version 10 had suggested the most optimal parameters condition with the optimal percentage of factors for this study as shown in Figure 4.13. To guide the estimation of desirability, a linear ramp has been created by the software between the minimum value and the maximum value of factors and parameters. By referring to Figure 4.13, it shows that the optimal percentage of *Curcuma longa* and *Moringa oleifera* were 1.00 and 17.10 respectively with the optimum percentage of protein requirement, lipid requirement, mineral requirement and carbohydrate requirement were 70.5539, 72.8604, 396.597 and 129.423 respectively. The overall desirability value was 0.738 that near to 1.00 indicates that it was a quite desirable formulation to fulfill the nutrient requirement of *Macrobrachium rosenbergii* larvae.

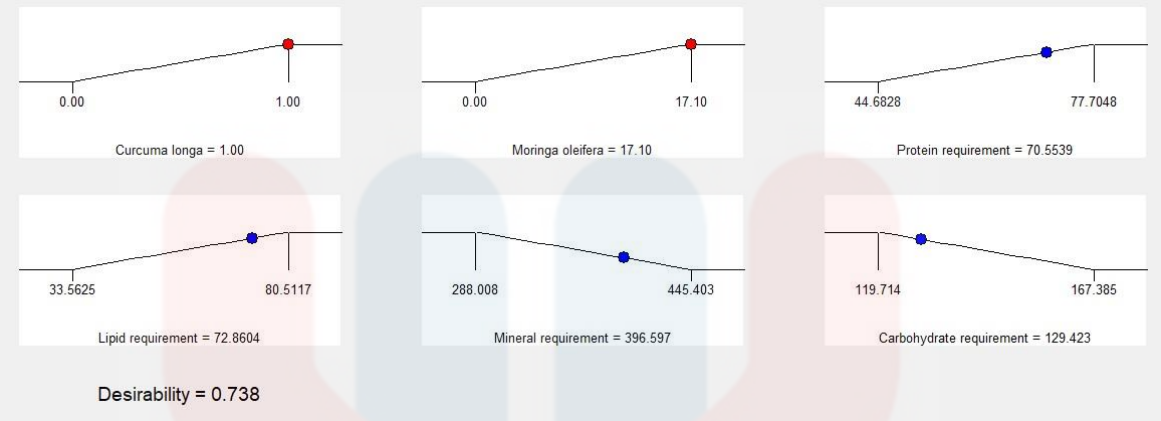

Figure 4.13: The desirability function ramp for all factors and parameters

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

An experimental design called Response Surface Methodology (RSM) was used to investigate the relationship between the used of *Curcuma longa* and *Moringa oleifera* in egg custard formulation with the percentage of required nutrient of *Macrobrachium rosenbergii* which was protein, lipid, mineral and carbohydrate based on the obtained nutrient composition of each formulations by employing central composite design (CCD).

In this study, the correlation coefficient R^2 of all responses, protein requirement, lipid requirement, mineral requirement and carbohydrate requirement were 0.7004, 0.2288, 0.5148 and 0.2186 respectively. All the R^2 value was lesser than 1.00 revealed that there was less relevance of the dependent variables in the model.

The actual versus predicted data by Design Expert Software version 10 revealed that the highest protein requirement percentage was 77.90 (predicted value) and 77.70 (actual value) for run order 2 which consist of 97 % egg custard, 3 % egg shells, 0.00 % *C. longa* and 0.00 % *M. oleifera*. Predicted value for lipid requirement was 80.82 for run order 2 which differ to the highest value for actual, 80.51 for run order 8 which consist of 40 % egg custard, 3 % egg shells, 0.50 % *C. longa* and 8.55 % *M. oleifera*. Next, for

mineral percentage, the highest actual and predicted value stated on run 5 consist of 40 % egg custard, 3 % egg shells, 0.50 % *C. longa* and 8.55 % *M. oleifera* with 445.40 and 418.16 respectively. For carbohydrate percentage, the highest value for actual and predicted was 167.38 and 140.06 respectively for run order 10 that consists of 40 % egg custard, 3 % egg shell, 1.00 % *C. longa* and 0.00 % *M. oleifera*.

On the other hand, ramp graph of desirability function generated by Design Expert Software version 10 suggested the most desirable percentage off all parameters and response were 0.738 with the percentage of *C. longa* was 1.00 % and *M. oleifera* was 17.10 %. In conclusion, the most desirable formulation was formulation 9 which contain 40 % egg custard and 3 % egg shell for overall feed formulation and 1.00 % *C. longa* and 17.10 % *M. oleifera* from the suggested formulation percentage by Design Expert Software version 10.

5.2 Recommendation

Feeding trial should be done in the next study to observe the potential of application of *Curcuma longa* and *Moringa oleifera* in egg custard formulation towards the nutrient requirement of *Macrobrachium rosenbergii* larvae. This is because feeding trial may result in more scientific and significant result in order to promotes the growth rate and reduce the mortality rate of *M. rosenbergii* larvae. The final recommendation is to explore more designs of Response Surface Methodology (RSM) such as two level full factorial design, Box-Behnken Designs (BBD) and three level full factorial design in Design Expert Software.

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APPENDIX

APPENDIX A

Figure A1: Preparation of *Moringa oleifera* powder

Figure A2: Preparation of *Curcuma longa* powder

Figure A3: Preparation of egg shells powder

Figure A4: Cooked egg custard

Figure A5: 13 egg custard formulation with egg shells, *Moringa oleifera* and *Curcuma longa*

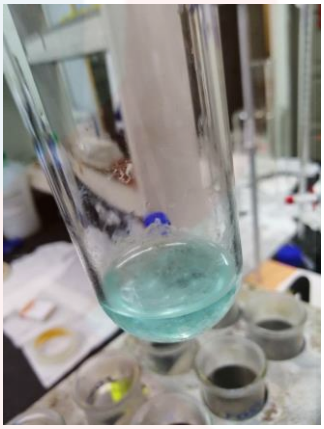

Figure A6: Protein analysis of formulated feed

Figure A7: Fat analysis by using Soxtec machine

Figure A8 (a): Fiber analysis machine

Figure A8 (b): Fiber bags with samples