The Potential of Thiosulfinates in Garlic Extract as Bio-Inhibitor in Urea Fertilizer

by

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Dissertation submitted in partial fulfilment of

the requirements for the

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(Chemical Engineering)

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CERTIFICATION OF APPROVAL

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Approved by,

_____

(Dr. Nurlidia binti Mansor)

UNIVERSITI TEKNOLOGI PETRONAS

TRONOH, PERAK

September 2012

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

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12198

CHEMICAL ENGINEERING

THE POTENTAL OF THIOSULFINATES IN GARLIC EXTRACT AS BIO-INHIBITOR IN UREA FERTILIZER

ABSTRACT

Urea is extensively used as fertilizer in the agricultural industry based on its suitability for all types of crops. The hydrolysis of urea fertilizer produces ammonia (NH_3) and carbon dioxide (CO_2). However, up to 40% of NH_3 release affects the efficiency of urea fertilizer. By introducing inhibitors into the urea enzymatic reaction, the NH_3 emission problem can be solved. Unfortunately, current inhibitors are usually chemical based and non-biodegradable. Several complaints and accidents have been reported when handling chemical based inhibitors especially for surface application. Research on garlic or A*llium savatium* has been conducted to ensure its inhibitory effects as potentially safe and biodegradable inhibitor. From previous research, thiosulfinates (TS) contained in garlic extract proved to inhibit platelets aggregation in medical applications. TS is obtained by extracting garlic cloves. In this study, the inhibitory effect is determined by analyzing NH_3 concentration in urease-garlic mixture and standard urea assay mixtures using UV-VIS spectrophotometer device. The Beer's Lambert law is used for calculating the concentration with an aid of the standard NH_3 calibration graph. Based on previous research, the NH_3 concentration is predicted to decrease with urease-garlic mixture amount and time.

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ABBREVIATIONS AND NOMENCLATURES

TS	Thiosulfinates
NBPT	n-(n-butyl) thiophosphoric triamide
UAN	Urea Ammonium Nitrate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
HXA	Hydroxamic acid
PPD	Phosphorodiamidates
MSDS	Material Safety Data Sheets
LD50	Lethal Dose 50%
NOHSC	National Occupational Health and Safety Commission (Australia)
UV-VIS	Ultraviolet-Visible

CHAPTER 1
INTRODUCTION

1.1 Background of Study

Urea, $CO(NH_2)_2$ is widely used in fertilizers as a major source of nitrogen. Since past decades, urea has nearly substitute ammonium nitrate, NH_4NO_3 as an agricultural fertilizer due to safety issues. In 1994, an accident occurred where ammonium nitrate solution exploded during its production and caused several injuries and death (Environmental Protection Agency, 2007). Besides that, the demand on urea increases because of its high nitrogen content (46%) with low production cost (C. Watson, 2005).

In the presence of water, urea fertilizers are rapidly transformed to ammonium types in soils (R. M. Gifford, 2004). Theoretically, there are two main products from this reaction: NH_3 and carbon dioxide. Loss of nitrogen as NH_3 gas has reduced the efficiency of urea. According to E. Funderburg (2009), up to 40% of nitrogen emission might happen due to incorrect application of urea in soils. Evidently, the nitrogen gas from urea contributes 50% of total world nitrogen consumption (C. Watson, 2005). Besides, nitrogen gas is essential component for DNA, RNA and amino acids for proteins which required for all living things including plants to exist and grow (Landscape Connection, 2012). In wet conditions, urea fertilizer may react as shown in the equation below:

$$\underset{\text{urea}}{CO(NH_2)_2} + \underset{\text{water}}{H_2O} \xrightarrow[\text{urease}]{} \underset{\text{ammonia}}{2NH_3} + \underset{\text{carbon dioxide}}{CO_2}$$

From the equation, one mol of urea fertilizer will react with one mol of water to produce two mols of NH_3 and one mol of carbon dioxide (CO_2). The urease enzyme allows the reaction to proceed at faster rates by reducing the energy of activation of the reaction. It may attack the nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end product which is NH_3 (virtual lab of Amrita University, 2012). In soil,

urease is contributed by plants, soil animals and microorganisms. As shown in Figure 1.1, consider the lock and key hypothesis where urea with water is the substrate and urease is the enzyme respectively. Throughout the reaction, if the shape of enzyme's active site exactly matches and fit with the shape of the substrate, enzyme-substrate complex will formed.

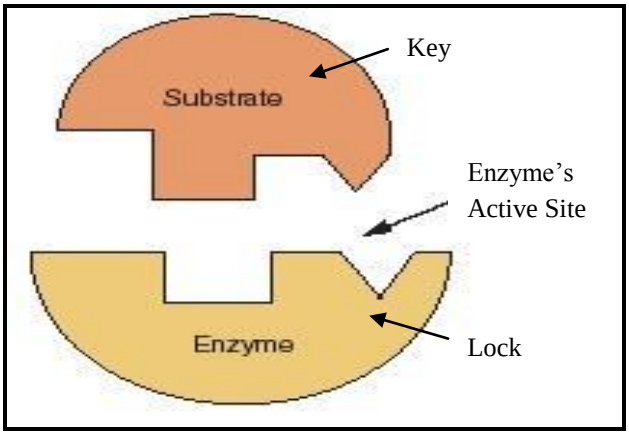

Figure 1.1: Lock (Enzyme) and Key (Substrate) Hypothesis

Furthermore, the active site of urease consists of two nickel (II) atoms linked together by Carbamate Bridge as shown in Figure 1.2 (Tatsuo Yoshinobu et al., 2003). Two imidazoles nitrogen atoms are bounded to each nickel atoms while a carboxylate group and a water molecule fill the remaining coordination site of the metal ion.

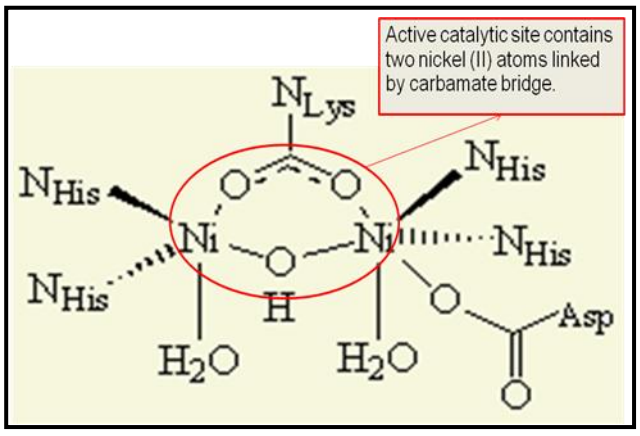

Figure 1.2: Urease Enzyme Structure

However, released of NH_3 gas is hazardous to workers who handle fertilizers at plantation sites. Based on Heber et al. (2004), at moderate concentration between 24 to 134 ppm, NH_3 is able to irritate eyes and respiratory tract of human beings. In order to prevent release of NH_3, researchers found a way to slow down the nitrogen released from urea fertilizer by applying inhibitors in the reaction. Inhibitor is a compound used to prevent the occurrence of enzymatic reaction. As mentioned before, urea fertilizer reaction only occurs if the substrate fits well with the enzyme. If an inhibitor were introduced into the urease active site, the urea enzymatic reaction does not take place and NH_3 gas will not been released.

To date, thousand of chemicals have been evaluated as potential urease inhibitors and can be classified according to their structures and interaction with urease (C. Watson, 2005). Urease inhibitors have been proved to slow down or prevent the reaction between substrate and enzyme (Zaborska et al., 2009). The inhibitor delays the conversion of urea to ammonium ion ($NH_4^{+)}$, hence reduces concentration of NH_4^+ in soil solution as well as the volatility of NH_3 (C. Watson, 2005). Jongeneel (2012) reported that, when urea is broadcast on the dry soil surface without any urease inhibitor, nitrogen losses start to increase after 3 days. After 10 days, about 30% of urea fertilizer function is lost.

C. Watson (2005) has proposed four main classes of inhibitor which are: reagents which interact with the sulphydryl groups (sulphyldryl reagents), hydroxamates (HXAs), agricultural crop protection chemicals and structural analogues of urea and related compound. Instead of n-(n-butyl) thiophosphoric triamide (NBPT), HXAs, phosphorodiamidates (PPDs), imidazoles and phosphazene related compounds are also types of current inhibitors (Z. Amtul et al., 2002). Most of these inhibitors are using chemical based inhibition techniques.

According to Fernandez (2011), nitrogen volatilation can be minimized by applying inhibitor called NBPT. This product is sold under trade name of Agrotain and marketed by IMC-Agrico Group (Varsa et al., 1998). Since 2007, Agrotain is the most commercial and popular technology which control nitrogen loss by blocking the enzyme urease. Agrotain is used in blending together with urea-based fertilizer products to create an enhanced efficiency fertilizer that controls surface loss by blocking enzyme urease (AGROTAIN International, 2012). With time, Agrotain will decompose and the enzyme's activity will resume as normal. Meanwhile, another inhibitor brand using NBPT formulation is Arborite, from Weyerhaeuser. Arborite is used either by coating with urea granules or mix with urea nitrate solutions (UAN) (Weyerhaeuser, 2012).

Obviously, most of the current inhibitors have negative side effects, not safe and have low efficiency (Zaborska et al., 2009). According to P.M.Tejo et al. (2011) research, NBPT found in Agrotain and Arborite has some visible effects. On the first week of treatment, the leaf tips transforms from green color to yellow. Besides that, plants treated with urea and NBPT were found to have higher urea content in their tissues and retard the plant growth (phytotoxicity effect). Based on Department of Health and Ageing (2010) draft report, 500 mg/kg/day of NBPT exposure for 15 days on rats cause decrease in total cholesterol, triglyceride, brain red blood cells and the target organ is the liver. Urine sample from tested animal was taken and two major matabolites of NBPT were identified: N-(n-butyl)-thiophosphoric diamide and the glucuronic acid conjugate of NBPT. In addition, there is evidence that Agrotain can affect fertility in animals. From the same report, NBPT proved to cause weight changes in reproductive organs in both males and females, and abnormalities in sperm assessments.

Bio-inhibitor is a new approach to replace current chemical based inhibitor. By producing an inhibitor from organic compound, the purpose of getting minimum NH_3 emission with lower costs, risks and hazards on environment can be achieved. There are many types of potential bio-inhibitor which have the same inhibitory effect with current

inhibitor. Garlic or *Allium savatium* is a local plant that has been commonly used in herbal medicine. According to Juszkiewicsz et al. (2004), this plant proved to inhibits platelet aggregation, reduce serum cholesterol and triglycerides effectively and lowers ocular pressure. Since inhibitors are crucial in fertilizer application, Juszkiewicsz et al. (2004) study can be used to support the facts where garlic has a potential to inhibit any enzymatic reaction. Thiosulfinates (TS) is the main active component in garlic extract. This compound attacks and reacts with the urease enzyme's active site therefore blocking the reactions. Allicin products are available in market for herbs and medicine purpose.

1.2 Problem Statement

The research on inhibitor for urease enzyme activity of fertilizers have been extensively conducted and already been applied on the agriculture field. Unfortunately, most of the current enzyme inhibitors are chemical based and lead many problems. Most inhibitors are also non-biodegradable and toxic. Any chemical component which reacts with the ecosystem may affect the ecosystem either directly and indirectly. Furthermore, NH_3 gas produced by the urea fertilizer reaction is flammable, toxic by inhalation, may cause burns, risk of serious damage to eyes and very toxic to aquatic organisms (MSDS Orica Chemicals, 2008). Based on various problems occurs while using current inhibitors in fertilizer, it is reasonable to state that current chemical based inhibitors causes issues in health and the environment.

This project is significant to reduce the level of NH_3 contamination in the atmosphere since most plants and trees are using urea as its fertilizer. Besides, by having organic compound to control NH_3 released, the hazard on ecosystem can be reduced.

1.3 Objectives of Study

Bio-inhibitor is a method of using green or organic element as an inhibitor. This project looks into garlic extract which consists of TS as a potential safe and environmental-friendly urease bio-inhibitor. The main intention of this research is to determine whether garlic extract has the potential to inhibit urease activity in urea fertilizer reaction and prevent NH_3 released. There will be no harm towards the environment since garlic is a natural plant compared to other chemical inhibitors with the same purpose. During accomplishment of the main objective, garlic inhibitory effect will be taken into consideration by observing the urease activity within a specific time interval. Overall, the objectives of this research are:

1. To determine the potential of garlic extract to inhibit urease activity in urea fertilizer reaction.
2. To examine the garlic inhibitory effect through time.

1.4 Relevancy of the Research

The availability of garlic in current market is enormous and the supply is no-end since garlic is extensively used in cooking spices. Garlic is easy to plant and grows throughout the year in mild climate. It is much easier to get supply of TS content in garlic rather than most organic compound. Therefore, this project is feasible to being conducted with availability of sources, equipments and reasonable cost.

CHAPTER 2
LITERATURE REVIEW

Fertilizer is necessary to replace crop nutrients that have been consumed by previous plant growth (Managing Agricultural Fertilizer Application, 2010). Urea is the first organic molecule synthesized in laboratory (Zahid & Raza, 2012). Based on V. Jais (2012) article, urea fertilizer is suitable for all types of crops with lower cost of production. This fertilizer is manufactured by combining CO_2, NH_3 and used nitrogen fertilizer and causes no harm to the environment (Urea Fertilizer and Agriculture, 2012). Besides, urea fertilizer has excellent shelf life and safe on storage purposes. The right amount of urea application lead to increase plant yield and productivity. On wet soil, urea fertilizer may take an irreversible reaction producing NH_3 and CO_2 with the help of urease as its catalyst. However, rapid emission of NH_3 gas as product of urea fertilizer reaction has reduced the efficiency of this fertilizer. In M. Robert (2009) study, up to 10 to 40% of NH_3 loss may be expected from remaining urea on the soil surface at certain period of time. NH_3 emission may affect the efficiency of urea fertilizer.

The study of urease inhibition to control NH_3 released on agricultural site is widely known. NPBT is an example of a chemical based urease inhibitor which reduces nitrogen loss from NH_3 volatilization of urea and dicyandiamide. In agriculture terms, it is also known as fertilizer additives. Unfortunately, NPBT cause many side effects on health, occupational and environmental exposure. For health, NPBT may cause weight changes in reproductive organs in both males and females, and abnormalities in sperm evaluations (Department of Health and Ageing, Australia, 2010). NPBT was widely found in commercial brands of Agrotain and Arborite. Agrotain contains 25% NPBT, faced comments on health of agricultural workers and consumers. It was found that worker handles with the compound experience serious damage to the eyes on 7 days exposure. An experiment tested on Fischer 344 rats gives clinical results that at high dose (LD_{50}), NBPT may cause mortality or death (NOHSC, 2004). Meanwhile, other urease inhibitors such as hydroxamic acid and phosphoroamides (Zaborska et al., 2009)

have been applied in medicine a long time ago but have many side effects. Until now, a safe and efficient urease inhibitor is still being sought (Zaborska et al., 2009).

Therefore, bio-inibitor based on natural plant is the ultimate way to solve problems on current chemical based urease inhibitor. Garlic or allium savatium is a strong antibacterial agent and acts as an inhibitor on both gram-positive and gram-negative bacteria (Juszkiewicz et al., 2004). Besides, this study also describes in between 2 and 5 mg/ml concentration of garlic extract is enough to inhibit the bacterial growth. The active component of garlic extract, alk(en)yl TS is formed from alk(en)ylcysteine sulfoxides in the enzymatic reaction after garlic crusing. Allicin, main compound in TS is rapidly formed from alliin (S-allyl-L-cysteine sulfoxide) by the catalytic action of alliinase (Analytical Biochemistry, 2005).

The inactivation or urease activity can be determined by measuring the sample absorbance using UV light in UV-VIS spectrophotometer device. This device was able to measure intensity of light in the function of wavelength. The urease activity is determined by evaluating the NH_3 concentration with time using Beer's Lambert Law. Palmer, Ross & Nutt (2001) study shows the UV light absorbance for NH_3 analyzer is calibrated within the range of 200–450 nm. Meanwhile, in T. Merian et al. (2009) research, the wavelength of the light source used in NH3 absorbance was fixed at 430 nm and experiments were performed at room temperature.

A spectrophotometer result from Juszkiewicz (2004) on residual activity explains that the urease activity reduced with additional amount of garlic. Half of the inhibitory concentration was achieved with 5.6 g/l of garlic extract and it is fully inhibited at 100 g/l of garlic extract. Furthermore, at the end of reaction, the TS concentration increases by 2 mM. Refer Figure 2.1 (Juszkiewicz, 2004).

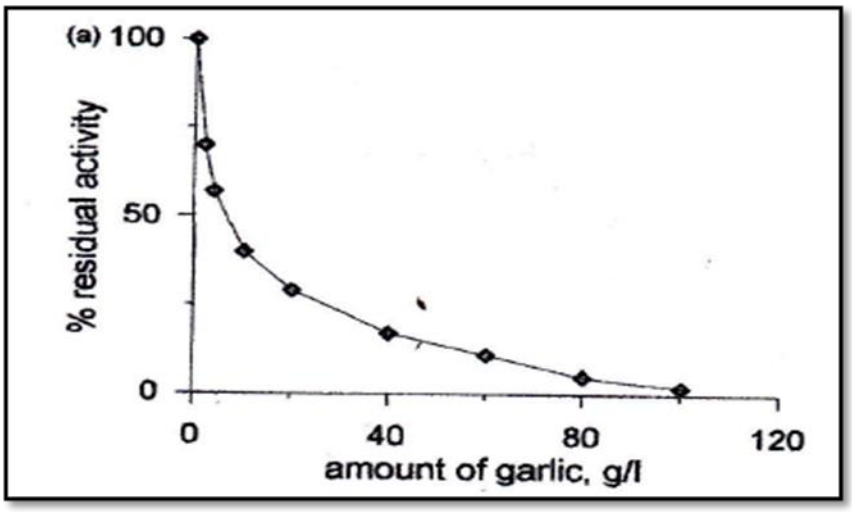

Figure 2.1: Residual activity of urease versus the amount of garlic

Besides, in Zaborska et al. (2009) study, the TS are active inhibitors in the garlic extract and the inhibition of urease is not depends on alk(en)yl group, but on the TS concentration and incubation time with the urease. The 18 minutes incubation of enzyme with the inhibitor causes total loss of urease activity or inactivation (Juszkiewicz et al., 2004). The comparison between garlic extract TS and synthetic allicin in urease activity is shown by Figure 2.2 below (Juszkiewicz et al., 2004).

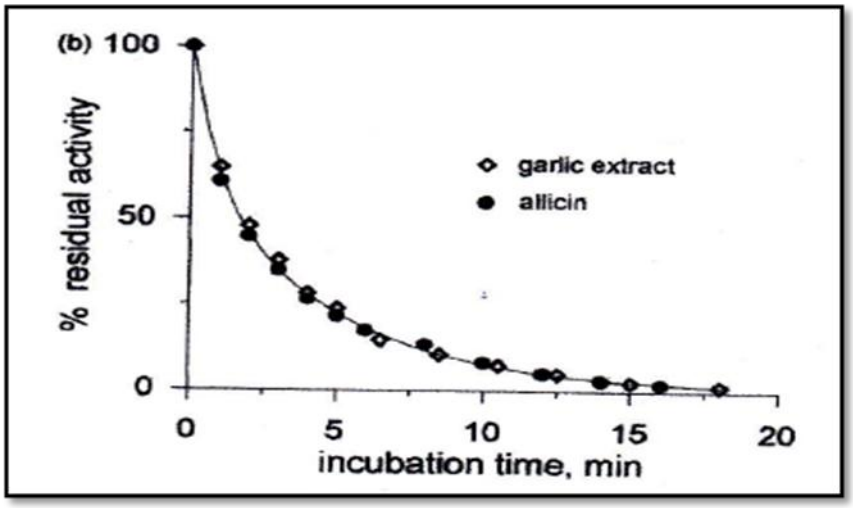

Figure 2.2: The comparison between synthetic allicin and organic TS reaction

The inhibitory strength of garlic extract is determined by storing that garlic extract at cold and room temperature. In Juzkiewicz et al., 2004 study, extract stored at cold temperature is relatively stable and manage to retain over 40% of its inhibitory effect. Meanwhile at room temperature, the extract showed only 3% of its inhibitory potential after 57 days. Refer Figure 2.3 (Juszkiewicz et al., 2004).

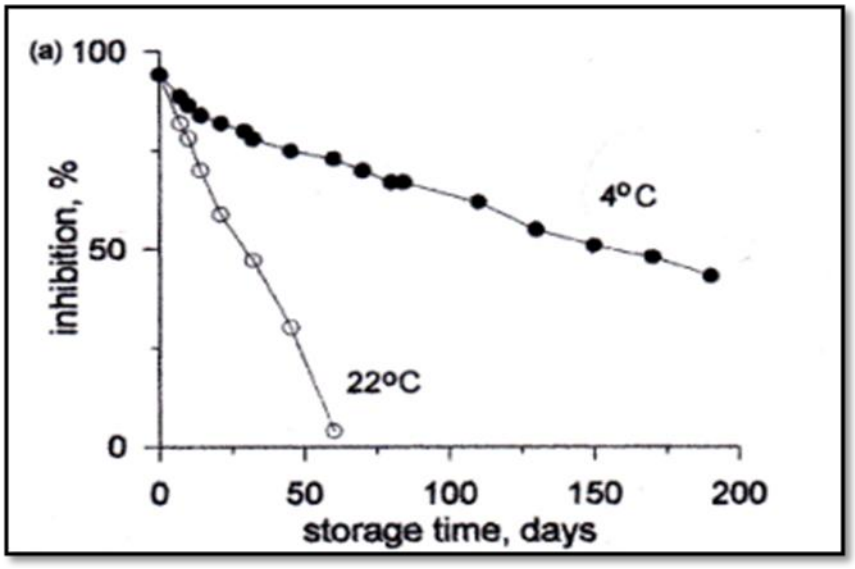

Figure 2.3: Temperature effect on inhibitory potential

These data provide evidence on actual inhibiting agents in the garlic extract is TS. According to Juszkiewicsz et al. (2004) study, TS is not stable and transform into more stable components which are polysulfides and thiosulphonates.

Moreover, the urease inhibition by TS is irreversible (Zaborska, 2009). In order to keep nutrient on plant after inhibition, thiol containing compound have been tested for its ability to reverse and protect against the inactivation of urease by garlic extract. As Karajewska, Zaborska & Chuddy (2004) re-cited again by Zaborska (2009) stated that monothiols such as cysteine, 2-ME, glutathione and dithiol (DTT) were added before incubation.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Garlic Extract Preparation

This experiment used the garlic extract available in market (Allimax) due to its known TS concentration. One capsule of Allimax contains 180 mg of TS. Garlic extract solution is prepared by weighing 6 gram of garlic extract powder from Allimax's capsule and diluted with 300 ml of distilled water (Z. Olech & W. Zaborska, 2012).

Figure 3.1: Garlic Extract from Allimax

Shake the solution using incubated shaker for 30 mins. After 30 mins, filter the garlic solution through filter net and take the extract. However, there are still some micro impurities inside the garlic extract. According to Z. Olech and W. Zaborska (2012), these impurities were removed by centrifugation at 300xg for 4 mins. After centrifugation, filter the extract again through filter paper. During handling this methodology, it is advice to use proper gloves and face mask since garlic extract produce strong smell of NH_3. Then the garlic extract is stored at two different temperatures: 22°C and 4°C.

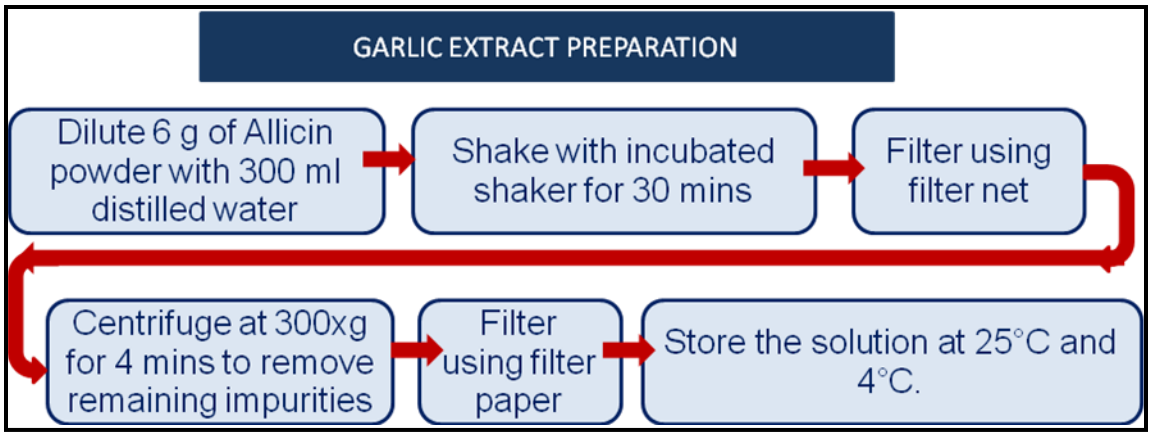

Figure 3.2: Summary of Garlic Extract Preparation

3.2 UV-VIS Spectrophotometer Device Start-Up

Almost all samples been examined for its absorbance using UV-VIS spectrophotometer device. This device is operated under wavelength range 190-800 nm and it detects sample's absorbance along these wavelengths. As a start, power ON the device and wait for 30 mins for its internal calibration. Then, place cuvette containing sample in device's sample slot as in Figure 3.3 and click 'Start' in computer screen. After a few minutes, the graph and data will display on screen as in Figure 3.4.

Figure 3.3: Sample Slots in UV-VIS Spectrophotometer Device

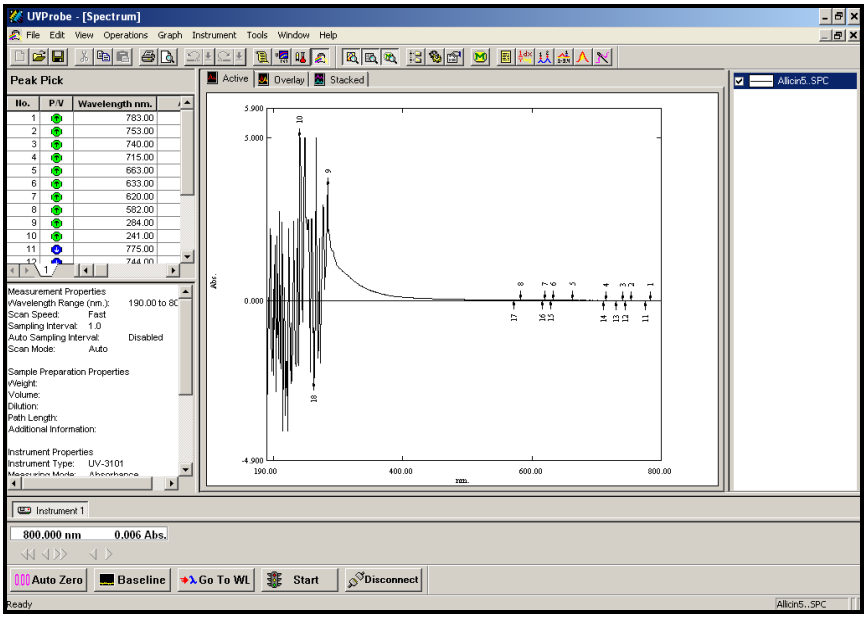

Figure 3.4: Example of Software Display for UV-VIS Analysis

3.3 Garlic Extract Analysis

Take a portion of Allimax's garlic extract sample and analyze for TS content using UV-VIS spectrophotometer. For accurate result, analyze stock garlic extract and some extract dilutions as in Table 3.1.

Table 3.1: Garlic Extract Dilution Ratio

Sample No.	Garlic Extract stock (ml)	Distilled Water (ml)
1	5	0
2	5	10
3	5	20
4	5	30

3.3 Inhibition Studies

For inhibition studies, two mixtures need to be prepared which are: standard assay mixture and urease mixtures. For standard assay, prepare the solution by mixing all material listed below:

1. 50 mM urea
2. 50 mM phosphate buffer of pH 7.8
3. 2 mM ethylenediaminetetraacetic acid (EDTA)

Next, prepare the urease solution by mixing equal volume of jack bean urease and garlic extract solution. Then, the mixture was incubated at 25°C. According to Z. Olech and W. Zaborska (2012), the mixture must always consist of:

1. 2 mg solid/ml jack bean urease
2. 2 mg solid/ml garlic extract
3. 50 mM phosphate buffer of pH 7.8
4. 2 mM EDTA

It is important to simulate the actual condition of urea fertilizer reaction in soils in order to achieve accurate result. Phosphate buffer is used to control and minimize the NH_3 released throughout reaction by lowering the pH value of mixture. Meanwhile, EDTA is used to ensure nutrients such as zinc, ion and copper available for long time.

The inhibition studies begin by taking 1 ml of urease mixture and place into the standard assay mixture. Stir the mixture gently and analyze the absorbance of mixture using UV-VIS spectrophotometer device. It is important to analyze the mixture immediately right after transferring urease into assay. After 2 mins, take another 1 ml of urease mixture and repeat the same procedure and observe the graph trends.

3.4 Key Milestone

Key milestone is known as the significant event happens in a project. The first milestone for this experiment is data analysis for garlic extract. Garlic extract was expected to contain TS compound. According to Dr. H. Hinna (2007), thiols chromophores can be determined at wavelength 210 nm by using UV-VIS spectrophotometer device. Then, the second milestone is on analyzing the inhibitory effect of garlic extract. The expected results from this milestone are:

1. The concentration of NH_3 decreases with increase amount of garlic mixture in reaction.
2. The concentration of NH_3 decreases with time.

3.5 Project Scheduling

No	Calendar Week	1	2	3	4	5	6	7	MIDSEM BREAK	8	9	10	11	12	13
1	Research Project Continuation														
2	Prepare Garlic Extract + Analyzing Sample using Spectrophotometer Machine														
3	Prepare Standard Solution from Garlic Extract - Different Concentration Being Used														
4	Analyzing Data for Standard Sample									★					
5	Chemicals Delivering - Avantis														
6	Mixture of Urease + Garlic Extract Solution Preparation														
7	Urease Solution Preparation														
8	Analyzing Inhibitory Effect on Urease using Spectrophotometer Machine											★	★		
9	Finalize Result														
10	Data and Report Compilation														

Experimental Work/Progress

★ Key Milestone

3.7 Equipments

A spectrophotometer is an instrument that measures the amount of light absorbed on a sample (Mostafa et al., n.d). This machine works by measuring the light intensity in a function of wavelength in a cuvette placed in spectrophotometer. According to the Beer-Lambert law, the amount of light absorbed at these wavelengths is directly proportional to the concentration of the solution. This machine has ability to measure the concentration of the solution, identify organic compound by determining absorption maximum, and color determination within the spectral range. The spectrophotometer will absorbed wavelength of the sample and reflected in a way to graph. From the graph, the amount of NH_3 will decrease with respect to time and during 18 minutes incubation, the NH_3 will be totally lost.

Centrifugal machine is another device that been used in this experiment. The purpose of usage is to remove the remaining materials after first filtration of garlic extract and allicin. This machine separates liquids at different densities by rotating the bottle contains liquids at high speed. Heavier materials are thrown out farther from the center of rotation and separate from lighter materials due to centrifugal force.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Temperature for Garlic Extract Storage

Garlic extracts produced need to be stored properly at right condition. Therefore, the comparison between two garlic extract stored at two different temperatures: 25°C (room temperature) and 4°C (refrigerator) is conducted. According to P. Helen (2012), when heat is applied, garlic changes its colors due to reaction of its own enzyme. The original color of garlic extract was light greenish. After one day, garlic extract stored at 25°C shows slight change in color, which is brownish and garlic sample stored at 4°C remain as it original color as shown in Figure 4.1.

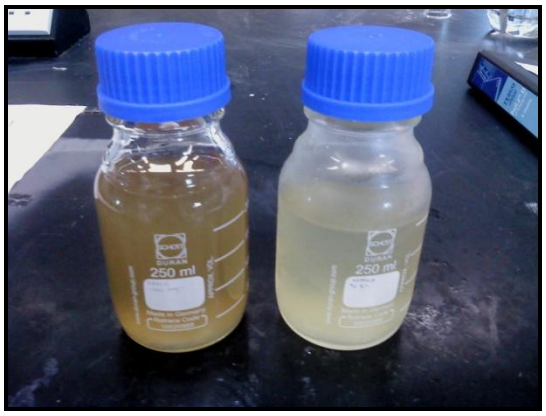

Figure 4.1: Garlic extract stored at 25°C (left) and 4°C (right) after 1 day

An aliquot of garlic extract stored at 25°C is being transferred into the UV-VIS Spectrophotometer device. As shown in Figure 4.2, the garlic extract stored at 25°C shows negative absorbance on sample test run. The negative value indicates no UV light being absorbed by the sample due to porous structure.

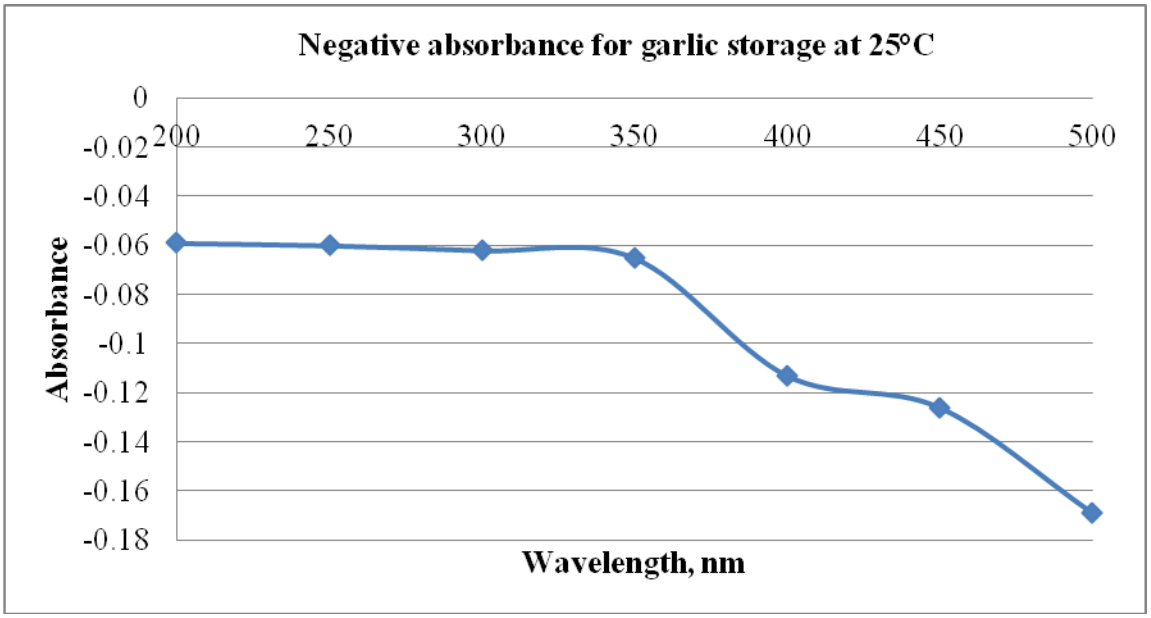

Figure 4.2: Absorbance result at 25°C garlic extract storage temperature

Therefore, the most suitable storage temperature for garlic extract is at 4°C. This temperature allows garlic extract to keep its strength without rusting the structure of the extract.

4.2 Garlic Extract Analysis

The availability of TS contents in Allimax's garlic extract is determined by comparing data from UV-VIS spectrophotometer and standard TS wavelength. Theoretically, in Beer's Lambert law, the absorbance versus wavelength chart is given in Equation 1 (S.Svanberg, 2006).

$$A = \varepsilon bc \quad . \quad . \quad . \quad . \quad (1)$$

Where: $A = absorbance$

$\varepsilon = molar\ absorptivity/constant$

$b = slit\ width$ (1 cm)

$c = concentration$ (mol/L)

In spectrophotometer device, liquid/solid sample absorbs some portions of ultraviolet light and transmits the remaining light to the detector. The detected light or transmittance will convert into absorbance and displayed it in the form of graph. The highest peak of absorbance at certain range of wavelength determined the functional group of components in sample.

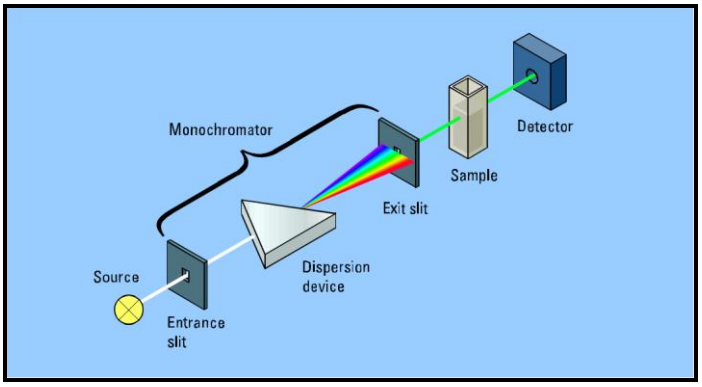

Figure 4.3: UV-VIS spectrophotometer arrangement

According to Shanghai Laser & Optics Century Co (2004), allicin compound found in TS falls in range of far ultraviolet and the laser types found between these ranges are:

1. Xenon Chloride (XeCl)
2. Argon Flouride (ArF)
3. Krypton Chloride (KrCl)
4. Krypton Flouride (KrF)

The absorbance data obtained from UV-VIS Spectrophotometer of stock garlic extract and garlic extract dilutions are shown in Table 4.1. Meanwhile, the graph to analyze the availability of TS content in garlic extract is shown in Figure 4.3.

Table 4.1: Allicin data at different controlled concentration

Wave length (nm)	Volume of Distilled Water			
	0ml	10ml	20ml	30ml
190	-4.00	2.88	1.94	0.50
200	0.34	-0.06	0.46	-2.09
210	2.74	-2.03	2.79	1.10
220	-1.86	0.50	-2.18	-2.47
230	-0.92	2.61	-2.15	2.12
240	0.50	-0.31	0.50	-0.48
250	2.56	2.79	5.00	-2.17
260	2.52	2.83	0.31	2.70
270	-1.32	-1.57	1.73	-2.07
280	0.44	0.76	3.12	0.28
290	1.57	1.02	0.54	0.37

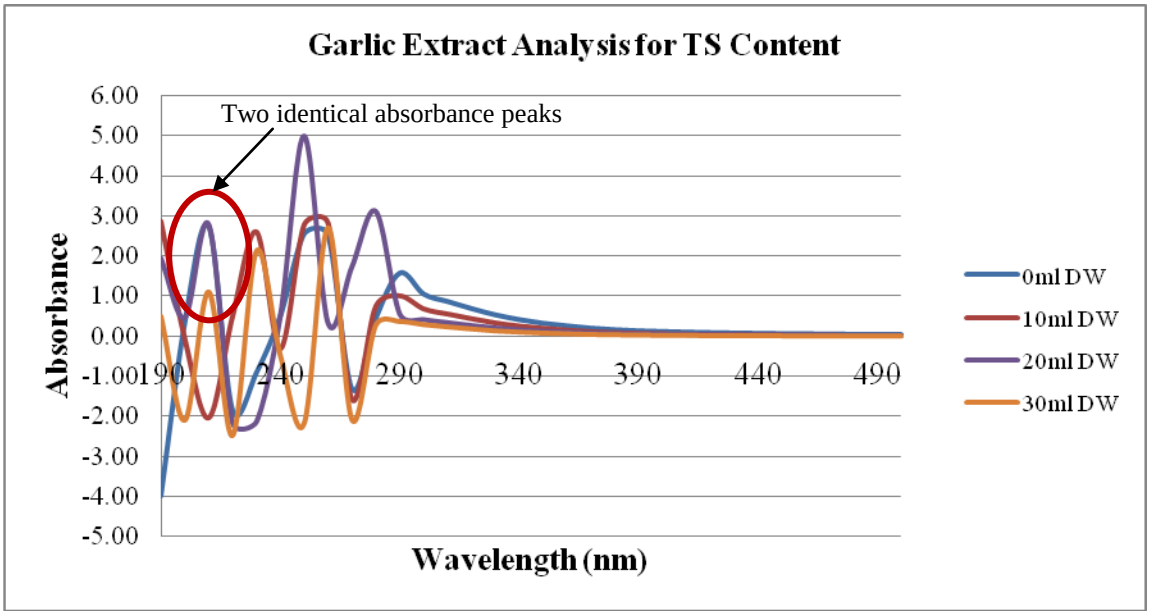

Figure 4.4: Graph for different concentration of allicin

Based on Figure 4.4, garlic extract's peak absorbance falls in ranges of 190 – 290 nm. There are identical absorbance peaks between stock garlic extract and 20ml dilution which falls at wavelength 210 nm. This data was identical with P. Bocchini et al. (2001) TS standard data. TS from thiol group contains allicin compound has its own absorbance characteristics. Table 4.2 shows the absorbance characteristics for some chromophores. Thiol chromophore wavelength found in Table 4.2 is 210 nm (Dr. H. Hinna, 2007).

Table 4.2: Absorbance characteristics for common chromophores

Chromophore	Transition	λmax	Log (e)
Nitrite	n - π*	160	<1.0
Alkyne	π - π*	170	3.0
Alkene	π - π*	175	3.0
Alcohol	n - σ*	180	2.5
Ether	n - σ*	180	3.5
Ketone	π - π*	180	3.0
	n - π*	280	1.5
Aldehyde	π - π*	190	2.0
	n - π*	290	1.0
Amine	n - σ*	190	3.5
Ester	n - π*	205	1.5
Amide	n - π*	210	1.5
Thiol	n - σ*	210	3.0
Nitro	n - π*	271	<1.0
Azo	n - π*	340	<1.0

Transition of thiol chromophores from n - σ* shows its electronic transition which is from non-bonding to antibonding. This transition occurs in saturated compound containing atoms with lone pairs. In inhibitory reaction, the lone pairs of TS will attack urease lone pairs and blocking the reaction to occurs.

To summarize, the analysis of Allimax's garlic extract detects high absorbance at wavelength 210 nm. This data is equivalent with TS standard data from Dr. H. Hinna (2007) and P. Bocchini et al. (2001). Therefore, Allimax's garlic extract contains thiols group and allicin compound is its active component.

4.3 Inhibition Studies

Main part of this research is to determine the potential of garlic extract to inhibit urease enzyme in urea fertilizer reaction. At beginning, two mixtures have been prepared: standard assay mixture containing urea and urease-garlic mixture. Both mixtures were incubated at room temperature. Based on urea fertilizer reaction equation, if garlic extract is introduced at urease enzyme's active site, the shape of urease enzyme is no longer match and fit with the shape of substrate (standard assay mixture) as in Figure 4.5.

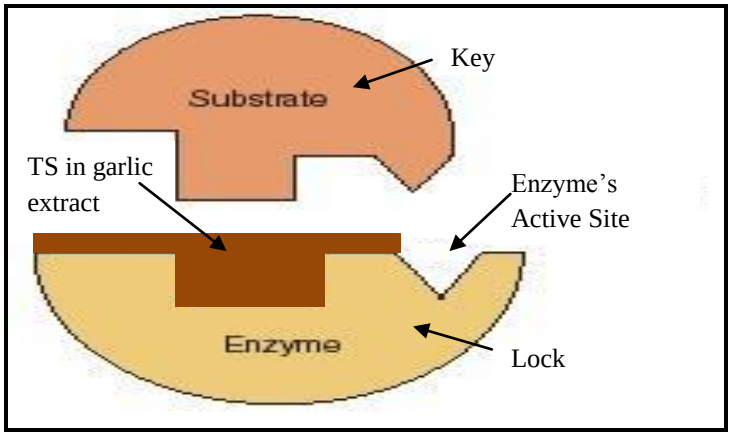

Figure 4.5: TS in garlic extract is introduce in urease enzyme's active site

Every 1 ml of urease-garlic mixture added into standard assay mixture shows different absorbance characteristics in UV-VIS spectrophotometer analysis. According to H. P. Dong et al. (2010), highest absorbance of free NH_3 is detected at wavelength 370 nm by using UV light. Figure 4.6 shows the standard calibration curve of the absorbance at 370 nm versus the concentration of free NH_3 (H. P. Dong et al., 2010).

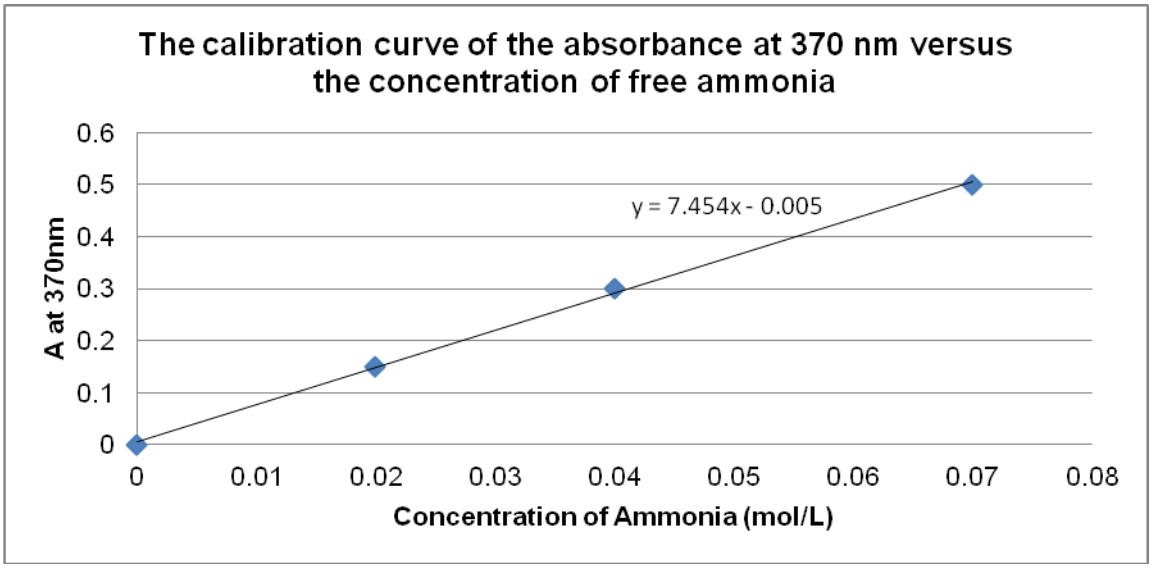

Figure 4.6: Standard Calibration Curve for Free NH_3

Beer's Lambert Law state that it has linear relationship between absorbance and concentration. Therefore, 7.454 is the slope value of the standard calibration curve and the value is equivalent with the molar absorptivity constant, ε of Equation 1. Thus, the new equation in determining NH_3 concentration is:

$$c = \frac{(7.454)(1)}{A} \quad . \quad . \quad . \quad . \quad (2)$$

The absorbance data of urea-urease-garlic reaction are varies from wavelength 190 nm to 800 nm. However, based on standard calibration graph for free NH_3, absorbance data recorded at 370 nm are only required for this inhibitory study as shown in Table 4.3.

Table 4.7: Raw Data of NH_3 Absorbance at 370 nm

Wave length (nm)	Amount of Urease-Garlic Mixture (ml)														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
350	0.148	0.126	0.122	0.120	0.111	0.108	0.106	0.105	0.103	0.104	0.105	0.103	0.103	0.035	0.069
360	0.142	0.123	0.121	0.118	0.107	0.105	0.105	0.104	0.101	0.103	0.101	0.102	0.100	0.03	0.034
370	0.135	0.120	0.116	0.113	0.103	0.101	0.101	0.100	0.098	0.098	0.096	0.083	0.067	0.029	0.008
380	0.129	0.114	0.111	0.108	0.098	0.095	0.096	0.095	0.093	0.093	0.092	0.084	0.091	0.024	0.024
390	0.131	0.115	0.113	0.109	0.101	0.098	0.098	0.097	0.095	0.095	0.094	0.082	0.093	0.027	0.093
400	0.128	0.113	0.110	0.107	0.098	0.095	0.096	0.094	0.093	0.093	0.091	0.090	0.090	0.025	0.091

Subsequently, Equation 2 is used to determine the concentration for each amount of urease-garlic mixture in standard assay solution. The result is shown in Table 4.4 and Figure 4.6.

Table 4.4: Result of NH_3 Concentration at 370 nm using Beer's Lambert Law

Urease-Garlic Mixture (ml)	Absorbance at 370 nm	Concentration (mol/L)
1	0.135	0.018
2	0.120	0.016
3	0.116	0.016
4	0.113	0.015
5	0.103	0.014
6	0.101	0.014
7	0.101	0.014
8	0.100	0.013
9	0.098	0.013
10	0.098	0.013
11	0.096	0.013
12	0.083	0.011
13	0.067	0.009
14	0.029	0.004
15	0.008	0.001

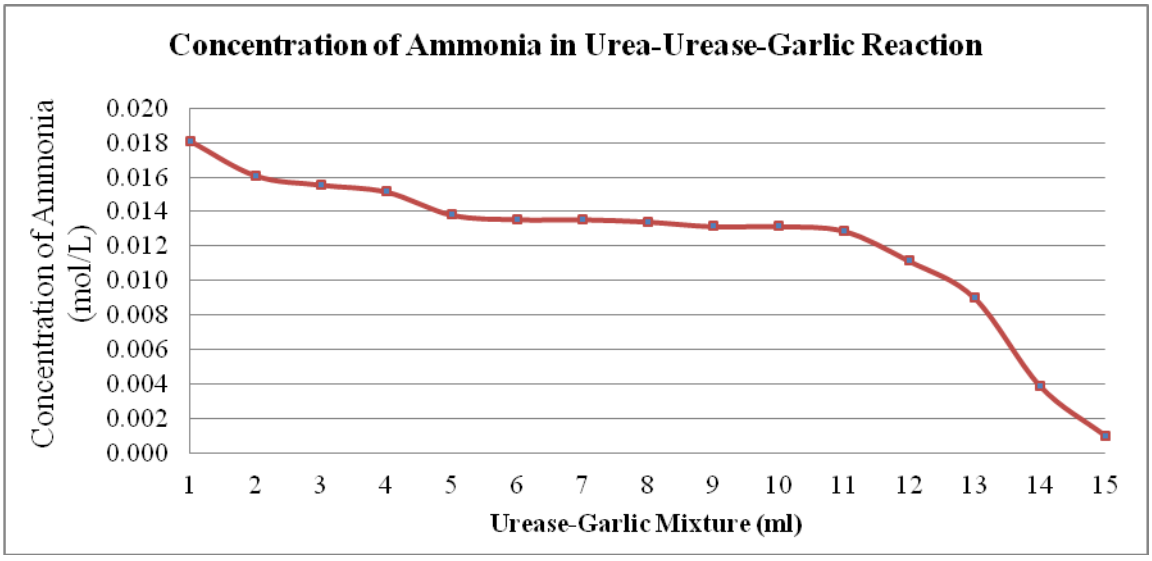

Figure 4.7: NH_3 concentration with increase amount of urease-garlic mixture

Figure 4.7 illustrate the effect of TS in urea-urease-garlic reaction on NH_3 release. With increase amount of urease-garlic mixture in urea or standard assay mixture shows reduction in NH_3 release from the reaction. The lowest concentration of NH_3 detected by UV-VIS spectrophotometer is at 15 ml of urease-garlic mixture which is 0.001 mol/L. In real situation, applied urea on wet plantation area was able to increase its efficiency by lowering the amount of NH_3 released. This is because the formation of NH_3 from urea fertilizer is delayed with additional bio-inhibitor into the reaction. Although the mechanism of inhibition is similar with current inhibitor, but the bio-inhibitor used is safe to health and environment.

Furthermore, 2 mins of incubation time for each amount of urease-garlic mixture added is important to integrate the urea-urease-garlic reaction. The result is shown in Table 4.5 and Figure 4.8.

Table 4.5: NH_3 Concentration at 370 nm with incubation time

Time (mins)	Concentration (mol/L)
2	0.018
4	0.016
6	0.016
8	0.015
10	0.014
12	0.014
14	0.014
16	0.013
18	0.013
20	0.013
22	0.013
24	0.011
26	0.009
28	0.004
30	0.001

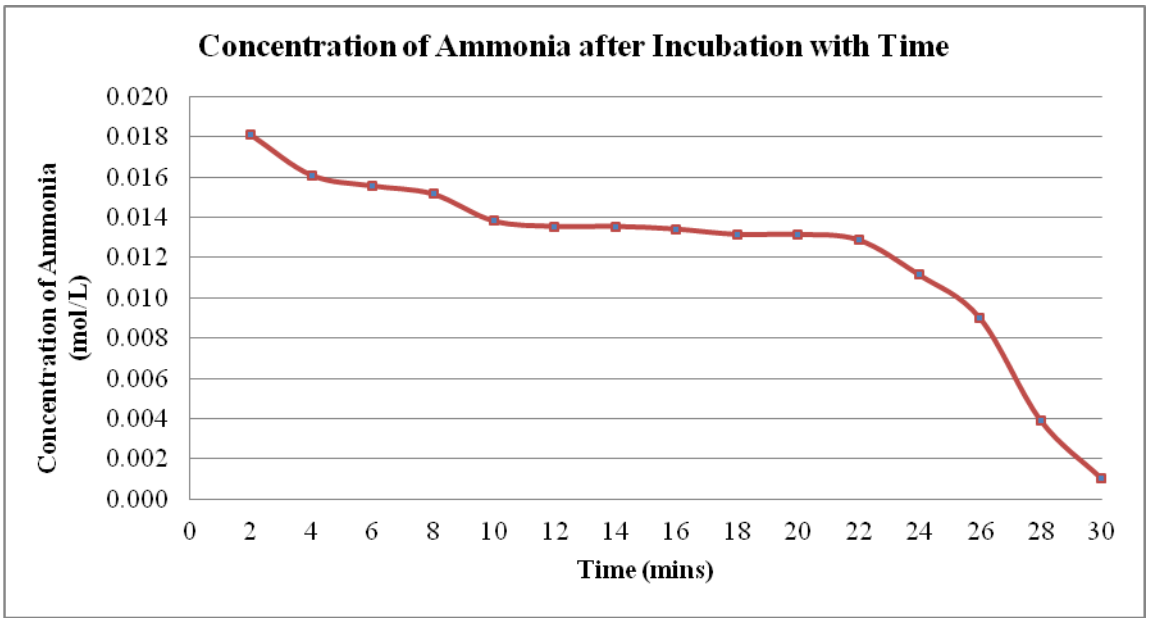

Figure 4.8: NH_3 concentration with time

In Figure 4.8, the urea-urease-garlic reaction takes 30 mins of incubation to achieve the lowest NH_3 emission. Consider this value and compare with Juszkiewicz et al., (2004) study which takes 18 mins of incubation. The time taken is different due to different mixture content used to determine the inhibitory effect.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Relevancy to Objective

The purpose of this research is to determine the potential of organic compound in garlic which is TS, as a new bio-inhibitor in agricultural industry. Current inhibitor products are chemical based and non-biodegradable which causes environmental issues. Agrotain and Arborite are examples of inhibitors containing NBPT which has been found unsafe to being applied at the surface of soil by Department of Health and Ageing, Australia.

Throughout the experiment, the garlic extract was stored at 4°C to maintain its strength without damaging the chemical structure of the garlic. UV-VIS spectrophotometer is the main device used to analyze the compound in sample. The absorbance of TS contained in Allimax's garlic extract shown its similarity with standard absorbance value which is 210 nm. Hence, this garlic extract brand is suitable to become a potential bio-inhibitor. Meanwhile, by observing NH_3 released at different amount of garlic decides whether TS has potential to block the reaction of urease in urea fertilizer. With increase amount of urease-garlic mixture, the concentration of NH_3 is decreasing. Therefore, garlic extract containing TS has shown its potential to become an effective bio-inhibitor in urea fertilizer application.

Besides, the consideration on urease activity with incubation time may support the result before. With time, the urease activities are decrease and stopped at certain period of time. This behavior shows TS has potential to to inhibit urease enzyme in 30 mins after application.

5.2 Future Recommendation

The most excellent condition to conduct the experiment is in a room with constant temperature and humidity. The temperature and humidity are found can affect the strength and color of inhibitor. Besides, the UTP laboratories should increase the unit of spectrophotometer device to have accurate measurement with respect to time. Scheduled calibration also needed.

Meanwhile, it is possible to conduct a research on determination of NH_3 for the standard curve rather than obtaining the universal standard. Besides, the research on inhibitory experiment can be improve further by using another type of organic component such as rosemary plant or camphor tree to observe the effectiveness of using another type of local plant.

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APPENDIX

Appendix 1: Raw data of NH_3 absorbance from 190 nm to 800 nm.

Wave length (nm)	**Amount of Urease-Garlic**														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
190	4.366	4.909	4.579	4.394	4.608	4.608	4.608	5	4.758	4.67	4.278	4.486	4.667	4.608	4.608
200	4.909	5	5	5	5	5	5	5	5	4.909	5	5	5	5	5
210	3.996	3.986	4.038	3.927	3.828	4.185	4.237	4.03	4.228	4.055	4.294	3.88	3.79	3.712	3.836
220	1.994	1.963	2.025	1.889	1.766	2.21	2.319	2.031	2.276	2.041	2.363	1.825	1.717	1.596	1.784
230	0.976	0.943	0.977	0.903	0.831	1.083	1.15	0.973	1.127	0.973	1.181	0.864	0.805	0.689	0.837
240	0.555	0.523	0.538	0.502	0.462	0.584	0.616	0.525	0.601	0.524	0.628	0.47	0.442	0.341	0.458
250	0.304	0.272	0.281	0.262	0.237	0.289	0.3	0.26	0.288	0.258	0.302	0.235	0.222	0.13	0.225
260	0.224	0.188	0.191	0.184	0.162	0.181	0.189	0.171	0.18	0.167	0.185	0.155	0.149	0.062	0.152
270	0.202	0.17	0.173	0.166	0.147	0.161	0.162	0.148	0.154	0.146	0.16	0.14	0.136	0.05	0.136
280	0.205	0.172	0.177	0.169	0.149	0.159	0.164	0.153	0.158	0.151	0.163	0.144	0.14	0.058	0.142
290	0.203	0.177	0.177	0.171	0.157	0.164	0.165	0.156	0.159	0.153	0.162	0.148	0.147	0.065	0.146
300	0.199	0.169	0.173	0.169	0.151	0.153	0.161	0.154	0.151	0.152	0.152	0.147	0.143	0.067	0.145
310	0.179	0.157	0.159	0.154	0.139	0.139	0.142	0.135	0.135	0.136	0.137	0.135	0.131	0.056	0.13
320	0.169	0.143	0.139	0.141	0.125	0.124	0.125	0.124	0.123	0.118	0.123	0.117	0.117	0.043	0.119
330	0.157	0.133	0.132	0.128	0.117	0.118	0.116	0.111	0.108	0.112	0.113	0.111	0.11	0.039	0.108
340	0.154	0.128	0.124	0.126	0.115	0.109	0.111	0.11	0.107	0.104	0.106	0.104	0.104	0.034	0.1
350	0.148	0.126	0.122	0.12	0.111	0.108	0.106	0.105	0.103	0.104	0.105	0.103	0.103	0.035	0.069
360	0.142	0.123	0.121	0.118	0.107	0.105	0.105	0.104	0.101	0.103	0.101	0.102	0.1	0.03	0.034
370	0.135	0.12	0.116	0.113	0.103	0.101	0.101	0.1	0.098	0.098	0.096	0.083	0.067	0.029	0.008

380	0.129	0.114	0.111	0.108	0.098	0.095	0.096	0.095	0.093	0.093	0.092	0.084	0.091	0.024	0.024
390	0.131	0.115	0.113	0.109	0.101	0.098	0.098	0.097	0.095	0.095	0.094	0.082	0.093	0.027	0.093
400	0.128	0.113	0.11	0.107	0.098	0.095	0.096	0.094	0.093	0.093	0.091	0.09	0.09	0.025	0.091
410	0.083	0.068	0.065	0.062	0.053	0.05	0.05	0.049	0.048	0.048	0.047	0.056	0.047	0.047	0.047
420	0.083	0.067	0.065	0.062	0.053	0.05	0.05	0.049	0.047	0.048	0.046	0.047	0.046	0.046	0.046
430	0.082	0.066	0.063	0.061	0.052	0.048	0.05	0.048	0.047	0.047	0.046	0.047	0.045	0.045	0.046
440	0.081	0.066	0.063	0.06	0.052	0.048	0.049	0.048	0.047	0.047	0.045	0.046	0.045	0.045	0.045
450	0.081	0.065	0.062	0.06	0.051	0.048	0.049	0.048	0.046	0.047	0.045	0.045	0.045	0.045	0.045
460	0.08	0.064	0.062	0.059	0.051	0.048	0.049	0.048	0.046	0.046	0.044	0.045	0.044	0.044	0.044
470	0.079	0.064	0.062	0.059	0.051	0.047	0.048	0.047	0.046	0.046	0.044	0.045	0.044	0.044	0.044
480	0.079	0.063	0.061	0.058	0.05	0.047	0.047	0.046	0.045	0.045	0.044	0.045	0.044	0.044	0.044
490	0.078	0.063	0.06	0.058	0.05	0.046	0.047	0.046	0.045	0.045	0.043	0.045	0.043	0.043	0.044
500	0.078	0.062	0.06	0.058	0.05	0.046	0.047	0.047	0.045	0.045	0.043	0.044	0.043	0.043	0.043
510	0.078	0.062	0.06	0.057	0.05	0.046	0.047	0.046	0.046	0.044	0.043	0.044	0.043	0.043	0.043
520	0.077	0.062	0.059	0.057	0.049	0.046	0.046	0.046	0.044	0.045	0.043	0.044	0.043	0.043	0.043
530	0.078	0.062	0.059	0.057	0.05	0.046	0.047	0.046	0.045	0.045	0.043	0.044	0.044	0.044	0.043
540	0.078	0.062	0.059	0.058	0.05	0.045	0.047	0.046	0.045	0.045	0.043	0.045	0.043	0.043	0.044
550	0.077	0.06	0.057	0.055	0.049	0.045	0.046	0.045	0.043	0.043	0.041	0.041	0.042	0.042	0.042
560	0.078	0.061	0.058	0.056	0.049	0.045	0.046	0.046	0.045	0.045	0.043	0.044	0.043	0.043	0.043
570	0.075	0.059	0.055	0.054	0.047	0.043	0.044	0.043	0.043	0.042	0.04	0.041	0.041	0.041	0.04
580	0.076	0.06	0.058	0.057	0.048	0.044	0.046	0.044	0.045	0.044	0.042	0.044	0.042	0.042	0.042
590	0.075	0.06	0.057	0.055	0.049	0.045	0.046	0.045	0.044	0.044	0.042	0.042	0.042	0.042	0.042
600	0.074	0.059	0.056	0.054	0.047	0.043	0.045	0.044	0.044	0.043	0.041	0.042	0.041	0.041	0.041
610	0.066	0.067	0.069	0.061	0.058	0.055	0.05	0.047	0.045	0.045	0.041	0.041	0.044	0.041	0.044

620	0.067	0.066	0.068	0.061	0.058	0.055	0.05	0.046	0.044	0.043	0.042	0.042	0.046	0.042	0.043
630	0.067	0.066	0.069	0.062	0.058	0.055	0.051	0.048	0.044	0.045	0.041	0.041	0.05	0.041	0.043
640	0.067	0.066	0.068	0.061	0.057	0.055	0.05	0.047	0.044	0.044	0.044	0.044	0.049	0.044	0.043
650	0.066	0.064	0.066	0.061	0.057	0.054	0.05	0.045	0.044	0.044	0.046	0.046	0.041	0.046	0.044
660	0.063	0.063	0.066	0.059	0.055	0.053	0.048	0.046	0.043	0.045	0.05	0.05	0.042	0.05	0.047
670	0.063	0.062	0.063	0.058	0.054	0.052	0.047	0.043	0.043	0.044	0.049	0.049	0.041	0.049	0.049
680	0.06	0.06	0.063	0.056	0.053	0.05	0.047	0.044	0.042	0.044	0.045	0.044	0.044	0.041	0.05
690	0.058	0.059	0.06	0.054	0.051	0.049	0.044	0.043	0.041	0.044	0.045	0.044	0.046	0.041	0.048
700	0.056	0.055	0.059	0.054	0.051	0.048	0.046	0.043	0.042	0.044	0.045	0.045	0.05	0.045	0.047
710	0.055	0.055	0.059	0.052	0.05	0.048	0.044	0.043	0.041	0.043	0.043	0.041	0.049	0.045	0.049
720	0.057	0.055	0.057	0.055	0.052	0.049	0.046	0.044	0.044	0.043	0.045	0.042	0.043	0.045	0.044
730	0.056	0.057	0.061	0.055	0.053	0.051	0.049	0.047	0.046	0.042	0.042	0.041	0.041	0.043	0.045
740	0.06	0.06	0.062	0.058	0.056	0.055	0.051	0.049	0.05	0.041	0.044	0.044	0.043	0.045	0.041
750	0.058	0.057	0.062	0.057	0.055	0.052	0.052	0.05	0.049	0.042	0.044	0.046	0.04	0.042	0.041
760	0.057	0.058	0.06	0.055	0.053	0.053	0.049	0.048	0.048	0.041	0.043	0.05	0.042	0.044	0.042
770	0.055	0.055	0.058	0.053	0.052	0.051	0.049	0.047	0.049	0.044	0.045	0.049	0.042	0.044	0.041
780	0.053	0.055	0.058	0.051	0.051	0.05	0.048	0.049	0.047	0.046	0.047	0.046	0.041	0.043	0.044
790	0.053	0.054	0.055	0.051	0.05	0.048	0.048	0.044	0.046	0.05	0.046	0.05	0.041	0.045	0.046
800	0.051	0.05	0.054	0.05	0.048	0.047	0.046	0.045	0.044	0.049	0.044	0.049	0.042	0.047	0.05