OPTIMIZATION OF ENCAPSULATION OF NATURAL DYE BY CYCLODEXTRIN USING RESPONSE SURFACE METHODOLOGY

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OPTIMIZATION OF ENCAPSULATION OF NATURAL DYE BY CYCLODEXTRIN USING RESPONSE SURFACE METHODOLOGY

NOR ASHIKIN BINTI ABDUL HAMID

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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MAY 2010

I declare that this thesis entitled "Optimization of Encapsulation of Natural Dye by Cyclodextrin Using Response Surface Methodology" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

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Special Dedication to my family members,
My fellow lecturers
my friends, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

Nowadays, cyclodextrin widely used in industries due to its ability in encapsulation process with natural dye. However, the production cost for cyclodextrin is very expensive. A study on how to reduce the production cost by optimization of pH, concentration of cyclodextrin and temperature effect on encapsulation of natural dye by cyclodextrin using Response Surface Methodology (RSM) was successfully done. During the preliminary experiment, one factor at a time was employed to screen the best range for pH, concentration of cyclodextrin and temperature. All the parameter ranges obtained were used in RSM. RSM software in Design Expert version 6.0.8 was used with Central Composite Design (CCD) mode. Seventeen sets of experiments with different parameter values were suggested by the software. The predicted optimum values for pH, concentration of cyclodextrin, temperature and concentration of encapsulation of natural dye-β-cyclodextrin were pH 3.11, 0.004 mol/L, 60 ^{o}C and 1.27763 g/L respectively. One set of experiment was run using the optimized parameter and as a result, 1.2534 g/L concentration on encapsulation of natural dye-β-cyclodextrin was recorded. Before optimization, concentration on encapsulation of natural dye-β-cyclodextrin was only 0.775 g/L and the concentration was increased by 38.17% after optimization. The optimization also reduces the cost and energy consumption as the pH reduced from pH 10.5 to pH 3.11, concentration of cyclodextrin reduced from 0.005 mol/L to 0.004 mol/L and temperature was reduced from 70 ^{o}C to 60 ^{o}C. As a conclusion, this study was successful to increase encapsulation of natural dye by cyclodextrin production, reduce the energy consumption and also be able to reduce the production cost of cyclodextrin.

ABSTRAK

Pada masa kini, siklodekstrin telah digunakan secara meluas dalam industri atas kebolehannya dalam proses pengkapsulan dengan pewarna semulajadi. Walaubagaimanapun, kos penghasilan siklodekstrin sangat mahal. Satu kajian tentang bagaimana mengurangkan kos pengeluaran dengan pengoptimuman pH, kepekatan siklodekstrin dan suhu terhadap pengkapsulan pewarna semulajadi dengan siklodekstrin menggunakan Kaedah Permukaan Tindak balas (RSM) telah berjaya dilakukan. Di awal peringkat eksperimen, kaedah satu faktor pada satu masa telah digunakan untuk saringan julat terbaik untuk pH, kepekatan siklodekstrin dan suhu. Kesemua julat parameter yang diperolehi digunakan dalam RSM. Perisian RSM dalam Design Expert versi 6.0.8 telah digunakan dengan mod Rekabentuk Komposit Pusat (CCD). Tujuh belas set eksperimen berlainan nilai parameter telah dicadangkan oleh perisian ini. Nilai optimum yang diramalkan untuk pH, kepekatan siklodekstrin, suhu dan kepekatan pengkapsulan terhadap pewarna semulajadi dengan siklodekstrin masing-masing pH 3.11, 0.004 mol/L, 60 ^{o}C dan 1.27763 g/L. Satu set eksperimen telah dijalankan bagi menguji parameter yang telah dioptimumkan dan sebagai keputusannya, 1.2534 g/L kepekatan pengkapsulan terhadap pewarna semulajadi dengan siklodekstrin telah direkodkan. Sebelum pengoptimuman, kepekatan pengkapsulan terhadap pewarna semulajadi dengan siklodekstrin hanya 0.775 g/L dan kepekatannya meningkat 38.17%. Pengoptimuman juga mengurangkan kos dan penggunaan tenaga seperti pH dikurangkan dari pH 10.5 kepada pH 3.11, kepekatan siklodekstrin dikurangkan dari 0.005 mol/L kepada 0.004 mol/L dan suhu telah direndahkan dari 70 ^{o}C kepada 60 ^{o}C. Sebagai kesimpulan, kajian ini telah berjaya meningkatkan penghasilan pengkapsulan pewarna semulajadi oleh siklodekstrin, mengurangkan penggunaan tenaga dan juga mampu mengurangkan kos pengeluaran siklodekstrin.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of variance
CCD	-	Central composite design
g	-	Gram
mL	-	Mililitre
g/L	-	Gram per liter
mol/L	-	Molar per liter
L	-	Liter
M	-	Molar (mol/L)
mg	-	Milligram
mM	-	Milimolar
MW	-	Molecular weight
OD_{315}	-	Optical density at 315nm
OFAT	-	One Factor at a Time
RSM	-	Response surface Methodology
rpm	-	Revolution per minutes
T	-	Temperature
U/mg	-	Unit enzyme per miligram
^{o}C	-	Degree Celcius
%	-	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cyclodextrins are among of the most remarkable monocyclic molecules, with significant theoretical and practical impacts in chemistry. Cyclodextrins are able to form host-guest complexes with hydrophobic molecules given the unique nature imparted by their structure. As a result, these molecules have found a number of applications in a wide range of fields and widely used in chemical, pharmaceutical, food and other technologies as enzymes mimic, enantioselective catalysts, drug carriers and odor tastes-masking compounds (Chankvetade *et al*., 1993).

Lei and Qing (2002) claims that taking advantage of their inclusion capacity, cyclodextrins are being used in various fields to improve stability of guest substances, example to protect any components that are unstable when exposed to light or oxygen, to prevent volatile components evaporating, to modify physicochemical properties or to eliminate unpleasant tastes or odor.

In addition, cyclodextrins are also utilized to control release of a fragrance or drug and the non-toxic character of it together with their applicability in pharmaceutical, food and cosmetic sciences is also important for their laboratory and environmentally friendly analytical applications (Lei and Qing, 2002). Therefore, natural dye are employed as carriers for biologically active substances, enzyme models, separating agents, catalysts, mass transfer promoters, additives in perfumes, cosmetics, aliments or food , environmental protection agents or sensors for organic molecules. Cyclodextrin are essentially inert to photochemical excitation but their chemical modification with chromophoric moities may associate spectroscopic properties to the inclusion of guest molecule. For this research, *curcuma longa* is used as the source of extraction in encapsulation of natural dye by cyclodextrins in order to get and maintain the color (Min *et al.*, 2009).

Importance of natural dyes is more relevant world wide in the context of increasing environment consciousness. The non-toxic, biodegradable and eco-friendly properties make them exceedingly popular amongst nature loving and health awarded people. In spite of all, the present international consumption natural dyes are about 1% of the synthetic dye consumption. It is all due to the existing limitations and technical drawbacks of these dyes, which restrict their usage in textile dyeing. Some of these are color yield, complexibility of dyeing process, limited shades, reproducible results, blending problems and inadequate fastness properties. Lack of standardized profiles for extraction and textile dyeing are also major constraints (Kiran and Kapoor, 2007).

Indian craftsmen had monopoly on natural dyes before the advent of synthetic dyes but the craft of natural dyeing was very secretive amongst the particular communities. They did not disclose their trade secrets to any one except their family members. These techniques varied a lot depending upon the communities practicing in the different regions of India. They had no communication system and no documentation was made. In the era of synthetic dyes, natural dyeing became uncompetitive with regards to limited shades, less fastness properties and higher cost and thus neglected. It forced the craftsmen to switch over to other professions thereby causing major set back to natural dyeing and the traditional knowledge became abdomened. Technical drawbacks and limitation of natural dyes can be solved through adequate scientific.

1.2 Problem Statement

Synthetic dyes have their own worth. Synthetic dyes for example hair dyes cannot maintain the color in long time compare to the natural dyes. It was reported that, the resulting dry of natural dye concentrated maintained its stability after sixteen and one half weeks of storage in dark and having good color. Natural dyes also give glow to skin and keep some harmful bacteria away from the body. Presently, the yellow pigments, turmeric and tartrazine are approved by the Food and Drug Administration (FDA) for use in foods and beverages. Tartrazine is a stable and water soluble synthetic dye. Turmeric, on the other hand is water insoluble and relatively unstable.

However, turmeric is a natural rather than a synthetic dye. Natural dyes have been tested and was reported that, it is hard to be absorbed and hard to be color on the clothes. So, the type of cyclodextrins has been used in this experiment because of their characteristics as the water soluble dyes in water based systems for natural dye. Cyclodextrin inclusion is a molecular phenomenon in which usually only the guest molecule interacts with the cavity of a cyclodextrin molecule to become entrapped and form a stable association. Molecules or functional groups of molecules those are less hydrophilic than water can be included in the cyclodextrin cavity in the presence of water. So, the molecules of natural dye solution can be fit, at least partly into cyclodextrin cavity and can help the coloring to be part of the cloth.

1.3 Objectives

The objective of this research is to optimize the encapsulation of natural dye by cyclodextrin using response surface methodology (RSM).

1.4 Scopes of Study

To achieve the objectives, scopes has been identified in this research. The scopes of this research are listed as below:

a) To compare the effect of cyclodextrin types in encapsulation of natural dye
b) To study the effect of pH, concentration and temperature on encapsulation of natural dye by cyclodextrin
c) To optimize the concentration, temperature and pH on encapsulation of natural dye by cyclodextrin using response surface methodology (RSM)

1.5 Significance of Study

Potential markets for natural dye by cyclodextrins in future are expected to be huge by researchers, although the current market is not so large and development efforts, exploring various exploring end uses, are currently going on worldwide.

Advantage of the research is that it provides a new, useful and unique product that should have a high market demand. The production of natural dye by cyclodextrins should be improved and developed because of the profitable for the economy. Therefore, findings the way to produce higher amount and quality of natural dye by cyclodextrins and their derivatives is important because of the expected consumption for the future (Lei and Qing, 2002).

It was reported that, the resulting dry of natural dye concentrated maintained its stability after sixteen and one half weeks of storage in dark and having a good color. Natural dye also can be fit and maintain the colour on the cloth in long time because of their characteristic as water soluble dyes in water based systems for natural dye.

CHAPTER 2

LITERATURE RIVIEW

2.1 Natural Dye

Today dyeing is important and popular amongst natural loving and health awarded people. Nearly all dyes is currently produced from synthetic compounds. This means that costs have been greatly reduced and certain applications far enhanced. Customers more aware of environmental issues are now demanding natural products, naturally sourced. If a fashion company introduces a new line of clothes produced with a natural fibre, the naturally sourced dye is needed to complete the green label. Natural dyes can offer not only a rich and varied source of dyes, but also the possibility of an income through sustainable harvest of the dye plants. They also have a far superior aesthetic quality, which is much more pleasing to the eye.

Nature provides a wealth of plants which will yield their colours for the purpose of dyeing. Most of the natural dyes are adjective in nature. They require a suitable fixing agent under dyeing process. There is a lot of possibilities to obtain numerous shades with good fastness properties by using suitable safe mordant. Natural dye is gaining popularity especially in the food, cloth and beverage sectors due to strong demand for more natural products by health-conscious consumers (Rebecca *et al*., 2008). Some examples of natural colorants which have already been used in the textile industry include anthocyanin, lycopene, paprika extract and curcumin.

In addition, natural colours and pigment from fruits and vegetables may contribute additional nutritional value to food coloured as observed in cactus fruits (Mohammer *et al.,* 2005). For this research, turmeric is used as the source of extraction in encapsulation of natural dye by clodextrins in order to get and maintain the color.

2.1.1 Turmeric

The experiment deals with standardization of extraction and dyeing profiles for traditionally known dye from the rhizomes of turmeric (*curcuma longa*). Turmeric (*curcuma longa*) is an importance spice used as a cosmetic and coloring agent has also been used Indigenous System of Medicine. Turmeric is mainly valued for its principle coloring constituent curcumin, which imparts the yellow color on textile fibers and food items. Rhizome, the main source of curcumin also contains various ingredients like protein, fat, fibers, carbohydrates, essential oil and others. Curcumin processes various bioactive properties and is used in modern system of medicine. It is well recognized for its anti-flammatory, hepatoprotective, anticancer, metabolic disorders, antimicrobial, antiviral and antioxidant activities (Herbach *et al.,* 2007).

The yellow pigmented fraction curcumin is isolated from the rhizomes of *curcuma longa* rhizomes are also used for diverse medicinal or food purposes. Turmeric is closely related to ginger and the methods by which ginger oleoresin is obtained may be applied to turmeric, the curcumin and the volatile-oil being both extracted by the same volatile-solvent. Until the late 1970s, turmeric oleoresin was prepared only in a few of the consuming countries, mainly in United States as well in the United Kingdom and manufacturers refuse to disclose their methods of processing. India, however, has now embarked upon commercial production of turmeric oleoresin following the investigation of and the publication of information on processing techniques by the Central Food Technological Research Institute in Mysore (Rohe *et al.,* 2006).

The food colour curcumin (turmeric yellow) is obtained by solvent extraction of turmeric which is the ground rhizomes of *curcuma longa*., with purification of the resultant extract by crystallization. The commercial product consists essentially of curcumins: the colouring principle (1,7-*bis* (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione) and its desmethoxy and bisdesmethoxy derivatives in varying proportions. The total content of colouring matter (*curcuminoids*) in *curcumin* is not less than 90%. The term "*curcumin*" in this monograph refers to the material for which specifications exist. The principal colouring component, 1,7-*bis* (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione, is often referred to as *curcumin* in the literature. To avoid confusion, the researcher decided that this report would not use the term of *curcumin* when referring to this substance. A common synonym for this substance is diferuloylmethane and this name will be used when it is necessary to refer to the principal colouring component of *curcumin* (Benford, 2001).

2.1.2 Dragon Fruit

Fruits of the genus *Hylocereus* (Berger) Briton and rose originated from Latin America and are known as red pitaya belonging to the *Cactaceae* family (Kugler *et al.,* 2007). This vine-like epiphytic cactus is also cultivated in Vietnam, Malaysia, Taiwan, China, Okinawa, Israel and Southern China. Producing a deep purple-coloured flesh comparable to red beet or amaranth, fruits from *Hylocereus polyrhizus* are highly appealing in the European and United States market.

Dragon fruit is one of the new focus for the next source of natural dye because it is rich in betalains which are the similar array of colour pigments found in beetroot. Beetroot has been the most important betalain source for natural red colouring and is mainly composed of the red-purple betanin and the C15-isomer isobetanin. However, there is a demand for alternative compounds because of the unfavourable earthy flavour caused by geosmin and pyrazine derivatives, as well as high nitrate concentrations associated with the formation of carcinogenic nitrosamines (Esquivel *et al.,* 2007).

Hence, fruits from the *Cactaceae* family have been suggested as a promising betalain source being devoid of the mentioned drawbacks. Betalains are nitrogenous vacuolar pigments and important chemotaxonomical markers found in 13 families within the plant kingdom and in some members of the Basidiomycetes (Kugler *et al.,* 2007). Betalains have never been found co-existing with the widely distributed plant pigment anthocyanon which explains their roles as markers.

Some advantages that betalains possess over anthocyanins include being more water soluble, a tinctorial strength up to three times higher than anthocyanins and a wider pH stability range from pH 3 to 7 making it suitable for application in a broad palette of low-acid and neutral food (Diego *et al*., 2008). Betalains are divided into the red-purple betacyanins and yellow-orange betaxanthins which comprise about 55 different structures and promise a great variation of colour array to the food industry. In dragon fruit alone, there are at least seven known betalain namely; betanin, isobetanin, phyllocactin, isophyllocactin, betanidin, isobetanidin and bougainvillein-r-I (Sadilova *et al.,* 2006) all of which have identical absorption spectra (AmaK) that contribute to the deep-purple colour observed in the fruit pulp.

2.2 Cyclodextrin

Cyclodextrin inclusion is a molecular phenomenon in which usually only one guest molecule interacts with the cavity of a cyclodextrin molecule to become entrapped and form a stable association. Molecules or functional groups of molecules those are less hydrophilic than water, can be included in the cyclodextrin cavity in the presence of water (Min *et al*., 2009).

In order to become complex, the "guest molecules" should fit, at least partly, into the cyclodextrin cavity. The cavity sizes as well as possible chemical modifications determine the affinity of cyclodextrins to the various molecules. In the case of some low molecular weight molecules, more than one guest molecule may fit into the cavity. On the opposite, some high molecular weight molecules may bind more than one cyclodextrin molecule (Min *et al*., 2009).

Cyclodextrins, as known today is called cellulosine. The history of the three naturally occurring cyclodextrins a, ß and γ has been identified by Schardinger, (1935). These compounds were therefore referred to as "schardinger sugars". For 25 years, between 1911 and 1935, Pringsheim in Germany was the leading researcher in this area, demonstrating that cyclodextrins formed stable aqueous complexes with many other chemicals. By the mid 1970's, each of the natural cyclodextrins had been structurally and chemically characterized and many more complexes had been studied. Others, usually smaller molecules (called guests) can enter their cavity forming inclusion complexes with these hosts. Since the 1970s, extensive work has been conducted by Szejtli (scientist) in 1970 and others exploring encapsulation by cyclodextrins and their derivatives for industrial and pharmacologic applications (Lajos *et al*., 1998).

Typical cyclodextrins are constituted by 6-8 glucopyranoside units can be topologically represented as toroids with the larger and the smaller openings of the toroid exposing to the solvent secondary and primary hydroxyl group respectively. Because of this arrangement, the interior of the toroids is not hydrophobic but considerably less hydrophilic than the aqueous environment and thus able to host other hydrophobic molecules. In contrast, the exterior is sufficiently hydrophilic to impart cyclodextrins or their complexes water solubility.

The formation of the inclusion compound greatly modifies the physical and chemical properties of the guest molecule, mostly in terms of water solubility. This is the reason why cyclodextrins have attracted much interest in many fields, especially pharmaceutical applications because inclusion compounds of cyclodextrins with hydrophobic molecules are able to penetrate body tissues. These can be used to release biologically active compounds under specific conditions. In most cases the mechanism of controlled degradation of such complexes is based on pH change of water solutions, leading to the cleavage of hydrogen or ionic bonds between the host and the guest molecules. Alternative means for the disruption of the complexes take advantage of heating or action of enzymes able to cleave α-1,4 linkages between glucose monomers (Dodziuk, 2006).

2.2.1 Advantages of Cyclodextrins

Cyclodextrins are able to form host-guest complexes with hydrophobic molecules given the unique nature imparted by their structure. As a result these molecules have found a number of applications in a wide range of fields. Cyclodextrins are widely applied in pharmaceutical formulations to enhance the solubility, stability and biovailability of drug molecule (Lei and Qing, 2002).

Cyclodextrins are able to form inclusion complexes with broad range hydrophobic molecules as poorly soluble drugs, rapidly deteriorating flavours, volatile fragrances, toxic pesticides or dangerous explosives, even gases, entrapping these substances in their inner cavities. For example, α-cyclodextrin forms inclusion complexes with both aliphatic hydrocarbons and gases, such as carbon dioxide. β-cyclodextrin typically forms complexes with small aromatic molecules. γ-cyclodextrin can accept more bulky compounds, including vitamin D2 or organic macro cycles (Chankvetade *et al*., 1993).

Cyclodextrins are used to maintain flavor and color as well as for the dietary supplements. The amino acids and vitamins can be stabilized and protected by cyclodextrins. The cyclodextrins are used in the cosmetics field to solubilize fragrances, to suppress their volatility and to allow perfume-containing to be sprayed as micropowders. The strong ability of complexing fragrances can also be used for another purpose. First dry, solid cyclodextrin microparticles are exposed to a controlled contact with fumes of active compounds, then they are added to fabric or paper products. Such devices are capable of releasing fragrances during ironing or when heated by human body. Such a device commonly used is a typical 'dryer sheet'. The heat from clothes dryer releases the fragrance into the clothing. Cyclodextrins are also used as stabilizers, emulsifiers and deodorants (Dodziuk, 2006).

In industry, cyclodextrin is used to produce the ink including the water soluble dyes in water based systems for ink jet printers, water resistance-fixing characteristics in ink jet printers, water resistance in thermal ink jet printing, aqueous composition or wettability in writing ustencils and greater florescent yield in highlighter pen (Demchuk *et al*., 2004).

Textile products with specific functions have been developed. Cyclodextrins are essential ingredients for the production of such new materials in the textile field. Cyclodextrins are mainly used for keeping moisture in squalance fibers, reducing odors in fibers containing an added antibacterial agent. The ability of cyclodextrins to form complexes with hydrophobic molecules has led to their usage in molecular chemistry (Lei and Qing, 2002).

2.3 Ultraviolet-Visible Spectroscopy

Application of ultraviolet-visible spectroscopy is routinely used in the quantitative determination of solutions of transition metal ions and highly conjugated organic compounds.

Solutions of transition metal ions can be colored (for example, absorb visible light) because the electrons within the metal atoms can be excited from one electronic state to another. The color of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulfate is a very light blue, adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λmax) (Kolbesen and Doll, 2005).

Organic compounds, especially those with a high degree of conjugation, also absorb light in the ultra violet or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water soluble compounds, or ethanol for organic-soluble compounds. Organic solvents may have significant ultra violet absorption, not all solvents are suitable for use in ultra violet spectroscopy. Ethanol absorbs very weakly at most wavelengths. Solvent polarity and pH can affect the absorption spectrum of an organic compound. Tyrosine, for example, increases in absorption maxima and molar extinction coefficient when pH increases from 6 to 13 or when solvent polarity decreases. While charge transfer complexes also give rise to colours, the colours are often too intense to be used for quantitative measurement (Kolbesen and Doll, 2005).

The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, ultra-violet spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from molar extinction coefficients or more accurately, determined from a calibration curve. Figure 1 below show the prism of ultra-violet spectroscopy.

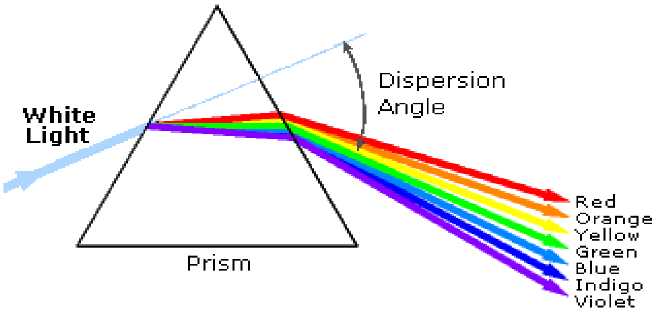

Figure 2.1 : Prism of ultra-violet spectroscopy

Employment of ultra-violet spectroscopy in encapsulation natural dye by cyclodextrins (CDs) production has been reported by Min *et. al*., (2009). From their findings, the formation of complex of natural dye with the types of cyclodextrin in aqueous solution was characterized by ultra-violet spectroscopy. The absorption spectra of natural dye in the absence of cyclodextrin are showed through the absorption peaks. The absorption increasing with the increasing of concentration of cyclodextrin (Min *et al*., 2009).

2.4 Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. The most extensive applications of RSM are in the particular situations where several input variables potentially influence some performance measure or quality characteristic of the process. Thus performance measure or quality characteristic is called the response. The input variables are sometimes called independent variables and they are subject to the control of the scientist or engineer. The field of response surface methodology consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables or optimization methods for finding the values of the process variables that produce desirable values of the response (Kathleen *et al*., 2004).

Response Surface Methodology (RSM) has been extensively applied to optimize culture medium and other process parameters for the production of lipase (Helen *et al*., 2010; Chien *et al*., 2006 ; He and Tan, 2006), tannase (Manjit *et al*., 2010), α-amylase (Shaktimay *et al*., 2009), β-cyclodextringlucanotransferase (Ibrahim *et al*. 2005), dextran dextrinase (Naessens *et al*., 2004), chitinase (Nawani and Kapadnis, 2005) and cyclodextrin glucanotransferase from alkalophilic *Bacillus sp*.(Salwanis *et al*., 2007). In their findings, the production has been increased as compared to the production before the optimization after using the response surface methodology (RSM).

Chen *et al*. (1992) studied the optimization of β- galactosidase production using a statistical and mathematical approach which also known as response surface methodology. This research used a central composite design to optimize the medium composition for lactase production by *Kluyveromyces marxianus* and found the optimal medium composition as 80.6 g/L lactose, 107.7 g/L corn steep liquor, 4.1 g/L glucose and 9.6 g/L glycerol. Becerra and Siso, (1996) used a full-factorial design to optimize β-galactosidase production by *Kluyveromyces lactis* in solid-state fermentations on corn grits or wheat bran moistened with deproteinized milk whey. In the research, the production was increased and parameter of temperature and pH was successfully optimized and improved in term of production and energy consumption by using Response Surface Methodology (RSM).

2.5 Encapsulation of Natural Dye

The dyeing step of the process of the invention preferably comprises the addition of the dye, as a liquid or a solution, to a stirred mixture of the treated or untreated microbes in an aqueous liquid and separating the dyed microbes from the resulting mixture. The aqueous liquid is preferably water but it may contain organic solvents such as lower alcohols (ethanol) and its pH may be adjusted, depending on the nature of the dye, to optimize the dyeing of the microbes. The encapsulated substance may be released from the microbial capsules when desired by, for instance, chemical biodegradation or mechanical rupture of the microbial cell wall or by subjecting the capsules to an environment in which the substance diffuses gradually out through the pores in the microbial cell wall (Yuliani *et al.,* 2006).

The product of the invention allows the substance to be retained within the microbial capsules, identifiable by their colour until its release by biodegradation, rupture or diffusion. The product of the encapsulation may be employed in any application for which known microcapsules are conventionally used either as a free-standing product or adhered to a substrate.

Suitable applications include for example, use in foodstuffs to give a delayed release of flavour when the foodstuff is eaten, striped toothpaste to immobilise a given flavour in a coloured stripe, confectionery, personal hygiene products, the advertising of perfumes by providing small samples of microcapsules adhered to paper, copying paper and tamper-evident applications where a colour or odour produced on rupturing the product indicates tampering (Hobson *et al.,* 1996).

Cyclodextrins (CDs) are interesting microvessels capable of embedding appropriately sized molecules and the resulting supramolecules can serve as excellent miniature models for enzyme-substrate complexes. Owing to their great usefulness in the areas of synthetic, analytical and pharmaceutical chemistry a number of studies have been undertaken to understand the nature of the complexes of

CDs. The cyclodextrin molecules have an internal cavity accessible to guest molecules of proper dimension through an opening 0f 4.5, 7.0, 8.5 Å for α-CD, β-CD and γ-CD respectively. The depths of all the CDs are more or less the same (7.9 Å). Thus, depending on the cavity size, CDs are capable of encapsulating different guest of molecules. The reduced polarity and the restricted space provided by the CD cavity markedly influence a number of photophysical or photochemical processes (Arabinda *et al.*, 2005).

Cyclodextrins (CDs) are chemically and mechanically stable cyclic oligosaccharides consisting of glucopyranose units. These oligosaccharides have cone or doughnut shaped structures with monomolecular hosts in a supramolecular chemical with a hydrophilic surface and a hydrophobic cavity space. Similar to cellulose or chemically modified cellulose, hydroxyl or alkylhydroxyl functional groups glucopyranose units are capable of reacting with other chemicals through various chemical reaction processes to alter properties such as solubility, chemical reactivity and the color of the compound (Jozsef, 2003).

Molecules with adequate size, shape and compatible chemical functionality can be held, hosted or encapsulated within the cavity. Forces creating such encapsulation may be electrostatic, van der waals, hydrophobic interactions or hydrogen bonding. The molecules, generally referred to as "inclusion compound", held within the cavity may be released under appropriate conditions such as temperature gradient or pressure change, depending on the specific encapsulation and the nature of the chemicals that are encapsulated. Due to the hydrophilic property of the surface structure, most cyclodextrins (CD) have a relatively high solubility in water carrier (Ming, 2005).

The encapsulation process which introduces disperses or sublimation dyes into the hosting cyclodextrins (CD) cavities can be carried out in either a solvent system or aqueous system with cosolvent so that a certain solubility of the dyes can be achieved prior to the encapsulation into the hosting structure. For example, a mixture of cyclohexane-pyrrolidinone (CPY) and dimethylformalmide (DMF) may be used for the process, followed by separating the completed reactive dyed and sublimation dye encapsulated cyclodextrins (CD) from the solvent mixture. Direct sublimation processes may also be used, but caution must be taken in order to allow sufficient time that a controlled amount of disperse or sublimation dyes can be hosted to completion. Pressurized reactors may be used to maintain the sublimation dye gas concentration for a period of time (Francisco *et al*., 2006).

The extra amount of unhosted dyes may be removed through various washing techniques. The encapsulation process of the present invention is useful for creating unique colors of the ink and the image on the final substrate by adjusting ratios between the reactive dyes bonded on the cyclodextrin and the hosted disperse or sublimation dyes. Expanded color or shade can be achieved, whereas they may not be obtained by either type of colorant alone. Furthermore, other inclusion compounds or colorant may also be encapsulated through similar processes for the purpose of enhancing the functionality of the final substrate with a printed image. For example, the interactions of six solvatochromic pyridiniophenolate dyes with α- and β-cyclodextrins were investigated with the aid of UV–vis and molecular dynamics simulations. The deduced mode of encapsulation of these dyes within the hydrophobic host cavity was employed as a measure of the relative contributions of the donor and acceptor moieties to their solvatochromic properties (Moses *et al.,* 2000).

2.6 Factor Affecting the Encapsulation of Natural Dye

Physical conditions during encapsulation of natural dye are very important. All parameters involved must be monitor closely to ensure the encapsulate of natural dye by cyclodextrin develop well and can produce maximum concentration of interest. There are several factors that affecting the encapsulation of natural dye by cyclodextrin.

2.6.1 Effect of Cyclodextrin's Types

Generally there are three different types of cyclodextrin (CD) compounds : α, β and γ –cyclodextrins (CD). Each has a different cavity size and therefore is only suitable for certain molecules through a size exclusion process. Furthermore, cyclodextrins (CD) are also categorized as either non-branched or branched cyclodextrin (CD), where one or more molecules of one or more oligosaccharides have been bonded with an extra glucose or similar unit with improved properties such as water solubility. Many different chemical functional groups or moieties can be introduced into the cyclodextrin (CD) structure through chemical reactions between the hydroxyl/alkylhydroxyl groups and other functional groups such as alkyl, amine, hydroxylalkyl, acetyl and the like. These modified cyclodextrin (CD) compounds possess different chemical and physical properties compared with the untreated cyclodextrins and generally are referred to as chemically modified cyclodextrin (CMCD).

All these cyclodextrins may be used either singly or in combination in the present invention and the term cyclodextrin (CD) is used to include all these variations in the following discussions. Solubilization of these dyes can also be achieved by introducing these dyes into cyclodextrin (CD) cavities with encapsulation processes and can be used in combination with reactive dyes. A subsequent heat activation may sublimate/heat-activated the dye onto synthetic materials such as polyester compound and/or bond to a compound with hydroxyl or other active hydrogen groups to form a covalent bond (Bharat *et al*., 2006).

The differential fluorimetric behavior of aqueous in different cyclodextrin environments has been rationalized from the variation of the relative dimensions of the probe and the cyclodextrin cavities. For example, in encapsulation of norharmane in cyclodextrin which formation of 1:1 and 1:2 inclusion complex, β-cyclodextrin was the best because having the appropriate cavity size for activating the complexation process and was formed resulting in an enhancement of the cationic fluorescence of norharmane. While the cavity size of α-cyclodextrin is insufficient for the formation of an inclusion complex with the molecular system and γ-cyclodextrin, in spite of having sufficient space for encapsulating the probe into it. It does not modify the absorption and fluorescence spectral behaviors of the fluorophore (Arabinda *et al.,* 2005).

2.6 2 Effect of pH

pH is an important parameter in the production of encapsulation of natural dye by cyclodextrin. It is noted that the formation constants are very sensitive to the changes of pH values. The binding constants for inclusion complexation interaction of natural dye-cyclodextrin with different pH values is in the order of pH 3 > pH 6.5 > pH 10.5. One of the major factors affecting the inclusion interaction is in the hydrophobic degree of the guest, which is related to the form of acidic. Acidic has four forms which they are three charged forms and one neutral. There existed following equilibrium in aqueous solution. In pH 2.0 to 3.5, the neutral form of acidic is predominant, while pH 5.5 to 7.4, the form is predominant gradually and pH more than 12.5, the form is predominant.

For example, in preparation and spectral investigation of inclusion complex of caffeic acid with hydroxypropyl-β-cyclodextrin, it was found that pH at acidic was the best to encapsulate with β-cyclodextrin. The normal of natural dye-cylodextrin are not charged (2<pH<11) and the major inclusion interactions are hydrophobic interactions between the guest and the cyclodextrin cavity and hydrogen bonding of the guest to –OH groups or other introduced groups on the CD rings. In acidic media, the neutral (uncharged) form of acidic is predominant, which is more hydrophobic than other forms, it is more easily to form the inclusion with natural dye-cyclodextrin (Min *et al*., 2009).

2.6.3 Effect of Concentration

Concentration measurements were based on the phase-solubility technique established by Higuchi and Connors (1965). For that purpose, aqueous solutions of cyclodextrins with different increasing concentrations were prepared, excess amount of natural dye were added to each solution of cyclodextrins, the solution were reacted completely by ultrasonic for 1 hour and equilibrated for 24 hours, centrifuged and filtered. Their absorption was measured by UV spectrophotometer (315nm). The phase-solubility profile was therefore obtained by plotting the absorption versus concentration of cyclodextrin types. For example, in research of β-cyclodextrin inclusion complex of *curcumin*, the fluorescence intensity was found to increase with increasing concentration of β-cyclodextrin. The higher value of concentration indicates that there are more than one kinetic steps involved in complex formation (Swaroop *et al*., 2006).

2.6.4 Effect of Temperature

The temperature does not only affect the production on encapsulation of natural dye by cyclodextrin, but it can also have a marked effect of extraction and dyeing conditions for traditional turmeric dye. For example, the observation of dyeing trials under various conditions conclude that 2% dye-bath maintained at 50-55 ºC and it was the optimize condition. The result was observed due to the dark and light color of natural dye. In the research of β-cyclodextrin inclusion complex of *curcumin*, the concentration was found to decrease with increasing temperature (Kiran and Kapoor, 2007).

CHAPTER 3

METHODOLOGY

3.1 The Overall Methodology

This chapter will elaborate on the materials and methods that have been applied in this experiment. The overall methodology involves all the steps in achieving encapsulation of natural dye by cyclodextrin. This method is divided into 4 step : Firstly, preparation of natural dye-clodextrin, second is preparation of standard curve, third is effect of cyclodextrin types, fourth is effect of pH, concentration, temperature and the fifth is optimization of these parameters using Response Surface Methodology (RSM). The first step of the methodolody was to encapsulate the process of natural dye-cyclodextrin. The second step was to obtain the standard curve of cyclodextrin concentration. The third step of experiment was to obtain the best types of cyclodextrin to be used for the next of experiment. The fourth step of the experiment was carried out to obtain the best range of cyclodextrin types, the best range of pH, concentration and temperature. Interaction of all parameters was analyzed at this step. One factor at a time method (OFAT) was applied at this step. For the fifth step of this experiment, Response Surface Methodology (RSM) was used to further optimize using the data gained from the fourth step of experiment. Interaction of all parameters was analyzed at this step. Figure 3.1 shows the flow diagram that simplified the strategies of this research.

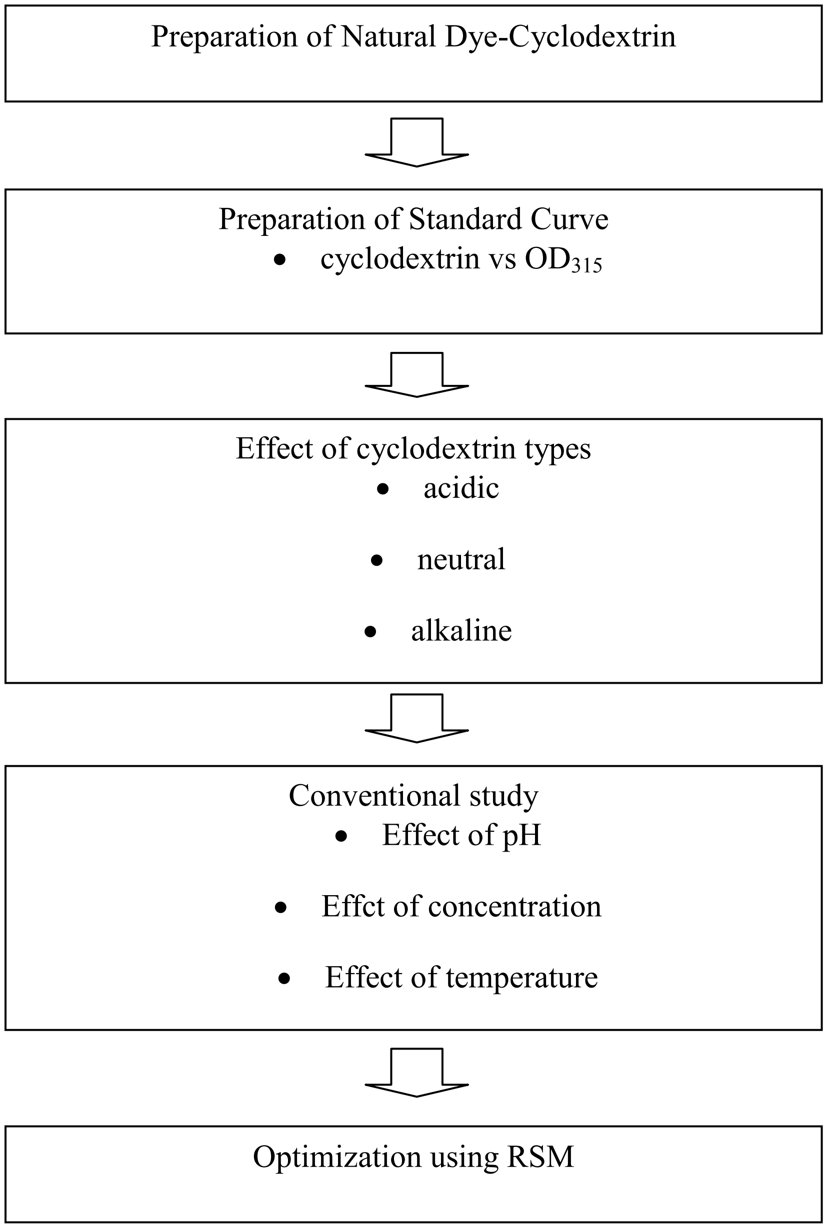

Figure 3.1: Research step for optimization on encapsulation of natural dye by cyclodextrin

3.2 Chemicals Involved

All chemicals used in this research were α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin was bought from Dinschem Technologies Sdn.Bhd while 0.10 M citric acid, 0.10 M natrium hydroxide, 0.10 M potassium phosphate monobasic (KH_2PO_4) and 0.20 M sodium hydroxide used in this research was provided by Laboratory of Faculty of Chemical Engineering and Natural Resources (FKKSA), UMP.

3.3 Extraction of Turmeric

Extraction of natural dyes has been done by using turmeric as the source of natural dye. The raw material was ground to small particles about 1 mm. The natural dyes then extracted with blender by applying ratio 1:20, corresponding to a ratio of 1g of raw material to 20 mL of water.

3.4 Filtration/Separation

The insoluble residue in natural dye was then separated by using filter paper to separate the natural dyes from their fibre. The filtration was used as pre-treatment for the process before the natural dyes can be used for further synthesis.

3.5 Preparation of Natural Dye-Cyclodextrin

The experiment was started by prepared the natural dye-cyclodextrin to study the process of encapsulation on natural dye-cyclodextrin. The stock solution of $1.0x10^{-3}$ mol/L of natural dye was prepared by dissolving it in water and diluted by water. Phosphate buffer solution (0.2 mol/L) was used to control the pH-value of the media. All other reagents were of analytical-reagent grade and were used without purification. Doubling distilled water was used throughout. All the experiments were carried out at room temperature. 1 ml aliquot of the stock solution (1.0×10^{-2} mol/L) of natural dye was transferred into 10 ml volumetric flask and 1 mL of α-cyclodextrin (1.0×10^{-2} mol/L) was added.

The pH was controlled by 0.2 mol/L phosphate buffer solution. An appropriate amount of citric acid and natrium hydroxide is added to control the desired pH. The mixed solution was diluted to final volume with distilled water and shaken thoroughly, followed by ultrasonic for 30 min at 20 ^{o}C. The solution is heat on the heater using thermometer to control the desired temperature. All the measurements of absorption and fluorescence were made against the blank solution treated in the same way using natural dye with α-cyclodextrin. The procedure of experiment was repeated using the different types of cyclodextrin (β-cyclodextrin and γ-cyclodextrin).

3.6 Determination of The Best Cyclodextrin Types on Encapsulation of Natural Dye by Cyclodextrin

Determination of natural dye-cyclodextrin stability in term of cyclodextrin types is important to use for the further steps in experiment. Determination of encapsulation reaction was done during the binding of natural dye with cyclodextrin cavity. During this study, natural dye reacted with different types of cyclodextrin which the pale colour and light colour of natural dye had been seen. Manipulation of all types of cyclodextrin during the reaction is neccessary to determine the best conditions for natural dye-cyclodextrin reaction. The three types of cyclodextrin involed were α, β and γ-cyclodextrin had been tested to obtain the best types of cyclodextrin on encapsulation of natural dye by cyclodextrin.

3.7 Selection of The Best Range of Parameters Using Conventional Method

Natural dye-cyclodextrin was encapsulated at different pH, concentration of cyclodextrin and temperature. One factor at a time method (OFAT) was applied for this step. To obtain the best range of pH, the rate must be varied while the concentration of cyclodextrin and temperature must be set constant. The experiment was repeated by tested on the parameter of concentration and temperature with the varied value. The maximum concentration of encapsulation of natural dye by cyclodextrin was selected for each of all the parameters.

The value of pH was carried out at 2, 2.5, 3 and 3.5. During this step, the concentration of cyclodextrin and temperature were set constant at 0.003 mol/L and 55 °C respectively. The best pH was determined by plotting the graph of concentration versus pH.

Second step was to determine the best concentration of cyclodextrin. The concentration of cyclodextrin was tested at 0.001 mol/L, 0.002 mol/L, 0.003 mol/L, 0.004 mol/L and 0.005 mol/L. The pH and temperature were set constant at pH 3 and 55°C respectively. The best concentration was determined by plotting the graph concentration of cyclodextrin versus concentration of natural dye-cyclodextrin.

To determine the best range of parameter, it were manipulated using 25 ºC, 35 ºC, 45 ºC, 50 ºC, 55 ºC, 60 ºC, 65 ºC and 70 ºC. The pH and concentration were set constant at pH 3 and 0.003 mol/L respectively. The best range parameters were determined for further used in optimization process.

3.8 Optimization of pH, Concentration and Temperature on Encapsulation of Natural Dye by Cyclodextrin Using Response Surface Methodology (RSM)

Based on the previous result of one at a time method, the best range of pH, concentration of cyclodextrin and temperature were used for further study using Response Surface Methodology (RSM). For optimization of parameters, the design was made up of a full 2^3 factorial with a total of 17 experiments using Central Composite Design (CCD). Among of the 17 experiments, there are 14 star point and 3 replicates at the centre points. The value of alpha was set at 1.68179. The value of alpha determines the location of the star points in a central composite design. Table 3.1 shows the initial data used in RSM.

Table 3.1: Initial data used in RSM

Factors	**Symbol**	**Units**	**Low Level**	**High Level**
pH	A		2	4
Concentration	B	mg/L	0.002	0.004
Temperature	C	^{o}C	50	60

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Fluorescence Study

The maximum excitation and emission wavelength can be observed from the absorption peaks. The fluorescence intensity was enhanced and the emission wavelengths have blue shifts with increasing concentration of cyclodextrin types. These data suggested that stable complexes were formed between cyclodextrins and natural dye. The cyclodextrins cavity provided a polar environment for natural dye molecule and thus increased the quantum yield of the fluorescence of natural dye (Min *et al.,* 2009).

4.2 Effect of Cyclodextrin Types and pH on Encapsulation of Natural Dye Using Conventional Method

Studies on the effect of cyclodextrin types and pH on encapsulation of natural dye were carried out by using the conventional method. This study was done to select the best types of cyclodextrin and the best range of pH for further optimization process. The types of cyclodextrin used were α, β and γ-cyclodextrin and the pH used were at pH 3, 7 and 10.5.

4.2.1 Effect of Cyclodextrin Types on Encapsulation of Natural Dye

The effect of cyclodextrin types on encapsulation of natural dye by cyclodextrin was determined by study the each of cylodextrin types and the data obtained from the experiment is shown in Figure 4.1. The types of cyclodextrin used were α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin.

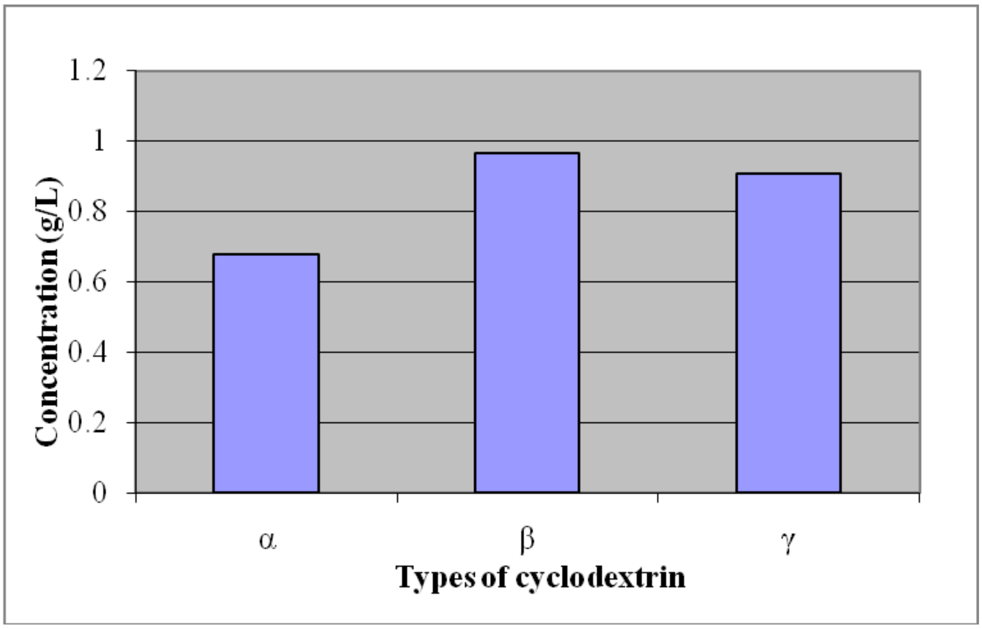

Figure 4.1: Effect of cyclodextrin types on encapsulation of natural dye by cyclodextrin

From the figure, the highest concentration was obtained at β-cyclodextrin with 0.966 g/L while α-cyclodextrin produced 0.681 g/L and γ-cyclodextrin produced 0.9073 g/L. Therefore, β-cyclodextrin has been used for the next study.

In encapsulation of norharmane in cyclodextrin, Arabinda *et al.* (2005) also reported that encapsulation of norharmane in cyclodextrin was produced using β-cyclodextrin. It was reported that the cavity size of α-cyclodextrin is insufficient for the formation of an inclusion complex with the molecular system. β-cyclodextrin has the appropriate cavity size for activating the complexation process. This leads to the remarkable modification in the fluorometric behavior of the fluorophore. However, γ-cyclodextrin, in spite of having sufficient space for encapsulating the probe into it, does not modify the adsorption and fluorescence spectral behaviors of the fluorophore. This suggests the inclusion of some water molecules within the cyclodextrin cavity along with the probe molecule.

4.2.2 Effect of pH on Encapsulation of Natural Dye

The variables of pH for acid, neutral and base has been used with value 3, 7 and 10.5 respectively while temperature, concentration and types of cyclodextrin were kept constant at 55 ºC, 0.003 mol/L and β-cyclodextrin respectively. The data obtained from the experiment is shown in Figure 4.2.

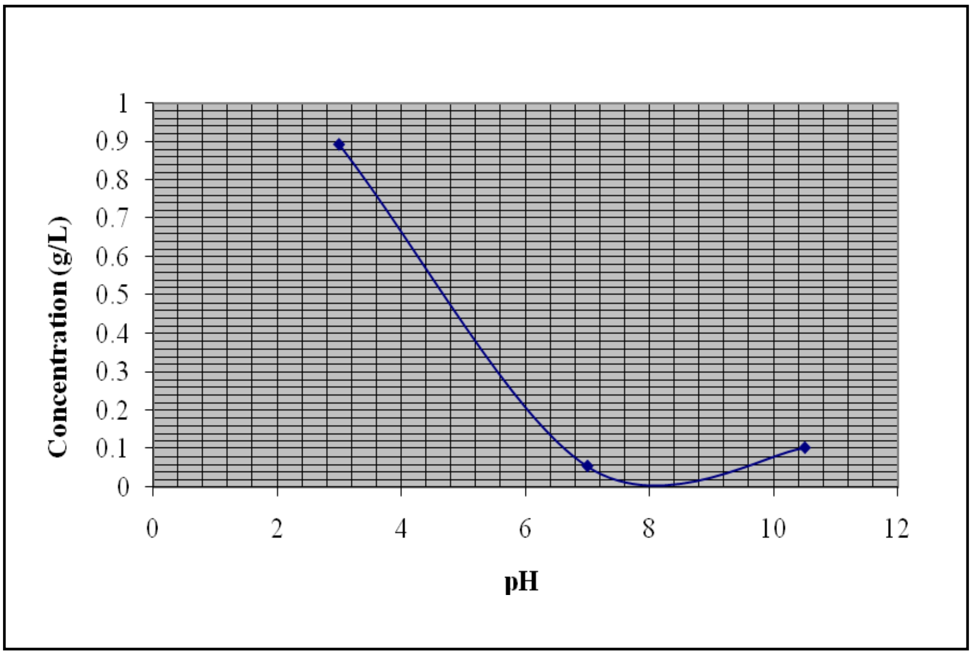

Figure 4.2: Effect of pH on encapsulation of natural dye-β-cyclodextrin

From Figure 4.2, it is noted that the interactions are very sensitive to the change of pH values. The highest concentration was obtained at pH 3 which value 0.893 g/L. One of the major factor affecting the inclusion interaction is the hydrophobic degree of the guest, which is related to the form of acid. Acidic has four forms: three charged forms and one neutral form. This fact also was supported by previous researcher which the pH of 3 has been used for preparation and spectral investigation of inclusion complex of caffeic acid with hydroxypropyl-β-cyclodextrin by previous researcher (Min *et al*., 2009).

This study also reported that, in pH 2-3.5, the neutral form of acid is predominant, while pH 5.5-7.4, the charged form also predominant, while pH more than 8.5, the charged form is predominant gradually. In acidic media, the neutral (uncharged) form of acid is predominant, which is more hydrophobic than other forms, so it is more easily to form the inclusion with natural dye-β-cyclodextrin. The acidic condition was selected for the next experiment and the range of acid at 2, 2.5, 3, 3.5 and 4 has been focused to obtain the optimum pH.

4.3 Screening of The Best Range of pH, Concentration and Temperature on Encapsulation of Natural Dye-β-Cyclodextrin Using Conventional Method

Studies on the parameters that influenced the interaction of natural dye-β-cyclodextrin were carried out by using the conventional method. The method used was the one factor at a time. This study was done to determine the best range for all parameters for further optimization process. Basically, the low and high levels or the best range of every parameter were obtained before and after peak values respectively in the appropriate study and this value will be used in optimization process. The parameters involved in this study were pH, concentration and temperature.

4.3.1 The Effect of pH on Encapsulation of Natural Dye-β-Cyclodextrin

The effect of pH was study to determine the best range of pH using a constant concentration of cyclodextrin at 0.003 mol/L and temperature at 55^{o}C on encapsulation of natural dye-β-cyclodextrin. The pH was manipulated at pH 2, 2.5, 3, 3.5 and 4. The result from the experiment was shown in Figure 4.3.

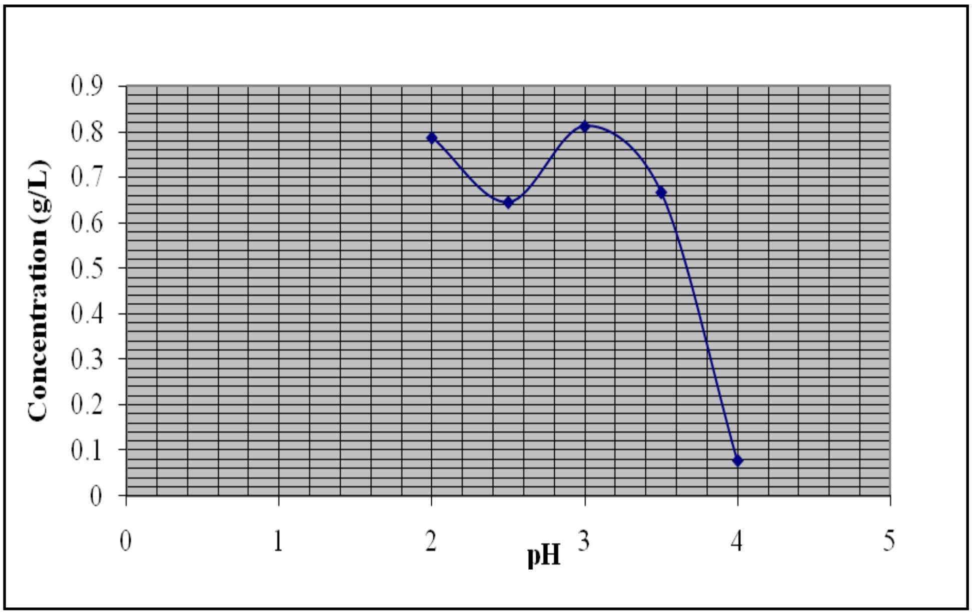

Figure 4.3: Effect of pH on encapsulation of natural dye-β-cyclodextrin

From Figure 4.3, the highest concentration has achieved at pH 3 with 0.812 g/L. The lowest concentration was obtained at pH 4 with 0.077 g/L. The concentration was decreased while pH was increased. The result shows the best range of pH between 2.5 until 3.5 and this range will be used for further study using Response Surface Methodology.

In medium optimization for polysaccharide production of *cordyceps sinensis*, Chienyan *et al.,* (2007) was reported that when pH was controlled at a higher level such as pH 5.0, cell growth and polysaccharide production were inhibited. This study was reported that a low pH of 2.85 was required for maximum production of polysaccharides.

It also was reported by several researcher (Lajos *et al*., 1998 ; Lying and Passos, 2005) which pH 3 is an optimal pH for stabilization and solubilization of natural colorants with cyclodextrins. It was noted that the solubilization of natural colorants and stabilization of encapsulation became pure and most effectively achieved at pH 3 value. The best value of pH obtained will be used for the next experiment in order to study the effect of concentration on the encapsulation of natural dye-β-cyclodextrin.

4.3.2 The Effect of Concentration on Encapsulation of Natural Dye-β-Cyclodextrin

The effect of concentration was studied to determine the best range of cyclodextrin concentrations while pH and temperature were kept constant at pH 3 and 55ºC respectively to use for the next step of experiment. The concentration of cyclodextrin was manipulated at 0.001 mol/L, 0.002 mol/L, 0.003 mol/L, 0.004 mol/L and 0.005 mol/L. The data obtained from the experiment is shown in Figure 4.4.

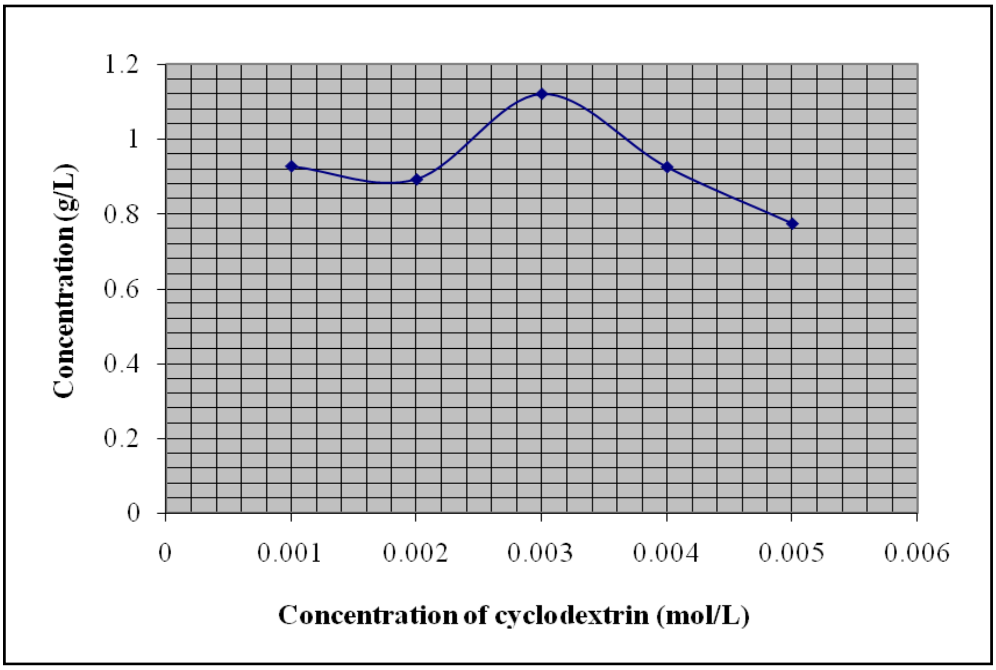

Figure 4.4: Effect of cyclodextrin concentrations on encapsulation of natural dye-β-cyclodextrin.

From Figure 4.4, the highest concentration of cyclodextrin can be achieved at 0.003 mol/L with concentration of 1.1214 g/L. The concentration of cyclodextrin was increased proportionally with the increment of concentration on encapsulation of natural dye-β-cyclodextrin. The lowest concentration was obtained at 0.005 mol/L with concentration of 0.775 g/L. The best range from the graph is at range 0.002 mol/L until 0.004 mol/L and has been used to the further study in Response Surface Methodology.

This fact also was suggested by Singhal *et al*., (2009) due to the enhance production of laccase by *Cryptococcus albidus* and its application for bioremediation of chemicals. In this study, the production of laccase was inhibited with increasing copper sulphate (CuS) concentration from 0.002 mol/L to 0.008 mol/L. It was stated that The production of laccase was enhanced seven times by optimizing the growth media using small concentration of 0.002 mol/L copper sulphate. In investigation on cyclodextrin production with cyclodextrin glucanotransferase from *bacillus* *megaterium,* Boriana *et al.,* (2008) was reported that the high β-cyclodextrin amount inhibit cyclodextrin glucanotransferase.

From the figure, concentration was increased at initial and start decrease after reach at equilibrium. This study was supported by Swaroop *et al*. (2007) in β-cyclodextrin inclusion complex of *curcumin*, which solubility of natural dye increased as the concentrations of β-cyclodextrin increased and the linear concentration of cyclodextrin inhibit the solubility of natural dye as well.

4.3.3 The Effect of Temperature on Encapsulation of Natural Dye-β-Cyclodextrin

The effect of temperature was studied to determine the best range of temperature on natural dye-β-cyclodextrin. The temperature was manipulated at 25, 35, 45, 50, 55, 60, 65 and 70^{o}C while the pH and concentration of cyclodextrin was kept constant at pH 3 and 0.003 mol/L respectively. The data obtained from the experiment is shown in Figure 4.5.

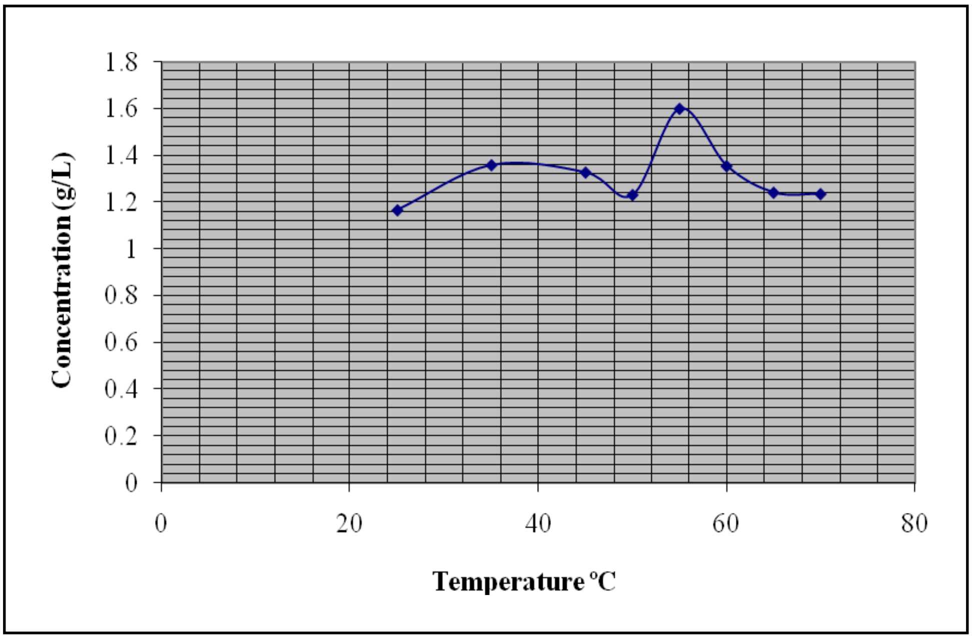

Figure 4.5: Effect of temperature on encapsulation of natural dye-β-cyclodextrin

From the graph, the highest concentration was obtained at 55°C with 1.6 g/L of concentration. The lowest concentration was 1.1645 g/L at 25°C of temperature. The result shows the best range of temperature between 50^{o}C until 60^{o}C and it was found to be the best range of temperature. This range was selected to be used in further studies using response surface methodology (RSM).

This study also was supported by Ralf *et al.*, (2009) in the development and optimization of low temperature enzyme-assisted liquefaction for the production of colouring foodstuff from purple pitaya (*Hylocereus sp*. [Weber] Britton & Rose). It was stated that conducting an enzyme screening, the most suitable enzyme preparation for the liquefaction of pitaya pulp at low temperatures was selected for the production of colouring foodstuff from purple pitaya.

In isolation of cyclodextrin producing thermotolerant *Paenibacillus sp*. from waste of starch factory, Porntida *et al*., (2006) also suggested that 50-55°C was the best range for dextrinizing activity. It was noted that most cyclodextrin glycosyltransferase producing bacteria are mesophilic and their enzymes mostly function at moderate temperature. Besides, this fact also was supported by Kiran and Kapoor (2007) which dyeing at 50-55 °C was most appropriate temperature to obtain a good dyeing results for encapsulated with cyclodextrin.

As conclusion, the best range of pH, concentration of cyclodextrin and temperature on encapsulation of natural dye-β-cyclodextrin is pH 2-4, 0.002-0.004 mol/L and 50-60^{o}C respectively. Those values will be used to be further study in Response Surface Methodology (RSM).

4.4 Analysis of the Optimum pH, Concentration and Temperature on Encapsulation of Natural Dye by Cyclodextrin Using Response Surface Methodology

Optimization of pH, concentration and temperature on the encapsulation of natural dye was carried out using response surface methodology (RSM). The parameters involved were pH, concentration and temperature. By using the Central Composite Design (CCD), the experiment with different combination of pH, concentration and temperature were performed. The combination of data was arranged using Design Expert Software was listed in Table 4.1.

Table 4.1: Optimization of pH, concentration and temperature on encapsulation of natural dye-β-cyclodextrin.

Standard	A : pH	B : Concentration (mol/L)	C : Temperature (oC)	Concentration (g/L)	Actual Value	Predicted Value
1	2.0	0.002	50	0.9726	0.9726	0.9750
2	4.0	0.002	50	1.0574	1.0574	1.0272
3	2.0	0.004	50	1.0970	1.0970	1.0587
4	4.0	0.004	50	1.0900	1.0900	1.1054
5	2.0	0.002	60	0.6920	0.6920	0.6713
6	4.0	0.002	60	0.7960	0.7960	0.8291
7	2.0	0.004	60	1.0431	1.0431	1.0680
8	4.0	0.004	60	1.2280	1.2280	1.2203
9	1.3	0.003	55	0.4260	0.4260	0.4423
10	4.7	0.003	55	0.6230	0.6230	0.6142
11	3.0	0.001	55	0.9530	0.9530	0.9596
12	3.0	0.005	55	1.3580	1.3580	1.3589
13	3.0	0.003	47	1.3264	1.3264	1.3540
14	3.0	0.003	63	1.2154	1.2154	1.1953
15	3.0	0.003	55	0.8734	0.8734	0.8721
16	3.0	0.003	55	0.9125	0.9125	0.8721
17	3.0	0.003	55	0.8316	0.8316	0.8721

The regression equation (Equation 4.1) was obtained from the analysis of variance and all term regardless of their significance are included in the following equation.

$$1/Y = +0.87 + 0.051\,A + 0.12\,B - 0.047\,C - 0.12\,A^2 + 0.10\,B^2 + 0.14\,C^2 - 1.362 \times 10^{-3}\,A.B + 0.026\,A.C + 0.078\,B.C$$ **(Equation 4.1)**

Y is the predicted response, A is coded value for pH, B is coded value for concentration of cyclodextrin and C is coded value for temperature. The predicted levels of natural dye- β-cyclodextrin and effect of pH, concentration of cyclodextrin and temperature at each experimental point using Equation were given in Table 4.1 along with the experimental result. The highest production of encapsulation natural dye-β-cyclodextrin which produced 1.358 g/L of concentration was read on Standard no. 12. The parameters of Standard no. 12 were pH 3, 0.005 mol/L of concentration and 55 ºC of temperature. The lowest concentration was recorded at Standard no. 9 with 0.426 g/L of concentration. The parameters for Standard no. 9 were pH 1.3, 0.003 mol/L concentration of cyclodextrin and 55 °C of temperature.

The coefficient values of Equation 4.1 calculated using Design Expert Software and *P*-value of every term and the interaction were listed in Table 4.2. Based on Table 4.2, the linear term of pH (A), squared terms of pH (A^2), concentration (B) and squared terms of concentration (B^2), temperature (C) and squared terms of temperature (C^2) were significant model terms that influence the encapsulation of natural dye by cyclodextrin due to the *P*-value less than 0.05. The interaction term of AB and AC seem to be insignificant and BC was significant model.

Table 4.2: Regression coefficients and *P*-value calculated from the model

Factor	**Coefficient**	**Prob > F**
Intercept	0.85	< 0.0001
A- pH	0.039	0.0014
B- Concentration	0.091	< 0.0001
C- Temperature	-0.036	0.0022
A^2	-0.093	< 0.0001
B^2	0.078	< 0.0001
C^2	0.11	< 0.0001
AB	-1.00E-03	0.9234
AC	0.02	0.0835
BC	0.06	0.0006

Values of *P*-value less than 0.0500 indicate model terms are significant

Table 4.3: Regression analysis

R-Squared	0.99038
Adj R-Squared	0.97802
Pred R-Squared	0.93854
Adeq Precision	32.1818

Table 4.2, 4.3 and 4.4 shows the ANOVA and regression for the encapsulation of natural dye-β-cyclodextrin. Determination of coefficient (R^2) and correlation coefficient (R) indicates the precision of a model. Determination coefficient (R^2) implies that the independent variables tested were attributed by the sample variation of 99.04% for natural dye-β-cyclodextrin production. The R^2 value also indicates that only 6.49% of the total variation was not explained by the model. Normally, a regression model having an R^2 value higher than 0.9 is considered to have a very high correlation (Haaland, 1989). A better correlation between the

experimental and predicted values indicated by the value of *R* (correlation coefficient) which closer to 1. From R^2 value, the value of *R* (0.9385) for Equation 4.1 indicates a close agreement between the experimental results and the theoretical values predicted by the model equation. According to the software evaluation, the adjusted R^2 (coefficient of determination) was calculated to be 97.80%. The value indicates a good agreement existed between the experimental and predicted values of concentration.

When significance of the regression model was tested, it was found that *P*-values obtained were small than 0.0001 (Table 4.4) compared to a desired significance level which is 0.05. It is indicates that the regression model was accurate in predicting the pattern of significance to the production of encapsulation of natural dye-β-cyclodextrin.

Table 4.4: ANOVA for response surface quadratic model for the production of encapsulation of natural dye-β-cyclodextrin

Source	**Sum of Squares**	**Degree of freedom**	**Mean**	**F Value**	**p-value Prob > F**	**R^2**
Model	0.99	9	0.11	80.10	< 0.0001	0.9904
Residual	9.654E-03	7	1.379 E-03			
Lack of Fit	6.380E-03	5	1.276 E-03	0.78	0.6449	
Pure Error	3.274E-03	2	1.637 E-03			
Correlation Total	1	16				

To further investigate the effect of all parameters on encapsulation of natural dye by cyclodextrin, a three dimension (3D) plot was performed by the software. Figure 4.6, and 4.7 were the response surface curves for all parameters involved. The 3D plot representing the concentration on encapsulation of natural dye-β-cyclodextrin. The 3D plot is very useful as it can show the interaction of all parameters involved. Figure 4.6 shows the response surface plot and interactive effect of concentration and pH on encapsulation of natural dye-β-cyclodextrin.

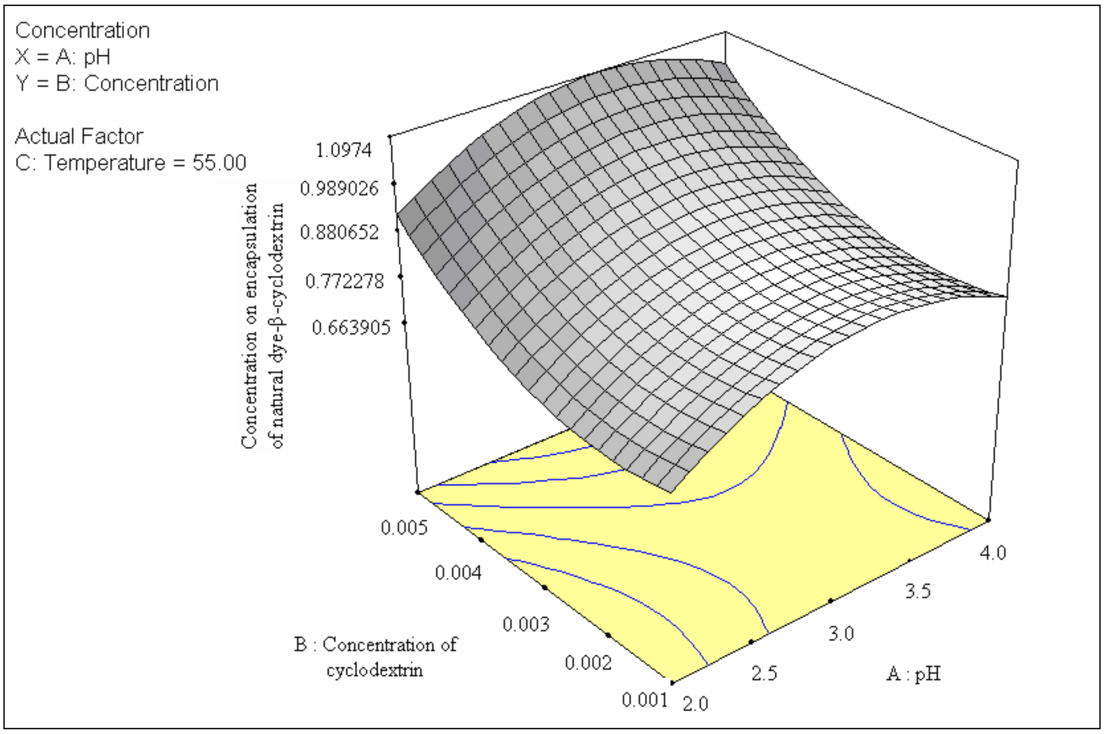

Figure 4.6: Response surface plot on encapsulation of natural dye-β-cyclodextrin production : concentration of cyclodextrin vs. pH

From the figure, concentration on encapsulation of natural dye-β-cyclodextrin decreased initially with increases concentration of cyclodextrin from 0.001 to 0.003 mol/L. After reached equilibrium, concentration on encapsulation of natural dye-β-cyclodextrin decreased with increases of cyclodextrin concentration. The highest concentration was observed at pH 3.0 and concentration of cyclodextrin at 0.003

mol/L. The lowest concentration was observed at 0.005 mol/L of cyclodextrin with 0.775 g/L of concentration on encapsulation of natural dye-β-cyclodextrin.

The concentration obtained was supported by Nicolle *et al*., (2009) which stated that progressive increases in adsorption of cationic dyes from aqueous medium with increasing the concentration of cyclodextrin. However, the concentration of encapsulation of natural dye-β-cyclodextrin was declined after reached at the equilibrium.

This small concentration also was used by Pourjavadi *et al*., (2007) in the optimization of synthetic conditions carboxymethyl cellulose (CMC-*g*-poly) (acrylic acid)/Celite composite superabsorbent which used small amount of 0.004 mol/L methylene bisacrylamide (MBA). This value had the greatest effect to obtain the optimum of synthetic conditions carboxymethyl cellulose.

Figure 4.7 shows the interactive effect of two variables pH and temperature on encapsulation of natural dye-β-cyclodextrin.

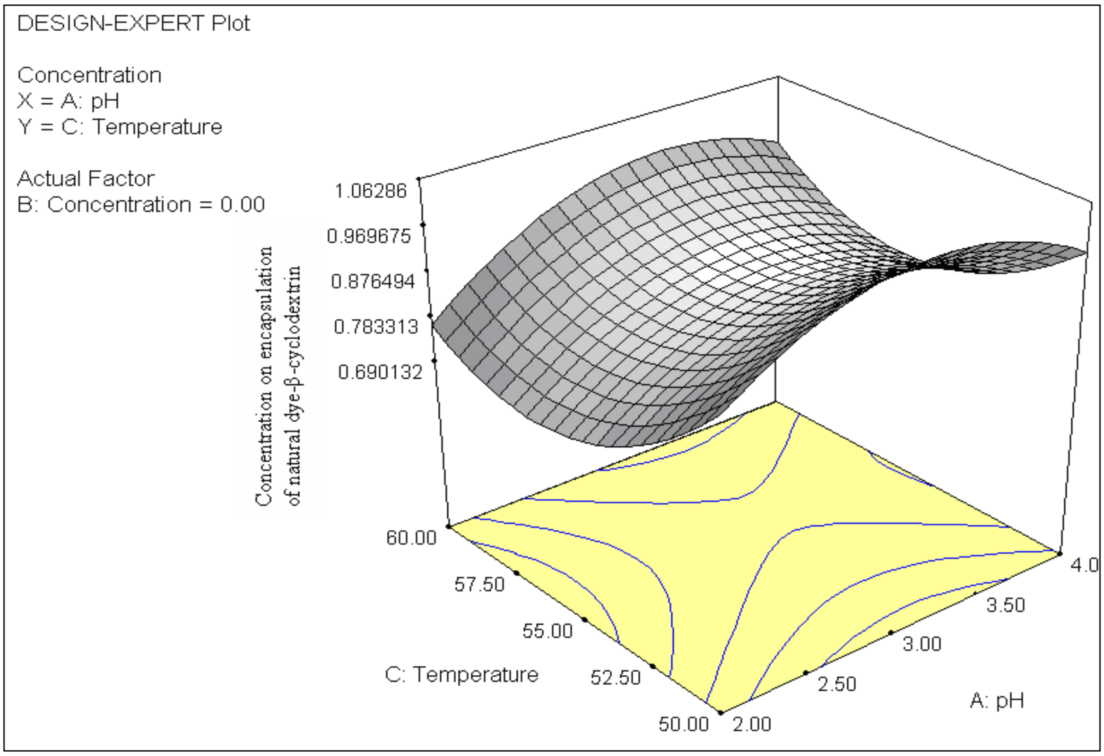

Figure 4.7: Response surface plot on encapsulation of natural dye-β-cyclodextrin production : temperature vs. pH

In the figure, the concentration on encapsulation of natural dye-β-cyclodextrin was decreased with increase of pH from 2.0 to 4.0. Maximum concentration was observed at pH 3.0. High concentration on encapsulation of natural dye-β-cyclodextrin observed at pH 3.0 may be attributed due to the cationic interactions between acid and cyclodextrin molecules.

Sunsanee and Thomas (2009) also reported that the low pH was used in natural dyeing of wool and hair with indigo carmine. It was noted that indigo carmine dyed samples show darker colour at low pH. The earlier studies also supported the best pH was at ranges pH 2 to 4 and pH 3 was the best pH for encapsulation of natural dye-β-cyclodextrin (Arabinda *et al*., 2005 ; Min *et al*., 2009 ; Lajos *et al*., 1998 ; Lying and Passos, 2005).

Using the same figure 4.7, concentration of encapsulation of natural dye-β-cyclodextrin increased with increase of temperature from 50 °C to 55 °C. After reached equilibrium, concentration decreased with increases of temperature. It was also observed that concentration is high at higher temperature at 55 °C than at lower temperature 50 °C and it was decreased at 60 ºC, so decreasing trend of concentration is slow at higher temperature 60 °C than at lower temperature 50 °C with increase in pH of the solution. Maximum concentration of encapsulation of natural dye-β-cyclodextrin was observed at 55 °C.

The study on temperature effect has further strengthened through the research in production of cyclodextrin using raw corn starch by Tae *et al.*, (1997) which stated that the ranges of temperature on raw corn starch from 50 °C to 65 °C was used for cyclodextrin production. This study observed that optimum temperature was obtained at 55 °C. This temperature obtained also was suggested by Bora and Kalita (2007) in production and optimization of thermostable lipase from a thermophilic *bacillus* sp. which enzyme was found to be most stable at low temperature and most suitable for enzyme production.

The earlier studies also supported the effect of temperature on encapsulation of natural dye-β-cyclodextrin (Kiran and Kapoor, 2007). It was noted that a decrease in concentration of natural dye-β-cyclodextrin with the rise in temperature may be due to either damage of active binding sites in interactions which molecules of cyclodextrin cannot bind and do not fit into the cavity of natural dye due to the oxygen transfer rate and transfer charged.

4.5 Optimization of pH, Concentration of Cyclodextrin and Temperature on Encapsulation of Natural Dye-β-Cyclodextrin Using RSM

The optimum parameters can be predicted using response surface plots that were employed by the software using the Equation 4.1. From the plots in Figure 4.6 and 4.7, the effect of the parametes on encapsulation of natural dye-β-cyclodextrin can be determined. By choosing the appropriate target at the numerical criteria in the software interface, highest desirability of pH, concentration of cyclodextrin and temperature was able to determine.

Result from the response surface analysis shown the optimum levels of the predicted variables for maximum concentration on encapsulation of natural dye-β-cyclodextrin were pH 3.11, 0.004 mol/L and 60^{o}C for pH, concentration and temperature respectively. The highest concentration that can be achieved by the optimized parameters was predicted as 1.27253 g/L. In order to verify the predicted value of parameters, an experiment of rechecking was performed according to the proposed parameter. Before optimization, concentration on encapsulation of natural dye-β-cyclodextrin was only 0.775 g/L. The maximum value of the concentration achieved after optimization of parameter is 1.2534 g/L.

Based on Table 4.5, the parameters on encapsulation of natural dye by cyclodextrin were successfully optimized and the concentration was increased by 38.17 % after optimization. The predicted and experimental value of concentration that was optimized by parameters is detailed in Table 4.5.

Table 4.5: Summary of the optimized parameters for encapsulation of natural dye-β-cyclodextrin.

<table>
<tr><th rowspan="3">Parameters</th><th colspan="2">Before Optimization</th><th colspan="3">After Optimization</th></tr>
<tr><th rowspan="2">Parameter Values</th><th rowspan="2">Concentration (g/L)</th><th rowspan="2">Parameter Values</th><th colspan="2">Concentration (g/L)</th></tr>
<tr><th>Predicted</th><th>Experi-mental</th></tr>
<tr><td>pH</td><td>10.5</td><td rowspan="3">0.775</td><td>3.11</td><td rowspan="3">1.27253</td><td rowspan="3">1.2534</td></tr>
<tr><td>Concentration</td><td>0.005 mol/L</td><td>0.004 mol/L</td></tr>
<tr><td>Temperature</td><td>70 ºC</td><td>60 ºC</td></tr>
</table>

From Table 4.5, the value of the pH, concentration of cyclodextrin and temperature was increased after the optimization compared to before optimization. This shows the overall improvement in term of natural dye-β-cyclodextrin production. This fact also was supported by Salwanis *et al.,* (2007) in production of cyclodextrin glucanotransferase (CGTase) from alkalophilic *Bacillus sp*. This study shows a progressive result which the CGTase production increase by 14.54% of increment as compared to the production before the optimization.

CHAPTER 5

CONCLUSION AND RECOMENDATION

5.1 Conclusion

The research on the optimization of pH, concentration of cyclodextrin and temperature on encapsulation of natural dye by cyclodextrin was successfully carried out. By using conventional method, one factor at a time (OFAT), the best range of pH, concentration of cyclodextrin and temperature was able to be determined. The best range of pH is 2 to 4, concentration of cyclodextrin is 0.002 to 0.004 mol/L and temperature is 50^{o}C to 60^{o}C. All the values will be used for further optimization using Response Surface Methodology (RSM).

The objective of this research which is to determine the optimum pH, concentration of cyclodextrin and temperature on the encapsulation of natural dye by cyclodextrin using response surface methodology (RSM) was achieved. The optimized pH, concentration of cyclodextrin and temperature are pH 3.11, 0.004 mol/L and 60^{o}C respectively. The initial concentration on encapsulation of natural dye by β-cyclodextrin is 0.775 g/L. By using the optimized conditions, the concentration on encapsulation of natural dye-β-cyclodextrin is 1.2534 g/L and predict concentration is 1.2776 g/L. The concentration was increased 38.17% than initial concentration on encapsulation of natural dye-β-cyclodextrin.

In term of reduction of energy consumption, 14.3% energy for heating can be reduced due to reduction of temperature from 70^{o}C to 60^{o}C. The reduction for the concentration of cyclodextrin from 0.005 mol/L to 0.003 mol/L leads to 40% reduction of concentration for cyclodextrin and the reduction of pH from pH 10.5 to pH 3.11 leads to 70.4% of reduction. The reduction of energy consumption and concentration of cyclodextrin can reduce the operating cost because cyclodextrin is quite expensive in cost. Therefore, the encapsulation of natural dye by β-cyclodextrin was successfully optimized and improves in term of production and stability by using Response Surface Methodology (RSM).

5.2 Recommendation

To obtain more significant increment in optimization of encapsulation of natural dye by cyclodextrin, additional of more parameter such as duration for encapsulation of natural dye-β-cyclodextrin, acid concentration, different ratio of cyclodextrin to natural dye and amount of natural dye and cyclodextrin may increase the encapsulation production. Optimization of many parameters will give the best condition for encapsulation of natural dye with cyclodextrin expression.

For the further study of optimization on concentration effect of natural dye-β-cyclodextrin, selected raw material by using different natural colorants such as pitaya fruit, anthocyanin, beetroot red, caramel, lycopene and paprika extract may increase pure coloring in natural dye because of their pure extraction. Using complexation of *curcumin*, the superoxide scavenging ability of curcumin on complexation with β-cyclodextrin also may increase as compared to uncomplexed *curcumin*. To obtain the accurate data and result for concentration on encapsulation of natural dye by cyclodextrin, using HPLC (High Performance Liquid Chromatography) also may increase the production as compared by using UV-Vis Spectroscopy.

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APPENDIX A

MATERIALS AND METHODS

Appendix A1

Phosphate buffer solution preparation at pH 7 :

Chemical Involved:

0.10 M   Potassium phosphate monobasic (KH_2PO_4)

850 mL   Distilled water

0.20 M   Sodium Hydroxide

Prepare 0.10 M potassium phosphate monobasic (KH_2PO_4) solution by dissolving 3.40 g in 250 mL distilled water. Prepare 0.20 M sodium hydroxide solution by dissolving 0.8 g in 100 mL distilled water. Mix 250 mL of the 0.10 M potassium phosphate solution and 73 mL of 0.20 M sodium hydroxide solution, then dilute to 500 mL of distilled water.

Appendix A2

Extract using water extraction process:

Before extraction of natural dyes, the raw materials is ground to small particles about 1 mm. The natural dyes then extracted with boiling water by applying ratio 1:20, corresponding to a ratio of 1 g of raw material to 20 mL of water. The duration of the extraction fixed at 60 min.

Appendix A3

Natrium hydroxide solution preparation:

Chemical Involved:

0.10 M Natrium hydroxide

Solving 5.38474 mL of natrium hydroxide in 1000 mL of distilled water. Stir the solution until the material dissolved. The volume of the solution is adjusted to 1 L. The solution should be stored in sumehood.

APPENDIX B

RESULT AND DISCUSSION

Appendix B1

Experiment Data

Table B-2: Types of cyclodextrin

Cyclodextrin types	**OD Blank**	**OD natural dye-β-cyclodextrin**	**Encapsulation of natural dye-β-cyclodextrin concentrations (g/L)**
α	0.06	0.703	0.681
β	0.10	0.921	0.966
γ	0.09	0.876	0.9073

Table B-2 : Determination of the best pH

pH types	**OD Blank**	**OD natural dye-β-cyclodextrin**	**Encapsulation of natural dye-β-cyclodextrin concentrations (g/L)**
3 (acidic)	0.139	0.865	0.893
7 (neutral)	0.113	0.222	0.054
10.5 (alkaline)	0.120	0.259	0.102

Table B-2 : OFAT on pH

pH	OD Blank	OD natural dye-β-cyclodextrin	Encapsulation of natural dye-β-cyclodextrin concentrations (g/L)
2	0.21	0.784	0.787
2.5	0.12	0.675	0.645
3	0.26	0.803	0.812
3.5	0.15	0.692	0.667
4	0.09	0.240	0.077

Table B-2 : OFAT on concentration

Concentration (mol/L)	OD Blank	OD natural dye-β-cyclodextrin	Encapsulation of natural dye-β-cyclodextrin concentrations (g/L)
0.001	0.28	0.892	0.9282
0.002	0.21	0.865	0.8930
0.003	0.34	1.040	1.1214
0.004	0.27	0.890	0.9260
0.005	0.19	0.775	0.7750

Table B-2 : OFAT on temperature

Temperature	OD Blank	OD natural dye-β-cyclodextrin	Encapsulation of natural dye-β-cyclodextrin concentrations (g/L)
25	0.12	1.073	1.1645
35	0.24	1.221	1.358
45	0.23	1.197	1.3264
50	0.07	1.123	1.230
55	0.34	1.405	1.600
60	0.17	1.218	1.354
65	0.18	1.130	1.240
70	0.28	1.126	1.234

Table B-3 : Experiment data based on RSM suggested parameter

Standard	pH	Concentration (mol/L)	Temperature (°C)	Concentration (g/L)
1	2.0	0.002	50.0	0.9726
2	4.0	0.002	50.0	1.0574
3	2.0	0.004	50.0	1.0970
4	4.0	0.004	50.0	1.0900
5	2.0	0.002	60.0	0.6920
6	4.0	0.002	60.0	0.7960
7	2.0	0.004	60.0	1.0431
8	4.0	0.004	60.0	1.2280
9	1.3	0.003	55.0	0.4260
10	4.7	0.003	55.0	0.6230
11	3.0	0.001	55.0	0.9530
12	3.0	0.005	55.0	1.3580
13	3.0	0.003	46.6	1.3264
14	3.0	0.003	63.4	1.2154
15	3.0	0.003	55.0	0.8734
16	3.0	0.003	55.0	0.9125
17	3.0	0.003	55.0	0.8316

Appendix B2

RSM Analyzing

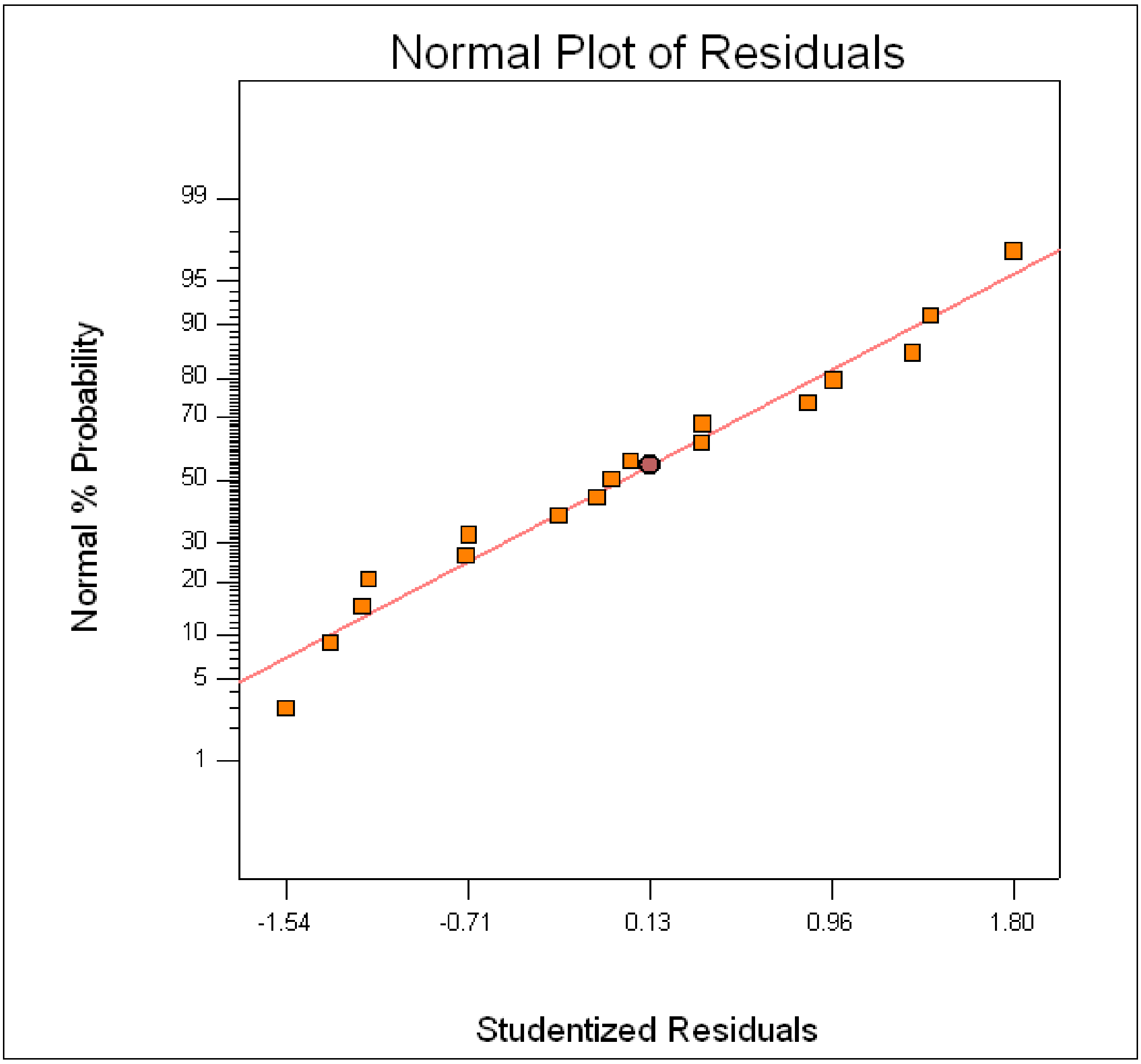
Table B-4 : ANOVA data

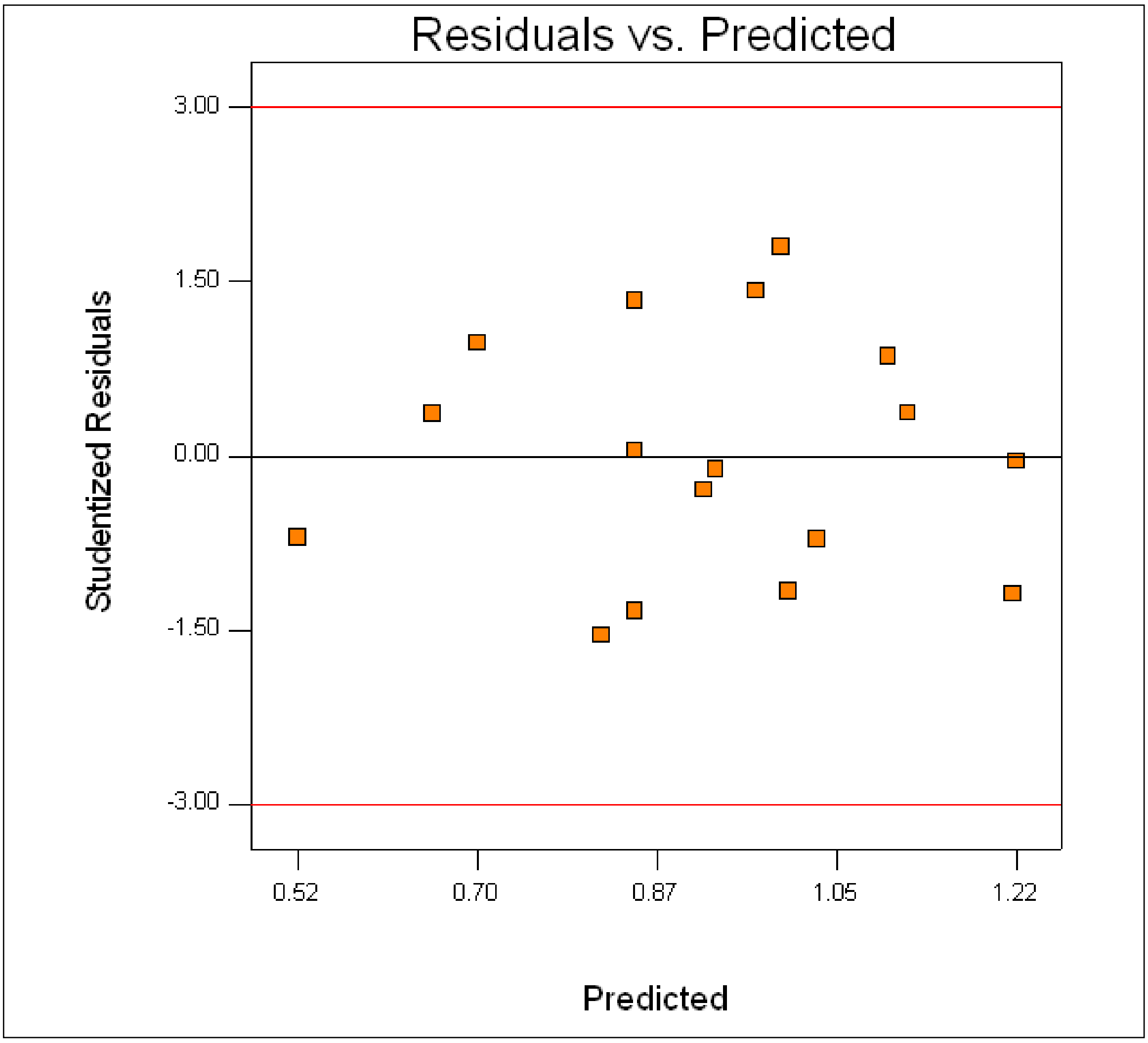
Analysis of variance table [Partial sum of squares]

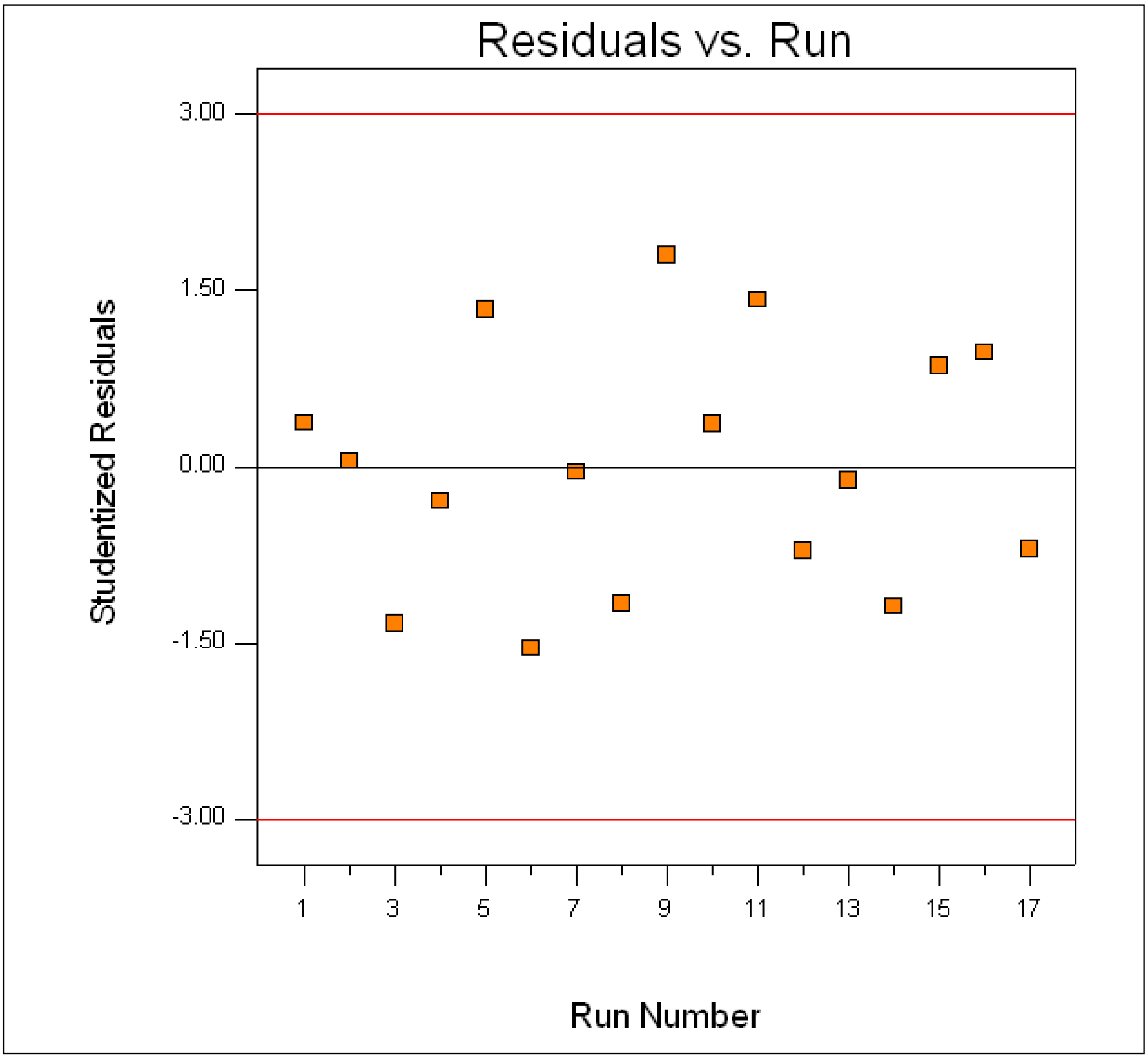
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	0.994199259	9	0.110467	80.10094	< 0.0001	significant
A - pH	0.035676035	1	0.035676	25.86922	0.0014	
B - Concentration	0.192458234	1	0.192458	139.5543	< 0.0001	
C - Temperature	0.030422972	1	0.030423	22.06014	0.0022	
A2	0.166593473	1	0.166593	120.7994	< 0.0001	
B2	0.116208449	1	0.116208	84.26445	< 0.0001	
C2	0.228372646	1	0.228373	165.5964	< 0.0001	
AB	1.48513E-05	1	1.49E-05	0.010769	0.9203	
AC	0.005570401	1	0.00557	4.03918	0.0844	
BC	0.049000151	1	0.049	35.53073	0.0006	
Residual	0.009653646	7	0.001379			
Lack of Fit	0.006380026	5	0.001276	0.779568	0.6449	not significant
Pure Error	0.00327362	2	0.001637			
Cor Total	1.003852905	16				

Appendix B3

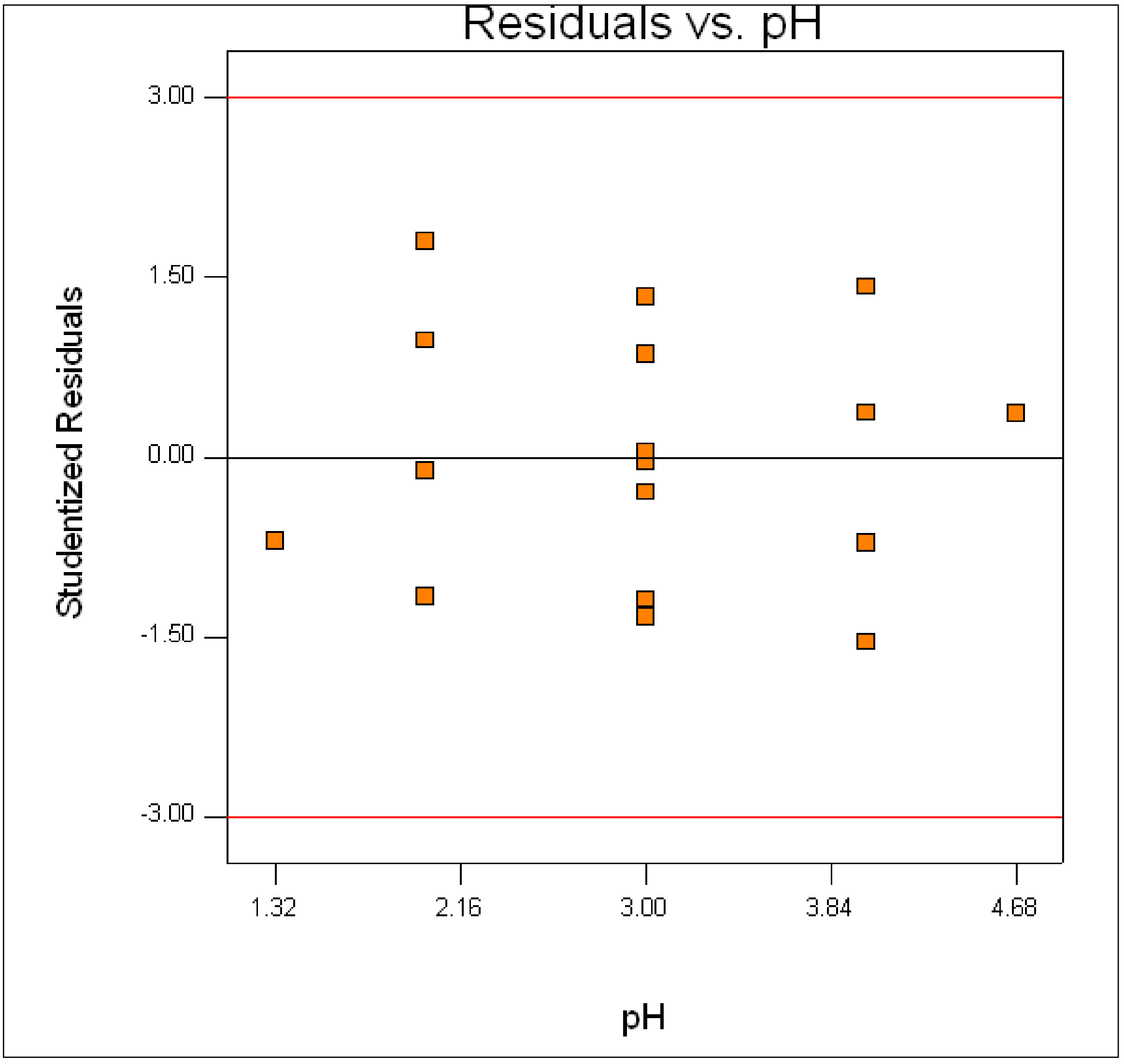
DESIGN- EXPERT PLOT

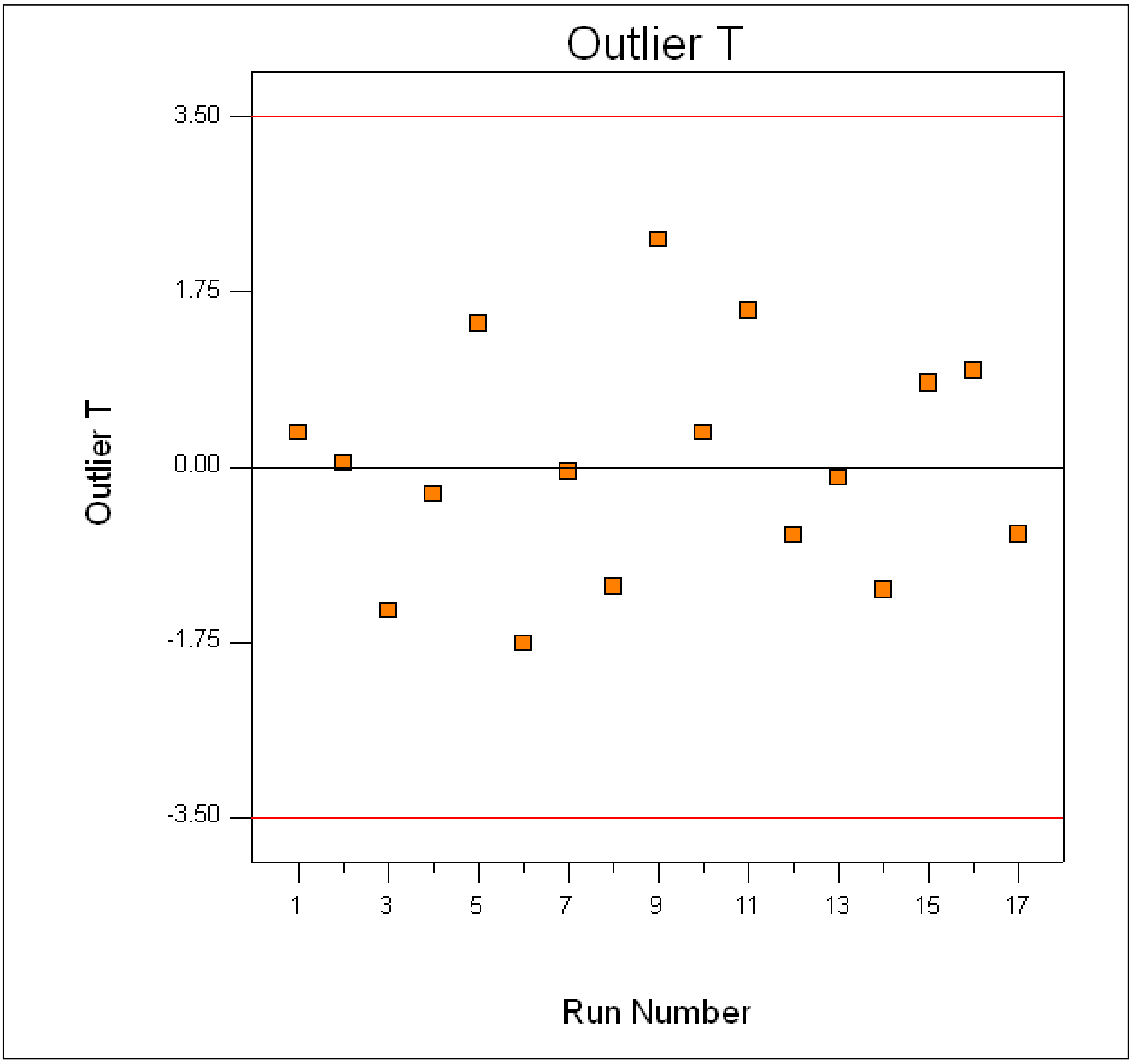



Residuals vs. Predicted
Studentized Residuals
3.00
1.50
0.00
-1.50
-3.00
0.52
0.70
0.87
1.05
1.22
Predicted

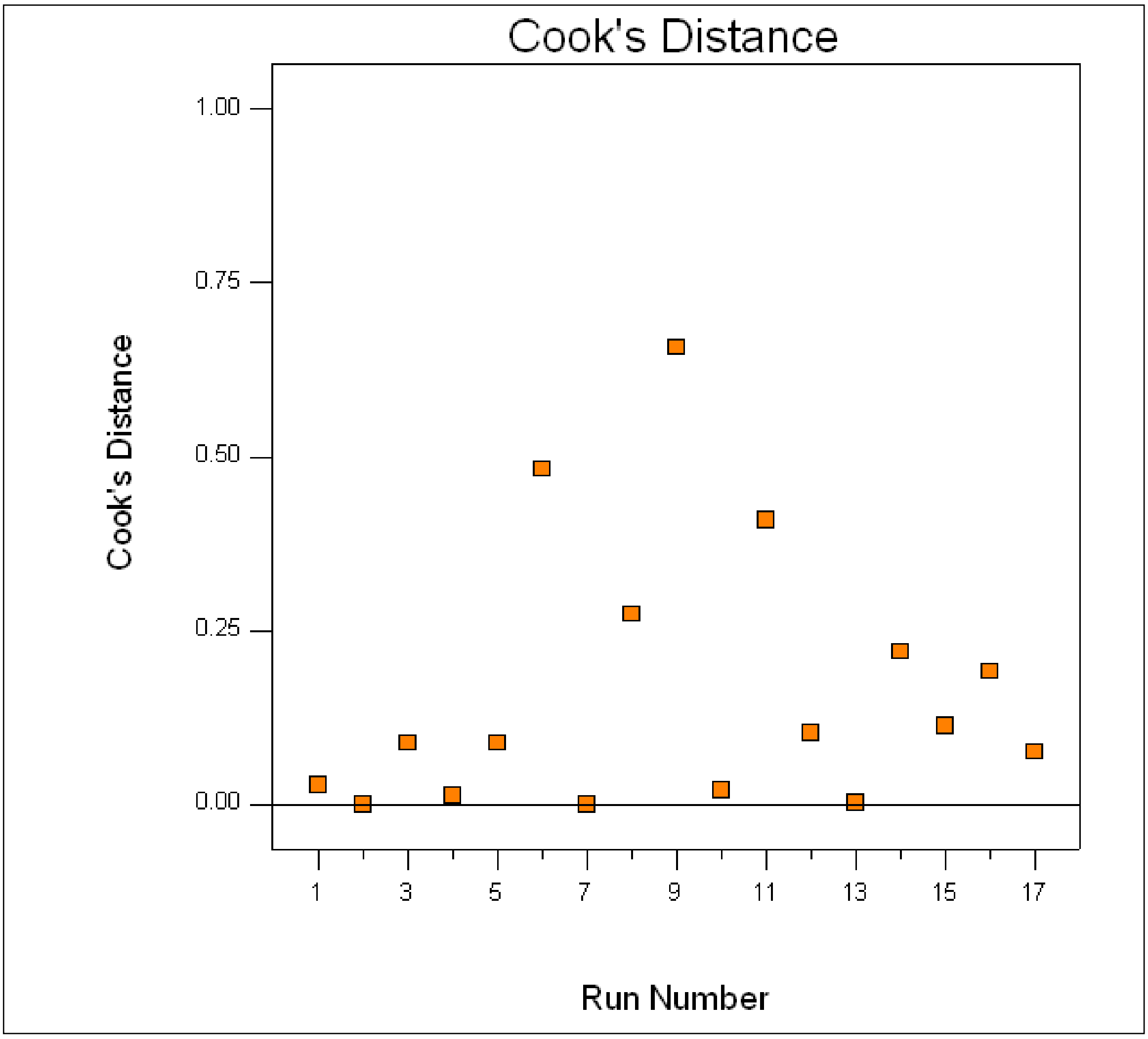


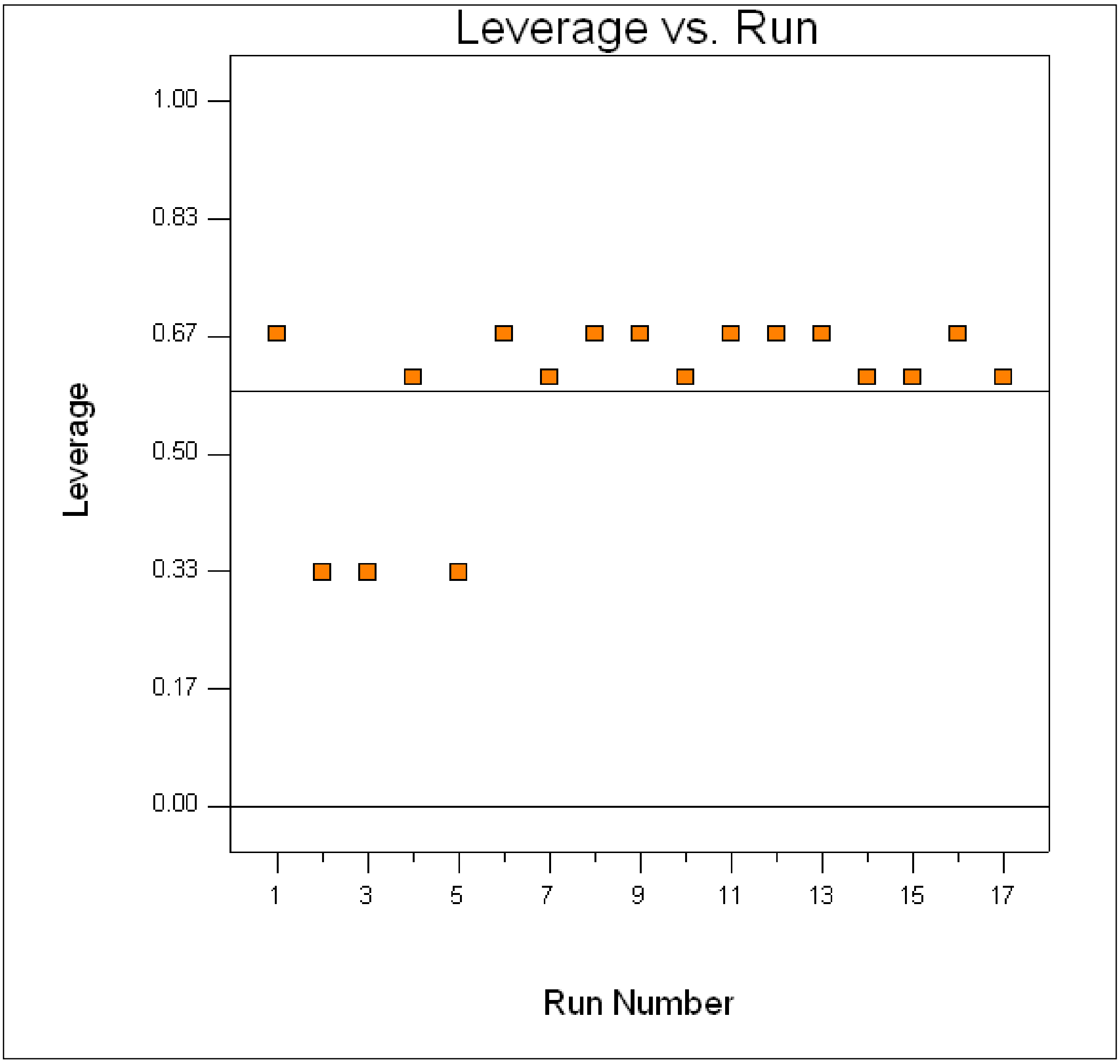
Residuals vs. Run
Studentized Residuals
3.00
1.50
0.00
-1.50
-3.00
1
3
5
7
9
11
13
15
17
Run Number

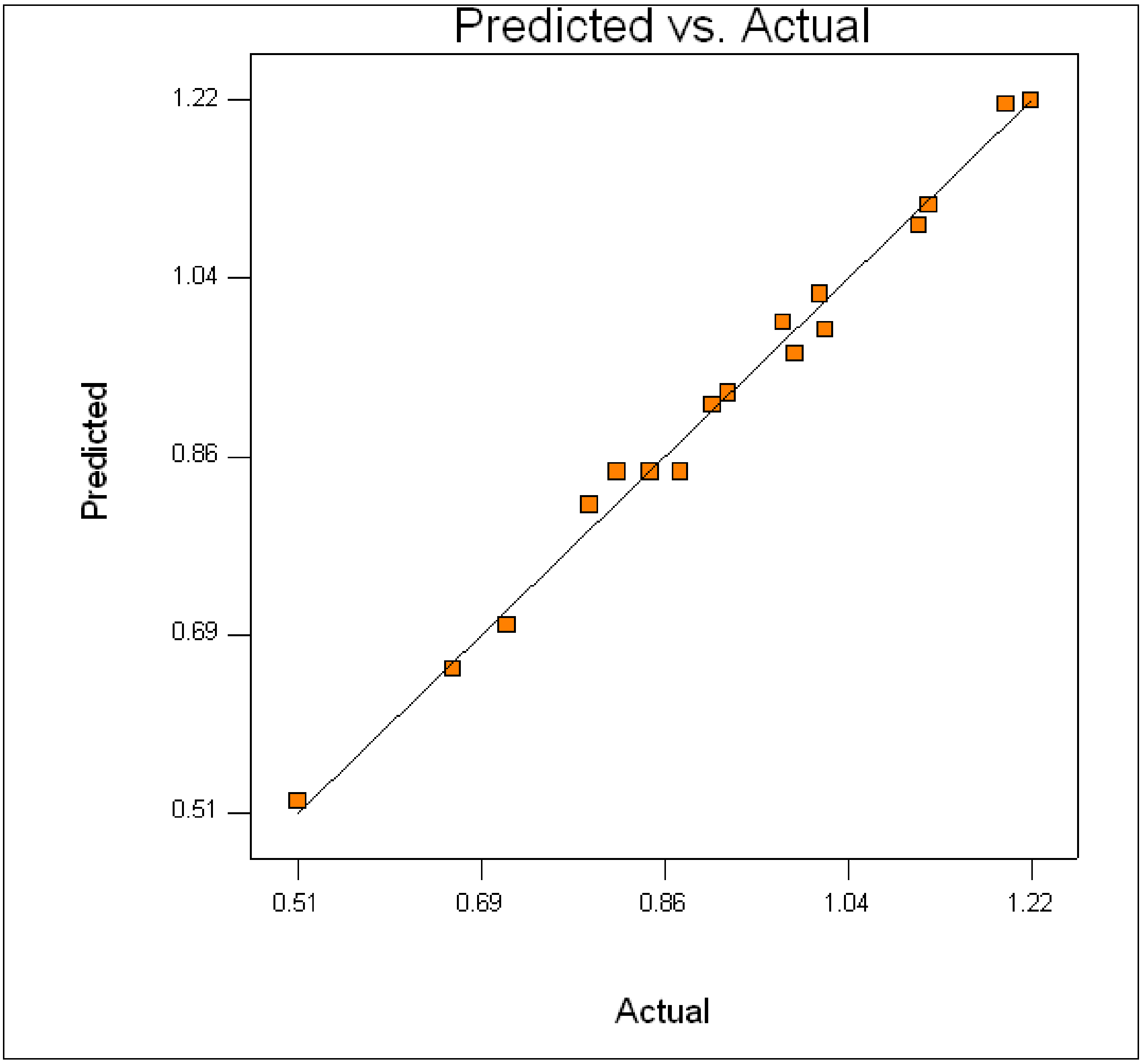

Residuals vs. pH
Studentized Residuals
3.00
1.50
0.00
-1.50
-3.00
1.32
2.16
3.00
3.84
4.68
pH



Outlier T
3.50
1.75
0.00
-1.75
-3.50
Outlier T
1
3
5
7
9
11
13
15
17
Run Number


Cook's Distance
1.00
0.75
0.50
0.25
0.00
Cook's Distance
1
3
5
7
9
11
13
15
17
Run Number


Leverage vs. Run
Leverage
1.00
0.83
0.67
0.50
0.33
0.17
0.00
1
3
5
7
9
11
13
15
17
Run Number


Predicted vs. Actual
Predicted
1.22
1.04
0.86
0.69
0.51
0.51
0.69
0.86
1.04
1.22
Actual

Box Cox Analysis

DESIGN-EXPERT Plot
Abs

Lambda
Current = 1
Best = 1.19
Low C.I. = 0.46
High C.I. = 1.99

Recommend transform:
None
(Lambda = 1)

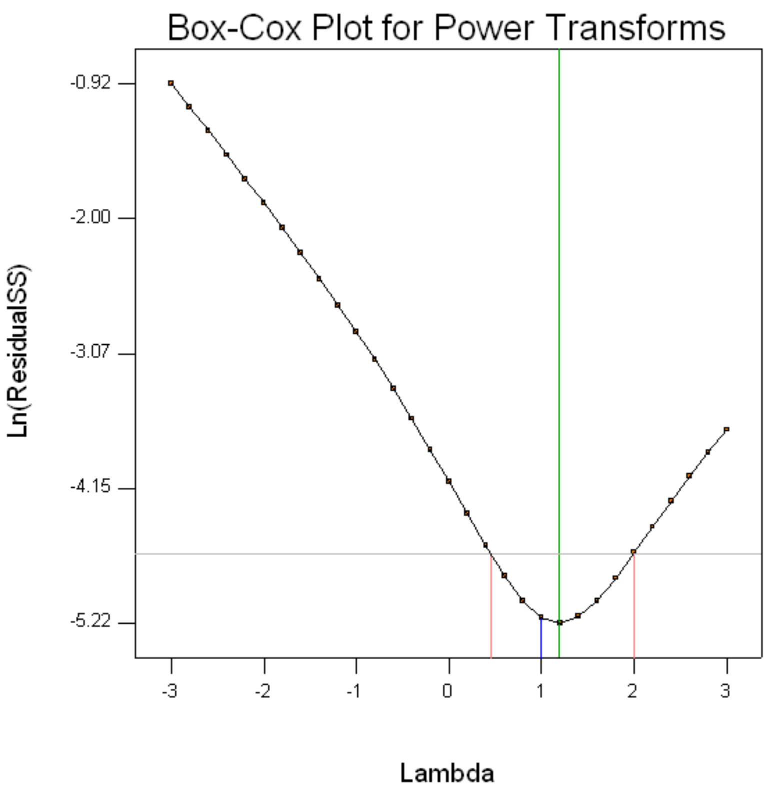


Table B-4 : Regression Analysis

Std. Dev.	0.0371361	**R-Squared**	0.990383
Mean	0.9703176	**Adj R-Squared**	0.978019
C.V.	3.8272139	**Pred R-Squared**	0.93854
PRESS	0.0616973	**Adeq Precision**	32.18181

Appendix B4

RSM Analyzing Report

Table B-4: Diagnostic Report

Diagnostics Case Statistics								
Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	0.9726	0.975031	-0.00243	0.669865	-0.11394	0.002634	-0.10558	5
2	1.0574	1.027203	0.030197	0.669865	1.415223	0.406393	1.550743	4
3	1.097	1.058654	0.038346	0.669865	1.797116	0.655313	2.267041	9
4	1.09	1.105376	-0.01538	0.669865	-0.7206	0.105363	-0.69336	8
5	0.692	0.671335	0.020665	0.669865	0.9685	0.190325	0.963534	1
6	0.796	0.829056	-0.03306	0.669865	-1.54922	0.486992	-1.76935	14
7	1.0431	1.068008	-0.02491	0.669865	-1.16733	0.276491	-1.20429	12
8	1.228	1.220279	0.007721	0.669865	0.361833	0.026565	0.338169	2
9	0.426	0.442282	-0.01628	0.607498	-0.69984	0.075806	-0.67186	16
10	0.623	0.614198	0.008802	0.607498	0.378321	0.022153	0.353894	15
11	0.953	0.959592	-0.00659	0.607498	-0.28334	0.012425	-0.26383	11
12	1.358	1.358888	-0.00089	0.607498	-0.03819	0.000226	-0.03536	3
13	1.3264	1.354018	-0.02762	0.607498	-1.18706	0.218095	-1.22972	17
14	1.2154	1.195263	0.020137	0.607498	0.865536	0.115951	0.847991	6
15	0.8734	0.872072	0.001328	0.33203	0.043748	9.51E-05	0.040509	7
16	0.9125	0.872072	0.040428	0.33203	1.332003	0.088192	1.427269	10
17	0.8316	0.872072	-0.04047	0.33203	-1.33346	0.088386	-1.42937	13