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Kinetics of Water Disinfection with *Moringa Oleifera* Seeds Extract

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Abstract
Moringa seeds extract was prepared using de-fatted seed cake and aqueous extraction. One mL of Moringa extract was added to 10mL of the cell cultures and incubated for 0, 30min, 60min, 90min, and 120min without agitation. Cell survival was assessed by making dilution series of bacterial suspensions obtained after each incubation period, plating on non-selective LB medium agar dishes, and incubating for 48hours at 37^{o}C. Duplicates were made of every individual assay. Colonies were counted on dishes and the cell survival ratio was estimated by comparison to a control experiment where no Moring extract was added (0 min incubation time). Pseudo-first-order kinetics was fitted to the data. The log-inactivation of the "viable" organisms at time t (N_t) divided by the number of "viable" organisms at time zero (N_0) was plotted as a function of chemical dose, Ct (mg min L $^{-1}$). The coefficient of specific lethality, $\Lambda_{cw,}$ was determined by linear regression using MINITAB for Windows Release 11.2 32Bit. A kinetic model was developed for the application of Moringa Oleifera seeds extract in water disinfection. The coefficient of specific lethality (Λ_{cw}) was determined as 3.76 L mg^{-1} min^{-1} for E.Coli inactivation using Moringa Oleifera seeds extracts. The mode of attack of the Moringa seeds extract on the E.Coli cell was explained as by rupturing the cell and damaging the intercellular components, when water dips in to the cell which causes it to swell more and burst leading to death.
Keywords: Moringa Oleifera, Seed Extract, Antimicrobial Activity, Disinfection Kinetics.

1. Introduction

1.1 Disinfection and Disinfectants

Although the addition of halogens readily comes to mind when talking about disinfection, other unit processes also provide rather substantial degree of disinfection. Robeck *et al.* (1962) for instance, found that up to 98% of poliovirus type I was removed in a coal and sand filter and up to 99% was removed when alum dose was increased and conventional flocculation and sedimentation carried out ahead of the filter. Alum coagulation was also found to remove 95% to 99% of Coxsackie virus, and Ferric chloride was found to remove 92% to 94% of the same Virus (Berg 1967; Fair *et al*. 1958). Although these methods cannot be relied upon to provide the only means of pathogenic removal, Hudson (1962) noted that they perform an important role in assuring the ability of other disinfectants to completely disinfect the water.

Traditionally, water disinfection has been achieved by the use of chlorine in many water supply agencies. However, the production of by-products of chlorination such as halogenated organic compounds has been shown to be associated with various ailments in humans (Hwang *et al*. 2008). Goveas *et al.* (2010) for instance, reported that although chlorine is widely used as an inexpensive and potent disinfectant in the United States for drinking water, it has the potential of forming carcinogenic and mutagenic disinfection by-products (DBPs). These, and other similar problems like the high cost of chlorine in developing countries, necessitates the search for natural disinfectants that are safer and cheaper to use.

Humeirah *et al.* (2010) reported that the essential oils from the twig and root of *Goniothalamus macrophyllus* (Annonaceae) obtained from Pasoh Forest, Malaysia exhibited the most notable inhibitory activity (0.3 mg/ml) against Vancomycin intermediate-resistance *Staphylococcus aureus* (*VISA 24)*, *Staphylococcus epidermidis* and *Candida albicans*. Oluseyi *et al*. (2009) also reported that *Buchholzia coriacea* (wonderful kola) posses antimicrobial properties. The report showed that the fresh kola, its hexane extract, and its methanolic extracts showed inhibitory zone of 62mm, 21mm, and 30mm respectively with E. Coli.

A lot of work has been done on the use of *Moringa Oleifera* seeds extract in Coagulation and Softening (Folkard *et al*. 1989; Jahn 1988; Bina, 1991; Okuda, et al 1999; Buthelezi, et al 2009; Okuda, et al, 2001a, b). It

has also been found to contain an active anti-microbial agent (Eilert *et al*, 1981; Madsen *et al*. 1987; Fisch, *et al*. 2004; Suarez *et al*. 2005; Bukar *et al*. 2010, and Walter *et al.* 2011).

Moringa Oleifera seeds extract, having been shown to possess some anti-microbial properties by earlier researchers, needs further investigation in terms of evaluating the best method of processing the seeds for its application in the disinfection of portable water supplies, and understanding the mechanism of its disinfection action (Suarez *et al.* 2005).

Suarez *et al* (2003) had earlier reported that Moringa seeds protein may be a viable alternative to chemicals commonly used as food preservatives or for water disinfection. They are unlikely to have toxic effects as the seeds are currently used for the treatment of drinking water and for preparation oils for various foods. Another advantage reported is their biodegradability, unlike other chemicals (e.g. Aluminium salts and chlorine) which remain as contaminants of treated waters and of the sediments.

Bichi (2011) has shown that its highest disinfection action was achieved with the use of de-fatted seed cake and extracting the active ingredients by aqueous extraction. Bichi (2012) also found that the optimal conditions for the extraction of the bioactive compounds to be 31 minutes mixing time, 85 rpm mixing speed and 3.25 mg/mL Moringa dosage. This research further developed a Quadratic model that can be used to optimize the process of antimicrobial bioactive compound extraction from de-fatted Moringa Oleifera seeds.

The aim of this segment of the research is to develop the kinetic equation for the application of Moringa Oleifera seeds extract in water disinfection, and investigate the mode of attack of the extract on the microbes using E. Coli as an indicator organism.

1.2 Rate of Disinfection

Under ideal conditions, all cells of a single species of organisms are discrete units equally susceptible to a single species of disinfectant; both cells and disinfectants are uniformly distributed (or dispersed) in the water, the disinfectant stay. Substantially unchanged in chemical composition and substantially constant in concentration throughout the period of contact; and the water contains no interfering substances. Under such conditions according to Metcalf & Eddy (1991), the rate of disinfection is a function of (i) time of contact, (ii) Concentration of Disinfectant, (iii) Concentration of organisms and (iv) Temperature of Disinfection.

1.2.1 Concentration of Organism: Chick's Law

The specific mechanism of microorganism inactivation during chemical disinfection is not well understood (Fair *et al*. 1958; MetCalf & Eddy 2003). It was, however, understood to be a function of chemical disinfection agent, properties of the microorganism, and properties of the water. One simple kinetic model is widely used, but many other models describe the mechanism of disinfection. According to Dr. Harriet Chick - Chick's Law (1908) – disinfection could be modeled as a pseudo-first-order reaction with respect to the concentration of the organisms. The time rate of kill follows chick's law of disinfection. This states that *Y*, the number of organisms destroyed in unit time, is proportional to *N*, the number of organism remaining, the initial number being *No*. The rate of change of organisms with time, $\frac{dy}{dt}$, is given by equation (1).

$$\frac{dy}{dt} = k\,(N_0 - y) \qquad (1)$$

Where, *k* represents the Chick's law rate constant (t^{-1}).

Integrating between the limits $y = 0$ at $t = 0$ and $y = y$ at $t = t$ we obtain equation (2) which is simplified into equations (3a) and (3b).

$$In\,[(N_0 - y)/N_0] = In\,(\frac{Nt}{N0}) = -\,kt \qquad (2)$$

$$(\frac{Nt}{N0}) = exp\,(-kt) \qquad (3a)$$

$$(\frac{Nt}{N0}) = e^{-kt} \qquad (3b)$$

A plot of log N_t/N_0 Vs t traces a straight line with slope of $-klog\,e = -k^1$ and an intercept of 1 (or 100%) at t = 0. When $kt = 1$ or $k^1 t = 0.4343$, the surviving fraction is 0.368 (Fair *et al*. 1958; MetCalf & Eddy 2003).

1.2.2 Concentration of Disinfectant: Watson Law

Watson (1908) reported that the microbial inactivation rate increased with disinfectant concentration. The disinfecting efficiency of a disinfectant is given by the relationship in equation (4).

$$C^n t_p = Constant \qquad (4)$$

Where, *C* represents the Concentration of disinfectant (mg/L), t_p is the time required to effect a constant percentage kills of organisms, *n* is the coefficient of dilution, and the *constant* is a value of given percentage of inactivation. When $n > 1$, the efficiency of the disinfectant decreases rapidly as it is diluted; when $n < 1$, time of contact is more important than dosage.

1.2.3 Kinetics of Disinfection – Chick-Watson Law

Since the early 1900s, it was discovered that disinfectant concentration and contact time were the primary variables affecting microbial inactivation kinetics and efficiency (Cunningham *et al.* 2008). Chick (1908) reported that for a given concentration of disinfectant, longer contact times resulted in an increased microbial

death. Additionally, Watson (1908) reported that the microbial inactivation rate increased with disinfectant concentration. Combining the expressions of Chick and Watson yields the equation that is known as the "Chick–Watson Law":

Taking logs on both sides of equation (4) yields equations (5a) and combining this with the Chicks' equation (3a) produces equation (5b).

$$n.\ log(C) + log(t) = log(constant) \quad (5a)$$

$$\frac{dN}{dt} = -\Lambda_{cw} C^n N = -K_{cw} N \quad (5b)$$

The parameters are as previously defined. After integration we obtain the Chick's-Watson equation (6).

$$In\ (\frac{N}{No}) = -\Lambda_{cw} Ct \quad (6)$$

The constant Λ_{cw} is an empirical constant known as the Chick–Watson coefficient of specific lethality.

Many other researchers have since modified the Chick–Watson Law to account for specific features of the dose response relationships such as lag or tailing (Haas 1980; Lambert & Johnson 2000).

1.3 Inactivation Mechanism

Cunningham *et al*. (2008) have documented the evidence of Chlorine inactivation of bacteria by many researchers. The current understanding among many researchers is that a major mechanism of bacterial inactivation by free chlorine is the alteration of cell permeability, resulting in the leakage of intracellular materials such as proteins, RNA and DNA (Venkobachar *et al*. 1977; Haas & Engelbrecht 1980). In addition, damage to nucleic acids and enzymes, oxidation of sulph-hydryl groups, damage to iron-sulphur centres, disruption of nutrient transport and inhibition of cell respiration have been reported (Hoyana *et al*. 1973; Barrette *et al.* 1988; Leyer & Johnson 1997). The mechanisms of microbial inactivation by iodine, like chlorine, are not clearly understood (Gottardi 1999). Although various forms of iodine have been shown to possess antimicrobial properties, it is believed by most researchers that molecular iodine (I_2) is the chemical form most responsible for bacterial inactivation (Gottardi 1999).

Silver has also been shown to be an effective disinfectant against bacteria (Zhao & Stevens 1998). However, the biocidal action of silver requires contact times on the order of hours and therefore it has been primarily used as a bacterial inhibitory compound for water storage. The current understanding of silver bacterial inactivation involves the reaction between silver ion (Ag^{3+}) and thiol groups of amino acids and key enzyme functional groups (Liau *et al.* 1997). Furthermore, Batarseh (2004) concluded that intracellular silver complexes resulted in DNA unwinding and were the primary causes for bacterial inactivation

Myriam *et al.* (2010) has documented the mechanism of inactivation of UV light. The effectiveness of UV light in biological inactivation arises primarily from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at 260 nm (Byrd *et al*. 1990). This absorption creates damage in the DNA by altering nucleotide base pairing, thereby creating new linkages between adjacent nucleotides on the same DNA strand. If the damage goes unrepaired, the accumulation of DNA photoproducts can be lethal to cells through the blockage of DNA replication and RNA transcription, which ultimately result in reproductive cell death (Zimmer & Slawson 2002).

The antimicrobial activity of Moringa extracts was previously attributed to plant-produced benzyl isothiocynate derivatives (Eilert *et al.* 1981). Suarez *et al.* (2003) showed that at least part of the antimicrobial activity of Moringa seeds extract may stem from Flo-like polypeptides. Antimicrobial peptides have attracted increasing attention recently because they can efficiently kill fungi and bacteria that are otherwise resistant to many commonly used antibiotics. According to Zasloff (2002), they act by forming essential enzymes, leading to cell deaths. Suarez *et al* (2003) reported that the antimicrobial action of Flo might results from similar activities or from its bacterial flocculation effect. The report further noted that the former is more likely because for example, Flo concentration required to obtain half-mixed effect on E.Coli are 0.1 mg/L for bacteriostatic action, 1.0mg/L for bacterial activity, and 6.0mg/L for cell flocculation. The study concluded that visual inspection of Flo-incubated E.Coli revealed that the peptide aggregated the bacteria, as indicated by defined particles or flocs.

Chuang *et al.* (2007) has studied the mode of attack of Moringa Oleifera seeds extract on fungus. The results showed that the cytoplasmic membrane of the fungal cell was ruptured and the intercellular components were seriously damaged after treatment with *M. Oleifera* seed crude extract. However, the intercellular components did not leak out. Based on previous studies of cell lysis pathways of antimicrobial peptides on bacteria (Cham *et al.* 1998; Chen *et al*. 2003), this indicated that extracted compounds interacted with the lipid bilayers in membranes leading to the separation of the two membranes (outer and inner). Subsequently, water dips in to the cell, which causes cell to swell more and leads to death.

Structural morphological study of microbes after treatment with antimicrobial agents is an important parameter in understanding the mechanism of action of these agents (Kitajim *et al*. 1998). However, not much

review is available on the mechanistic microscopic study of *Moringa oleifera* (Jabeen *et al*. 2008). Microscopic evaluation of E.Coli strains were carried out after treatment with Moringa Oleifera seeds extract.

2. Materials and Methods

2.1 Preparation of Moringa Oleifera Seeds Extract

Good quality dry *M. Oleifera* seeds were selected and the seed coat and wings were removed manually. The kernel was ground to fine powder using the coffee mill attachment of the Moulinex domestic food blender. The ground powder was then sieved through 210 µm sieve. The seed powder was de-fatted using hexane in electro-thermal Soxhlet extractor.

The best method of Moringa seed processing determined by (Bichi 2011) was used for this study. Measured quantities of the de-fatted Moring seed powder was dissolved in a beaker and made up to 1000mL with distilled water. The active ingredients were extracted by mixing with a stirrer at a pre-set mixing speed and for a pre-set mixing time as outlined in the experimental design. The mixture was filtered through No.1 whatt-man filter paper and the extract used for the disinfection studies.

2.2 Preparation of E. Coli Culture

The E.Coli culture was prepared as described in Obire *et al.* (2005). Nutrient broth (130.0gm) was dissolved in 1000mL distilled water by heating slightly. The mixture was sterilized at 130^{o}C for 15minutes at 15 SPT in autoclave. The sterilized broth was cooled to room temperature and was used to prepare the *E.Coli* culture. Escherchia Coli (ER2566) strain was grown in 10mL broth at 37^{o}C overnight to obtain an exponential growth phase. This was then used for the disinfection studies.

2.3 Disinfection Studies

The procedure for the disinfection study was as described in Suarez *et al*. (2003) and Fisch *et al.* (2004). The Moringa Oleifera dosage obtained from the optimization studies (3.25mg/L, 31min mixing time, and 85rpm mixing speed) was used for this study (Bichi 2012).

One mL of Moringa extract was added to 10mL of the cell cultures and incubated for 0, 30min, 60min, 90min, and 120min without agitation. Cell survival was assessed by making dilution series of bacterial suspensions obtained after each incubation period, plating on non-selective LB medium agar dishes, and incubating for 48hours at 37^{o}C. Duplicates were made of every individual assay (Suarez *et al*. 2003). Colonies were counted on dishes and the cell survival ratio was estimated by comparison to a control experiment where no Moring extract was added (0 min incubation time).

2.4 Inactivation kinetics

Pseudo-first-order kinetics was determined using the method described by Cunningham *et al*. (2008). The log-inactivation of the "viable" organisms at time t (N_t) divided by the number of "viable" organisms at time zero (N_0) was plotted as a function of chemical dose, *Ct* (mg min L $^{-1}$). The coefficient of specific lethality, Λ_{cw}, was determined by linear regression using MINITAB for Windows Release 11.2 32 Bit (Minitab Inc 1996).

2.5 Model Verification

The kinetic model equation was verified using surface water collected from Rimin Gado dam reservoir about 15Km from the Bayero University New Campus. The procedure was as described in Suarez *et al*. (2003), Fisch *et al*. (2004), and Gan *et al*. (2011) as earlier presented above.

2.6 Investigation of Mode of Attack of Moringa Extract on E.Coli

This was based on the microscopic observation of bacterial cell morphological changes after treatment with Moringa seeds extract. The procedure is as described in the Standard Methods (APHA 1990). Moringa seeds extract was added to a 24 hr E.Coli culture and incubated for 0, 60, 90, 120 minutes. A smear of the culture at the different stages was made on a slide to which few drops of crystal violet were added for 60 seconds. The slide was washed off with water and Gram Iodine was added for 60 seconds. The slide was de-colourized with alcohol until no colour appears. It was then immediately washed with water and counter-stained with safranin for 60 seconds. It was then washed with water and blotted dry with filter paper. The morphological changes in the structure of the E.Coli cell at the various stages of its destruction were examined under microscope (Nikon, Japan), and photographs of these slides were captured by camera (Nikon FDX-35).

3. Results and Discussions

3.1 Kinetics of Disinfection of Moringa Oleifera Seeds Extract

Table 1 gives the results of the disinfection studies using synthetic water. Figure 1a shows the log-inactivation plot of the E.Coli and the microbial consortium exposed to Moringa seeds extract for synthetic water.

The regression equation (7) is given below:

$$In\ (N_0/N_t) = 0.0746 - 0.00423\ Ct \qquad (7)$$

The correlation coefficient is 99.2% and the Adj R^2 was 98.9%, indicating goodness of fit. The normal plot of residuals (Figure 1b) also indicated good fit. The analysis of variance (Table 2) indicated no significant lack of fit (P> 0.1). The slope of line gives the Chicks-Watson coefficient of specific lethality. The coefficient of specific lethality (Λ_{cw}) was 3.77 L mg^{-1} min^{-1} for an E.Coli in synthetic water.

Table 3 gives the results of the disinfection studies using surface water obtained from Rimin Gado Dam reservoir. Figure 3 shows the log-inactivation plot of the E.Coli and the microbial consortium exposed to Moringa seeds extract for the surface water. Figure 2a shows log-inactivation plot of the E.Coli and the microbial consortium exposed to Moringa seeds extract for surface water. The regression equation (8) is given below:

$$In\ (N_t/N_o) = 0.080 - 0.00418\ Ct \qquad (8)$$

The correlation coefficient is 95.2% and the Adj R^2 was 93.7%, indicating goodness of fit. The normal plot of residuals (Figure 2b) also indicated good fit. The analysis of variance (Table 4) indicated no significant lack of fit with P=0.57 (P> 0.1). The slope of line gives the Chicks-Watson coefficient of specific lethality. The coefficient of specific lethality (Λ_{cw}) was 3.75 L mg^{-1} min^{-1} for an E.Coli in surface water. Thus the average Coefficient of lethality for Moringa disinfection (Λ_{cw}) was 3.76 L mg^{-1} min^{-1}.

The disinfection kinetic on E.Coli was found to be consistent with those determined by earlier researchers using chlorine. Butterfield *et al*. (1943) reported a coefficient of specific lethality (Λ_{cw}) of 3.75 L mg^{-1} min^{-1} for an E.Coli exposed to chlorine while Cunningham *et al.* (2008) reported a Λ_{cw} value of 4.71 L mg^{-1} min^{-1} for an E.Coli exposed to chlorine.

3.2 Mode of Attack of Moringa Disinfection Extract on E.Coli

The antimicrobial activity of Moringa extracts was previously attributed to plant-produced benzyl isothiocynate derivatives (Eilert *et al*. 1981). Suarez *et al.* (2003) later showed that at least part of the antimicrobial activity of Moringa seeds extract may stem from Flo-like polypeptides. Antimicrobial peptides act by forming essential enzymes, leading to cell deaths.

The results obtained are shown in Plates 3a – 3d. The Plates shows the changes in the E.Coli cell morphology from the active cell (Plate 3a) to the cell condition after the Moringa extract application for 1 hour (Plate 3b), then cell rupture after Moringa application for $1\frac{1}{2}$ hrs (Plate 3c), and finally the destroyed cells after Moringa extract was applied for 2 hours Plate (3d). The result of the study on the mode of attack showed that the cytoplasmic membrane of the E.Coli bacterial cell was ruptured and the intercellular components were seriously damaged after treatment with *M. Oleifera* seed crude extract. Plate 3d indicated that the intercellular components leaked out after cell burst. Based on previous studies of cell lysis pathways of antimicrobial peptides on bacteria (Cham *et al*. 1998; Chen *et al*. 2003), this indicated that extracted compounds interacted with the lipid bi-layers in membranes leading to the separation of the two membranes (outer and inner). Subsequently, water dips in to the cell, which causes cell to swell more and burst leading to death. Thus the results obtained agreed with the explanations given by earlier researchers.

4. Conclusion

The coefficient of specific lethality (Λ_{cw}) was 3.75 – 3.77 L mg^{-1} min^{-1}, with an average value of 3.76 L mg^{-1} min^{-1} for E.Coli inactivation using Moringa Oleifera seeds extracts. The mode of attack of the Moringa seeds extract on the E.Coli cell was explained as by rupturing the cell and damaging the intercellular components, allowing water to dip in to cell which causes it to swell more and burst leading to death.

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Table 1: Determination of Chiks-Watson Coefficient (Λ_{cw}) Using Synthetic Water

Time (mins)		**0**	**30**	**60**	**90**	**150**
E.Coli Count (N_t) (X 10^5 cfu/mL)	**Plate 1**	1048	840	450	336	134
	Plate 2	1008	728	624	300	150
	Plate 3	1064	832	495	270	133
	Average	1039	800	523	302	141.66
Ct (mg/min L^{-1})		0	97.5	195.0	292.5	487.5
N_t/N_0		1	0.9699	0.5033	0.2907	0.1363
In (N_t/N_0)		0	-0.260	-0.686	-1.230	-1.990

Table 2: Regression Analysis and ANOVA for Kinetic Equation for Synthetic Water

Predictor	**Coef**	**Std Dev**	**T**	**P**
Constant	0.07457	0.05970	1.25	0.300
Ct (mg/min mL^{-1})	-0.0042320	0.0002192	-19.30	0.000

S = 0.08223 R-Sq = 99.2% R-Sq(adj) = 98.9%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	2.5198	2.5198	372.62	0.000
Error	3	0.0203	0.0068		
Total	4	2.5401			

No evidence of lack of fit (P > 0.1)

Table 3: Determination of Chiks-Watson Coefficient (Λ_{cw}) Using Surface Water

Time (mins)		**0**	**30**	**60**	**90**	**120**
E.Coli Count (N_t) (X 10^5 cfu/mL)	**Plate 1**	1048	840	450	336	134
	Plate 2	1008	728	624	300	150
	Plate 3	1064	832	495	270	133
	Average	1039	800	523	302	141.66
Ct (mg/min L^{-1})		0	97.5	195.0	292.5	390
N_t/N_0		1	0.9699	0.5033	0.2907	0.1363
In (N_t/N_0)		0	-0.260	-0.686	-1.230	-1.990

Table 4: Regression Analysis and ANOVA of Kinetic Equation for Surface Water

The regression equation is

In (N/Nt) = 0.080 - 0.00418 Ct (mg/min mL)

Predictor	**Coef**	**StDev**	**T**	**P**
Constant	0.0802	0.1288	0.62	0.578
Ct (mg/min mL^{-1})	-0.0041815	0.0005391	-7.76	0.004

S = 0.1662 R-Sq = 95.2% R-Sq(adj) = 93.7%

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	1	1.6622	1.6622	60.15	0.004
Error	3	0.0829	0.0276		
Total	4	1.7451			

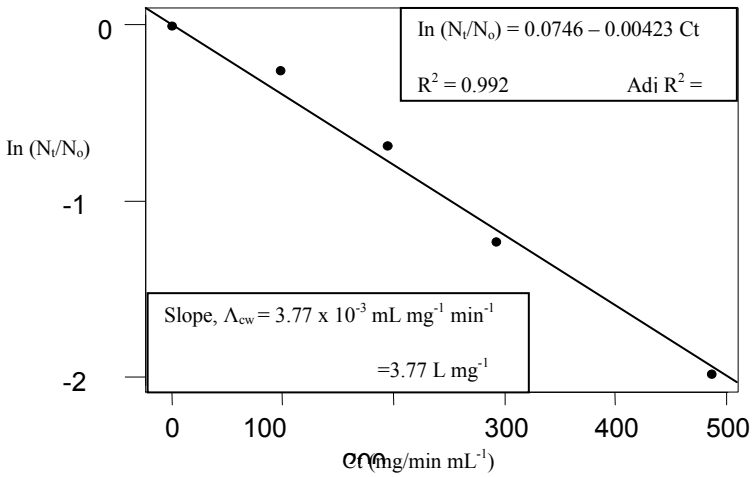

Figure 1a: Determination of Chick-Watson Coefficient for Synthetic Water

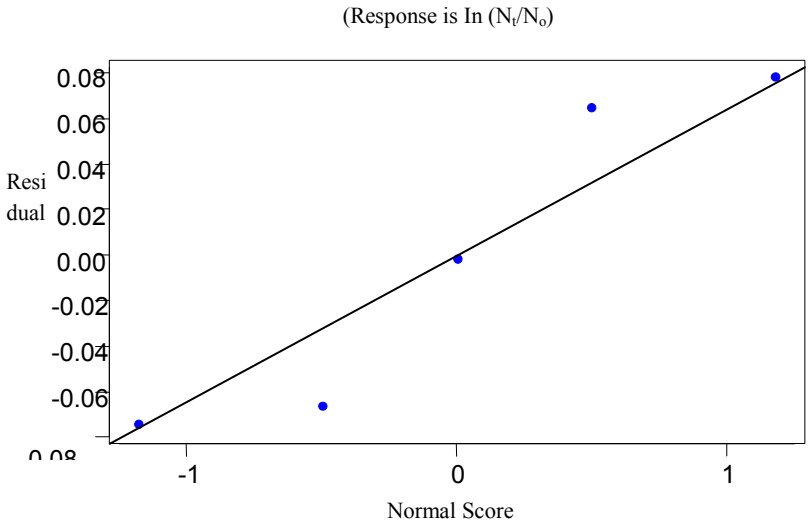

Figure 1b: Normal Probability Plot of the Residuals for

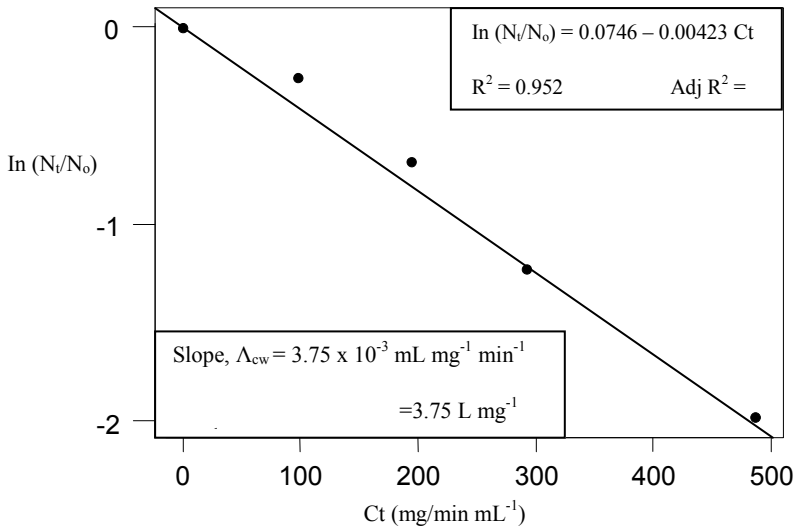

Figure 2a: Determination of Chick-Watson Coefficient for Surface Water

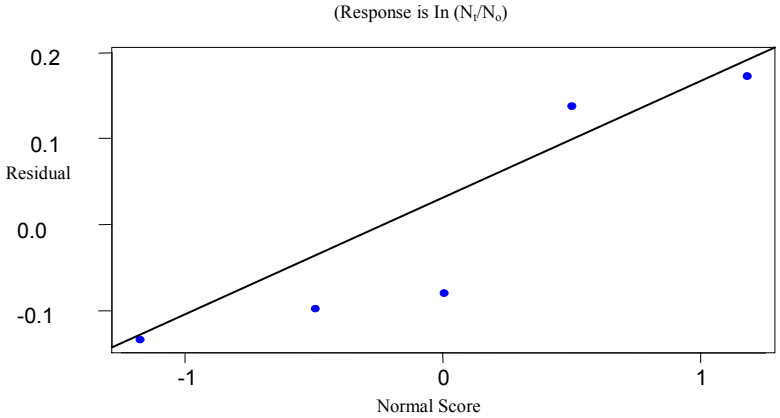

Figure 2b: Normal Probability Plot of the Residuals for Surface Water

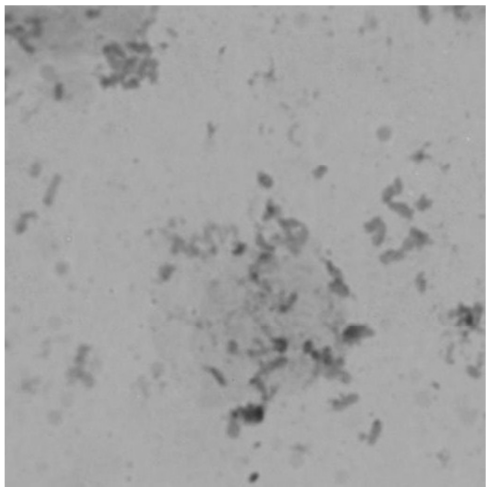

PLATE 3a: E. Coli Without Extract

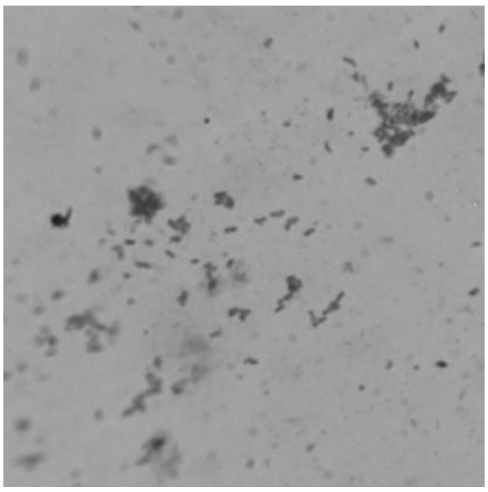

PLATE 3b: E. Coli With Extract after 1 hr

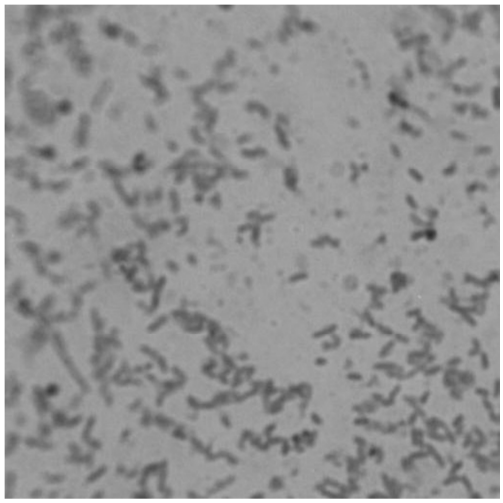

PLATE 3c: E. Coli With Extract after $1\frac{1}{2}$ hr

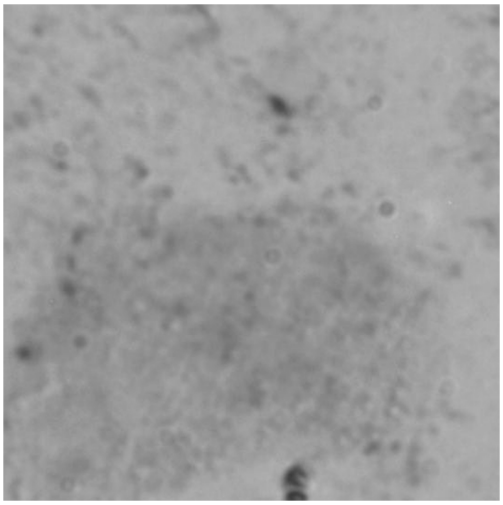

PLATE 3d: E. Coli With Extract after 2 hrs