The Study of the Biodiversity in Local Bio-Extract and the Treatment of Community Wastewater at Laboratory Scale: Wastewater from Restaurants

BY

SUPATCHAYAPORN NITSUWAT

A special project submitted to the School of Biotechnology,
Assumption University in part of fulfillment of the requirement
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Level of study	:	**Bachelor of Science**
Department	:	**Food Technology**
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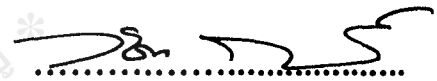

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Ms.Supatchayaporn Nitsuwat

ABSTRACT

Bio-extract is a solution composed of a diverse variety of microbes coexisting together, aiding the metabolism of each other. Nowadays bio-extract is widely use for many purposes and one of the popular usage is for wastewater treatment. In this experiment, the biodiversity of the bio-extract was determined by selective and non-selective enrichment mediums. For the treatment of restaurant wastewater (collected from local department store food court and steak restaurant), 0 (control), 0.25, 0.5 and 1 ml of bio-extract were inoculated per liter of wastewater. In the treatment procedure, light (with light and without light), time (24 hours and 48 hours), and oxygen (with oxygen and without oxygen) were varied. After treatment, the wastewater sample's chemical and microbiological properties were tested. The chemical properties measured were total solid (TS), BOD, total suspended solid (TSS), total dissolved solid (TDS), pH and grease and oil. The microbiological properties were measured by MPN method and total plate count method. The bio-extract biodiversity was found to contain *Bacillus spp.* 3.00×10^3 CFU/ml, mold 3.63×10^3 CFU/ml, lactic acid bacteria 4.35×10^4 CFU/ml, Actinomycetes 1.27×10^5 CFU/ml, and yeast 1.35×10^5 CFU/ml. When using bio-extract to treat restaurant wastewater, there were significant reduction of TS and grease and oil at 53.07% and 69.89% respectively. The best condition for restaurant wastewater treatment was 0.25 ml of bio-extract per liter of wastewater without oxygen and light for 48 hours. However, the quality of treated wastewater was still above the standard required therefore further experiment will be needed to improve the quality of water before discard.

Keywords: Bio-extract, biodiversity, TS, BOD, Grease and oil, MPN

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INTRODUCTION

Water is essential for every living organism. High amount of water is being used each day for many purposes ranging from drinking, cleaning to agricultural use. These activities resulted in high amount of discharged water contaminated physically, chemically and biologically. Wastewater is defined as water that is contaminated, undesirable or unsuitable for consumption. The wastewater could contaminate and cause quality depletion of natural water resources. Restaurant wastewater is one type of community wastewater with the characteristic of greasy, oily and contaminated with organic scraps and detergent from washing and cleaning (1). According to the control standards of the restaurant wastewater, the discharge must have a pH of 5 to 9 with biochemical oxygen demand (BOD) of ≤ 200 mg.L^{-1}, total suspended solid (TSS) of ≤ 60 mg.L^{-1} and grease and oil content of ≤ 100 mg.L^{-1}. The wastewater must be treated before discharging and one of the methods to treat the wastewater is by using the bio-extract (2 pp. 8-9).

Bio-extract is the product from fermentation of plants, fruits or animal with sugar or molasses also called as effective microorganisms (EM). The microbes present will utilize the nutrients to increase its population and variety (3). The microbes that can be found in the bio-extract are *Bacillus spp.*, Lactic acid bacteria, actinomycetes, purple non-sulphur bacteria, yeast and mold. Generally, the bio-extract has the pH of 3.5 to 5.6 (4). The most abundant microbe in the bio-extract according to former research is lactic acid bacteria, yeast and mold (5). Bio-extract has gained much attention since its discovery. Its benefits have been widely studied and the usage of bio-extract in wastewater treatment is one of them.

In this research, the local bio-extract was used to study the biodiversity of microbes and their efficiency in treating restaurant wastewater was determined in different physical conditions.

OBJECTIVE

1. To study the efficiency of the local bio-extract in restaurant wastewater treatment by monitoring the chemical and microbiological changes after treatment.

2. To study the biodiversity of the local bio-extract.

LITERATURE REVIEW

Wastewater

Wastewater is the water that has been used in certain activities in households and industries causing the contamination of impurities making the water undesirable, unsuitable for further usage and deplete the natural water sources if discharged (1), (6). The wastewater contaminated with impurities can come from either nonpoint source or point source. Nonpoint source usually involves a large space with wide range of activities and the source of the discharge cannot be determined. Point source on the other hand can be determined easily as of where the discharges are from such as hospitals, factories and farms etc. As for the impurities that contaminate the wastewater, they can be classified as the followings:

1. Microorganisms

Wastewater from households, hospitals, hotels and restaurants are often highly contaminated with pathogenic microbes due to the water was utilized in human activities that can cause contamination of microbes or organic substances that acts as energy source for the microbes. The majority of the microbes found in the wastewater are the nonpathogenic type but pathogenic microbes are still a concern. The best known microbe in the wastewater field is the fecal coliform which is commonly associated with feces and widely used as the indicator microorganism. Indicator microbes are not dangerous themselves but its presence indicates health risk since feces may contain pathogens that can contaminate the water and cause illness to its consumers (7). Possible illnesses transmitted via body discharges from an ill person are such as typhoid fever, dysentery, cholera, hepatitis and other intestinal infections (8).

2. Organic matters

Organic matters are carbon-based and are found generally in the environment normally as a combination of carbon, hydrogen, nitrogen and other elements. In the wastewater, organic matters are originated from plants, animals and synthetic organic compounds. They contaminated into the water in human waste, paper products, detergents, food scraps, agricultural and industrial sources. Biodegradable organic matters are often utilized by the microbe via its metabolism such as amino acids, proteins, carbohydrates etc. Some are harder to degrade and requires more specific bacteria to degrade. Excess amounts of organic matters can deplete the water quality due to the microbes will utilize dissolved oxygen present in the water to break down the organic wastes causing lack of oxygen supply in the water needed by the aquatic life.

The oxygen needed by the organisms to break down waste in the water can be referred to as biochemical oxygen demand (BOD) and is one of the measurements used to assess overall wastewater strength which will be discussed later on. In addition, synthetic organic matters such as insecticides and herbicides, generally containing benzene and toluene, are highly toxic to living organisms. It can kill or contaminate the fish causing them unfit for consumption (9).

3. Suspended solids (TS)

All particles that are suspended in the water and cannot pass through a filter are considered as suspended solids. High levels of suspended solid can cause loss of ability to support the aquatic diversity. Suspended solids absorb heat thus the temperature increase causing the levels of dissolved oxygen to decrease and photosynthesis decreases because less light can penetrate which affects the production of oxygen by plants and algae that exist in the water. Finally, aquatic lives that are sensitive to changes in dissolved oxygen level will die out. The suspended solids that come from point sources can be easily managed by using adequate treatment consisting of settling prior to discharging the wastewater. This will allow the solids to sink to bottom and removed. As for nonpoint sources, control measures must be implemented to reduce the suspended solid in water run-off such as using silt fences and sedimentations for construction sites or using retention ponds and regular street sweeping in urban areas (10).

4. Trace organics

Due to modern treatment plant efficiencies, only small concentrations of organic compounds remain in the wastewater. Even in small amounts, these trace organics still pose some threat due to the long life. Accumulation of trace organics in the tissue of mammals or plants could pass along the food chain and eventually reaching humans. Trace organics can affect the water quality in terms of smell, taste or color even if present in trace amounts. The majority of trace organics found in wastewater are phenolic compounds, phthalate esters, naphthalenes, monocyclic aromatic hydrocarbons, polycyclic aromatic hydrocarbons, halogenated ethers, polychlorinated biphenyls (PCB's), nirogenous organics, halogenated aliphatic hydrocarbons and organochloride pesticides (11).

5. Toxic substances

Toxic substances can exist as an inorganic or an organic compound such as heavy metals (ex. cadmium, lead etc.), pesticides and herbicides. Toxic substances generally come from

industrial wastewater and agricultural wastewater and run-offs while domestic wastewater rarely becomes the source of toxic substances. Toxic substances deplete the water quality and are harmful to the aquatic life and even to humans if consumed via water consumption or from contaminated fish.

6. Color and turbidity

The water color can indicate the conditions and degree of impurity of the wastewater. Wastewater will have undesirable colors according to its contaminants. Wastewater with light brown color is found to be discharge from less than 6 hours, water with light to medium grey is found to have undergone certain degree of decomposition under anaerobic conditions or was in the system for more than 6 hours. While the wastewater that is found to be dark grey to black has undergone extensive decomposition by bacteria under anaerobic conditions. Blackening of wastewater is usually due to the sulphides, particularly ferrous sulphide, formed from the combination of hydrogen sulphide and divalent metal (such as iron). Water turbidity corresponds to the amount of light that can penetrate through affecting the aquatic life's ability to photosynthesize. In water that has high turbidity, less light will be able to penetrate causing lack of light for photosynthesis. If the aquatic life dies out, this will increase the amount of organic compounds that the microbes can degrade which will cause dissolved oxygen to be utilized lowering the dissolved oxygen levels causing a chain of bad effects to the wastewater (12).

7. Nitrogenous and phosphorus compounds

The forms of nitrogen found in the wastewater includes organic nitrogen, nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_4^-) and nitrogen gas (N_2) which are all interconvertible. These compounds can cause growth acceleration of aquatic plants such as algal blooms. Too much aquatic plants can cause lack of dissolved oxygen affecting aquatic life. The organic nitrogen is the principle nitrogen constituent in feces. It includes urea (H_2NCONH_2), the principal compound of urine. It must be converted to nitrate for the plants to utilize. Nitrate is the most oxidized specie and can be utilized by plants. Nitrate is negatively charged thus does not bind to soil which is also negatively charged giving it the ability to pass through soil straight to the groundwater. By this, regulatory agencies may require an onsite system that can reduce the nitrogen in the effluent. Nitrite is converted to nitrate therefore not usually observed in water sources. It is found to be toxic to most aquatic species and can be oxidized by chlorine which may affect the cost in disinfection due to dosage requirements in treating wastewater that contain nitrite. For ammonia, can be found predominantly as ammonium ion (NH_4^+) when the pH is

below 9.3 and predominantly as ammonia gas (NH_3) when the pH is above 9.3. Unlike nitrate, ammonia binds to the soil since it is positively charged thus plants can utilize ammonia as nitrogen source.

As for phosphorus, it also has many forms including soluble orthophosphate ion (PO_4^{-3}), organically bound phosphate (from excretia and food residue) and other phosphorus/oxygen forms. Phosphorus, like nitrogen, causes eutrophication in the surface water bodies. Eutrophication is the over enrichment of an ecosystem by chemical nutrients or compounds that contains nitrogen and/or phosphorus causing excessive algae growth which will cause excessive amounts of organic matter when it dies. Excessive amount of organic matter leads to excessive dissolved oxygen utilized by microbes to decompose them and thus leads to death of other aquatic life due to lack of oxygen. However, phosphorus can rapidly combine with other compounds such as limestone to form calcium phosphate stopping the phosphorus from migrating to the water bodies (9), (13).

8. Grease and oil

This term applies to fats, oils, waxes and other related constituents. Lipid impurities can be easily removed if the lipid floats above the water but they may harden at the water surface or in cases where surfactants are involved, the lipids may be suspended in the water. Lipid cannot be quickly broken down by bacteria and can cause pollution because it increases the BOD levels and prevents oxygen from reaching the water. Grease and oil can trap trash, plants and other matters causing foul odors, attracting pests and disease vectors. In warm and greasy wastewater, the grease and oil will not separate from the water fast enough. Once it flows through septic tanks and into the soil, it can solidify and clog the soil pores ruining the drainage system or may accumulate in layers which will require more pumping. These will result in high expenses therefore grease traps are mandatory for restaurants and food service facilities. Pertroleum-based waste is hazardous and should be especially put into considerations for proper separation from the wastewater, collection and disposal.

9. Floating matters

Floating matters can either be in liquid or in solid form. The floating matters that are in liquid form are such as grease and oil and certain solvents while the floating matters that are in solid form are such as small pieces of wood or paper and garbage.

10. Volatile matters

Volatile matters that can evaporate at low temperatures are volatile fatty acids, volatile organic carbon and some gases (ex. hydrogen sulfide and ammonia). These volatile matters will cause the wastewater to have unpleasant odor. Ammonia is found generally in domestic wastewater. Ammonia can be caused from the breakdown of urea excreted by animals and humans by microbes or from protein breakdown. The oxidation of ammonia, as called nitrification, can stimulate excessive growth from providing nitrates and nitrites as nutrients (14). Hydrogen sulfide or as known as sewage gas can naturally occur in crude petroleum, natural gas, or from organic matter bacterial breakdown and from human and animal waste as well as from industrial activities. It is colorless and flammable under normal conditions. Exposure to low concentrations of hydrogen sulfide can cause eye irritation, sore throat, breath shortage and fluid in the lungs which will eventually go away after a few weeks. As for long term exposure, it can result in fatigue, loss of appetite, headaches and even dizziness (15). At very extreme concentrations, inhalation can cause sudden death which can be seen in news regarding workmen trying to clean the sewers and ends up losing consciousness leading to death (16).

Wastewater quality parameters

1. Total solids

Total solids ($mg.L^{-1}$) are the impurities that are left after the water has evaporated out. Total solid is made up of dissolved solids, suspended solids and settleable solids.

Dissolved solids are particles that can pass through 2-micron filters such as calcium, nitrate, phosphorus and other ions.

Suspended solids are particles that cannot pass through 2-micron filters and suspend itself in the water body such as silt, algae and other particulate matters.

Settleable solids are particles that cannot pass through 2-micron filters and is heavy enough to settle at the bottom when left to settle.

Water with low levels of solids will cause organisms to swell up and vice versa for high levels of solids due to the concentration of the solids outside and inside of the cell is not equilibrium causing loss of stability in maintaining the cell density and may cause it to sink or float into conditions unfit for survival. High levels of solid can clog the irrigation system, as well as reduce the efficiency of wastewater treatment. High total suspended solids also cause damage by attracting toxic substances and act as a carrier, introducing it into water sources. The amount of light that can penetrate through the water is affected by high total solids. It slows down the

photosynthesis process of aquatic plants causing the water to hold up heat affecting the aquatic life that live on low temperature waters (17).

2. pH

The value of pH indicates the concentration of hydrogen ions in the water. The water is considered to be acidic if the pH exceeds 7 and basic if the pH is below 7. The pH is an important chemical component of the wastewater that will affect the treatability of the wastewater especially the optimum performance of certain chemical reactions and optimum conditions for the organism used for biological wastewater treatment etc (18).

3. Biochemical oxygen demand

The biochemical oxygen demand (BOD) values can indicate the wastewater's degree of impurities in terms of the demand for oxygen. The microbes that can degrade organic matters will degrade them under aerobic conditions, most are considered to be heterotrophic. The standard conditions used in the analysis of BOD are as follows:

- Constant temperature of 20°C.
- Incubation period of 5 days.
- Under aerobic conditions
- Sufficient amount of nutrients for microbial growth.
- Microbes analyzed must exist in sufficient amounts with ability to grow in the wastewater.
- Absence of substances that is toxic to the microbes.

BOD can be either analyzed by determining the soluble BOD or determining the total BOD. The difference is that in preparing the sample for determining the soluble BOD, the wastewater sample must be filtered to remove the insoluble organics. But for determining the total BOD, the wastewater sample must be homogeneously mixed to prevent sedimentation causing the values read lower than the actual value.

4. Grease and oil

Grease and oil is a type of organic substance that is water insoluble causing it to float as a layer on the surface. It is harder to degrade than other types of organic substances. Grease and oil causes inconvenience in the wastewater treatment process in terms of acting as a barrier decreasing the amount of oxygen penetration and sedimentation etc. Thus the amount of grease and oil is another factor that needs considerations before choosing the suitable treatment method.

5. Microorganisms

There are two general types of microbes existing in wastewater, normal flora and pathogenic microbes. Source of pathogenic microbial contamination are mostly wastewater discharged from communities and hospitals. Analysis of the amount of pathogenic microbes can be done directly and indirectly. The direct method involves detecting the pathogen itself while the indirect method involves detecting indicators of pathogens presence. The most typical indicator used to determine the presence of pathogenic microbes is the presence of fecal coliforms. Fecal coliforms are bacteria that reside in the intestinal tract of warm-blooded animals thus excreted out via feces. An example of fecal coliform widely known is *Escherichia coli* or *E.coli.* Fecal coliforms can be detected by membrane filters, standard plate count method and by most probable number method (MPN). Most probable number method (MPN method) involves three processes as follows (19):

5.1 Presumptive test – series of lauryl tryptose broth (with bromocresol purple) tubes are inoculated with measured amounts of water sample. The series of tubes can be consisting from three to five or more. In this experiment five tubes were used which gives more sensitivity than using three tubes. Positive result indicating the presence of coliforms is marked by the color change of the bromocresol purple, an indicator in the medium, from purple to yellow due to acid production after 48 hours of incubation at approximately 35°C. By referring to the MPN table, estimated number or "most probable number" of coliforms in 100 mL of water sample is obtained.

5.2 Confirmed test – Positive tubes are inoculated into EC broth to selectively culture fecal coliforms at approximately 44.5°C. Positive results are indicated by gas production seen from the gas bubbles trapped in the durham tube. By referring to the MPN table, estimated number of fecal coliforms in 100 mL of water sample is obtained.

5.3 Complete test – Positive tubes are inoculated into eosin methylene blue (EMB) agar plates in order to confirm that the culture in the positive tubes from the confirm test are *E.coli*. The methylene blue in EMB agar will selectively inhibit gram positive organisms while allowing the growth of gram negatives. In this step, *E.coli* can be identified from its unique green metallic sheen produced after incubating at approximately 35°C, indicating positive fecal contamination. The positive colonies are then inoculated onto a nutrient agar slant, gram stained and observed under the microscope. If it appears to be a gram-negative, nonspore-forming rod then it is *E.coli* positive.

Wastewater classification

Wastewater can be classified based on the source (domestic wastewater, agriculture wastewater and industrial wastewater) and by the type of the impurities (organic wastewater and inorganic wastewater).

- **Classification by source**
  - Industrial wastewater

Industrial wastewater is the wastewater that is contaminated with different types of impurities at different levels of concentration depending on the type of industry, the raw materials used, as well as processing steps. The impurities can be classified into inorganic and organic substance. Industrial wastewater generally results from both the production steps inside the factory such as cleaning and cooling of the machines as well as from the activities done by its employees. Some industry may separate these two sources from one another and treat them separately while others threat them altogether. Industry wastewater differs in characteristics from one source to another. The composition is influenced by the type of manufacturing for example, food industries wastewater will contain high organic residues while high contents of metal is present in plating industries. Table 1 shows general information of the wastewater compositions from different types of industries (12).

Industry	BOD (mg/L)	TSS (mg/L)	Grease & Oil (mg/L)	Metals Present	Volatile Compounds Present
Oil Refinery	100 to 300	100 to 250	200 to 3,000	Arsenic, Iron	Sulphides
Tanneries	1000-3000	4000-6000	50-850	Chromium	Sulphides Ammonia
Bottling Plant	200 to 6,000	0 to 3,500			
Molasses/ Sugar Factory	600 to 32,000	200 to 30,000			Ammonia 5 to 400
Food Processing	100 to 7,000	30 to 7,000			
Paper Factory	250 to 15,000	500 to 100,000		Selenium, Zinc	
Chemical Plant	500 to 20,000	1,000 to 170,000	0 to 2,000	Arsenic, Barium, Cadmium	

Table 1. The compositions of the wastewater from different types of industry.

Source: **Alturkmani, Abdulrzzak.** Industrial Wastewater. *Environmental Engineering.* [Online] [Cited: September 24, 2013.] www.researchgate.net/publication/249656190_INDUSTRIAL_WASTEWATER/file/e0b4951e6739795ffb.pdf.

- Agriculture wastewater

Agricultural wastewater is the wastewater that is released from all agricultural activities, including animal farming areas and plant cultivation areas. This type of water generally contaminated mainly with organic matters from animal feces and feeds causing the wastewater to have high concentrations of organic compounds as well as insoluble solids. As for the agricultural wastewater that is from plant cultivation areas are mainly contaminated with chemical compounds, fertilizers, herbicides and pesticides used in cultivation areas.

- Domestic wastewater

Domestic wastewater is the wastewater that comes from households, restaurants, shops, buildings, hotels and etcetera. The cause of the discharge usually comes from human activities such as cooking, cleaning, and consuming. Therefore the wastewater will compose mainly of organic waste with surfactants and presence of microbes with absence of hazardous compounds. The important factors that affect the quality of the domestic wastewater are the type of usage purpose, type of wastewater collection pipe, the primary wastewater treatment system and the condition of the wastewater collection pipe. The usage purpose influences the wastewater composition such as in restaurant wastewaters the level of grease and oil as well as the BOD_5 will be much higher than the wastewater from households or government buildings which less activities that cause contaminations are implemented. The type of collection pipe used influences the degree of contamination. If the collection pipe is not separated from other pipes, the wastewater will be collected together with less contaminated water such as rain water and will be more diluted unlike separated collection pipes. The primary wastewater treatment will help lower the degree of contamination. If the community provides a primary wastewater treatment such as septic tank or cesspool, the wastewater will be less contaminated than those that do not use primary wastewater treatments. As for the conditions of the wastewater collection pipe, pipes that are in poor conditions or not according to construction standards will cause less contaminated water to pass through the pipes some may leak out while some may remain and accumulate in the pipes causing fermentation and degradation inside the pipes (20).

- **Classification by type of impurities**
  - Organic wastewater

Wastewater contaminated with organic waste that can be degraded and utilized by microbes are such as wastewater from domestic use, food industries, and agricultural uses etc. As explained earlier, this type of wastewater normally does not contain toxic substances but the organic waste can cause undesirable water characteristics as well as water quality depletion when degraded by microbes present in the environment.

- Inorganic wastewater

Unlike organic, inorganic cannot be degraded and utilized by the microbes. Sources of inorganic wastewater are such as wastewater from petrochemical industries, chemical industries and dye industries etc. This type of water often contains toxic chemicals and substances (6).

Restaurant wastewater

Restaurant wastewater is classified under domestic wastewater and organic wastewater type. The common characteristic of the restaurant wastewater is higher surge volumes in the peak hours, generally high temperatures and high levels of grease and oil as well as food scraps which can cause BOD increase. Grease and oil will cause clogging problems since it is in its liquid state at high temperatures but can later solidify. Shown in table 2 are the results from a study conducted by the Universities of Washington and Wisconsin in the 80's to characterize restaurant wastewater, along with results of general domestic wastewater to compare (21).

Type of Wastewater	BOD mg/l	Oil and Grease mg/l	TSS mg/l
Raw restaurant wastewater (Washington Study)	1000 – 2000	100 – 300	300 – 625
Pretreated restaurant wastewater (Wisconsin Study- 12 restaurants)	101 – 880 avg = 365	24 – 144 avg = 63	
Pretreated restaurant wastewater (Wisconsin Study-6 selected full-service restaurants)	245 – 880 avg = 506	40 – 144 avg = 83	65 – 372 avg = 177
Domestic Wastewater	100 – 400 avg < 230	16 – 65	100 – 350

Table 2. Restaurant wastewater characteristics compared with general domestic wastewater.

Source: **Barnstable County Department of Health and Environment.** Grease and Oil in Restaurant Wastewater. *Barnstable County Department of Health and Environment* . [Online] [Cited: October 3, 2013.] http://www.barnstablecountyhealth.org/ia-systems/information-center/compendium-of-information-on-alternative-onsite-septic-system-technology/grease-and-oil-in-restaurant-wastewater.

Restaurant wastewater standards and specifications

Control Standards of Wastewater Discharged from Buildings of Different Types and Sizes						
Water Quality Index	**Unit**	**Maximum limit according to the types of control standards of wastewater discharge**				
		A	**B**	**C**	**D**	**E**
1. pH	-	5-9	5-9	5-9	5-9	5-9
2. BOD	mg/L	≤ 20	≤ 30	≤ 40	≤ 50	≤ 200
3. Suspended solids	mg/L	≤ 30	≤ 40	≤ 50	≤ 50	≤ 60
4. Settleble solids	mg/L	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	-
5. Total dissolved solids	mg/L	≤ 500*	≤ 500*	≤ 500*	≤ 500*	-
6. Grease and oil	mg/L	≤ 20	≤ 20	≤ 20	≤ 20	≤ 100

Table 3. Wastewater discharged from buildings of different types and sizes control standards.

Note: * = the value that increased from normal total dissolved solid levels in water.
Building type A – Restaurants with total service area including all floors or group of buildings from 2500 sq.m. and over.
Building type B – Restaurants with total service area including all floors or group of buildings from 500 sq.m. but not over 2500 sq.m.
Building type C – Restaurants with total service area including all floors or group of buildings from 250 sq.m. but not over 500 sq.m.
Building type D – Restaurants with total service area including all floors or group of buildings from 100 sq.m. but not over 250 sq.m.
Building type E – Restaurants with total service area including all floors less than 100 sq.m.

Source: **Ministry of Natural Resources and Environment.** Control Standard Specification: Wastewater Discharged from Buildings of Certain Size. *Royal Thai Government Gazette.* Bangkok : s.n., 2005. 122.

Type	**Control Standard Limits**
Coliform	No more than 20,000 MPN/100 mL
Fecal Coliform	No more than 4,000 MPN/100 mL
E.coli	No more than 406 *E.coli*/100 mL

Table 4. Control standards limits of total coliform*, fecal coliform* and *E.coli***.

Source: * = **National Environment Board**. Control Standard Specification of Surface Water Sources. *Royal Thai Government Gazette.* Bangkok : s.n., 1994. 111.
** = *Bacteria in Surface Water.* **New Hampshire Department of Environmental Services.** New Hampshire : New Hampshire Government, 2011. WD-BB-14

Wastewater treatment

Wastewater treatment aims to allow community and industrial effluents to be in a generally acceptable condition for disposal with minimum danger to human and the environment. Wastewater treatment involves four main processes of preliminary treatment, primary treatment, secondary treatment and tertiary treatment (22).

In the preliminary treatment process coarse solids and large materials are removed to increase the efficiency of the operation and for maintaining the conditions of treatment units. The process involves mostly physical treatments such as coarse screening, grease traps, grit chambers and comminuting of large objects etc.

Primary treatment removes organic and inorganic solids by sedimentation and flotation processes. Primary treatment reduces the velocity to allow objects that are denser than water to settle and the ones that are less dense than water to float and disperses the flow of the wastewater. The effluent from primary treatment contains mainly colloidal and dissolved organic and inorganic solids since most of the suspended solids has been removed.

Secondary treatment provides additional removal of organics by biological treatments using different types of microbes in a controlled environment favorable to the microbe's growth. The mixed population of microbes will utilize the colloidal and dissolved organics as nutrients to obtain energy. With presence of oxygen, the end product will include carbon dioxides, water, sulfates, nitrates and phosphates. Before discharging, the biological mass must be separated from the wastewater to ensure proper degree of treatment. If not, the effluent quality will decrease and the BOD will be high (23).

Secondary treatments commonly used are activated sludge processes, trickling filters or bio-filters, oxidation ditches, and rotating biological contactors (RBC).

Tertiary treatment is applied on cases that specific wastewater constituents that must be removed cannot be removed by secondary treatment. Individual removal processes are applied and sometimes combined with primary and secondary treatment processes (22).

Wastewater treatment processes are classified into four general categories; physical, chemical, biological and physical-chemical treatment process (24).

Physical process may include screening, grit removal, primary settling tank, secondary settling tank and grease trap. Screening can either be coarse screening or fine screening. Coarse screening will help remove large particles, preventing clogging while fine screening will screen out suspended solids that are less than 1 inch in size from the wastewater. Grit removal aims to remove suspended solids that are classified as grit, sand etc. by settling method. There are two types of grit removal chambers which are conventional grit chamber and aerated grit chamber.

Primary settling tank and secondary settling tank differs with the settling target. Primary settling tank aims to remove organic suspended solids ex. food scraps. Secondary settling tank aims to remove microbial sludge after the wastewater passes through an aeration tank. Grease trap can be classified into baffle grease traps and dissolved-air flotation or DAF. It aims to remove grease and oil from the wastewater by allowing them to suspend on the surface.

Chemical process may include pH adjustment, coagulation and flocculation, precipitation, ion exchange and disinfection. For pH adjustment, it aims to adjust the wastewater's pH to be suitable for the next process. Coagulation and flocculation involves addition of chemicals to cause the flocculation of suspended solids, color and turbidity making it easier to settle. General coagulation and flocculation involves fast and slow mixing. Fast mixing aims to mix the wastewater and added chemicals together, while slow mixing aims to cause flocculation. Precipitation involves the addition of chemical substances to precipitate the hazardous soluble solids ex. zinc, copper and chromium usually in industry wastewaters. Ion exchange uses resin as the exchanger in a cylindrical column. This method solves the water hardness which is a problem generally found in industrial wastewaters. Ion exchanging is used after the water has pass through the precipitation process to prevent depletion of the resin's conditions. As for disinfection process, chemicals (usually chlorine) ozone and ultraviolet light is added to disinfect the wastewater.

For the biological treatment process, microbes or plants are used to treat the wastewater mainly by decreasing the organic compounds. It can either be anaerobic or aerobic treatment. Anaerobic treatment deals with microbes that can survive under anaerobic conditions. This type of treatment utilizes less energy and the by product is methane which can be used as household gas and electricity production. The well known methods are anaerobic pond, septic tank, anaerobic filter, upflow anaerobic sludge blanket (UASB) and anaerobic digester. As for the aerobic treatment, it deals with microbes that can survive under aerobic conditions. These aerobes will utilize the organic compounds into ammonia, nitrate, sulfate, phosphate and new cells. The pros are this method can treat the wastewater to be according to the standards and reduce the foul odors etc. But the cons are the high cost and no usable by products made. Methods used for aerobic treatment are stabilization pond, aerated lagoon, activated sludge (AS), trickling filter, rotating biological contractor (RBC) and construction wetland etc. (24)

Bio-extract

Bio-extract or commonly known as "Effective Microorganism (EM)", is the product from the fermentation of plants, fruits or animal with sugar or molasses. The molasses will cause the organic substances inside the cell of plants and animals to lysis out of the cell. There are two types of fermentation: with oxygen (open lid) and without oxygen (closed lid). The microbes present will utilize the nutrients to increase its population and variety (3). The microbes that can be found in the bio-extract are *Bacillus spp.*, Lactic acid bacteria, actinomycetes, purple non-sulphur bacteria, yeast and mold. Generally, the bio-extract has the pH of 3.5 to 5.6 (4).

Bacillus spp. is a rod-shaped, gram-positive, aerobe with some facultative anaerobes. It has the ability to form resistant spore coats and product heat-resistant endospores. *Bacillus spp.* are mesophilic with a growth range between 10°C to 48°C, optimally at 28°C to 35°C at pH range of 4.9 to 9.3. *Bacillus spp.* can produce protease and amylase. Protease will catalyze the protein hydrolysis reaction which will degrade protein to peptides (25). Amylase will catalyze the hydrolysis of starch into dextrin and saccharides (26).

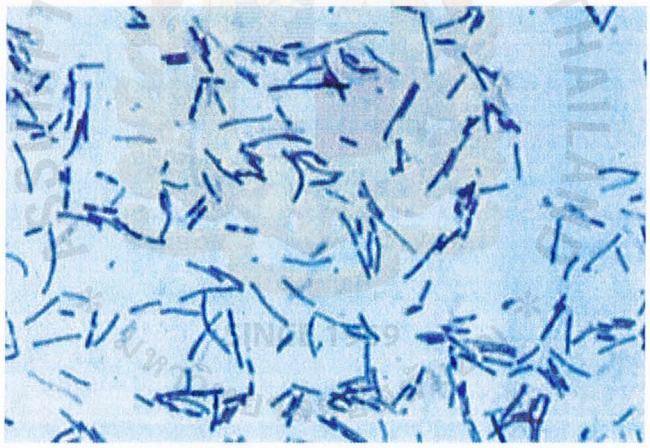

Figure 1. Gram-stain of *Bacillus subtilis* one of the many in *Bacillus spp.*
Source: http://oomycota.blogspot.com/2010/08/bacillus-subtilis.html

Lactic acid bacteria are gram-positive fermenting-bacteria. Via fermentation, it can convert sugar into organic acids such as lactic acid and has the ability to produce bacteriocins which suppresses bacterial growth. The acid production will cause the lowering of pH in wastewater thus suppressing growth of most microbes (27). From fermentation, carbon dioxide (CO_2) is formed and acts as an energy source to other microbes e.g. phototrophic bacteria (28).

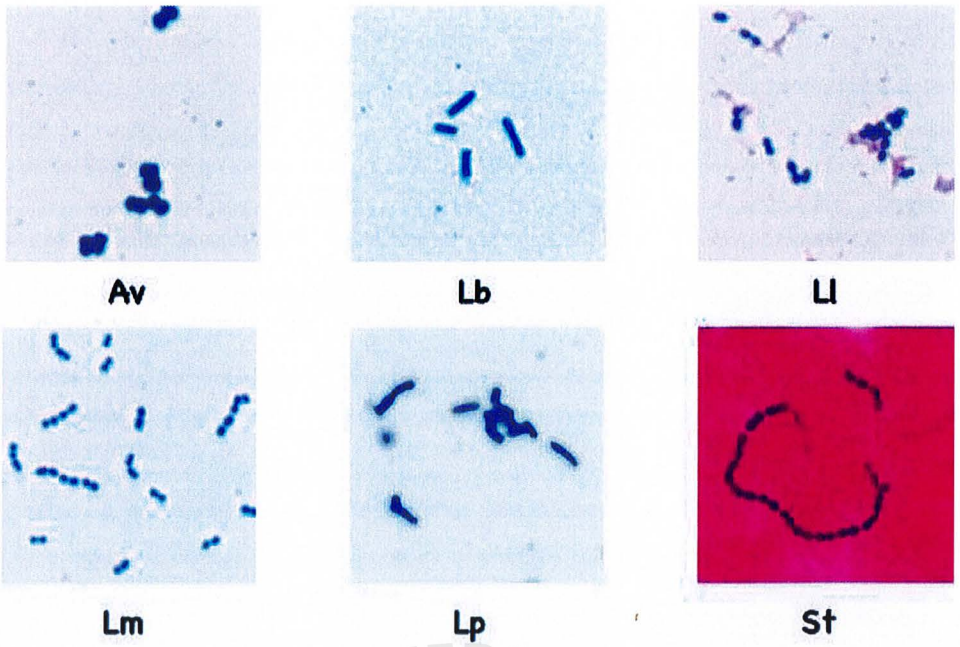

Figure 2. Gram stain microscopic views of various lactic acid bacteria (from left to right: Av, *Aerococcus viridans*; Lb, *Lactobacillus bulgaricus*; Ll, *Lactococcus lactis*; Lm, *Leuconostoc mesenteroides*; Lp, *Lactobacillus plantarum*; St, *Streptococcus thermophilus*.)

Source: http://inst.bact.wisc.edu/inst/index.php?module=book&func=displayarticle&art_id=278

Purple non-sulfur bacteria, on the other hand, is a phototrophic anaerobe which utilizes hydrogen sulfide and volatile organic compounds as an electron donor for photosynthesis. As well as using volatile organic compounds and alcohol as a carbon source. Its ability to utilize toxic hydrogen sulfide and convert it to a non-toxic form is very beneficial in industry wastewater treatments (29).

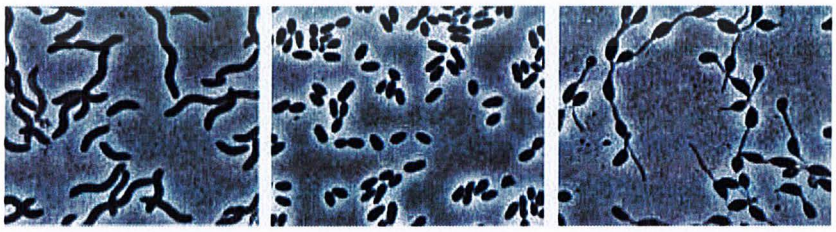

Figure 3. Microscopic photos of purple non-sulfur bacteria

Source: http://textbookofbacteriology.net/themicrobialworld/procaryotes.html

Yeast and mold can utilize organic compounds as its carbon source. Molds are multicellular, mostly aerobic and capable of growing at low moisture. Yeasts are unicellular and can be oxidative and fermentative and just like mold, it can be both mesophilic and psychrotrophic.

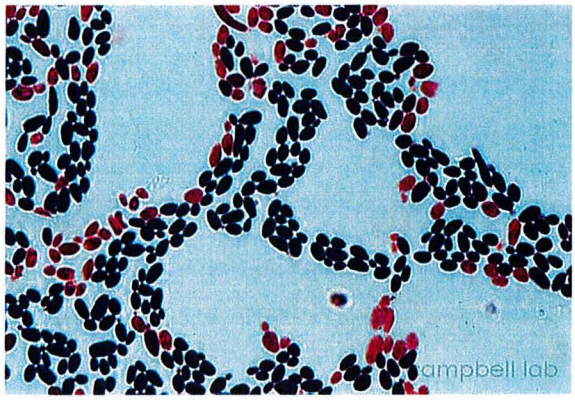

Figure 4. Microscopic photo of yeast

Source: http://eyemicrobiology.upmc.com/Default.htm

Figure 5. Microscopic photo of *Rhizopus* a genus of mold

As for actinomycetes, they are gram positive bacteria with the ability to form spores. They are found to be able to degrade starch, cellulose, hydrocarbon and lignin etc. as well as heavy metals which will be beneficial for organic-rich and industrial wastewaters (5), (30)- (31).

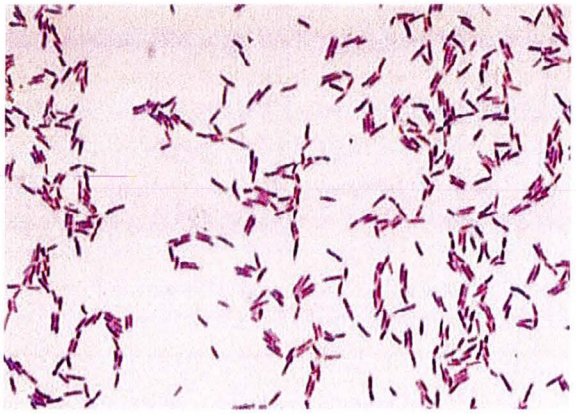

Figure 6. Microscopic photo of *Actinomyces viscosus* a member of the *Actinomyces* genus.

Source: http://phil.cdc.gov/phil/details_linked.asp?pid=1256

The most abundant microbe in the bio-extract according to previous research is lactic acid bacteria, yeast and mold (5).

Benefits and applications of bio-extract

Bio-extract has been widely used since its discovery. It was found to be beneficial and applicable in many fields as follows:

1. Agriculture

1.1. Crops

According to previous researches, bio-extract had shown to be capable of improving the soil quality and health as well as the growth, yield and quality of the crops. Though it cannot substitute other management process but it serves as an additional dimension that if used efficiently can enhance the beneficial effects to soil and crop management practices such as crop rotations, use of organic amendments, conservation tillage, crop residue recycling and bio-control of pests (32).

1.2. Livestock

The bio-extract is applied to help reduce the odor from urine and fecal matter by reducing the concentrations of ammonia gas produce from urine which can cause respiratory stress to workers and animals. The microbes in the bio-extract will break down ammonia. As for fecal matter, the bio-extract will act as an antioxidant that will resist the putrefaction and prevent odors, suppressing the growth of pathogens by the growth of beneficial microbes.

1.3. Composting

Composting is a great way to use organic waste to create a valuable product. But they produce foul odor from ammonia and mercaptans as well as proliferation of harmful microbes. The bio-extract helps prevent anaerobic pockets from putrefaction reducing the foul odor and harmful microbes.

2. Household usage

2.1. Cleaning

The bio-extract can be used as a substitute of chemical cleaners such as bleacher and synthetic detergents to avoid chemical components that can be absorbed through the skin, leading to health problems and pollute the environment. It functions in cleaning by degrading the organic matter.

2.2. Food waste

Food waste can be reduced by transforming them into fertilizer for using in the garden. The bio-extract will ferment the food waste, eliminating odor and attraction of flies.

3. Environment

3.1. Sewage treatment

The bio-extract helps reduce the foul odor and sludge of sewage. It prevents the corrosion and deterioration of the sewer systems and tanks by suppressing the activity of the free radicals saving maintenance cost. As it enters the natural water sources, it creates a condition that aids the propagation and revitalizes the plants preventing erosion.

3.2. Water purification

Application of bio-extract in water purification can help reduce the foul odor and sludge. The bio-extract can be used to revive the aquatic ecosystem which is damaged by the pollution and sludge accumulation that decreases the diversity in the ecosystem from decrease in nutrition necessary for the function and purification of the system (33).

Bio-extract, especially in the form of a ball, was widely discussed during Thailand's big flood incident in the year 2011 due to its ability to treat wastewater. The discussions were mainly debates on whether the bio-extract works or not. While many supported the fact that the bio-extract can beneficially treat the flood waters that were starting to deplete in terms of quality, some opposed. But conclusions came to the fact that the bio-extract does help treat wastewater but under certain conditions (34)- (35).

Previous research

Bio-extract was long since discovered by Dr.Teruo Higa and was generally named "Effective Microorganism" or "EM". Since then, many studies were conducted to test its efficiency on various applications. Examples of research conducted are as follows:

1. "The effect of bio-extract from cabbage waste on growth, yield and quality of volatile oil extracted from *Mentha spicata* and *Mentha arvensis var. Piperascens*" by Faculty of Pharmacy Srinakharinwirot University and Rajamangala University of Technology Thanyaburi. The objective was to observe the affect of the bio-extract produced from cabbage waste on the leaf biomass, yield and chemical composition of the volatile oils from both types of spearmint. Three fertilizers composing of cabbage waste bio-extract, sulfur fertilizer and a combination of both were tested. Results indicate that the cabbage waste bio-extract acted as an effective source of nutrients for cultivation of both spearmint types. In spearmint, the cabbage waste bio-extract also effectively promoted the yield of the volatile oil which also had the greatest carvone content. For Japanese spearmint, the combination of cabbage bio-extract and sulfur fertilizer was more efficient in enhancing the biomass.

2. "The Use of Chemical Fertilizer and Bio-extract for Vegetable Plant Growth under Hydroponics Condition" by Rajamangala University of Technology Thanyaburi. The growth yield of vegetables grown in a nutrient film technique hydroponics system was compared between those using conventional solutions, chemical fertilizers and a mixture of the chemical fertilizer and bio-extract in 3 different ratios (3:1, 1:1 and 1:3 V/V). Results shows that the conventional method was most significant in growth and yield but the mixture also had a trend indicating high yield in certain vegetables as well as lower total nitrogen in plants and less production cost.

3. "The Use of Bio-extract Water for Environment at Schools and Other Organizations" by Tangon Munjaiton, School of Social and Environmental Development, National Institute of Development Administration. The project studied the usage of bio-extract in schools and organizations and obtained the findings that there was a decrease in the BOD level to be according to standards. As well as significant reduction in cost, dirtiness, unpleasant odor and pest problems.

4. "Production of Bio-extracts Using Effective Microorganisms from Shrimp-Cooking Water for Wastewater Treatment System of Frozen Seafood Industry" by Kanjana Sohurat, Prince of Songkla University. The research studied the production of shrimp-cooking water and its efficiency in treating wastewater from the frozen food industry. Results indicated that the microbes were able to utilize the nutrients in the shrimp-cooking water. The facultative anaerobes and yeast were more abundant in the bio-extract produced under aerobic conditions while for anaerobic conditions the lactic acid bacteria were more abundant. The bio-extract made from shrimp-cooking water could significantly reduce the COD, BOD, TKN and SS as significantly as the bio-extract made from molasses can.

From the previous research above, it demonstrates that the bio-extract can provide many benefits in many fields from agriculture to wastewater treatment. Therefore in order to study the microbes that coexist in the bio-extract solution and investigate their association with wastewater treatment as well as its efficiency in treating restaurant wastewater, this project was implemented.

EXPERIMENTAL DESIGN

1. COLLECTION OF LOCAL BIO-EXTRACT

The bio-extract was provided by NAVA Social Enterprise, Bangkok, Thailand. It was contained in a plastic bottle, and stored at room temperature, away from direct sunlight. A new bottle of bio-extract was used for each round of wastewater sample testing.

2. PREPARATION OF THE ENRICHMENT MEDIUM FOR MICROBIAL ANALYSIS OF LOCAL BIO-EXTRACT

The local bio-extract was examined for the presence of mold, yeast, lactic acid bacteria, actinomycetes, purple non-sulfur bacteria, *bacillus spp.* and total viable cells using RBA (Rose-Bengal Agar), YM (Yeast Mold) agar, MRS (de Man, Rogosa and Sharpe) agar, GYEA (Glycerol-Yeast Extract Agar), GM broth and PCA (Plate Count agar) respectively as the enrichment medium (PCA is used for both *bacillus spp.* and total viable cells). RBA and MRS are premixed by HIMEDIA, Mumbai while the other media were prepared according with appendix E and autoclaved (Hirayama, model HA300-mii) at 121°C for 15 minutes then poured into sterile petri dishes or conical flasks for GM broth.

3. MICROBIAL ANALYSIS OF LOCAL BIO-EXTRACT

The bio-extract was analyzed by spread plate method. All plates were incubated at room temperature for 24 hours. The plates were examined and counted to determine the colony forming units per ml of bio-extract (CFU/ml). *Bacillus spp.* was analyzed by boiling the bio-extract for 10 minutes before diluting and spread plating 0.1 ml onto the PCA plates. The plates were incubated at room temperature for 24 hours and the CFU/ml was determined. GM broth was used to isolate purple non-sulfur bacteria by incubating 0.5 ml of bio-extract in 20 ml GM broth at room temperature for 5 days. After 5 days, the culture was examined through a microscope by gram staining technique.

4. COLLECTION OF THE RESTAURANT WASTEWATER SAMPLE

The wastewater samples were collected from western style restaurant and at the food court in a local department store using a Nansen bottle. The sample was stored in an opaque 20 L plastic container. The samples were tested on the same day of collection and the remaining stored under refrigerated conditions for not more than 5 days.

5. RESTAURANT WASTEWATER TREATMENT

The wastewater sample was treated by transferring 600 ml of the sample into a 1000 ml Erlenmeyer flask. The sample was then inoculated with the bio-extract varying in amounts of 0.25, 0.5 and 1.0 ml/L of wastewater. The sample is stirred to disperse the bio-extract for 7

minutes. The conditions of the treatment are varied between with exposure to light and without light as well as with oxygen and without oxygen. The samples are left under the varied conditions for 24 hours and for 48 hours. Control was created by treating wastewater sample (without inoculation of the bio-extract) under the same conditions.

6. PREPARATION OF THE ENRICHMENT MEDIUM FOR MICROBIAL ANALYSIS OF RESTAURANT WASTEWATER SAMPLE

The LT (Lauryl Tryptose) broth with bromocresol purple, EC (*Escherichia coli*) broth, NA (Nutrient Agar), EMB (Eosin-methylene Blue Agar) and peptone broth enrichment mediums were prepared and autoclaved at 121°C for 15 minutes except for LST broth which was autoclaved at 115°C for 13 minutes. The agar mediums were then poured into sterile petri dishes.

7. MICROBIAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

The microbial properties of the treated wastewater were analyzed by quantifying the CFU/ml of sample and by using standard qualitative analysis to quantify the coliform and fecal coliform present in the treated wastewater with untreated wastewater and water treated at the same conditions without inoculation of the bio-extract as control.

The CFU/ml of sample was determined by drop plate technique using nutrient agar (NA) as enrichment media. Three 20 ml of each dilutions of the treated wastewater was dropped onto the nutrient agar and incubated at room temperature for 12 hours. The CFU/ml was recorded. This step was repeated for every conditions and controls.

The presumptive tests was implemented by inoculating 0.01, 0.1 and 1 ml of the treated wastewater sample into 5 tubes of LT broth (with bromocresol purple) and incubated at 37°C (Jouan, model N 39105293) for 24-48 hours. Positive results were detected by the color change from purple to yellow. One loop of the positive tube is transferred to EC broth and incubated (Memmert, model INE 600) at 45°C for 24 hours. The positive results were detected from the presence of gas production and the MPN of coliform bacteria can be determined. The confirm test was then implemented by streak plating a loop of the positive EC broth tubes onto an EMB agar followed by incubation (Jouan, model N 39105293) at 37°C for 24-48 hours. Positive results are the presence of green metallic sheen colonies indicating the presence of *E.coli*. A complete test was implemented by randomly transferring 10 of the positive colonies onto a NA slants and incubate (Jouan, model N 39105293) at 37°C for 24-48 hours followed by using gram stain technique to observe the physical characteristics under the microscope. Positive *E.coli* colonies will be stained red and are bacilli (rod-shaped).

8. CHEMICAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

The chemical analysis of the restaurant wastewater after 24 hours or 48 hours treatment with the bio-extract is implemented by measuring the total solid (TS), the total suspended solid (TSS), the total dissolved solid (TDS), the pH, the biological oxygen demand (BOD) and the grease and oil content. The untreated wastewater and the wastewater treated with the same conditions but without inoculations of the bio-extract was used the controls.

The total solid (TS) is measured by drying 20 ml of the treated wastewater sample in a known weight aluminum pan at 130°C until constant weight is gained. The TS weight was recorded by subtracting the final weight with the weight of the aluminum pan.

$$\text{TS (mg/L)} = \frac{weight\ of\ TS \times 1000}{sample\ volume\ (mL)}$$

The total suspended solid (TSS) is measured by filtering 20 ml of the treated wastewater sample with a known weight filter paper (no.1, 7 cm diameter) and dry at 130°C until constant weight is gained. The weight of the TSS was recorded by subtracting the final weight with the weight of the filter paper. The TSS per liter can be calculated with the following formula:

$$\text{TSS (mg/L)} = \frac{weight\ of\ TSS \times 1000}{sample\ volume\ (mL)}$$

The TDS and pH was measured by using a multi-parameter meter and recorded. The BOD was determined by applying air through an aquarium air pump to the treated wastewater sample for 30 minutes. Then the DO was measured and referred to as "DO_0". The treated wastewater was then transferred into a BOD bottle and closed tightly avoiding presence of air bubbles. The BOD bottle was stored at room temperature without exposure to sunlight for 5 days. The DO was then measured again after 5 days and referred to as "DO_5". The BOD was calculated by subtraction of DO_0 with DO_5.

The grease and oil content was determined by adding 10 ml of the treated wastewater sample into a separatory funnel, followed by the addition of 1 ml of 1% ammonia, 10 ml of 95% ethyl alcohol and 25 ml of diethyl ether. The separatory funnel was shook, occasionally opening the valve to release the air inside. The separatory funnel was left for the separation to occur. After separating into two layers, the upper layer was collected into a known weight empty beaker. Then 25 ml of petroleum ether was added and the separatory funnel was shaken, occasionally opening the valve to release the air inside. The separatory funnel was left for the separation to occur. After separating into two layers, the upper layer was collected in the beaker. The beaker was left to dry in a fume hood until constant weight is

gained. The weight of the grease and oil was recorded by subtracting the final weight with the weight of the empty beaker.

RESULT AND DISCUSSIONS

1. MICROBIAL ANALYSIS OF LOCAL BIO-EXTRACT

From analyzing the local bio-extract, total aerobic bacteria count in the local bio-extract was found to be up to 1.22×10^{11} CFU.mL^{-1} with most of it composing of actinomycetes and yeast as shown in table 5. The results were according to previous studies except for Actinomycetes, lactic acid bacteria and *Bacillus spp.* which were found in higher amounts in this study (36-38). This can help give higher biodegradation while not causing the dissolved oxygen content in the wastewater to deplete too much since Actinomycetes and lactic acid bacteria are generally anaerobes while *Bacillus spp* on the other hand, are facultative anaerobes as stated earlier in the literature review.

Table 5. The groups of the microorganisms found in the bio-extract.

Description	Colony Forming Unit per mL (mean ± SD)
Total Viable Cell	$1.22\times10^{11}\pm2.12\times10^{11}$
Actinomycetes	$1.26\times10^{5}\pm6.86\times10^{4}$
Yeast	$1.35\times10^{5}\pm2.82\times10^{3}$
Lactic Acid Bacteria	$4.34\times10^{4}\pm5.01\times10^{4}$
Bacillus spp.	$3.48\times10^{4}\pm4.50\times10^{4}$
Mold	$3.62\times10^{3}\pm4.49\times10^{3}$

Note: The experiments were done for at least 2 replicates.

The result for the purple non-sulfur bacteria was primarily observed under the microscope by gram stain technique, its quantity unknown. Although unconfirmed as purple non-sulfur bacteria, the morphology of the microbe found from culturing the bio-extract in the GM broth was gram positive bacillus as seen in figure 7.

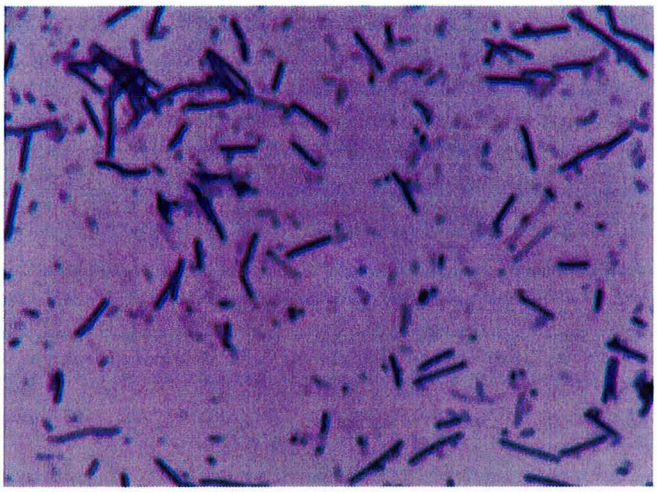

Figure 7. Morphology of the culture found in GM broth hypothesized as purple non-sulfur bacteria.

2. MICROBIAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

The total aerobic bacteria in wastewater after treated with the bio-extract were analysed. Total aerobic bacteria from the wastewater treated for 24 or 48 hours were not significantly different ($p > 0.05$) from the total aerobic bacteria of the untreated wastewater (raw) and the control of each condition as seen in figure 8. However, the wastewater was treated for 48 hours, the total aerobic bacteria showed an increase in number. This indicates that the bio-extract does not cause an increase in the microbial load at 24 hours which may be due to the microbes are still in lag phase where they are only adapting to the changing environmental conditions. As for 48 hours, it cannot be determined whether the microbes that increased are in fact, the beneficial kind or not. Therefore whether this increase in number is bad or good cannot yet be decided. The standard deviations are quite high due to the difference of the properties between the two wastewater samples.

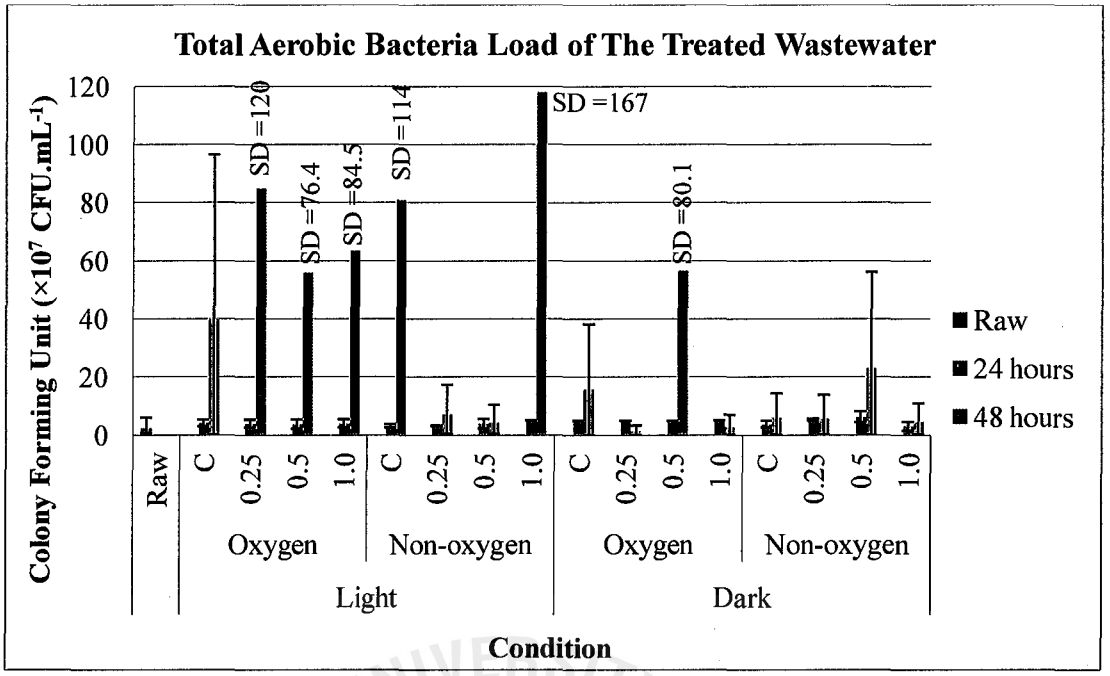

Figure 8. The total aerobic bacteria in the wastewater sample after treating with the bio-extract under varied conditions.

The treated wastewater was analysed to determine its quality by detection of the indicator bacteria for poor sanitation. The total coliform and total fecal coliform bacteria of the sample were found to be over 16,000 MPN per 100 mL (MPN/100mL) for all conditions (36). According to the surface water standards, the surface water should contain no more than 20,000 MPN/100 mL of total coliform. But due to the underestimation in the scale analyzed, further investigation will be needed to assess the total coliform bacteria in the sample. As for the total fecal coliform bacteria, the treated wastewater sample was over the standard limit of 4,000 MPN/100 mL (37).

For the *E. coli*, the result in figure 9 indicates reduction of *E. coli* after treatment both in the wastewater treated with the bio-extract and in the control that did not use the bio-extract. This may be due to the nature of the wastewater sample such as the presence of SDS (Sodium Dodecyl Sulfate) from detergents used in cleaning that can cause destruction of the microbes' cell membrane. Another assumption is that the reduction was because of the natural life span of fecal coliforms which is from 30 to 60 days in the natural water resources and wastewater (38). Also, it is possible that the bio-extract could have created a competition effect that caused the reduction of *E.coli* similar to a case from previous study that detected the absence of *E.coli* in the wastewater after primary and secondary wastewater treatment that involved treating with activated sludge (39). Finally, the initial

E.coli load were also already within the standards of surface water which is within 406 *E. coli* per 100 mL of recreational water causing the wastewater to be according to the standards even before treatment as expected from restaurant wastewater that was used generally for washing and cleaning (40).

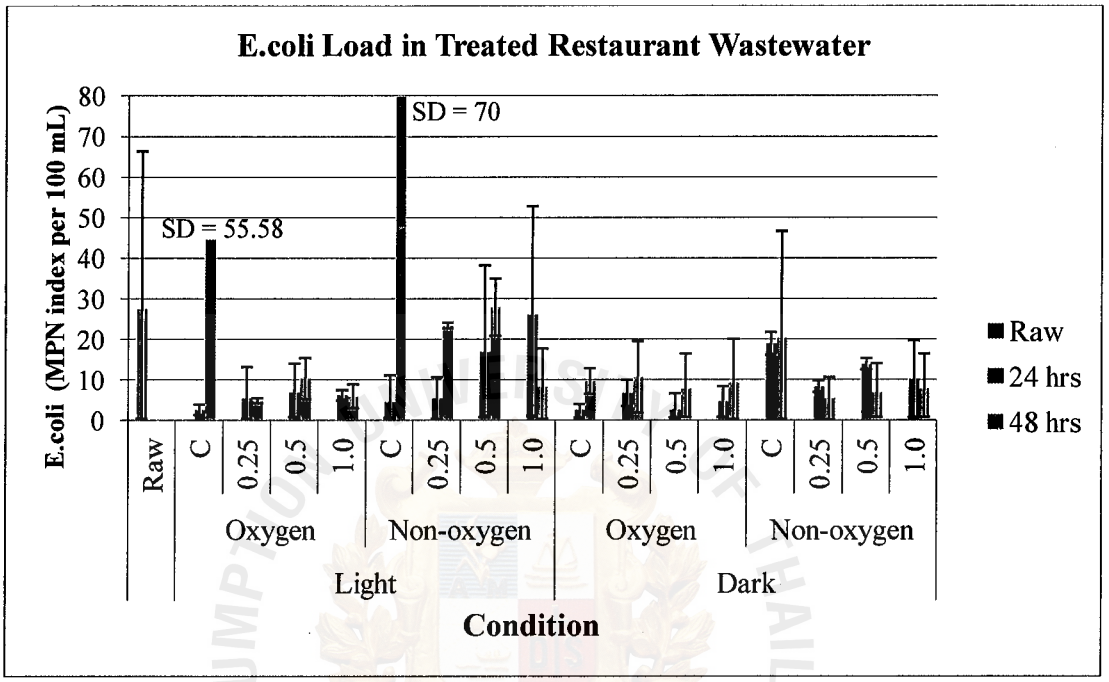

Figure 9. The *E.coli* load in the restaurant wastewater sample after treating under varied conditions.

3. CHEMICAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

From figure 10, the total solid content of restaurant wastewater treated for 48 hours shows significant reduction from the untreated wastewater ($P < 0.05$). However, the control also showed significant reduction in the total solid content. The bacteria that were originally in the wastewater may have been responsible for this reduction, as seen when considered the number of total aerobic bacteria in control that also increased after 48 hours even without the addition of the bio-extract. The dissolved solids and suspended solids, the composition of total solids, were also measured as seen in figure 11 and figure 12. The dissolved solids and suspended solids did not show significant difference between the raw, control and treated wastewater. Even though not significant, there was a reduction in the suspended solids. This may be due to the degradation of the suspended solids into dissolved solids which may have cause the slight reduction of the suspended solids but the constant level of dissolved solids content (41).

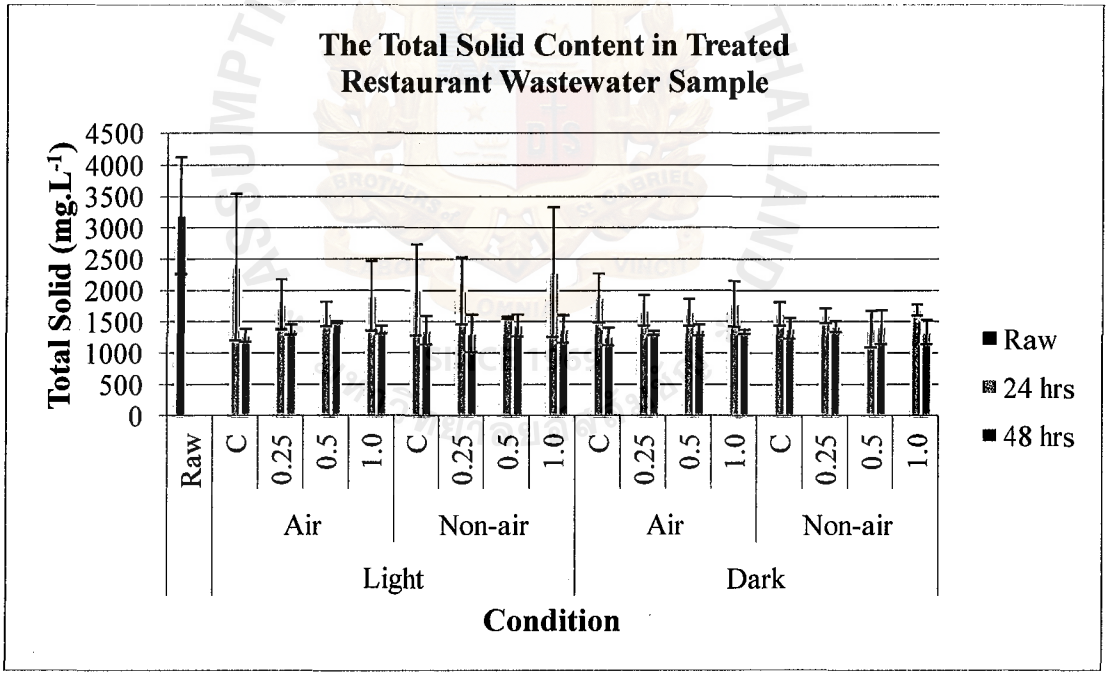

Figure 10. The total solid content in restaurant wastewater treated under varied conditions.

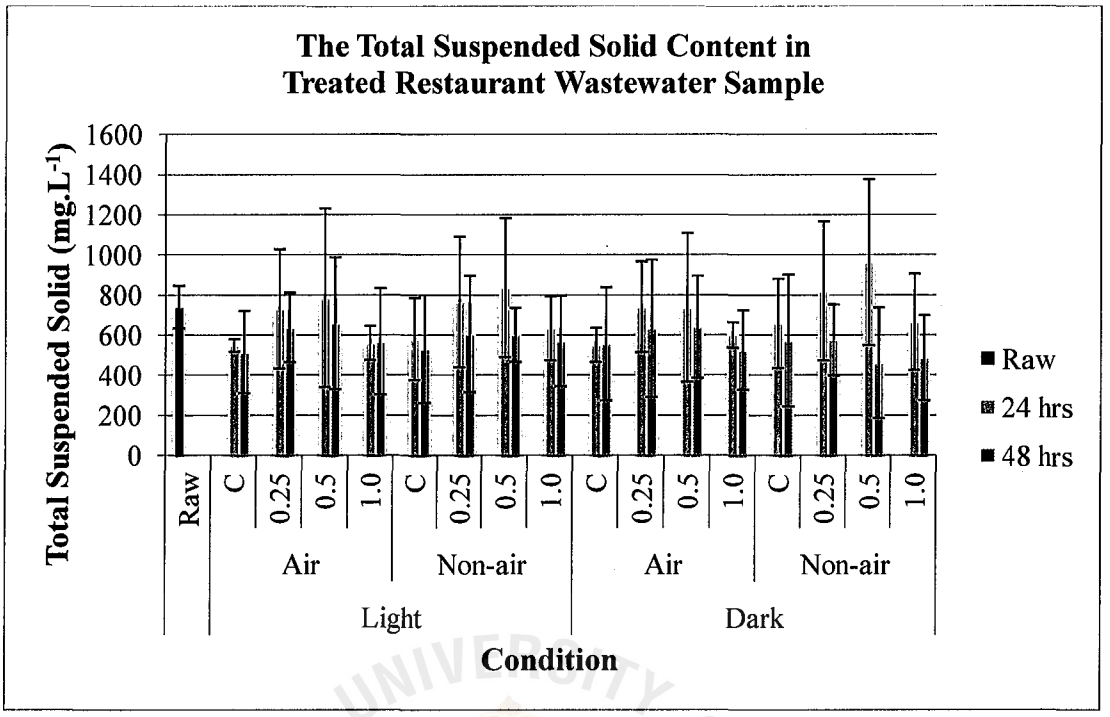

Figure 11. The total suspended solid content in restaurant wastewater treated under varied conditions.

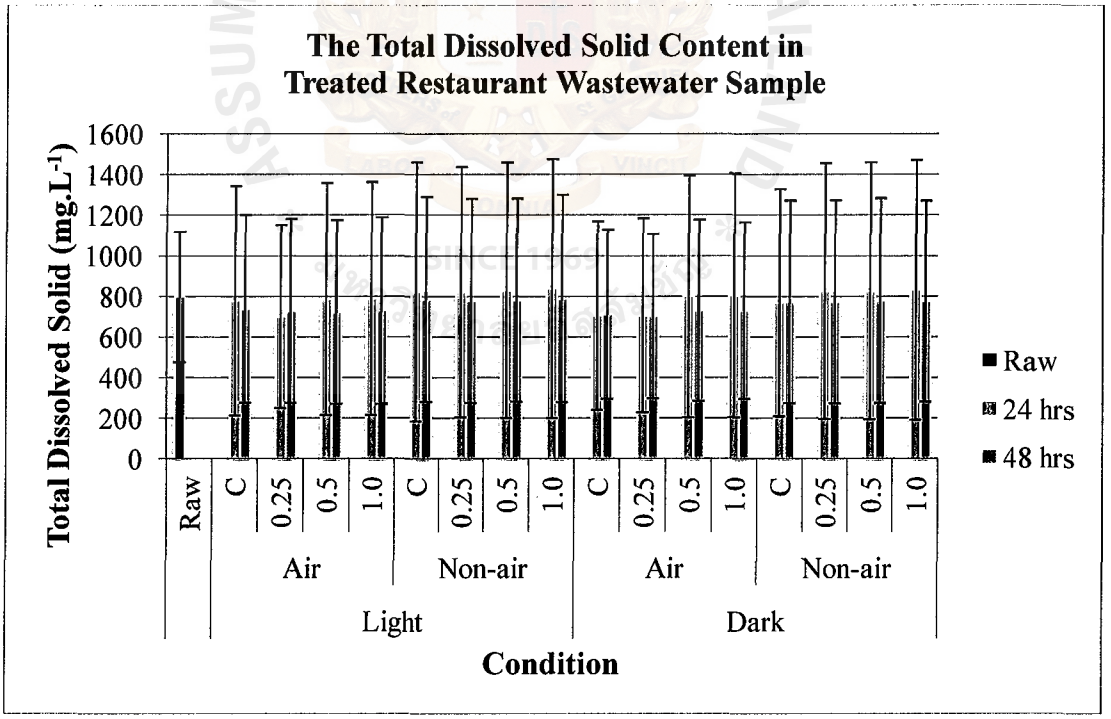

Figure 12. The total dissolved solid content in restaurant wastewater treated under varied conditions.

In figure 13, grease and oil content was generally found to have reduced at 48 hours of treatment especially under dark, non-oxygen conditions with addition of bio-extract. The reduction at 48 hours might have been due to the lack of organic compounds that can be readily degraded at 48 hours forcing the microbes to utilize the harder to degrade organic compounds like grease and oil as energy source instead.

According to the results from a study of the biodiversity in the bio-extract (Sanjaya and Kunathigan, work in progress) high percentage of lipid degrading bacteria were isolated, which could have lead to this reduction of the grease and oil content. The reduction is also consistent with previous researches that study the application of the bio-extract (42)- (43).

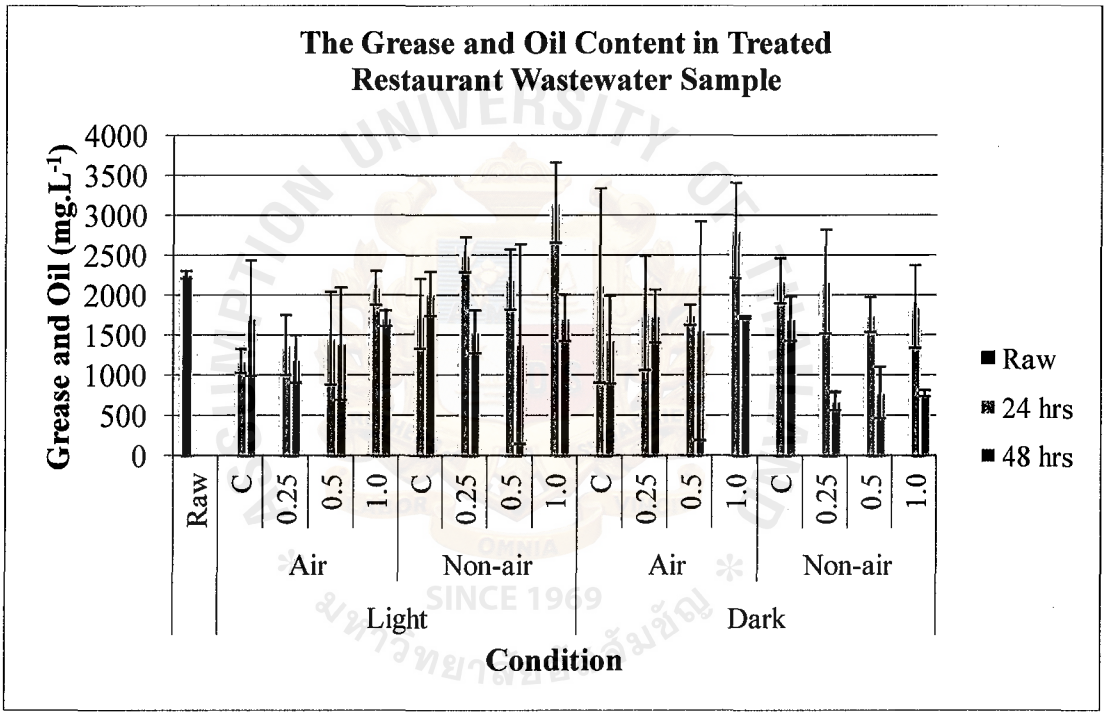

Figure 13. Grease and oil content in the restaurant wastewater treated under varied conditions.

However, the grease and oil content was still over the standards of $\leq$ 100 $mg.L^{-1}$ even after treating with the bio-extract (2). Therefore other methods should be used to primarily lower the grease and oil content before further treatments with the bio-extract such as using a grease trap or increasing the treatment time.

For the BOD, as seen in figure 14, the BOD was found to be within the range of approximately 1.8 to 3.2 $mg.L^{-1}$, and increases accordingly with the time. The wastewater treated under the time condition of 24 hours shows lower BOD than those treated under the time condition of 48 hours and the raw wastewater sample. Reasons for the increased

biochemical oxygen demand could be due to at 24 hours the microbes were in lag phase and was still adapting to the changing environment. Initially the value was already within the standards, causing the BOD values after treatment to be according to the standards of ≤ 200 mg.L^{-1} (2).

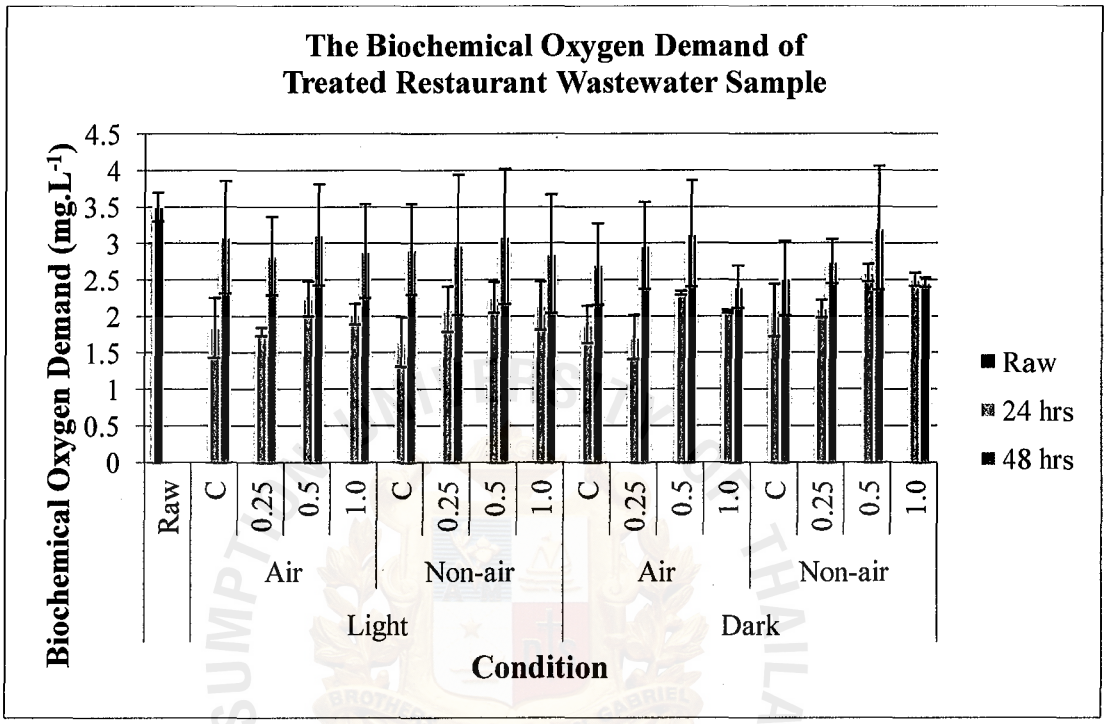

Figure 14. Biochemical oxygen demand in the restaurant wastewater treated under varied conditions.

From figure 15, the pH was found to be within the range of approximately 5.3 to 6. The initial values were within the standards of 5 to 9 pH and because the pH value was generally constant throughout all treatment conditions with the bio-extract, it was found to be according to the standards (2).

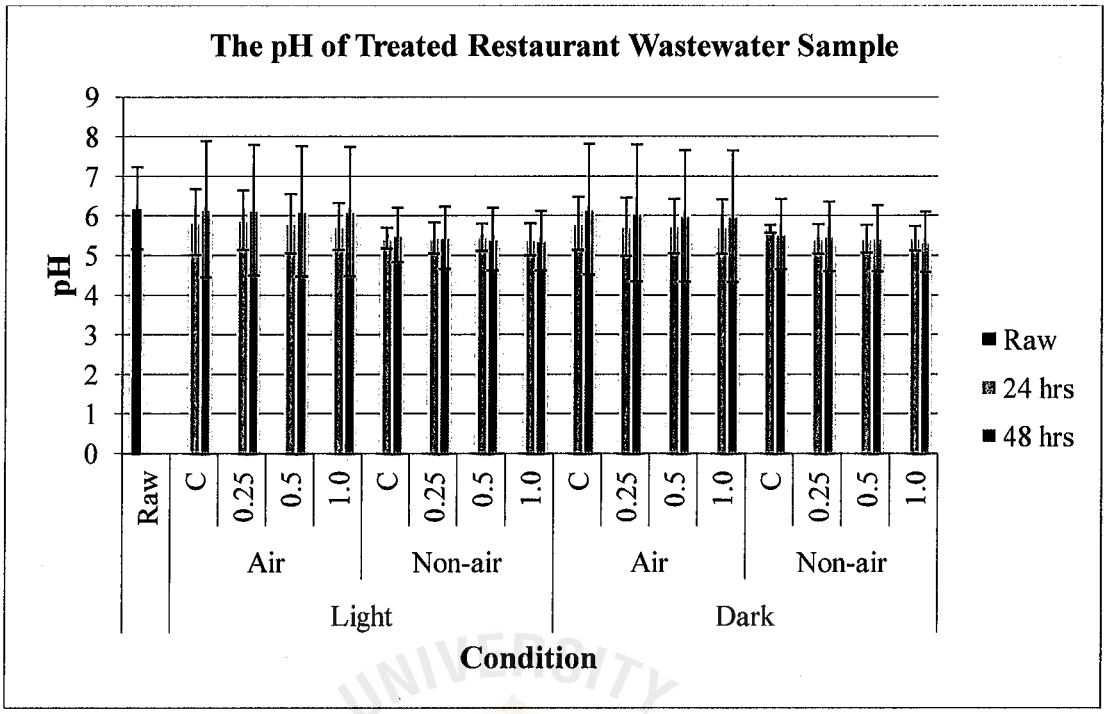

Figure 15. The pH of the restaurant wastewater treated under varied conditions.

From analyzing the results, the treatment condition selected as the optimum treatment condition is 0.25 mL of bio-extract per liter of wastewater for 48 hours without light and oxygen. This condition led to the reduction of TS and grease and oil by 53.07% and 69.89% respectively. While the *E.coli* load, total aerobic bacteria load, TSS, TDS, pH and BOD were at an acceptable level.

Further experiments are needed in order to control the quality of the local bio-extract to be consistent in every batch as well as create a custom made bio-extract composed of the beneficial microbes that are suitable for certain types of wastewater treatment as well as how to effectively integrate the bio-ferment solution with other wastewater treatment methods to create an efficient wastewater treatment system.

CONCLUSION

1. The bio-extract contained total viable cells of up to 1.22×10^{11} $CFU.mL^{-1}$ mainly composing of *Actinomycetes* and yeast.
2. The optimum treatment condition was treatment with 0.25 mL of bio-extract per liter of wastewater for 48 hours without light and oxygen.
3. The treated wastewater from this condition showed the total solid reduction of 53.07% and the grease and oil reduction of 69.89% as well as the reduction of *E.coli* load.
4. Detected duration is a major factor for influencing change in the wastewater more than light and oxygen.

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APPENDIX

A. RAW DATA FROM MICROBIAL ANALYSIS OF LOCAL BIO-EXTRACT

Table 6. The groups of the microorganisms found in the bio-extract.

Replication	1	2	3	4	5	Mean	SD
Mold	6.80×10^{3}	N/A	N/A	4.50×10^{2}	N/A	3.62×10^{3}	4.49×10^{3}
Actinomycetes	1.75×10^{5}	N/A	N/A	7.80×10^{4}	N/A	1.26×10^{5}	6.86×10^{4}
LAB	4.00×10^{4}	2.70×10^{3}	1.15×10^{5}	1.61×10^{4}	N/A	4.34×10^{4}	5.01×10^{4}
Yeast	1.33×10^{5}	N/A	N/A	1.37×10^{5}	N/A	1.35×10^{5}	2.82×10^{3}
Bacillus spp.	3.00×10^{3}	N/A	N/A	N/A	6.67×10^{4}	3.48×10^{4}	4.50×10^{4}
Total Cell	6.00×10^{6}	N/A	N/A	2.50×10^{8}	3.67×10^{11}	1.22×10^{11}	2.12×10^{11}

Note: N/A = data unavailable due to error.

B. RAW DATA FROM MICROBIAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

Table 7. The total aerobic bacteria in restaurant wastewater treated under varied conditions.

Condition				CFU/ml		
				Replication		Mean±SD
Time	Light/Dark	Air/Non-air	Concentration (mL/Liter of wastewater)	1	2	
Raw Waste water				$>5.00\times10^{7}$	5.28×10^{5}	$2.53\times10^{7}\pm3.50\times10^{7}$
24 hrs.	Light	Air	C	3.38×10^{7}	$>5.00\times10^{7}$	$4.19\times10^{7}\pm1.14\times10^{7}$
			0.25	3.12×10^{7}	$>5.00\times10^{7}$	$4.06\times10^{7}\pm1.33\times10^{7}$
			0.5	2.73×10^{7}	$>5.00\times10^{7}$	$3.87\times10^{7}\pm1.60\times10^{7}$
			1.0	3.07×10^{7}	$>5.00\times10^{7}$	$4.03\times10^{7}\pm1.37\times10^{7}$
		Non-air	C	2.73×10^{7}	3.62×10^{7}	$3.18\times10^{7}\pm6.25\times10^{6}$
			0.25	3.10×10^{7}	3.25×10^{7}	$3.18\times10^{7}\pm1.06\times10^{6}$
			0.5	2.73×10^{7}	5.07×10^{7}	$3.90\times10^{7}\pm1.65\times10^{7}$
			1.0	$>5.00\times10^{7}$	5.08×10^{7}	$5.04\times10^{7}\pm5.89\times10^{5}$
	Dark	Air	C	$>5.00\times10^{7}$	$>5.00\times10^{7}$	$>5.00\times10^{7}\pm0$
			0.25	$>5.00\times10^{7}$	$>5.00\times10^{7}$	$>5.00\times10^{7}\pm0$
			0.5	$>5.00\times10^{7}$	$>5.00\times10^{7}$	$>5.00\times10^{7}\pm0$
			1.0	$>5.00\times10^{7}$	$>5.00\times10^{7}$	$>5.00\times10^{7}\pm0$
		Non-air	C	3.00×10^{7}	4.65×10^{7}	$3.83\times10^{7}\pm1.17\times10^{7}$
			0.25	$>5.00\times10^{7}$	5.57×10^{7}	$5.28\times10^{7}\pm4.01\times10^{6}$
			0.5	$>5.00\times10^{7}$	7.67×10^{7}	$6.33\times10^{7}\pm1.89\times10^{7}$
			1.0	1.97×10^{7}	4.15×10^{7}	$3.06\times10^{7}\pm1.54\times10^{7}$
48 hrs.	Light	Air	C	1.52×10^{6}	8.00×10^{8}	$4.01\times10^{8}\pm5.65\times10^{8}$
			0.25	2.50×10^{7}	1.70×10^{9}	$8.51\times10^{8}\pm1.20\times10^{9}$
			0.5	1.92×10^{7}	1.10×10^{9}	$5.60\times10^{8}\pm7.64\times10^{8}$
			1.0	3.90×10^{7}	1.23×10^{9}	$6.36\times10^{8}\pm8.45\times10^{8}$
		Non-air	C	4.02×10^{6}	1.62×10^{9}	$8.10\times10^{8}\pm1.14\times10^{9}$
			0.25	3.15×10^{6}	1.45×10^{8}	$7.40\times10^{7}\pm1.00\times10^{8}$
			0.5	2.23×10^{6}	8.67×10^{7}	$4.45\times10^{7}\pm5.97\times10^{7}$
			1.0	4.45×10^{5}	2.37×10^{9}	$1.18\times10^{9}\pm1.67\times10^{9}$
48 hrs.	Dark	Air	C	2.38×10^{6}	3.17×10^{8}	$1.60\times10^{8}\pm2.22\times10^{8}$
			0.25	2.90×10^{6}	2.83×10^{7}	$1.56\times10^{7}\pm1.80\times10^{7}$
			0.5	2.63×10^{5}	1.13×10^{9}	$5.67\times10^{8}\pm8.01\times10^{8}$
			1.0	1.82×10^{5}	5.83×10^{7}	$2.93\times10^{7}\pm4.11\times10^{7}$
		Non-air	C	3.15×10^{6}	1.20×10^{8}	$6.16\times10^{7}\pm8.26\times10^{7}$
			0.25	2.68×10^{6}	1.15×10^{8}	$5.88\times10^{7}\pm7.94\times10^{7}$
			0.5	2.28×10^{5}	4.65×10^{8}	$2.33\times10^{8}\pm3.29\times10^{8}$
			1.0	2.95×10^{6}	9.17×10^{7}	$4.73\times10^{7}\pm6.27\times10^{7}$

Table 8. The total coliform and fecal coliform of restaurant wastewater treated under varied conditions.

Condition				MPN/100 mL of wastewater	
Time	Light/Dark	Air/Non-air	Concentration (mL/Liter of wastewater)	Total Coliform	Total Fecal Coliform
Raw				>16000±0	>16000±0
24 hrs.	Light	Air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0
		Non-air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0
	Dark	Air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0
		Non-air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0
48 hrs.	Light	Air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0
		Non-air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	10650±7566.04
			1	>16000±0	>16000±0
	Dark	Air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0
		Non-air	C	>16000±0	>16000±0
			0.25	>16000±0	>16000±0
			0.5	>16000±0	>16000±0
			1	>16000±0	>16000±0

Table 9. Most probable number of *E.coli* per 100 mL of restaurant wastewater treated under varied conditions.

Condition				*E.coli* (MPN/100 ml of wastewater)		Mean±SD
Time	Light/Dark	Air/Nonair	Concentration (mL/Liter of wastewater)	Replication		
				1	2	
Raw				18	550	284±376.18
24 hrs.	Light	Air	C	36	18	27±12.73
			0.25	18	110	64±65.05
			0.5	180	120	150±42.43
			1.0	72	54	63±12.73
		Non-air	C	18	93	55.5±53.03
			0.25	18	92	55±52.33
			0.5	18	320	169±213.55
			1.0	72	450	261±267.29
	Dark	Air	C	37	18	27.5±13.44
			0.25	91	45	68±32.53
			0.5	18	55	36.5±26.16
			1.0	20	74	47±38.18
		Non-air	C	170	210	190±28.28
			0.25	74	94	84±14.14
			0.5	150	130	140±14.14
			1.0	36	170	103±94.75
48 hrs.	Light	Air	C	54	840	447±555.79
			0.25	54	45	49.5±6.36
			0.5	68	140	104±50.91
			1.0	40	81	60.5±28.99
		Non-air	C	130	310	220±127.28
			0.25	230	240	235±7.07
			0.5	230	330	280±70.71
			1.0	18	150	84±93.34
	Dark	Air	C	75	120	97.5±31.82
			0.25	45	170	107.5±88.82
			0.5	18	140	79±86.27
			1.0	18	170	94±107.48
		Non-air	C	18	390	204±263.04
			0.25	18	92	55±52.33
			0.5	18	120	69±72.12
			1.0	18	140	79±86.27

C. RAW DATA FROM CHEMICAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

Table 10. Total solids in the restaurant wastewater before and after treatment under variable conditions.

Condition			Total Solid (mg/L)					
			Replication		Mean±SD	Replication		Mean±SD
Raw			1			2		
			2535.0000			3855.0000		3195.0±933.4
Time			24 hours			48 hours		
		Concentration (mL/L)	1	2		1	2	
Light	Air	0 (control)	1545	3200	2372.5±1170.26	1355	1210	1282.5±102.53
		0.15	1495	2060	1777.5±399.52	1435	1320	1377.5±81.32
		0.3	1485	1760	1622.5±194.45	1505	1480	1492.5±17.68
		0.6	1515	2300	1907.5±555.08	1415	1325	1370.0±63.64
	Non-air	0 (control)	1495	2525	2010.0±728.32	1530	1205	1367.5±229.81
		0.15	1615	2375	1995.0±537.40	1530	1105	1317.5±300.52
		0.3	1550	1580	1565.0±21.21	1565	1320	1442.5±173.24
		0.6	1560	3025	2292.5±1035.9	1540	1230	1385±219.20
Dark	Air	0 (control)	1605	2155	1880.0±388.91	1360	1165	1262.5±137.89
		0.15	1510	1855	1682.5±243.95	1340	1295	1317.5±31.82
		0.3	1495	1800	1647.5±215.67	1430	1305	1367.5±88.39
		0.6	1530	2045	1787.5±364.16	1365	1320	1342.5±31.82
	Non-air	0 (control)	1760	1495	1627.5±187.38	1515	1280	1397.5±166.17
		0.15	1680	1515	1597.5±116.67	1485	1360	1422.5±88.39
		0.3	1590	1180	1385.0±289.91	1605	1225	1415.0±268.70
		0.6	1625	1750	1687.5±88.39	1470	1195	1332.5±194.45

Table 11. Total suspended solids in the restaurant wastewater before and after treatment under variable conditions.

Condition			Total Suspended Solid (mg/L)					
			Replication		Mean±SD	Replication		Mean±SD
Raw			1			2		
			815			665		665.0±106.07
Time			24 hours			48 hours		
		Concentration (mL/L)	1	2		1	2	
Light	Air	0 (control)	525	570	547.5±31.82	370	660	515.00± 205.06
		0.15	520	940	730.0±296.98	515	760	637.50±173.24
		0.3	470	1100	785.0±445.48	425	890	657.50±328.80
		0.6	620	500	560.0±84.85	380	755	567.50± 265.17
	Non-air	0 (control)	435	725	580.0±205.06	340	720	530.00±268.70
		0.15	535	995	765.0± 325.27	400	810	605.00±289.91
		0.3	590	1080	835.0±346.48	505	695	600.00±134.35
		0.6	520	745	632.5± 159.10	410	730	570.00± 226.27
Dark	Air	0 (control)	490	610	550.0± 84.85	355	755	555.00±282.84
		0.15	580	900	740.0± 226.27	390	875	632.50± 342.95
		0.3	475	1000	737.5± 371.23	460	820	640.00± 254.56
		0.6	555	645	600.0± 63.64	385	665	525.00±197.99
	Non-air	0 (control)	500	815	657.5±222.74	340	805	572.50±328.80
		0.15	575	1065	820.0±346.48	450	700	575.00±176.78
		0.3	670	1255	962.5±413.66	265	655	460.00±275.77
		0.6	495	835	665.0±240.42	335	635	485.00±212.13

Table 12. Total grease and oil content in the restaurant wastewater before and after treatment under variable conditions.

Condition			Grease and Oil (mg/L)					
			Replication		Mean±SD	Replication		Mean±SD
Raw			1			2		
			2220			2290		2255.00±49.50
Time			24 hours			48 hours		
		Concentration (mL/L)	1	2		1	2	
Light	Air	0 (control)	1280	1070	1175.00±148.49	2220	1200	1710.00±721.25
		0.15	1640	1110	1375.00±374.77	1410	990	1200.00±296.98
		0.3	1870	1050	1460.00±579.83	1890	900	1395.00±700.04
		0.6	2240	1940	2090.00±212.13	1780	1640	1710.00±98.99
	Non-air	0 (control)	1460	2080	1770.00±438.41	2210	1820	2015.00±275.77
		0.15	2350	2660	2505.00±219.20	1730	1350	1540.00±268.70
		0.3	1930	2460	2195.00±374.77	2270	510	1390.00±1244.51
		0.6	2800	3510	3155.00±502.05	1920	1510	1715.00±289.91
Dark	Air	0 (control)	2980	1260	2120.00±1216.22	1830	1050	1440.00±551.54
		0.15	1270	2280	1775.00±714.18	1970	1500	1735.00±332.34
		0.3	1840	1660	1750.00±127.28	2520	590	1555.00±1364.72
		0.6	3230	2390	2810.00±593.97	1710	1730	1720.00±14.14
	Non-air	0 (control)	1980	2380	2180.00±282.84	1900	1510	1705.00±275.77
		0.15	1710	2630	2170.00±650.54	760	600	680.00±113.14
		0.3	1600	1910	1755.00±219.20	1010	560	785.00±318.20
		0.6	1490	2220	1855.00±516.19	750	800	775.00±35.36

Table 13. The biochemical oxygen demand in the restaurant wastewater before and after treatment under variable conditions.

Condition			Biochemical Oxygen Demand (mg/L)					
			Replication		Mean±SD	Replication		Mean±SD
Raw			1			2		
			3.36			3.64		3.50±0.198
Time			24 hours			48 hours		
		Concentration (mL/L)	1	2		1	2	
Light	Air	0 (control)	2.13	1.55	1.84±0.41	2.54	3.63	3.09 ±0.77
		0.15	1.75	1.83	1.79 ±0.06	2.45	3.21	2.83 ±0.54
		0.3	2.07	2.41	2.24 ±0.24	2.63	3.61	3.12 ±0.69
		0.6	1.93	2.13	2.03 ±0.14	2.44	3.35	2.90 ±0.64
	Non-air	0 (control)	1.89	1.41	1.65±0.34	2.48	3.36	2.92±0.62
		0.15	1.88	2.32	2.10±0.31	2.3	3.66	2.98±0.96
		0.3	2.11	2.41	2.26±0.21	2.44	3.75	3.10±0.93
		0.6	1.91	2.38	2.15±0.33	2.28	3.43	2.86± 0.81
Dark	Air	0 (control)	2.07	1.71	1.89± 0.25	2.32	3.11	2.72±0.56
		0.15	1.93	1.5	1.72±0.03	2.55	3.39	2.97±0.59
		0.3	2.34	2.3	2.32±0.30	2.62	3.65	3.14±0.73
		0.6	2.05	2.08	2.07±0.02	2.2	2.61	2.41± 0.29
	Non-air	0 (control)	1.83	2.34	2.09± 0.36	2.16	2.88	2.52±0.51
		0.15	2.02	2.19	2.11±0.12	2.54	2.97	2.76±0.30
		0.3	2.51	2.68	2.60±0.12	2.61	3.81	3.21±0.85
		0.6	2.44	2.57	2.51±0.09	2.51	2.42	2.47± 0.06

Table 14. Total dissolved solids in the restaurant wastewater before and after treatment under variable conditions.

Condition			Total Dissolved Solid (mg/L)					
			Replication		Mean±SD	Replication		Mean±SD
Raw			1			2		
			1024.10			570.00		797.05±321.10
Time			24 hours			48 hours		
		Concentration (mL/L)	1	2		1	2	
Light	Air	0 (control)	1178.10	379.40	778.75±564.77	1065.40	412.30	738.85±461.81
		0.15	1019.20	381.50	700.35±450.92	1047.90	407.40	727.65±452.90
		0.3	1189.30	382.20	785.75±570.71	1043.00	403.20	723.10±452.41
		0.6	1194.20	382.90	788.55±573.68	1054.90	405.30	730.10±459.34
	Non-air	0 (control)	1274.70	371.70	823.20±638.52	1142.40	429.10	785.75±504.38
		0.15	1257.20	384.30	820.75±617.23	1134.00	422.80	778.40±502.89
		0.3	1274.70	385.00	829.85±629.11	1135.40	428.40	781.90±499.92
		0.6	1288.70	387.80	838.25±637.03	1150.80	428.40	789.60±510.81
Dark	Air	0 (control)	1033.20	376.60	704.90±464.29	1005.20	417.90	711.55±415.28
		0.15	1043.70	368.20	705.95±477.65	987.70	415.80	701.75±404.39
		0.3	1220.10	378.70	799.40±594.96	1046.50	415.10	730.80±446.47
		0.6	1229.90	380.10	805.00±600.90	1038.10	422.80	730.45±435.08
	Non-air	0 (control)	1164.10	373.10	768.60±559.32	1126.30	421.40	773.85±498.44
		0.15	1271.20	380.10	825.65±630.10	1127.00	420.00	773.50±499.92
		0.3	1274.70	379.40	827.05±633.07	1135.40	422.10	778.75±504.38
		0.6	1283.10	378.70	830.90±639.51	1125.60	425.60	775.60±494.97

Table 15. The pH of the restaurant wastewater before and after treatment under variable conditions.

Condition			pH					
			Replication		Mean±SD	Replication		Mean±SD
Raw			1			2		
			5.46			6.93		6.195±1.04
Time			24 hours			48 hours		
		Concentration (mL/L)	1	2		1	2	
Light	Air	0 (control)	6.43	5.25	5.84±0.83	7.38	4.95	6.17±1.72
		0.15	6.42	5.36	5.89±0.75	7.31	4.98	6.15±1.65
		0.3	6.33	5.27	5.80±0.75	7.28	4.95	6.12±1.65
		0.6	6.15	5.31	5.73±0.59	7.26	4.95	6.11±1.63
	Non-air	0 (control)	5.62	5.25	5.44±0.26	6	5.03	5.52±0.69
		0.15	5.72	5.16	5.44±0.40	6	4.89	5.45±0.78
		0.3	5.7	5.21	5.46±0.35	5.97	4.85	5.41±0.79
		0.6	5.69	5.12	5.41±0.40	5.9	4.84	5.37±0.75
Dark	Air	0 (control)	6.28	5.33	5.81±0.67	7.33	5	6.17±1.65
		0.15	6.24	5.2	5.72±0.74	7.29	4.85	6.07±1.73
		0.3	6.22	5.25	5.74±0.69	7.17	4.82	6.00±1.66
		0.6	6.21	5.24	5.73±0.69	7.16	4.81	5.99±1.66
	Non-air	0 (control)	5.74	5.6	5.67±0.10	6.16	4.91	5.54±0.88
		0.15	5.68	5.15	5.42±0.37	6.1	4.86	5.48±0.88
		0.3	5.67	5.17	5.42±0.35	6.02	4.84	5.43±0.83
		0.6	5.65	5.21	5.43±0.31	5.88	4.8	5.34±0.76

D. SAS OUTPUT OF THE CHEMICAL ANALYSIS OF TREATED RESTAURANT WASTEWATER

The SAS System 05:15 Thursday, November 21, 2013 121

The GLM Procedure

Dependent Variable: TS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	32	10246395.45	320199.86	1.89	0.0365
Error	33	5586500.00	169287.88		
Corrected Total	65	15832895.45			

R-Square	Coeff Var	Root MSE	TS Mean
0.647159	25.17890	411.4461	1634.091

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Con	3	259840.625	86613.542	0.51	0.6771
Oxygen	1	3906.250	3906.250	0.02	0.8802
Light	1	367539.062	367539.062	2.17	0.1501
Time	1	3014564.063	3014564.063	17.81	0.0002
Con*Oxygen	3	90003.125	30001.042	0.18	0.9111
Con*Light	3	55226.563	18408.854	0.11	0.9544
Oxygen*Light	1	22126.563	22126.563	0.13	0.7200
Con*Oxygen*Light	3	56914.063	18971.354	0.11	0.9524
Con*Time	3	626745.313	208915.104	1.23	0.3129
Oxygen*Time	1	38514.062	38514.062	0.23	0.6365
Con*Oxygen*Time	3	182507.813	60835.938	0.36	0.7827
Light*Time	1	267806.250	267806.250	1.58	0.2173
Con*Light*Time	3	104234.375	34744.792	0.21	0.8920
Oxygen*Light*Time	1	85556.250	85556.250	0.51	0.4821
Con*Oxyge*Light*Time	3	45759.375	15253.125	0.09	0.9650

Least Squares Means

Time	TS LSMEAN	LSMEAN Number
0	3195.00000	1
1	1802.34375	2
2	1368.28125	3

Least Squares Means for effect Time
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: TS

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

Tukey's Studentized Range (HSD) Test for TS

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	169287.9
Critical Value of Studentized Range	5.86560
Minimum Significant Difference	1706.5

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Trt
A	3195.0	2	0
A			
B A	2372.5	2	1
B A			
B A	2292.5	2	19
B A			
B A	2010.0	2	3
B A			
B A	1995.0	2	15
B A			
B A	1907.5	2	13
B A			
B A	1880.0	2	5
B A			
B A	1787.5	2	25
B A			
B A	1777.5	2	9
B A			
B A	1687.5	2	31
B A			
B A	1682.5	2	21
B A			
B A	1647.5	2	23
B A			
B A	1627.5	2	7
B A			
B A	1622.5	2	11
B A			
B A	1597.5	2	27
B A			
B A	1565.0	2	17
B A			
B A	1492.5	2	12
B			
B	1442.5	2	18
B			
B	1422.5	2	28
B			
B	1415.0	2	30
B			
B	1397.5	2	8
B			
B	1385.0	2	20
B			

Tukey Grouping	Mean	N	Trt
B	1385.0	2	29
B			
B	1377.5	2	10
B			
B	1370.0	2	14
B			
B	1367.5	2	24
B			
B	1367.5	2	4
B			
B	1342.5	2	26
B			
B	1332.5	2	32
B			
B	1317.5	2	22
B			
B	1317.5	2	16
B			
B	1282.5	2	2
B			
B	1262.5	2	6

Dunnett's t Tests for TS

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	169287.9
Critical Value of Dunnett's t	3.22560
Minimum Significant Difference	1327.2

Comparisons significant at the 0.05 level are indicated by ***.

Trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
1 - 0	-822.5	-2149.7	504.7	
19 - 0	-902.5	-2229.7	424.7	
3 - 0	-1185.0	-2512.2	142.2	
15 - 0	-1200.0	-2527.2	127.2	
13 - 0	-1287.5	-2614.7	39.7	
5 - 0	-1315.0	-2642.2	12.2	
25 - 0	-1407.5	-2734.7	-80.3	***
9 - 0	-1417.5	-2744.7	-90.3	***
31 - 0	-1507.5	-2834.7	-180.3	***
21 - 0	-1512.5	-2839.7	-185.3	***
23 - 0	-1547.5	-2874.7	-220.3	***
7 - 0	-1567.5	-2894.7	-240.3	***
11 - 0	-1572.5	-2899.7	-245.3	***
27 - 0	-1597.5	-2924.7	-270.3	***
17 - 0	-1630.0	-2957.2	-302.8	***
12 - 0	-1702.5	-3029.7	-375.3	***
18 - 0	-1752.5	-3079.7	-425.3	***
28 - 0	-1772.5	-3099.7	-445.3	***
30 - 0	-1780.0	-3107.2	-452.8	***
8 - 0	-1797.5	-3124.7	-470.3	***
20 - 0	-1810.0	-3137.2	-482.8	***
29 - 0	-1810.0	-3137.2	-482.8	***
10 - 0	-1817.5	-3144.7	-490.3	***
14 - 0	-1825.0	-3152.2	-497.8	***
24 - 0	-1827.5	-3154.7	-500.3	***
4 - 0	-1827.5	-3154.7	-500.3	***
26 - 0	-1852.5	-3179.7	-525.3	***
32 - 0	-1862.5	-3189.7	-535.3	***
22 - 0	-1877.5	-3204.7	-550.3	***
16 - 0	-1877.5	-3204.7	-550.3	***
2 - 0	-1912.5	-3239.7	-585.3	***
6 - 0	-1932.5	-3259.7	-605.3	***

The GLM Procedure

Dependent Variable: TS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	32	10246395.45	320199.86	1.89	0.0365
Error	33	5586500.00	169287.88		
Corrected Total	65	15832895.45			

R-Square	Coeff Var	Root MSE	TS Mean
0.647159	25.17890	411.4461	1634.091

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Con	3	259840.625	86613.542	0.51	0.6771
Oxygen	1	3906.250	3906.250	0.02	0.8802
Light	1	367539.062	367539.062	2.17	0.1501
Time	1	3014564.063	3014564.063	17.81	0.0002
Con*Oxygen	3	90003.125	30001.042	0.18	0.9111
Con*Light	3	55226.563	18408.854	0.11	0.9544
Oxygen*Light	1	22126.563	22126.563	0.13	0.7200
Con*Oxygen*Light	3	56914.063	18971.354	0.11	0.9524
Con*Time	3	626745.313	208915.104	1.23	0.3129
Oxygen*Time	1	38514.062	38514.062	0.23	0.6365
Con*Oxygen*Time	3	182507.813	60835.938	0.36	0.7827
Light*Time	1	267806.250	267806.250	1.58	0.2173
Con*Light*Time	3	104234.375	34744.792	0.21	0.8920
Oxygen*Light*Time	1	85556.250	85556.250	0.51	0.4821
Con*Oxyge*Light*Time	3	45759.375	15253.125	0.09	0.9650

Least Squares Means

Time	TS LSMEAN	LSMEAN Number
0	3195.00000	1
1	1802.34375	2
2	1368.28125	3

Least Squares Means for effect Time

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: TS

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		<.0001
3	<.0001	<.0001	

Tukey's Studentized Range (HSD) Test for TS

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Trt
	A	3195.0	2	0
	A			
B	A	2372.5	2	1
B	A			
B	A	2292.5	2	19
B	A			
B	A	2010.0	2	3
B	A			
B	A	1995.0	2	15
B	A			
B	A	1907.5	2	13
B	A			
B	A	1880.0	2	5
B	A			
B	A	1787.5	2	25
B	A			
B	A	1777.5	2	9
B	A			
B	A	1687.5	2	31
B	A			
B	A	1682.5	2	21
B	A			
B	A	1647.5	2	23
B	A			
B	A	1627.5	2	7
B	A			
B	A	1622.5	2	11
B	A			
B	A	1597.5	2	27
B	A			
B	A	1565.0	2	17
B	A			
B	A	1492.5	2	12
B				
B		1442.5	2	18
B				
B		1422.5	2	28
B				
B		1415.0	2	30
B				
B		1397.5	2	8
B				
B		1385.0	2	20
B				
B		1385.0	2	29
B				
B		1377.5	2	10
B				
B		1370.0	2	14
B				
B		1367.5	2	24
B				
B		1367.5	2	4
B				

Tukey Grouping	Mean	N	Trt
B	1342.5	2	26
B			
B	1332.5	2	32
B			
B	1317.5	2	22
B			
B	1317.5	2	16
B			
B	1282.5	2	2
B			
B	1262.5	2	6

Dunnett's t Tests for TS

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	169287.9
Critical Value of Dunnett's t	3.22560
Minimum Significant Difference	1327.2

Comparisons significant at the 0.05 level are indicated by ***.

	Trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
	1 - 0	-822.5	-2149.7	504.7	
	19 - 0	-902.5	-2229.7	424.7	
	3 - 0	-1185.0	-2512.2	142.2	
	15 - 0	-1200.0	-2527.2	127.2	
x	13 - 0	-1287.5	-2614.7	39.7	
	5 - 0	-1315.0	-2642.2	12.2	
	25 - 0	-1407.5	-2734.7	-80.3	***
	9 - 0	-1417.5	-2744.7	-90.3	***
	31 - 0	-1507.5	-2834.7	-180.3	***
	21 - 0	-1512.5	-2839.7	-185.3	***
	23 - 0	-1547.5	-2874.7	-220.3	***
	7 - 0	-1567.5	-2894.7	-240.3	***
	11 - 0	-1572.5	-2899.7	-245.3	***
	27 - 0	-1597.5	-2924.7	-270.3	***
	17 - 0	-1630.0	-2957.2	-302.8	***
	12 - 0	-1702.5	-3029.7	-375.3	***
	18 - 0	-1752.5	-3079.7	-425.3	***
	28 - 0	-1772.5	-3099.7	-445.3	***
	30 - 0	-1780.0	-3107.2	-452.8	***
	8 - 0	-1797.5	-3124.7	-470.3	***
	20 - 0	-1810.0	-3137.2	-482.8	***
	29 - 0	-1810.0	-3137.2	-482.8	***
	10 - 0	-1817.5	-3144.7	-490.3	***
	14 - 0	-1825.0	-3152.2	-497.8	***
	24 - 0	-1827.5	-3154.7	-500.3	***
	4 - 0	-1827.5	-3154.7	-500.3	***
	26 - 0	-1852.5	-3179.7	-525.3	***
	32 - 0	-1862.5	-3189.7	-535.3	***
	22 - 0	-1877.5	-3204.7	-550.3	***
	16 - 0	-1877.5	-3204.7	-550.3	***
	2 - 0	-1912.5	-3239.7	-585.3	***
	6 - 0	-1932.5	-3259.7	-605.3	**

The GLM Procedure

Dependent Variable: FAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	32	18280727.27	571272.73	1.94	0.0312
Error	33	9712850.00	294328.79		
Corrected Total	65	27993577.27			

R-Square	Coeff Var	Root MSE	FAT Mean
0.653033	31.15494	542.5208	1741.364

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Con	3	1796931.250	598977.083	2.04	0.1280
Oxygen	1	85556.250	85556.250	0.29	0.5934
Light	1	158006.250	158006.250	0.54	0.4689
Time	1	5141556.250	5141556.250	17.47	0.0002
Con*Oxygen	3	626156.250	208718.750	0.71	0.5535
Con*Light	3	667581.250	222527.083	0.76	0.5268
Oxygen*Light	1	3213056.250	3213056.250	10.92	0.0023
Con*Oxygen*Light	3	767806.250	255935.417	0.87	0.4667
Con*Time	3	1691156.250	563718.750	1.92	0.1463
Oxygen*Time	1	1494506.250	1494506.250	5.08	0.0310
Con*Oxygen*Time	3	611131.250	203710.417	0.69	0.5634
Light*Time	1	551306.250	551306.250	1.87	0.1804
Con*Light*Time	3	475156.250	158385.417	0.54	0.6595
Oxygen*Light*Time	1	47306.250	47306.250	0.16	0.6911
Con*Oxyge*Light*Time	3	409381.250	136460.417	0.46	0.7096

Least Squares Means

Time	FAT LSMEAN	LSMEAN Number
0	2255.00000	1
1	2008.75000	2
2	1441.87500	3

Least Squares Means for effect Time

Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: FAT

i/j	1	2	3
1		0.5722	0.0655
2	0.5722		0.0003
3	0.0655	0.0003	

Least Squares Means

Oxygen	Light	FAT LSMEAN
0	0	2255.00000
1	1	1514.37500
1	2	1863.12500
2	1	2035.62500
2	2	1488.12500

Oxygen*Light Effect Sliced by Oxygen for FAT

Oxygen	DF	Sum of Squares	Mean Square	F Value	Pr > F
0	0	0	.	.	.
1	1	973013	973013	2.47	0.1209
2	1	2398050	2398050	6.10	0.0164

Least Squares Means

Oxygen	Time	FAT LSMEAN
0	0	2255.00000
1	1	1819.37500
1	2	1558.12500
2	1	2198.12500
2	2	1325.62500

Oxygen*Time Effect Sliced by Oxygen for FAT

Oxygen	DF	Sum of Squares	Mean Square	F Value	Pr > F
0	0	0	.	.	.
1	1	546013	546013	1.61	0.2098
2	1	6090050	6090050	17.92	<.0001

Tukey's Studentized Range (HSD) Test for FAT

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	Trt
	A	3155.0	2	19
	A			
B	A	2810.0	2	25
B	A			
B	A	2505.0	2	15
B	A			
B	A	2255.0	2	0
B	A			
B	A	2195.0	2	17
B	A			
B	A	2180.0	2	7
B	A			
B	A	2170.0	2	27
B	A			
B	A	2120.0	2	5
B	A			
B	A	2090.0	2	13
B	A			
B	A	2015.0	2	4
B	A			
B	A	1855.0	2	31
B	A			
B	A	1775.0	2	21
B	A			
B	A	1770.0	2	3
B	A			
B	A	1755.0	2	29
B	A			
B	A	1750.0	2	23
B	A			

Tukey Grouping		Mean	N	Trt
B	A	1735.0	2	22
B	A			
B	A	1720.0	2	26
B	A			
B	A	1715.0	2	20
B	A			
B	A	1710.0	2	2
B	A			
B	A	1710.0	2	14
B	A			
B	A	1705.0	2	8
B	A			
B	A	1555.0	2	24
B	A			
B	A	1540.0	2	16
B	A			
B	A	1460.0	2	11
B	A			
B	A	1440.0	2	6
B	A			
B	A	1395.0	2	12
B	A			
B	A	1390.0	2	18
B	A			
B	A	1375.0	2	9
B	A			
B	A	1200.0	2	10
B	A			
B	A	1175.0	2	1
B				
B		785.0	2	30
B				
B		775.0	2	32
B				
B		680.0	2	28

Dunnett's t Tests for FAT

Comparisons significant at the 0.05 level are indicated by ***.

Trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
19 - 0	900.0	-850.0	2650.0
25 - 0	555.0	-1195.0	2305.0
15 - 0	250.0	-1500.0	2000.0
17 - 0	-60.0	-1810.0	1690.0
7 - 0	-75.0	-1825.0	1675.0
27 - 0	-85.0	-1835.0	1665.0
5 - 0	-135.0	-1885.0	1615.0
13 - 0	-165.0	-1915.0	1585.0
4 - 0	-240.0	-1990.0	1510.0
31 - 0	-400.0	-2150.0	1350.0
21 - 0	-480.0	-2230.0	1270.0
3 - 0	-485.0	-2235.0	1265.0
29 - 0	-500.0	-2250.0	1250.0
23 - 0	-505.0	-2255.0	1245.0
22 - 0	-520.0	-2270.0	1230.0
26 - 0	-535.0	-2285.0	1215.0
20 - 0	-540.0	-2290.0	1210.0
2 - 0	-545.0	-2295.0	1205.0
14 - 0	-545.0	-2295.0	1205.0
8 - 0	-550.0	-2300.0	1200.0
24 - 0	-700.0	-2450.0	1050.0
16 - 0	-715.0	-2465.0	1035.0
11 - 0	-795.0	-2545.0	955.0
6 - 0	-815.0	-2565.0	935.0
12 - 0	-860.0	-2610.0	890.0
18 - 0	-865.0	-2615.0	885.0
9 - 0	-880.0	-2630.0	870.0
10 - 0	-1055.0	-2805.0	695.0
1 - 0	-1080.0	-2830.0	670.0
30 - 0	-1470.0	-3220.0	280.0
32 - 0	-1480.0	-3230.0	270.0
28 - 0	-1575.0	-3325.0	175.0

The GLM Procedure

Dependent Variable: BOD

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	32	15.93510303	0.49797197	2.07	0.0207
Error	33	7.94520000	0.24076364		
Corrected Total	65	23.88030303			

R-Square	Coeff Var	Root MSE	BOD Mean
0.667291	19.55832	0.490677	2.508788

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Con	3	1.60621875	0.53540625	2.22	0.1038
Oxygen	1	0.09000000	0.09000000	0.37	0.5451
Light	1	0.00902500	0.00902500	0.04	0.8477
Time	1	9.95402500	9.95402500	41.34	<.0001
Con*Oxygen	3	0.15472500	0.05157500	0.21	0.8858
Con*Light	3	0.15035000	0.05011667	0.21	0.8900
Oxygen*Light	1	0.04515625	0.04515625	0.19	0.6678
Con*Oxygen*Light	3	0.08331875	0.02777292	0.12	0.9505
Con*Time	3	0.61795000	0.20598333	0.86	0.4738
Oxygen*Time	1	0.22800625	0.22800625	0.95	0.3376
Con*Oxygen*Time	3	0.03816875	0.01272292	0.05	0.9837
Light*Time	1	0.50055625	0.50055625	2.08	0.1588
Con*Light*Time	3	0.31996875	0.10665625	0.44	0.7238
Oxygen*Light*Time	1	0.09610000	0.09610000	0.40	0.5319
Con*Oxyge*Light*Time	3	0.01512500	0.00504167	0.02	0.9958

Least Squares Means

Time	BOD LSMEAN	Number
0	3.50000000	1
1	2.08343750	2
2	2.87218750	3

Least Squares Means for effect Time

Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: BOD

i/j	1	2	3
1		<.0001	0.0519
2	<.0001		<.0001
3	0.0519	<.0001	

Tukey's Studentized Range (HSD) Test for BOD

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Trt
A	3.5000	2	0
A			
A	3.2100	2	30
A			
A	3.1350	2	24
A			
A	3.1200	2	12
A			
A	3.0950	2	18
A			
A	3.0850	2	2
A			
A	2.9800	2	16
A			
A	2.9700	2	22
A			
A	2.9200	2	4
A			
A	2.8950	2	14
A			
A	2.8550	2	20
A			
A	2.8300	2	10
A			
A	2.7550	2	28
A			
A	2.7150	2	6
A			
A	2.5950	2	29
A			
A	2.5200	2	8
A			
A	2.5050	2	31
A			
A	2.4650	2	32
A			
A	2.4050	2	26
A			
A	2.3200	2	23
A			
A	2.2600	2	17
A			
A	2.2400	2	11
A			
A	2.1450	2	19
A			
A	2.1050	2	27
A			
A	2.1000	2	15
A			
A	2.0850	2	7
A			
A	2.0650	2	25
A			

Tukey Grouping	Mean	N	Trt
A	2.0300	2	13
A			
A	1.8900	2	5
A			
A	1.8400	2	1
A			
A	1.7900	2	9
A			
A	1.7150	2	21
A			
A	1.6500	2	3

Dunnett's t Tests for BOD

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	0.240764
Critical Value of Dunnett's t	3.22560
Minimum Significant Difference	1.5827

Comparisons significant at the 0.05 level are indicated by ***.

Trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
30 - 0	-0.2900	-1.8727	1.2927	
24 - 0	-0.3650	-1.9477	1.2177	
12 - 0	-0.3800	-1.9627	1.2027	
18 - 0	-0.4050	-1.9877	1.1777	
2 - 0	-0.4150	-1.9977	1.1677	
16 - 0	-0.5200	-2.1027	1.0627	
22 - 0	-0.5300	-2.1127	1.0527	
4 - 0	-0.5800	-2.1627	1.0027	
14 - 0	-0.6050	-2.1877	0.9777	
20 - 0	-0.6450	-2.2277	0.9377	
10 - 0	-0.6700	-2.2527	0.9127	
28 - 0	-0.7450	-2.3277	0.8377	
6 - 0	-0.7850	-2.3677	0.7977	
29 - 0	-0.9050	-2.4877	0.6777	
8 - 0	-0.9800	-2.5627	0.6027	
31 - 0	-0.9950	-2.5777	0.5877	
32 - 0	-1.0350	-2.6177	0.5477	
26 - 0	-1.0950	-2.6777	0.4877	
23 - 0	-1.1800	-2.7627	0.4027	
17 - 0	-1.2400	-2.8227	0.3427	
11 - 0	-1.2600	-2.8427	0.3227	
19 - 0	-1.3550	-2.9377	0.2277	
27 - 0	-1.3950	-2.9777	0.1877	
15 - 0	-1.4000	-2.9827	0.1827	
7 - 0	-1.4150	-2.9977	0.1677	
25 - 0	-1.4350	-3.0177	0.1477	
13 - 0	-1.4700	-3.0527	0.1127	
5 - 0	-1.6100	-3.1927	-0.0273	***
1 - 0	-1.6600	-3.2427	-0.0773	***
9 - 0	-1.7100	-3.2927	-0.1273	***
21 - 0	-1.7850	-3.3677	-0.2023	***
3 - 0	-1.8500	-3.4327	-0.2673	***

The GLM Procedure

Dependent Variable: TDS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	112723.521	6630.795	0.03	1.0000
Error	48	9226514.994	192219.062		
Corrected Total	65	9339238.515			

R-Square	Coeff Var	Root MSE	TDS Mean
0.012070	57.01487	438.4279	768.9712

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Con	3	11778.65062	3926.21687	0.02	0.9960
Oxygen	1	55072.35562	55072.35562	0.29	0.5949
Light	1	1960.27563	1960.27563	0.01	0.9200
Time	1	22597.60563	22597.60563	0.12	0.7332
Con*Light	3	5233.84312	1744.61437	0.01	0.9988
Con*Time	3	6723.19813	2241.06604	0.01	0.9983
Light*Time	1	22.32562	22.32562	0.00	0.9914
Con*Light*Time	3	2534.80063	844.93354	0.00	0.9996

Tukey's Studentized Range (HSD) Test for TDS

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	279264.8
Critical Value of Studentized Range	5.86560
Minimum Significant Difference	2191.8

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Trt
A	838.3	2	19
A			
A	830.9	2	31
A			
A	829.9	2	17
A			
A	827.1	2	29
A			
A	825.7	2	27
A			
A	823.2	2	3
A			
A	820.8	2	15
A			
A	805.0	2	25
A			
A	799.4	2	23
A			
A	789.6	2	20
A			

Tukey Grouping	Mean	N	Trt
A	788.6	2	13
A			
A	785.8	2	4
A			
A	785.8	2	11
A			
A	781.9	2	18
A			
A	778.8	2	30
A			
A	778.8	2	1
A			
A	778.4	2	16
A			
A	775.6	2	32
A			
A	773.9	2	8
A			
A	773.5	2	28
A			
A	768.6	2	7
A			
A	738.9	2	2
A			
A	730.8	2	24
A			
A	730.5	2	26
A			
A	730.1	2	14
A			
A	727.7	2	10
A			
A	723.1	2	12
A			
A	711.6	2	6
A			
A	711.6	2	0
A			
A	706.0	2	21
A			
A	704.9	2	5
A			
A	701.8	2	22
A			
A	700.4	2	9

Dunnett's t Tests for TDS

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	279264.8
Critical Value of Dunnett's t	3.22560
Minimum Significant Difference	1704.6

Comparisons significant at the 0.05 level are indicated by ***.

Trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
19 - 0	126.7	-1577.9	1831.3
31 - 0	119.4	-1585.2	1823.9
17 - 0	118.3	-1586.3	1822.9
29 - 0	115.5	-1589.1	1820.1
27 - 0	114.1	-1590.5	1818.7
3 - 0	111.7	-1592.9	1816.2
15 - 0	109.2	-1595.4	1813.8
25 - 0	93.5	-1611.1	1798.0
23 - 0	87.9	-1616.7	1792.4
20 - 0	78.0	-1626.5	1782.6
13 - 0	77.0	-1627.6	1781.6
4 - 0	74.2	-1630.4	1778.8
11 - 0	74.2	-1630.4	1778.8
18 - 0	70.4	-1634.2	1774.9
30 - 0	67.2	-1637.4	1771.8
1 - 0	67.2	-1637.4	1771.8
16 - 0	66.9	-1637.7	1771.4
32 - 0	64.0	-1640.5	1768.6
8 - 0	62.3	-1642.3	1766.9
28 - 0	62.0	-1642.6	1766.5
7 - 0	57.0	-1647.5	1761.6
2 - 0	27.3	-1677.3	1731.9
24 - 0	19.3	-1685.3	1723.8
26 - 0	18.9	-1685.7	1723.5
14 - 0	18.6	-1686.0	1723.1
10 - 0	16.1	-1688.5	1720.7
12 - 0	11.6	-1693.0	1716.1
6 - 0	0.0	-1704.6	1704.6
21 - 0	-5.6	-1710.2	1699.0
5 - 0	-6.6	-1711.2	1697.9
22 - 0	-9.8	-1714.4	1694.8
9 - 0	-11.2	-1715.8	1693.4

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The GLM Procedure

Dependent Variable: pH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	32	5.36614848	0.16769214	0.17	1.0000
Error	33	33.43390000	1.01314848		
Corrected Total	65	38.80004848			

R-Square	Coeff Var	Root MSE	pH Mean
0.138303	17.63257	1.006553	5.708485

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Con	3	0.14676719	0.04892240	0.05	0.9857
Oxygen	1	3.79762656	3.79762656	3.75	0.0615
Light	1	0.00743906	0.00743906	0.01	0.9322
Time	1	0.34662656	0.34662656	0.34	0.5626
Con*Oxygen	3	0.00720469	0.00240156	0.00	0.9998
Con*Light	3	0.03269219	0.01089740	0.01	0.9984
Oxygen*Light	1	0.04357656	0.04357656	0.04	0.8370
Con*Oxygen*Light	3	0.00452969	0.00150990	0.00	0.9999
Con*Time	3	0.00442969	0.00147656	0.00	0.9999
Oxygen*Time	1	0.43725156	0.43725156	0.43	0.5158
Con*Oxygen*Time	3	0.00862969	0.00287656	0.00	0.9998
Light*Time	1	0.00237656	0.00237656	0.00	0.9617
Con*Light*Time	3	0.01895469	0.00631823	0.01	0.9993
Oxygen*Light*Time	1	0.00082656	0.00082656	0.00	0.9774
Con*Oxyge*Light*Time	3	0.01902969	0.00634323	0.01	0.9993

Tukey's Studentized Range (HSD) Test for pH

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	1.013148
Critical Value of Studentized Range	5.86560
Minimum Significant Difference	4.1748

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	Trt
A	6.195	2	0
A			
A	6.165	2	2
A			
A	6.165	2	6
A			
A	6.145	2	10
A			
A	6.115	2	12
A			
A	6.105	2	14
A			
A	6.070	2	22
A			

Tukey Grouping	Mean	N	Trt
A	5.995	2	24
A			
A	5.985	2	26
A			
A	5.890	2	9
A			
A	5.840	2	1
A			
A	5.805	2	5
A			
A	5.800	2	11
A			
A	5.735	2	23
A			
A	5.730	2	13
A			
A	5.725	2	25
A			
A	5.720	2	21
A			
A	5.670	2	7
A			
A	5.535	2	8
A			
A	5.515	2	4
A			
A	5.480	2	28
A			
A	5.455	2	17
A			
A	5.445	2	16
A			
A	5.440	2	15
A			
A	5.435	2	3
A			
A	5.430	2	31
A			
A	5.430	2	30
A			
A	5.420	2	29
A			
A	5.415	2	27
A			
A	5.410	2	18
A			
A	5.405	2	19
A			
A	5.370	2	20
A			
A	5.340	2	32

Dunnett's t Tests for pH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	33
Error Mean Square	1.013148
Critical Value of Dunnett's t	3.22560
Minimum Significant Difference	3.2467

Comparisons significant at the 0.05 level are indicated by ***.

Trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
2 - 0	-0.0300	-3.2767	3.2167
6 - 0	-0.0300	-3.2767	3.2167
10 - 0	-0.0500	-3.2967	3.1967
12 - 0	-0.0800	-3.3267	3.1667
14 - 0	-0.0900	-3.3367	3.1567
22 - 0	-0.1250	-3.3717	3.1217
24 - 0	-0.2000	-3.4467	3.0467
26 - 0	-0.2100	-3.4567	3.0367
9 - 0	-0.3050	-3.5517	2.9417
1 - 0	-0.3550	-3.6017	2.8917
5 - 0	-0.3900	-3.6367	2.8567
11 - 0	-0.3950	-3.6417	2.8517
23 - 0	-0.4600	-3.7067	2.7867
13 - 0	-0.4650	-3.7117	2.7817
25 - 0	-0.4700	-3.7167	2.7767
21 - 0	-0.4750	-3.7217	2.7717
7 - 0	-0.5250	-3.7717	2.7217
8 - 0	-0.6600	-3.9067	2.5867
4 - 0	-0.6800	-3.9267	2.5667
28 - 0	-0.7150	-3.9617	2.5317
17 - 0	-0.7400	-3.9867	2.5067
16 - 0	-0.7500	-3.9967	2.4967
15 - 0	-0.7550	-4.0017	2.4917
3 - 0	-0.7600	-4.0067	2.4867
31 - 0	-0.7650	-4.0117	2.4817
30 - 0	-0.7650	-4.0117	2.4817
29 - 0	-0.7750	-4.0217	2.4717
27 - 0	-0.7800	-4.0267	2.4667
18 - 0	-0.7850	-4.0317	2.4617
19 - 0	-0.7900	-4.0367	2.4567
20 - 0	-0.8250	-4.0717	2.4217
32 - 0	-0.8550	-4.1017	2.3917

E. FORMULA OF THE CULTURE MEDIA USED

YM (Yeast Mold) agar

Composition per liter:

Peptone	5.0	g
Glucose	10.0	g
Yeast extract	3.0	g
Malt extract	3.0	g
Agar	15.0	g

GYEA (Glycerol-Yeast Extract Agar)

Composition per liter:

Glycerol	5.0	mL
Yeast extract	2.0	g
Dipotassium hydrogen phosphate	1.0	g
Agar	15.0	g

PCA (Plate Count agar)

Composition per liter:

Tryptone	5.0	g
Glucose	1.0	g
Yeast extract	2.5	g
Agar	15.0	g
Water	1000	mL

NA (Nutrient Agar)

Composition per liter:

Peptone	5.0	g
Sodium chloride	5.0	g
Yeast extract	2.0	g
Beef extract	1.0	g
Agar	15.0	g

EMB (Eosin-methylene Blue Agar)

Composition per liter:

Tryptone	10.0	g
Lactose	5.0	g
Sucrose	5.0	g
Dipotassium hydrogen phosphate	2.0	g
Eosin Y	0.4	g
Methylene blue	0.065	g
Agar	13.5	g

LT (Lauryl Tryptose) broth with bromocresol purple

Composition per liter:

Tryptose	20.0	g
Lactose	5.0	g
Dipotassium hydrogen phosphate	2.75	g
Potassium dihydrogen phosphate	2.75	g
Sodium chloride	5.0	g
Sodium lauryl sulfate	0.1	g

EC (*Escherichia coli*) broth

Composition per liter:

Tryptose	20.0	g
Lactose	5.0	g
Bile salt	1.5	g
Dipotassium hydrogen phosphate	4.0	g
Potassium dihydrogen phosphate	1.5	g
Sodium chloride	5.0	g

GM broth

Composition per liter:

Sodium L-glutamic acid	3.8	g
DL-Malic acid	2.7	g
Yeast extract	2.0	g
Potassium dihydrogen phosphate	0.5	g
Dipotassium hydrogen phosphate	0.5	g
Diammonium hydrogen phosphate	0.8	g
Magnesium sulfate heptahydrate	0.2	g
Calcium chloride dihydrate	0.053	g
Nicotinic acid	0.001	g
Thiamine Hydrochloride	0.001	g
Biotin	0.01	g
Manganese sulfate pentahydrate	0.012	g
Ferric citrate	0.025	g
Cobalt chloride hexahydrate	0.95	g

Peptone broth

Composition per liter:

Peptone	1	g

F. GRAM STAIN TECHNIQUE

Reagents

Crystal violet
Gram's iodine
95% ethyl alcohol
Safranin

Equipments

Glass slide
Bunsen burner
Inoculation loop
Microscope

Method

1. Aseptically transfer one loop of water onto a clean glass slide.
2. Follow by aseptically transferring one loop of culture of interest onto the glass slide and in a circle motion, mix the water and the culture together spreading it on the glass slide.
3. Heat fix the smear and allow it to air dry.
4. Drop crystal violet onto the smear and let it stand for one minute.
5. Indirectly wash the smear with tap water and tap dry with tissue paper.
6. Repeat step 4 and 5 with gram's iodine.
7. Decolorize the stain by dropping the 95% ethyl alcohol drop by drop until run off is clear.
8. Indirectly wash the smear with tap water and drop safranin onto the smear.
9. After 45 seconds wash the safranin indirectly with tap water and pat dry.
10. Examine with the microscope under oil immersion.