Isolation of marine bacteria with bioremediation properties from the culture water of Tiger grouper (*Epinephelus fuscoguttatus*)

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Abstract

An experiment was conducted to isolate bacterial bioremediator from the culture water of Tiger Grouper (Epinephelus fuscoguttatus). In this study, the potential of the bacteria as ammonia degraders were investigated. This was done through enrichment assay using ammonium sulphate as carbon source. The bacteria were grown in minimal medium and sodium chloride. Through this assay, three distinct bacteria were isolated. Using the total ammonia-nitrogen (TAN) analysis, all three bacteria were able to degrade ammonium sulphate up to 0.04 ppm compared to the control of 0.54 ppm. All three bacteria were found to be Gram-positive bacteria by its distinctive purple color in Gram-staining method. Based on result, it can be concluded that the marine bacteria isolated from the culture water of Tiger grouper have the potential as bioremediator agent in aquaculture.

Keywords: Bioremediator, Tiger Groupper, Enrichment assay

1. Introduction

Epinephelus fuscoguttatus or Tiger Grouper are the most popular carnivorous fish with high market value in the world such as Kuwait, Indonesia, USA, Japan, Mexico, Taiwan, China, Philippines, Thailand and Malaysia. Global grouper production increased in recent years with 60 774 mt, 99 378 mt, 163 093 mt and 198, 690 mt in 1990, 2000, 2005, and 2007,respectively (FAO, 2005a,b; 2009). Brown-marbled grouper become an important mari-culture fish for intensive aquaculture, particularly in the Asia Pacific region because of their high consumer demand, desirable taste, hardiness in a crowd environment, fast growth, efficiency in feed conversion and rapid growth (Sim *et al.,* 2005; Chen and Tsai, 1994; Kohno *et al.,* 1993). Wild grouper culture become emerging as a viable venture besides from overexploitation in many countries. Because of the importance value as a cultured fish, grouper have become a challenging target research for aquaculture scientists (Kohno *et al.,* 1993).

Tiger Groupers are high priced and popular seafood fishes in Southeast Asia (Sarjito *et al.,* 2009). *Epinephelus* sp. is an aquaculture species that are widely used for intensive farming in world (Boonyaratpalin, 1997; Haemstra and Randall, 1993). However this intensive culture of grouper has been

severely hit by viruses and bacteria, which have caused serious economic losses (Chiu *et al.,* 2008) both in farms and hatcheries. Intracohort cannibalism usually results in mass mortality, during larviculture (Fukuda *et al.,* 1999 ; Watanabe *et al.,* 1991). Tiger Grouper farming has suffered several disease problem like nervous necrosis and sleepy disease as well as vibrosis caused by *Vibrio alginolyticus* (Fukuda *et al.,* 1993). Palanisamy *et al.* (1999) reported that grouper farm or hatcheries at Malaysia, commonly faces vibriosis disease caused by *Vibrio* spp.

Aquaculture has been supporting human demands for fish consumptions for a centuries and is an important industry around the world (Chopin and Yarish, 1998). Intensive scale fish aquaculture farming has caused many environmental problems to farmer (Wu, 1995). Usually modern intensive monoculture farm requires huge input of water, feeds, fertilizers and chemicals but produces a lot of wastes. Waste products from fish farms constitutes of nitrogen, phosphorus and carbon dioxide (Chung *et al.*, 2002). Many studies on biological nitrogen removal through nitrification, denitrification and anaerobic ammonium oxidation (annamox) have been done and many bacterial group have been described (Crooker and Contreras, 2010). But a lot more efforts must be put on bioremediation of aquaculture waste water which allow more environmental balanced aquaculture practices.

Therefore the objective of this study is to isolate bacterial bioremediator from the culture water of Tiger Grouper (*Epinephelus fuscoguttatus*).

2. Literature Review

2.1 *Epinephelus fuscoguttatus*

2.1.1 Taxonomy

Scientific classification of *Epinephelus fuscoguttatus*

Kingdom	: Animalia
Phylum	: Chordata
Class	: Actinopterygii
Order	: Perciformes
Family	: Serranidae
Genus	: *Epinephelus*
Species	: *fuscoguttatus*
Binomial name	: *Epinephelus fuscoguttatus*

The family Serranidae and subfamily Epinephelia, commonly known as tiger grouper, which are distributed world-wide in the tropical and subtropical waters. Species of the genus *Epinephelus* are present in the Atlantic Ocean and in the Mediterranean sea (Heemstra and Randall, 1993; Boglione *et al.,* 2005). Smith (1971) has given a detailed account of the groupers where as Heemstra *et al.,* (1991) has completed a taxonomic revision of fourteen species groupers that occur in the eastern Altantic Ocean and Mediterranean Sea. Heemstra and Randall (1993) has revised the Indo-Pacific groupers

consisting 110 species. There are compositions and phylogenetic relationship of the family Serranidae (Leis, 1986).

2.1.2 Importance in economic

Tiger grouper are economically valuable making up an important part of the catch of sport and artisanal fishers throughout their distribution. About 40 species of groupers occur in the Phillippines, where they are caught by small-scale fisherman with hook and line, bamboo traps, or dip net from estuaries and coral reefs (Kohno *et al.,* 1993). Global grouper production increased dramatically in recent years with 198,690 mt 2007 (FAO, 2009). Growth of the fishery has been observed from 1947 to 1972 with the highest yield about 21000 mt in the year 2000s (Burgos-Rosas and Perez-Perez, 2006).

In 1995, feral production of groupers reached 27,359 mt from the Phillippines, Taiwan, Thailand and Malaysia. While the total grouper production from the entire South China Sea area yielded 1348 mt from brackish water aquaculture and 771 mt from mariculture (SEAFDEC, 1997). However there is an insatiable demand for Tiger grouper as luxury protein such as Malaysia.

2.2 Effects of intensive fish farming to aquaculture

Intensive aquaculture fish farming activities have been one of the problem to eutrophication (Naylor *et al.,* 2000). Intensive fish farm can caused the formation of algal blooms and anoxia, due to excess feeds and fish excretory waste product (Wu, 1995). Consequences to the environment caused by the changes of pond from traditional to semi-intensive and lastly as a intensive fish farming practice (Rodrigueza and Montano, 2007). Localized fish have experienced death caused by overstocking, excessive feeding and low water quality in ponds. All of this make aquaculture scientists to propose various methods to achieve a sustainable fish farming practice (Neori *et al.*, 2004).

Water bloom can effects water quality which lead to a death of aquatic organism, but also decrease the esthethic value of pond (Yang and Abbaspour, 2007). Phosphorus and nitrogen are the main caused for the eutrophication problem in ponds. A method has been developed to eliminate the phosphorus and nitrogen which improve pond water quality (Wang *et al.*, 2007). Bioremediation is one of attractive strategy to clean up the contaminated areas (Segura *et al.*, 2007).

2.3 Bioremediation technology

The treatment of environmental pollution by microorganism is a safe and economic alternative way to be practiced in aquafarm.. Bioremediation has been defined as a biological response to environmental abuse (Hamer, 1993). This definition try to explain the differences between the use of microorganism to remediate contaminated sites and their application in biotreatment or biorecycle process designed to reduce organic and inorganic contaminant emission at sources. Bioremediation use a microbial metabolic potential for eliminating environmental pollutants. Microorganism that have the capability of mineralizing a variety of toxic compund such as ammonia has been isolated in laboratory conditions (Pieper and Reineke, 2000). Marine micro-organism can degrade a wide variety of contaminants (Harayama *et al.*, 2004).

Qian *et al.*, (1996) has done a studies on *Kappaphycus* sp. which are capable of assimilating nitogenous waste, ammonium in particular and exhibits high growth rates in ammonium-rich water (Li *et al.*, 1990). To apply this technology to the bioremediation of marine waters, the biocatalysts has to be active in the presence of NaCl and it also must be compatible with the organic solvent use. Nowadays, most highly solvent-tolerent bacteria were isolated from soils or continental waters, and very few isolated from marine water (Moriya and Horikoshi, 1993). Bioremediation is a relatively to restore and rehabiliate contaminated areas. Various method of dipersal, collection, removal, landfill disposal and incineration simply dilute or transfers them to another environmental medium (Bouwer, 1992). Bioremediation could be an effective and environmental friendly cleanup technology, since it result in the partial or complete bioconversion of organic pollutants to microbiol biomass and stable non-toxic endproducts (Baker and Herson, 1994).

3. Material and Methods

3.1 Preparation of minimal media

In distilled water of 500ml, sodium phosphate (Na_3PO_4) 32g; Pottasium dihydrogen phosphate (KH_2PO_4), 7.5g; Sodium chloride (NaCl), 1.25g; Ammonium chloride (NH_4Cl) 5.0g, were mixed togehter and stirred until it is fully dissolved. Meanwhile, 1M magnesium sulphate ($MgSO_4$) (mix 12.036g in 100ml H_2O); 20% Glucose (mix 10g in 40ml H_2O), and 1M of Calcium chloride (mix 14.702g in 100ml H_2O) was prepared separately and autoclaved it. After autoclaved, 100ml of minimal media, 400 ml distilled water and 7.5 agar base powder, mix evenly into minimal salt solution.

3.2 Enrichment Assays

Bacteria were cultured in 250ml Erlenmeyer flasks that contain 10 ml Marine broth and were incubated for 24 hours at 28^{o}C at 80rpm. Bacteria pellet were diluted at 10^9 cfu/ml with NaCl, after centrifugation at 3000rpm for 10 minutes. Different treatments were prepared:

Table 1. Potential ammonia degrader (10^6 cfu/ml) in different treatment

C	Bacteria + Minimal Media
T1	Bacteria + 20ppm $(NH_4)_2SO_4$ + 30 ppt NaCl
T2	Bacteria+ 20ppm $(NH_4)_2SO_4$ + Minimal Media

All of the treatments were incubated for three days at 28°C and 120 rpm. The bacterial present in the enrichment cultures was determined through streak plating method using Marine Agar plate. The plates were then incubated for 24 hours at 28°C. This process was repeated for three times using sub-culture techniques. Subculture were prepared by inoculation of 100µl of bacteria from the enrichment culture of the first cycle into 10ml Marine Broth in a 250ml Erlenmeyer flaks. Cultures were incubated for 24 hours at 28°C and 80rpm.

3.3 Total Ammonia-Nitrogen (TAN) Analysis

Total ammonia nitrogen was determined acording to Parson *et al.* (1984). Standard stock solution was prepared by weighing 9.343g of anyhydrous grade $(NH_4)_2SO_4$ (dried at 110°C for 1 hour, cooled in dessicator before weighing) and dissolving in 1000ml deionized water. From the stock solution (1000mgL^{-1} of total ammonia-nitrogen), a series of standard solutions (0.01, 0.03, 0.05, 0.07, 0.1, 0.03, 0.5 and 1.0 mgL^{-1}) were prepared by mixing with appropriate ratio of deionized water.

Sample and standard solution (10ml) were placed in test tube and 0.4ml of phenol solution (20g of analytical grade phenol was dissolved in 200ml of 95% v/v ethyl alcohol and 0.4ml of sodium nitroprusside; 1g of $Na_2[Fe(CN)_5NO]_2H_2O$, dissolved in 200ml of DDH_2O water) was added in sequences. Finally, 1ml of oxidizing solutions was added and allows cooling at room temperature (20-27 °C) for 1 hour. The test tube were covered with parafilm (the colour stable for 24 hours after the reaction period). The extinction was measured at 640nm using spectrophotometer (model UV-1601 Shidmazu, Kyoto, Japan). Oxidizing solution was prepared by mixing 100ml of alkaline reagent (dissolved 100g of sodium citrate and 5g of sodium hydroxide in 500ml of DDH_2O) and 25ml of sodium hypochlorite solution (commercial hypochlorite which should be about 1.5N).

3.4 Gram staining protocol

Gram staining protocol was determined according to Gephart *et al.* (1981). Sample was flood air-dried after heat-fixed with bunsen burner. The sample flooded for 1 minutes with crystal violet staining reagent. Slide was washed with gentle and indirect stream of tap water for 2 seconds. Slide was

then flooded with the mordant; Grams iodine for 1 minutes. After that, slide was washed with gentle and indirect stream of tap water for 2 seconds. Flooded slides was then treated with decolorizing agent drop by drop until clear. Afterwards, the slide was flooded with counterstain, sarafanin for 2 minutes. Lastly, the slide was washed with indirect stream of tap water until no colour appears in the effluent and was bloted dry with absorbent paper tissues. Samples were then observed under light microscope and using an immersion oil for 1000x magnification.

3.5 Statistical analysis

Data on total ammonia-nitrogena analysis were analyzed by using one-way analysis of varience (ANOVA). The level of significance was accepted at ($p<0.05$). All satistical analyses were performed using SPSS, Release 12, software (SPSS Inc., USA)

4.0 Results and Discussion

4.1 Enrichment assay

Enrichment assay was conducted to enrich bacterial bioremediator from the culture water of tiger grouper (*Epinephelus fuscoguttatus*). Based on Table 1, all three different treatments including control without any carbon sources, showed bacterial growth in the 1st and 2nd cycle of enrichment. On the 3rd cycle, no bacterial growth was observed in the control. Meanwhile, growth can be observed in the treatment with addition of ammonium sulphate as carbon sources. This shows that potential bacterial bioremediator can be isolated using this assay.

Table 2. Cycles of enrichment bacteria on different medium

		1st Cycle	**2nd Cycle**	**3rd Cycle**
C	Bacteria + Minimal Media	+	+	-
T1	Bacteria + 20ppm $(NH_4)_2SO_4$ + NaCl	+	+	+
T2	Bacteria+ 20ppm $(NH_4)_2SO_4$ + Minimal Media	+	+	+

* '-' no bacteria colony appear on Marine agar plate. '+' bacteria colony appear on Marine agar plate

The bacteria were cultured in ammonium sulphate to make the bacteria use the ammonia as a carbon sources. Commonly ammonium removal process use nitrification-denitrification reaction to complete (Kawagoshi *et al.*, 2010). Complete oxidation in nitrification reaction, ammonium will be converted to nitrite or nitrate and organic carbon source is required in denitrification reaction. In contrast to this, only half amount of ammonium is

oxidized to nitrite and no organic carbon source is required in nitrification, so it is possible to reduce requirement of carbon sources and bacteria need an organic carbon source to make them grow in certain medium. In this reasearch, bacteria T1 and T2 show a potential as bioremediator, cause can growth in absent of carbon organic matter and use ammonia as carbon source.

4.2 Isolation of potential bioremediator

There are many bacteria colonies from 3rd cycle treatment 2 (refer Table 2.) appeared on marine agar plate after incubation. However, only three distinctive different morphologies with yellow and orange pigmented colonies were selected. The bacteria has been selected due to a large colony form on marine agar plate compare to others and can be seen under microscope. The selected bacteria were named as D1,D2 and D3, respectively.

4.3 Ammonia degradation assay

Potential bioremediator (D1,D2 and D3) were subjected to ammonia degradation assay. In this assay, 0.5 ppm of ammonium sulphate was added to all treatments with and without bacteria. The results showed that all three potential bioremediator degraded ammonium sulphate after 24 hours of incubation (Table 2). The concentration of 0.5 ppm ammonium sulphate in the treatment with the bacteria decreased gradually after 24 hours of incubation to 0.048, 0.036 and 0.039 ppm, respectively. This indicates that bacteria D1,D2 and D3 has used 92% of ammonium sulphate as their organic carbon source.

Table 3. Ammonia degradation by bacteria in each treatment and control

	Sample	**Concentration (ppm)**
C	$(NH_4)_2SO_4$ + NaCl	0.54 ± 0.15^a
T1	Bacteria D1 + $(NH_4)_2SO_4$ + NaCl	0.05 ± 0.00^b
T2	Bacteria D2 + $(NH_4)_2SO_4$ + NaCl	0.04 ± 0.00^b
T3	Bacteria D3 + $(NH_4)_2SO_4$ + NaCl	0.04 ± 0.00^b

In aerobic condition, a bacteria will convert ammonium to nitrite and then to nitrate (Crooker and Contreras, 2010). This nitrification process need a lot of oxygen but it can lower dissolved oxygen in the pond. A bacteria can be used in reducing power of the nitogenous substrates to fix CO_2 via Calvin-Benson cycle as their organic carbon source. Because of that the nitrate and ammonia become a principal nitrogen sources for the growth of some microorganism especially marine bacteria.

4.4 Bacterial Gram stain

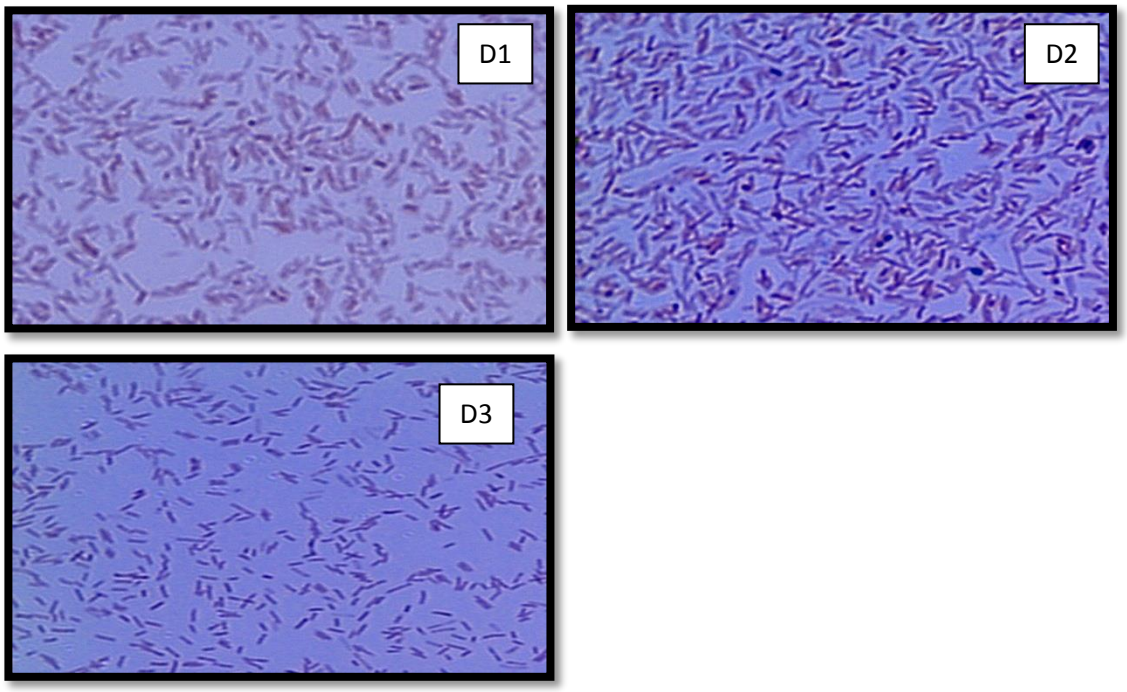

Figure 1. Gram-positive reaction in all bacteria **(D1,D2 & D3)** isolates from Tiger grouper culture water (1000x magnification)

The results of Gram-staining show that all bacteria from the Tiger grouper culture water were Gram-positive which are determined by observing purple stained rods (Figure 1). Gram-positive bacteria posses a thick mesh-like cell wall made of peptidoglycan which is 50%-90% of cell wall and Gram-negative bacteria have a thinner layer made of 10% of cell wall. For instance, Gram-positive bacteria retain their crystal violet-iodine (CV-I) complex, while Gram-negative bacteria can be colonized by ethanol treatment and can be counterstained to pink (Davis *et al.*, 1983). From previous study, *bacillus* is a well known genera that belong to Gram-positive bacteria. Laloo *et al.* (2007) has been carried out a research, where isolated *Bacillus* from *Cyprinus carpio* water culture to improve water quality. As a result three out of nine bacteria that had been isolated reduced ammonia and phosphate between 72%-76% respectively. This show a Gram-positive bacteria have high potential as probiotic in aquaculture since it can form endospores. Production of spore can response to starvation or harsh chemical or physical conditions (Laloo *et al.* 2007). This shows that Gram-positive bacteria are applicable for field applications and efficient in ammonia degradation.

5. CONCLUSION

This research was done to isolate marine bacteria with bioremediation properties from tiger grouper (*Epinephelus fuscoguttatus*) water culture. Different bacteria with bioremediation properties namely have been isolated using enrichment assays. The three distinctive bacteria were bacteria D1, D2, and D3 that are Gram-positive bacteria. From our preciminary study, three bacterial isolates has the potential as a bioremediator due to degradation of ammonia in TAN analysis. This bacteria can be used in bioremediation technology to treat the pollutant in waterbodies. Further studies using the bacterial isolates can be done through investigation on the effects of the isolates to Tiger grouper culture in *in vivo* setup. Furthermore, all the isolates can be identified using molecular technique such as 16S rRNA gene sequencing which would enable the bacterial identification up to species level.

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APPENDIX 1

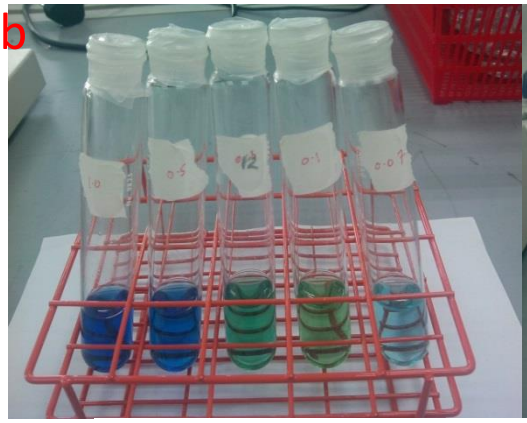
Solutions Formulations

Difco™ Marine Broth and Marine Agar 2216 (Sparks, USA)

1. Suspended the powder in 1L of purified water
    a. Difco Marine Agar 2216 : 55.1g
    b. Difco Marine Broth 2216: 37.4g
2. Heat using hot plate magnetic stirrer and boil until completely dissolved the powder
3. Autoclaved

APPENDIX 2

Colour of Standard solution and sample for TAN Analysis



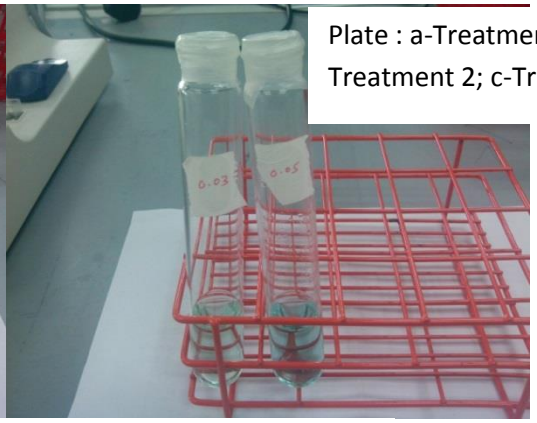
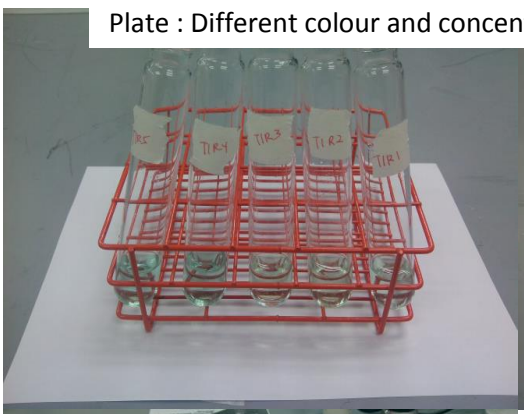
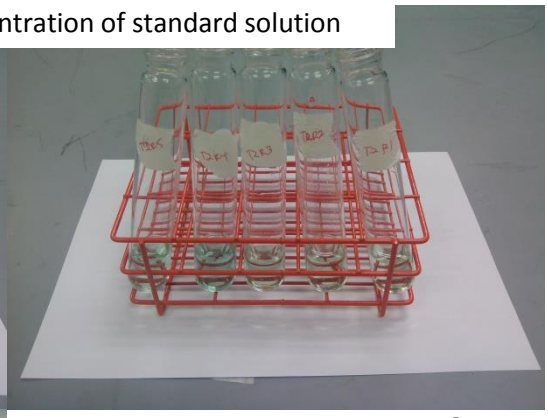


Plate : a-Treatment 1; b-Treatment 2; c-Treatment 3





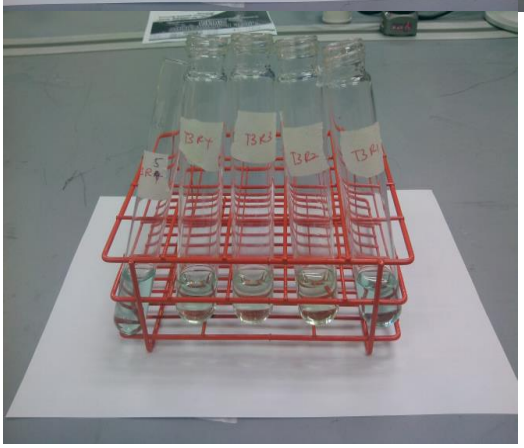


Plate : Different colour and concentration of standard solution

APPENDIX 3

Data of standard solution in TAN analysis using Lambda software

Date: 11/27/2012 Time: 5:54:35 PM
Instrument: PerkinElmer Lambda 25
Serial No: 502S09041519
Method: DINtan
Ordinate mode: Single wavelength
Baseline: No correction (0.00 0.00)
Analyst:

Wavelength(s)		Sample ID	Concentration		Ord. value	Comment
640.0	0.0	DINtan0.A01	0.0000	ppm	-0.000	
640.0	0.0	DINtan1.A02	0.0300	ppm	0.0776	
640.0	0.0	DINtan2.A03	0.0500	ppm	0.1417	
640.0	0.0	DINtan3.A04	0.0700	ppm	0.2252	
640.0	0.0	DINtan4.A05	0.1000	ppm	0.2617	
640.0	0.0	DINtan5.A06	0.3000	ppm	0.4832	
640.0	0.0	DINtan6.A07	0.5000	ppm	1.1483	
640.0	0.0	DINtan7.A08	1.0000	ppm	1.8333	

Equation: y = 1.916905e+00 * x
Residual error: 0.097963
Correlation coefficient: 0.988284

APPENDIX 4

Data of sample in TAN analysis using Lambda software

Date: 11/27/2012 Time: 6:05:59 PM
Instrument: PerkinElmer Lambda 25 Serial No: 502S09041519
Method: DINtan
Ordinate mode: Single wavelength
Slit: UV/VIS: 1.00 nm
Baseline: No correction (0.00 0.00)
Result Filename: DIN.RCO
Autozero performed: 11/27/2012 5:54:35 PM
Analyst:

Wavelength(s)		Sample ID	Ordinate	Factor	Concentration		Sample Info
640.0	0.0	C	0.1673	1.0000	0.0873	ppm	
640.0	0.0	C1	1.1376	1.0000	0.5934	ppm	
640.0	0.0	C2	1.1380	1.0000	0.5937	ppm	
640.0	0.0	C3	1.1506	1.0000	0.6002	ppm	
640.0	0.0	C4	0.3372	1.0000	0.1759	ppm	
640.0	0.0	C5	0.7999	1.0000	0.4173	ppm	
640.0	0.0	11	0.0832	1.0000	0.0434	ppm	
640.0	0.0	12	0.0780	1.0000	0.0407	ppm	
640.0	0.0	13	0.0634	1.0000	0.0331	ppm	
640.0	0.0	14	0.1112	1.0000	0.0580	ppm	
640.0	0.0	15	0.1245	1.0000	0.0650	ppm	
640.0	0.0	21	0.0551	1.0000	0.0287	ppm	
640.0	0.0	22	0.0587	1.0000	0.0306	ppm	
640.0	0.0	23	0.0001	1.0000	0.0001	ppm	
640.0	0.0	24	0.0015	1.0000	0.0008	ppm	
640.0	0.0	25	0.0936	1.0000	0.0488	ppm	
640.0	0.0	31	0.1201	1.0000	0.0627	ppm	
640.0	0.0	32	0.0577	1.0000	0.0301	ppm	

640.0 0.0 33 0.0664 1.0000 0.0346 ppm
640.0 0.0 34 0.0819 1.0000 0.0427 ppm
640.0 0.0 DINtan0 0.1231 0.0000 0.0642 ppm

APPENDIX 5

Standard Curve of Total Ammonia

Wavelength (nm)	Blank/Standard	Concentration (ppm)	Optical Density (OD)
640	Blank	0.0000	0.0000
640	A1	0.0300	0.0776
640	A2	0.0500	0.1417
640	A3	0.0700	0.2252
640	A4	0.1000	0.2617
640	A5	0.3000	0.4832
640	A6	0.5000	1.1483
640	A7	1.0000	1.8333
Residual Error : 0.097963			

Plate : The optical density (OD) for Total Ammonia Standard Curve

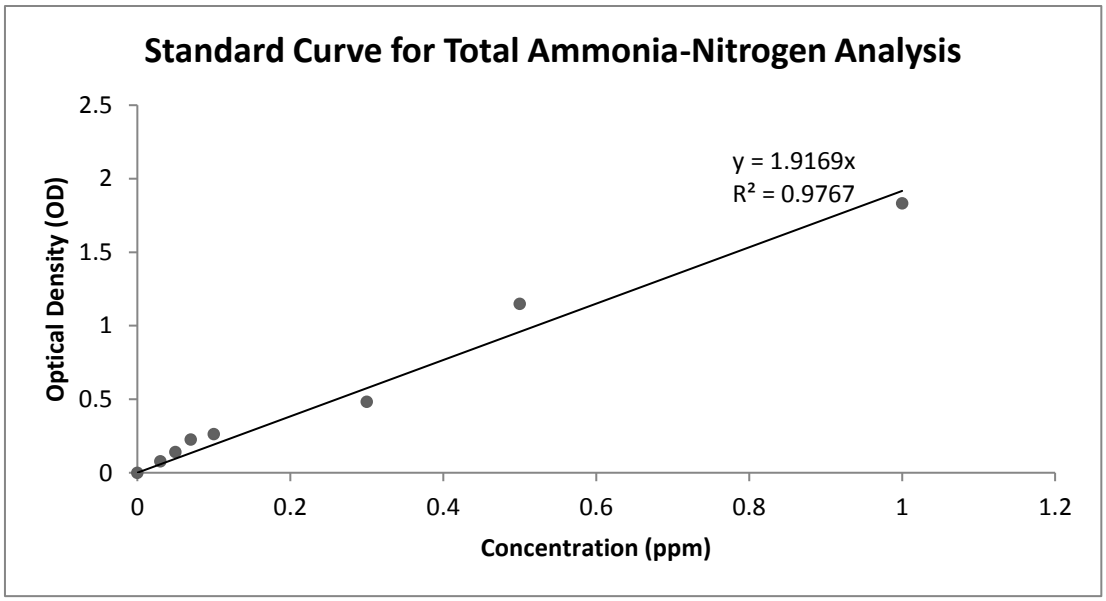


Plate : Standard curve of Total ammnia-nitrogen (TAN) analysis for standard solutio

APPENDIX 6

Data of Ammonia Degradation in SPSS Software One Way ANOVA

Descriptives

Concentration

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
C	5	.576060	.0340504	.0152278	.533781	.618339	.5173	.6000
T1	5	.048040	.0130906	.0058543	.031786	.064294	.0331	.0650
T2	5	.036000	.0085636	.0038298	.025367	.046633	.0287	.0488
T3	5	.038860	.0061825	.0027649	.031183	.046537	.0301	.0442
Total	20	.174740	.2384030	.0533085	.063164	.286316	.0287	.6000

ANOVA

Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.074	3	.358	992.927	.000
Within Groups	.006	16	.000		
Total	1.080	19			

Post Hoc Test

Multiple Comparisons

Dependent Variable:Concentration

	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	C	T1	.5280200*	.0120098	.000	.493660	.562380
		T2	.5400600*	.0120098	.000	.505700	.574420
		T3	.5372000*	.0120098	.000	.502840	.571560
	T1	C	-.5280200*	.0120098	.000	-.562380	-.493660
		T2	.0120400	.0120098	.750	-.022320	.046400
		T3	.0091800	.0120098	.869	-.025180	.043540
	T2	C	-.5400600*	.0120098	.000	-.574420	-.505700
		T1	-.0120400	.0120098	.750	-.046400	.022320
		T3	-.0028600	.0120098	.995	-.037220	.031500
	T3	C	-.5372000*	.0120098	.000	-.571560	-.502840
		T1	-.0091800	.0120098	.869	-.043540	.025180
		T2	.0028600	.0120098	.995	-.031500	.037220

*. The mean difference is significant at the 0.05 level.

Homogeneous subsets

Concentration

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	T2	5	.036000	
	T3	5	.038860	
	T1	5	.048040	
	C	5		.576060
	Sig.		.750	1.000
Duncan[a]	T2	5	.036000	
	T3	5	.038860	
	T1	5	.048040	
	C	5		.576060
	Sig.		.356	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.