



Faculty of Resource Science and Technology

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TEMPERATURE AND NUTRIENT***

Tan Wee Siong

Bachelor of Science With Honours
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P. KHIDMAT MAKLUMAT AKADEMIK
UNIMAS



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TAN WEE SIONG

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***Escherichia coli* Survival in Pig Farm Wastewater Temperatures Nutrient**

Tan Wee Siong

Resource Biotechnology Program
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

The survival of *Escherichia coli* (*E. coli*) in pig farm wastewater of different temperatures and nutrient conditions was studied. Non-sterile wastewater, non-sterile diluted wastewater, sterile wastewater and physiological saline (0.85%) inoculated with *E. coli* were incubated at 20°C and 30°C in triplicates for 21 days or until the bacteria were undetectable. The study showed that *E. coli* survived more than 21 days in non-sterile condition at 20°C. At 30°C, *E. coli* disappeared after day 21 in non-sterile wastewater and *E. coli* survived 16 days in diluted wastewater. *E. coli* mean decay rate in non-sterile wastewater at 20°C (k_{20}) was $0.3190 \pm 0.0807 \text{ d}^{-1}$ and k_{30} was $0.5543 \pm 0.1026 \text{ d}^{-1}$. In diluted wastewater, k_{20} and k_{30} was $0.4267 \pm 0.1380 \text{ d}^{-1}$ and $0.6630 \pm 0.2311 \text{ d}^{-1}$ respectively. *E. coli* multiplied in sterile wastewater but not physiological saline and maintained at high concentration of cells. Linear decay model fit the data well in non-sterile conditions. Results of this study indicated that temperature and nutrient were the primary factors affecting the survival of *E. coli* in wastewater. This information may assist the prediction of retention time for *E. coli* mortality in lagoon and the development of mathematic models.

Key words: *Escherichia coli*, wastewater, decay rate, lagoon.

ABSTRAK

Kemandiran *Escherichia coli* (*E. coli*) dalam air buangan ladang khinzir ditentukan pada suhu dan keadaan nutrien yang berbeza. *E. coli* diinokulumkan dalam air buangan bukan-steril, air buangan dicairkan, air buangan steril dan air garam (0.85%). *E. coli* dieramkan pada suhu 20°C dan 30°C dalam tiga replikasi untuk 21 hari atau sehingga tiada bakteria dikesan. Kajian ini menunjukkan bahawa *E. coli* hidup lebih daripada 21 hari dalam keadaan bukan-steril pada 20°C. Pada 30°C, *E. coli* mati selepas hari ke-21 dalam air buangan bukan steril dan *E. coli* hidup selama 16 hari dalam air buangan dicairkan. Min kadar kemortalan *E. coli* dalam air buangan bukan-steril pada 20°C (k_{20}) ialah $0.3190 \pm 0.0807 \text{ d}^{-1}$ dan k_{30} ialah $0.5543 \pm 0.1026 \text{ d}^{-1}$. Dalam air buangan dicairkan, k_{20} dan k_{30} masing-masing ialah $0.4267 \pm 0.1380 \text{ d}^{-1}$ dan $0.6630 \pm 0.2311 \text{ d}^{-1}$. Penggandaan *E. coli* berlaku dalam air buangan yang steril, bukannya di air garam, dan sel-sel dkekalkan pada kepekatan yang tinggi. Data bagi keadaan bukan steril menepati model kemortalan linear dengan baik. Keputusan kajian menunjukkan bahawa suhu dan nutrien adalah faktor-faktor utama yang mempengaruhi kemandiran *E. coli* dalam air buangan. Maklumat ini mungkin dapat membantu dalam ramalan masa penahanan untuk kemortalan *E. coli* dalam lagun dan perkembangan model-model matematik.

Kata kunci: *Escherichia coli*, air buangan, kadar kemortalan, lagun.

Introduction

The population growth has increased continuously and the occurrence of high global markets demands for the last twenty years has resulted in the replacement of the family sized farm with industrial livestock farms. These livestock farms would normally compose of sheds containing several hundreds to several thousands of pigs in overcrowded conditions (Kinson *et al.*, 2001). It was reported that the current practices of pig waste treatment are ineffective in Malaysia, with large amounts of the waste collected in waste lagoon, where it is poorly treated and discharged into the river system (Kinson *et al.*, 2001).

It has been identified that pig industry is one of the industries that caused serious water pollution in Malaysia (Kinson *et al.*, 2001). In order to control the released of pig wastes from farms in Sarawak, farms having more than 100 animals are required to obtain licenses to operate under the Natural Resources and Environment (Control of Livestock Pollution) rules (Kinson *et al.*, 2001). Farm operators must be prepared to treat pig wastes within four months after the issue of licenses. The Natural Resources Environment Board, Sarawak, Control of Livestock Pollution rules (1996) have set the standards for the permitted discharge of treated waste for existing farms and new farms based on Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Suspended Solid (TSS) (Kinson *et al.*, 2001). However, the microbial standard of effluent has not been proposed yet. Hence, the study of bacterial survival in pig waste lagoon becomes a key to predict the environmental risks due to the release of bacterial pathogens. *E. coli* is selected prior to the survival study due to its role of standard indicator for fecal contamination in aquatic environment and it displays a survival pattern similar to that of bacterial pathogens (Bitton, 1994). Its survivability can be studied through monitoring the environmental factors involved.

Objectives

The objective of this study was to investigate the die-off of *E. coli* in wastewater at two selective temperatures (20°C and 30°C). Under these temperatures, several conditions of wastewater were set: non-sterile wastewater, non-sterile diluted wastewater, sterile wastewater and sterile physiological saline (0.85%). The *E. coli* decay rates for those different conditions of wastewater were evaluated.

Literature Review

Lagoons System for Pig Wastes

Lagoons, also known as waste stabilization ponds or facultative ponds are the most common and inexpensive method of biological treatment of organic wastes where sufficient land is available (Martin, 1991; Taiganides, 1977). Lagoons are defined as earthen structures which are designed, constructed and operated to provide storage and/or treatment to wastes and wastewaters. According to Humenik *et al.* (1981), waste lagoons became popular for animal waste treatment as historic interest to utilize manure fertilizer constituents by direct land application was replaced by desires to have more convenient waste management systems. As in Malaysia, some farmers utilizing the wastes as manure for their vegetables farms (Kinson *et al.*, 2001).

In addition, survey of pig farms has shown that approximately 68% of the farms had constructed retaining ponds for the pig wastes but they were considered too small and thus overloaded and non-functional (Kinson *et al.*, 2001). Several reasons has been suggested for the poor planning, design and maintenance of the lagoon systems: lack of available land to build suitably sized lagoons, overstocking of livestock, lack of awareness, lack of finance and lack of interest.

Classification of Lagoon System

Waste lagoons are classified based on the mode of biodegradation (aerobic or anaerobic), a process that the stabilizing organics substances after the settling out of solids through sedimentation or chemical precipitation (Martin, 1991; Taiganides, 1977). Generally there are four types of lagoons: aerobic-algal lagoon, aerated lagoon, facultative lagoon and anaerobic lagoon (Taiganides, 1977). The depth of the lagoon is one the factors that determine the types of lagoon (Taiganides, 1977). The principle, characteristic, advantages and disadvantages of each lagoon can be summarized in Table 1.

Wastewater Characteristics

The important characteristics of wastewater from livestock include suspended solids, biodegradable organics, nutrients and pathogens (Peavy *et al.*, 1985). In confined commercial pig production, wastes consist of feces, urine, wasted feed, spilled water from the drinking nipples and leaking pipes and water used for cleaning the pens and cooling the pigs (Taiganides, 1992). These wastes are usually discharged into the waste lagoons. A mature pig produces three times the waste a person does (Kinson *et al.*, 2001). According to Kinson *et al.* (2001), manure production as excreted in kg/animal/day for mature pigs of average weight of 150 kg, would amount to approximately 21.1 kg/animal/day of solid material and 15.9 liters/animal/day of liquids. Total nitrogen and phosphorus production as excreted by mature pig are 0.033 and 0.026 kg/animal/day. Besides high nutrient level, Taiganides (1992) reported that raw pig wastewater contain high concentration of fecal coliforms which is around 8.1×10^7 CFU/100 mL. Fecal coliforms concentration per gram in pig feces is 3.3×10^6 and total bacterial population has been estimated to be equivalent to 1.8×10^{12} cells (Droste, 1997).

Table 1: Summary of the principle, characteristic, advantages and disadvantages of four types of lagoons: aerobic-algal lagoon, aerated lagoon, facultative lagoon and anaerobic lagoon that are commonly used for wastewater treatment.

	Aerobic-algal lagoon	Aerated lagoon	Facultative lagoon	Anaerobic lagoon
Principle of biodegradation	Based on a symbiotic mode of operation between bacteria and algae. Photosynthesis by algae produces oxygen for bacteria to undergo aerobic digestion of sludge (Metcalf & Eddy, 1991; Taiganides, 1997).	An artificial aeration achieved by either mechanical aerators or diffused aeration (Martin, 1991; Metcalf & Eddy, 1991).	Photosynthesis by algae provides oxygen for aerobic stabilization in upper layers; bottom layer of sludge undergoes anaerobic digestion (Metcalf & Eddy, 1991).	A deep holding unit in which the bottom anaerobic layers are the dominant feature (Metcalf & Eddy, 1991).
Depth (feet)	1 – 1.5 (Metcalf & Eddy, 1991)	6 – 20 (Metcalf & Eddy, 1991)	4 – 8 (Metcalf & Eddy, 1991)	8 – 16 (Metcalf & Eddy, 1991)
Advantages	Low capital cost, easy operation and low energy consumption for oxygen production (Bitton, 1994).	Best suited for feedlot wastes due to the elimination of odor problems (Taiganides, 1997).	Most biomass formed in the aerobic zone settle into anaerobic zone and less solids in the effluent expected; removal of additional carbon through methane formation in anaerobic zone (Taiganides, 1997).	Effective in bringing about rapid stabilization of strong organic wastes (Metcalf & Eddy, 1991); produces lower amounts of sludge (Bitton, 1994).
Disadvantages	Not well suited for feedlot wastes and the solids content of effluent is significant (Taiganides, 1997).	High energy consumption that necessary for oxygen production (Bitton, 1994).	Odor problems associated with growth of algae and mosquitoes (Bitton, 1994).	Lower process than aerobic digestion; start-up process requires long periods (Bitton, 1994).

***Escherichia coli* as Indicator of Fecal Contamination**

In microbiological water analysis, indicator microorganisms like *E. coli* and fecal coliforms are evaluated as to determine the presence of certain pathogens in wastewater. *E. coli* is a bacterium which belongs to the family Enterobacteriaceae whose normal habitat is the intestinal tract of human and animal (Cooke, 1974). As a member of fecal coliforms, *E. coli* has been effectively used for some time in Europe and has been incorporated into United State drinking water regulations as a specific indicator of fecal contamination (Hurst *et al.*, 1997). An emphasis has been placed on determining the presence of *E. coli* since it is considered the principal fecal coliforms (Fish & Pettibone, 1994). *E. coli* is not indigenous to water supplies and evaluating its survival in aquatic environments is vital, particularly with regard to interpretation of water quality data (Fish & Pettibone, 1994).

The Survival of Non-Indigenous Bacteria in Aquatic Environment

Survival of bacteria has been defined as the series of changes resulting from their stay in a hostile environment (Barcina, 1994). Understanding the fate of fecal indicator bacteria and pathogenic microorganisms after their release into water is important to address the potential impact of these microorganisms on the environment (Hurst *et al.*, 1997). Once they are released in alien environment, these bacteria need to cope with rapid changes of chemical and physical condition-in order to achieve a higher degree of metabolic adaptability. Thus, their physiological states are dependent on environmental factors. For instance, *E. coli*, a typical non-indigenous bacteria of natural waters do not multiply but they die-off eventually that due to environmental stresses (Cooke, 1974). In contrast, Barcina *et al.* (1997) have reviewed the studies regarding the survivability mechanisms of some allochthonous bacteria in aquatic systems. For example, *E. coli*, *Enterobacter faecialis* and *Vibrio cholera* encounter the viable

but non-culturable or dormant state in seawater; *E. coli* and marine bacteria undergoes starvation survival in seawater (Barcina *et al.*, 1997). The reason why some bacteria undergo viable but non-culturable state is unknown. However, studies have revealed that the bacterial starvation survival is due to the display of a high spontaneous mutation rate, changes in membrane fatty acids, cell wall amino acids, topology of the chromosomes and a general enhanced resistance to heat, oxidative and osmotic shock (Barcina *et al.*, 1997).

Factors that Affecting *E. coli* Survival in Aquatic Environment

Once *E. coli* are released into the aquatic environment, the study of its survival is important due to the interpretation of water quality data. Thus, the factors which affect the *E. coli* decay are of concern to environmental microbiologists with their contribution to the protection of surface and groundwater resources from enteric bacterial pollution. Such practices must be devised based on a sound knowledge of the fate of this bacterium in the environment (Crane & Moore, 1986). This valuable knowledge which is based on the interaction of environment factors against the decay rates is combined concisely into mathematical modeling in order to facilitate the prediction of bacterial population die-off in different conditions. However, factors affecting the mortality of *E. coli* in wastewater have not been adequately modeled (Mayo, 1995).

Nevertheless, a number of environmental factors have been suggested to have significant impact on the survival of bacteria in natural waters. These factors include light, temperature and grazing of bacteria by zooplankton (Scheuerman *et al.*, 1988). It has been shown that the temperature and substrate are among the factors that contribute to the bacteria survivability in aquatic environment (Tassoula, 1998; Crane & Moore, 1986). Besides, Flint (1987) has

established that temperature and competition for nutrients were the major factors influencing the survival of *E. coli* in freshwater. However, the significance of temperature and substrate that affect the survival rate of *E. coli* in pig waste lagoon has not been studied.

The factors affecting survival of microorganism in environment are numerous and complex (Edward, 1993). Temperature and substrate factors were studied and focused in this study and some other significant factors should be taken into account although they are not studied. These factors include protozoa predation (Barcina, 1994; Gurijala & Alexander, 1990; Mallory *et al.*, 1983; McCambridge & McMeekin, 1981; Scheuerman *et al.*, 1988), pH (Curtis *et al.*, 1992; Mayo, 1995; McFeters & Stuart, 1972; Reddy *et al.*, 1981), light radiation (Barcina, 1994; McCambridge & McMeekin, 1981), chemical composition (McFeters & Stuart, 1972) and oxygen (Curtis *et al.*, 1992).

Temperature

Temperature is a key factor in microbial survival. Water temperature above 10°C accelerates the growth of adapted organism with slow generation time (Letterman, 1997). Low water temperatures result in a precarious balance between new-cell development and the death of old cells (Letterman, 1997). Many studies have reported that the temperature is significantly affects the *E. coli*-survival. Terzieva & McFeters (1991) have found that the survival and injury of enteropathogenic bacteria which included *E. coli* in stream water were apparently affected by temperature (6°C and 16°C). *E. coli* injury occurred at the higher temperature (16°C) and this accelerated *E. coli* die-off. It can be indicated that *E. coli* persisted longer at 6°C than those at 16°C.

In order to study the temperature effect on *E. coli* survival, Flint (1987) has set up an experiment whereby untreated river water samples collected from above or below the sewage outfall were incubated at 4°C, 15°C, 25°C and 37°C in order to determine the die-off rate (k value) of *E. coli*. He reported that the decay rate of the untreated sample depended on temperature, the slowest decay rate was observed at the 4°C. This study indicates the unambiguous temperature effect on *E. coli* survival in aquatic environment. Increase of temperature is shown to lower the survival rate or increase the die-off (Reddy *et al.*, 1981). Reddy *et al.* (1981) in their review reported that the *E. coli* die-off rate approximately doubled with a 10°C rise in temperature (5 °C - 30°C).

Nutrient

Raw pig wastewater contains high concentration of substrate which includes nitrogen, phosphorus and organic matter (Kinson *et al.*, 2001). It has been reported that the availability of food and nutrient sources and the competition for them are the primary determinants of the rate of die-off (Droste, 1997). The nutrient supply and organic matter contained in water will affect the fate of bacterial die-off (Crane & Moore, 1986). Crane & Moore (1986) has reviewed previous studies regarding the effect of nutrient on fecal bacteria and they concluded that increased survival of fecal organisms is subjected to nutrient content of the water. This might also account for the extended bacterial survival that is found in concentrated waste storages (Crane & Moore, 1986). In terms of types of nutrient, nitrogen primarily affects the *E. coli* survival in lake water (Lim & Flint, 1989).

Tassoula (1998) has revealed that substrate and temperature are two of the most important parameters which act on the survival of *E. coli*. Tassoula (1998) has demonstrated the influence of temperatures at 4°C, 20°C, 37°C and 44°C on the *E. coli* survival rate in sewage (~ 200 mg COD/L) and in clean water (< 2 mg COD/L) for 28 days. The sewage was artificially constructed as a growth medium to exclude the *E. coli* survival experiment from the effects of both chemical substances and microorganisms. Clean water was made to investigate the starvation condition of *E. coli* at different temperatures. From the study, Tassoula (1998) concluded that *E. coli* multiplied at 20°C and 37°C and maintained at the high concentration of cells in artificial sewage. *E. coli* did not multiply in artificial sewage at 4°C but the cells concentration was maintained constantly. *E. coli* cells decreased significantly at 44°C in artificial sewage. In clean water, *E. coli* were maintained at high concentration at 4°C and 20°C. *E. coli* cells decreased at 37°C and 44°C in clean water. These conclusions showed that the interaction of temperature and nutrient have significantly affected the *E. coli* survival.

Other factors

There are some significant factors which have been proven to affect the survival of *E. coli* in freshwater, these include protozoan predation, pH, light radiation, chemical composition and oxygen. Although these factors are not investigated in this study, these factors should not be disregarded as these factors influence the survivability of *E. coli* in the natural environment.

pH

In a review "Modeling Enteric Bacterial Die-Off", Crane and Moore (1986) has reported that extremes in pH are detrimental to organisms survival; both acidic and alkaline conditions in aquatic system can greatly increases die-off rates whilst a neutral pH environment favors extended bacterial survival. It has been proved that the optimum pH for *E. coli* survival in fresh water is around 5.5 to 7.5 (McFeters & Stuart, 1972). Besides, Mayo (1995) has revealed that *E. coli* were removed rapidly at pH 9.3 in the waste stabilization ponds. Thus, it can be concluded that bacterial die-off rate increases due to the extreme pH of environment.

Protozoan Predation

Protozoan predation is an important biotic factor which affects the size population of introduced bacteria in the aquatic environment. A few identified protozoa have been known to feed bacteria actively, including flagellates and ciliates in estuarine water (Gonzalez, 1989); colpoda, which actively feeding on *E. coli* in freshwater (Levin *et al.*, 1992) and reduction of *E. coli* by grazing activities of amoebae in seawater (Gurijala & Alexander, 1990).

Several studies have provided evidences that bacteria die-off are significantly affected by protozoa predation. According to Scheuerman *et al.*, (1988), protozoa apparently are involved in elimination of many cells of tested bacteria in lake water, which include *E. coli*. The role of protozoa is evidenced by the increase in survival of bacteria in lake water after treatment by eukaryotic inhibitors, which removed the protozoa (Mallory *et al.*, 1983; Scheuerman *et al.*, 1988). In other study, Barcina (1994) has demonstrated the protozoan predation in microcosms of river water by marking the enteric bacteria with fluorochromes. This enabled the visualization of predation activities whereby the presence of labeled bacteria was found in

the interior of the digestive vacuoles. The ingestion rates, the total ingestion and digestion data corroborated that *E. coli* is predated quite efficiently, that is 45%. These information concluded that predation of *E. coli* by protozoa is significant.

Protozoan predation was a significant factor in the fall in bacterial population sizes because protozoa increased in numbers while the bacterial density fell and the bacterial elimination occurred constantly regardless of the existence of indigenous or non-indigenous protozoa population (Gurijala & Alexander, 1990). It has been reported that the high density of two bacterial densities (above 10^6 to 10^7 cells per mL of ciliates and amoebae) can be reduced rapidly as an alternative protozoa was added besides the indigenous protozoa in sewage (Mallory *et al.*, 1983).

Solar radiation

Solar radiation increases the rate of die-off of indicator microorganisms (Droste, 1997). The ultraviolet component of sunlight has relatively small effect on die-off because of its large attenuation coefficient in water and die-off rate is direct proportional to light intensity (Droste, 1997). Many studies have used solar radiation as the main parameter of determination of bacteria die-off in aquatic environment (Crane & Moore, 1986). McCambridge & McMeekin (1981) have intensively studied the effect of solar radiation on the survival of fecal bacteria. They discovered that the decline in the numbers of *E. coli* cells in estuarine water samples was significantly greater in the presence of both indigenous protozoa as well as solar radiation when each of these factors acted independently. The rate of decline is directly related to the total radiation received over a given time (Barcina, 1994; McCambridge & McMeekin, 1981).

The Modeling of Bacteria Survival in Aquatic Environment

Concepts of Modeling

The ideal of models is to predict enteric microorganisms in soil, water or waste management system without the need of expensive and time-intensive field activities (Crane & Moore, 1986). Designing modeling analysis is vital as model equations can be of great value in helping people understand the interactions which led to the observation and then providing a basis for anticipating the future outcome of similar situation (Hurst *et al.*, 1997).

Microbial fate studies are based on microbial population fate and their numbers are strictly dependent on environmental factors (Hurst *et al.*, 1997). Most often, the microbial populations that are released into the natural environment are fated to die, before they are able to establish suitable contact with indigenous microflora (Hurst *et al.*, 1997). According to Hurst *et al.*, (1992), the mortality of a microbial population can be described as analogous to the decay rates associated with radioisotopes, in that the frequency of individual death or decay events is assumed to occur at a statistically calculable rate. Thus, the microbial decay rate is termed to describe rapidity of the population members' die-off and expressed as a rate of function (Hurst *et al.*, 1997).

Equation for Microbial Survival

The following is a general exponential decay equation where the amounts of microbial titer decrease observed within a given time period is dependent on the number of organisms present at the beginning of that time period (Hurst *et al.*, 1997):

$$N_t / N_0 = e^{-Kt} \quad (1)$$

Where N_0 is the titer at the outset of the experiment and N_t represents the titer at some subsequent observation time, K represents a rate constant and t is time. This equation is based on model of Chick (1908) known as Chick's Law which presented the model of a simple first order reaction in chemical kinetics (Crane & Moore, 1986). The curve represent the equation (1) indicates the environment is totally unsuited for the indicator bacteria and die-off is constant with time (Crane & Moore, 1986). Logarithmic transformation of equation (1) yields equation (2):

$$\text{Log}_n(N_t / N_0) = -Kt \quad (2)$$

The equation (2) suggests that the rate of microbial die-off during the interval between times zero and t is log linear with constant value equal to K (Hurst *et al.*, 1997).

The relationship shown in equation (2) can be examined by using simple linear regression as defined by equation (3):

$$Y = B_0 + B_1X_1 \quad (3)$$

Whenever simple linear regression is used, Y represents the dependent or response variable; X represents the independent variable; B₁ is the coefficient (decay rate, k) associated with X₁, which results from regressing Y with respect to X₁; and B₀ represents the intercept value at y-axis, which can be viewed as error term (Hurst *et al.*, 1997). The advantage of the linear regression is simple to understand and produces the results that can be visualized and easily compared (Hurst *et al.*, 1997).

Once the K values for temperature factor are determined from equation (2), temperature coefficient is estimated based on the Arrhenius or van't Hoff function defined by the following equation (Mayo, 1995):

$$K_T = K_{20} [\theta]^{(T-20)} \quad (4)$$

Where T is water temperature (°C); K_T represents the first order bacterial mortality rate constant at temperature T (d⁻¹); K₂₀ is first order mortality rate constant at 20°C; and θ represents temperature coefficient. The θ value should be fallen between the ranges from 1.02 to 1.17 for accuracy (Reddy *et al.*, 1981). Once the K₂₀ and θ are determined, bacterial survivability in wastewater at any temperature can be predicted through equation (4).

Materials and Methods

Isolation and Confirmation Tests of *E. coli*

Wild strain of *E. coli* was isolated from fresh waste water and grown on Eosin Methylene Blue agar (Oxoid). All the plate media were incubated overnight at 37°C. *E. coli* grew as green metallic sheen colonies and was harvested and subcultured on a new EMB agar. After incubation, a single colony was scoped and streaked on a nutrient agar (Oxoid). The colonies from nutrient agar were used for API E20 confirmation test.

Inoculum Preparation

E. coli from nutrient slant agar were grown at 37°C, 200 rpm in 10 mL LB broth (Fluka). After growing for 4 hours (referred to the calibration growth curve), 1 mL of LB broth was transferred into a centrifuge tube and harvested by centrifugation (4000 rpm for 20 minutes) aseptically. The cell pellet was washed and resuspended in peptone water to the concentration of 10^8 cells/ml, so that when 1 mL was added to 100 mL of wastewater, it gave an initial viable count of approximately 10^6 cells/ml of wastewater. The calibration curve was constructed in order to obtain the approximate growth time of bacteria with the desired inoculum concentration.

The calibration curve, which is a growth curve of *E. coli*, was set. The universal bottles contained sterile LB broth was inoculated with the *E. coli* stock culture from slant agar for revival at 37°C, 200 rpm. The revived *E. coli* was transferred into a flask which containing fresh sterile LB broth by wire loops for bacteria growing. Sampling was done in six times with 1 hour interval. The blank LB broth (without inoculum) was prepared and followed by preparation of a sample for 0 hour as a starting point. The optical density (OD) value of

starting point was measured using ultraviolet visible spectrophotometer (Techcomp) and wave length was set to 650 nm. The flask was placed in the shaker (200 rpm at 37°C) prior to bacteria growth. The OD values for each hour were recorded. The concentration of bacteria was estimated by plating the culture on EMB agar using spread plate method (Kasing, 2001). Each of the plating was done in triplicate for selected dilution factors. Calibration graph was obtained by plotting the graph of concentration (log cfu+1/mL) against OD values.

Sample Collection

Wastewater was collected from a pig farm lagoon in Gedong, The samples were taken by using sterilize 2 L plastic bottles. Four liter of samples was collected for every replicate. The temperature and pH were determined by using a pH meter (CyberScan Waterproof) and dissolved oxygen for each replicate was measured by a DO meter (Jenway).



Figure 1: A typical pig waste lagoon which is found in Gedong, Sarawak. According to the correspond farmer, the pond depth is around 5 to 6 feet. This lagoon might be characterized as a facultative lagoon.

Experimental Design

The experimental design was two-factor factorial designs with 2 X 4 treatment combinations per replicate: two levels of temperature factor and four levels of nutrient factor. The nutrient factor was studied based on the nutrient distinction among the different condition of wastewater. Four 250 mL plastic beakers were prepared which contained: 100 mL of wastewater (WW), 100 mL of diluted wastewater (DW), 100 mL of sterile wastewater (SW) and 100 mL of physiological saline, 0.85% (PS). Wastewater was obtained from the sample without any chemical or physical treatment. Diluted wastewater (10%) was prepared by mixing of 90 mL sterile deionized water and 10 mL wastewater. The experiment control was the sterile wastewater, which was prepared by autoclaving the wastewater at 121°C for 15 minutes. Physiological saline 0.85% was prepared by dissolving 8.5g sodium chloride (Oxoid) into 1L of deionized water. The solution was then autoclaved at 121°C for 15 minutes.

One milliliter of inoculum was added to each beaker and stirred by in glass rod to ensure the uniformity of the samples. All beakers were maintained at pH 7.0 ± 0.5 . Water evaporated from samples was determined by weighing. The lost volume was replaced by adding distilled water. All the beakers were wrapped with aluminum foil for light exclusion. One ml of samples from wastewater and river water was pipetted from beakers for bacterial analysis. The bacterial analysis was conducted whereby the numbers of *E. coli* were determined. The experiment was conducted in triplicates at 20°C and 30°C. Sampling was done everyday for first week, followed by alternate day with a duration of 21 days or until the bacteria were undetectable.

Substrate Analysis

The nutrient and chemical characteristics of the samples were indicated by several parameters: Biochemical Oxygen Demand (BOD₅), Chemical Oxygen Demand (COD), Total Suspended Solid (TSS), Total Ammonia Nitrogen (TAN) and Total Phosphorus (TP). All parameters were determined according to the standard methods. The protocols of BOD₅ and TSS were based on Clesceri *et al.* (1998) while COD, TKN, TP and TAN were based on Hach (2000).

Bacterial Analysis

The *E. coli* population was estimated through spread plate count (Kasing, 2001). Serial dilution was conducted in 10⁻¹ to 10⁻⁶. One ml of samples was added to 9 mL of peptone water for six steps in tenfold series. Diluted samples with 0.1 mL were pipetted from at least two appropriate dilution factors were spread on EMB agar to determine the considerable bacterial counting. Bacteria cells in samples were allowed to grow on EMB agar on the petri dish to form green metallic sheen colonies. Assumption of counting colonies was made whereby each colony is derived from one cell. After incubation for 24 hours at 37°C, the colonies were then counted manually to get the total number of cells of the samples. Counting was preferably done on the petri dish that had 30 – 300 colonies.

Data Analysis

Analysis of interaction and significant difference in *E. coli* population numbers and decay rates between different levels of substrate and temperatures were carried out using two-way ANOVA and multiple comparison method. The mean die-off rate for different substrate and temperature were determined by using simple linear regression of bacteria \log_n concentration ($\log \text{cfu}+1/\text{mL}$) versus time (days). A graph logarithm of numbers of *E. coli* versus day was plotted for each replicates to determine the slope of the graph. Model fit for the conditions at different temperature were studied. Temperature coefficient (θ) was estimated in this study. Data was analyzed using SPSS 11.0.

Results

The standard graph for *E. coli* growth, log of bacteria concentration versus OD (Figure 2) and the log of bacteria concentration versus time (Figure 3) are shown below. The concentration of inoculum, 10^8 cfu/mL was obtained at OD 0.75 – 0.8 after 4 hours incubation.

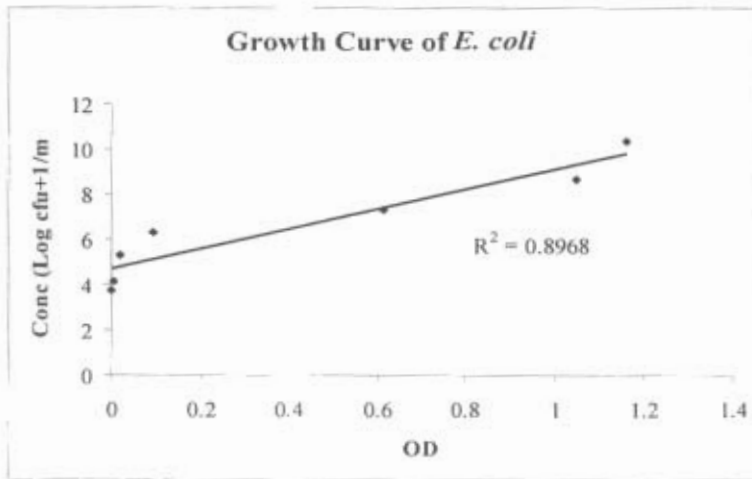


Figure 2: Graph Concentration (Log cfu+1/mL) versus OD.

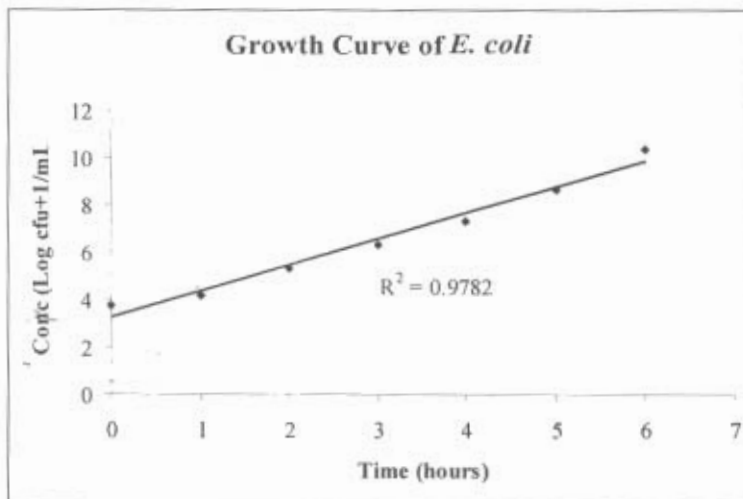


Figure 3: Graph Concentration (Log cfu+1/mL) versus time (hours).