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Nitrification of Fish Processing Waste Water using Mixed Cultures of *Nitrosomonas* and *Nitrobactor* for Ammonia Degradation (Phase-I)

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I. Introduction

Industrial fish processing generates large amounts of waste or residues of high nutrient content which, if not properly utilized or treated, is likely to be deposited in the environment creating pollution and health problems (Hwang and Hansen, 1998; Kotzamanis et al, 2001). Fish processing residues include scales, viscera, fish scrap, fat solids, proteins, fish rejects, and liquid stick waste water (UN Report, 1997; Hwang and Hansan, 1998).

The solid fish make up 30-40% of the total production, depending on the species processed. The waste water from seafood processing plant contains large amounts of organic matter, small particles of flesh, breading, soluble proteins and carbohydrates. Mauldin and Szabo (1974) reported that as much as 65% of the tuna is wasted in the canning process. The average daily waste flow is over 27,000 Lits ton^{-1} of fish, varying from 500 – 1550 mg Lit^{-1} of BOD; 1300 – 3250 mg Lit^{-1} of COD; and 17,000 mg Lit^{-1} of TSS of which 40% was organic. Steven (1981) surveyed the North Carolina seafood industry and stated that unloading, washing and separating ice, sorting grading and re-icing before shipping all constitute handling and sources of contamination form personnel. Results of the survey found that the average values for TSS was 2.4, ash 1.11, organic solids 1.30 and BOD 0.23 g kg^{-1} while water use was 1.51kg^{-1} of fish handled.

a) Characterization of fish processing waste water

The volume and concentration of wastewater from fish processing depends mainly on the raw fish composition, additives used, processing water source and the unit process. The main components of fish processing wastewater are lipids and protein (Gonzalez, 1996).

i. *pH*

Effluent pH from fish processing plants is usually close to neutral. The results obtained from four different fisheries from British Columbia showed pH in the range of 5.7 – 7.4 with an average pH of 6.48 (Technical Report Series FREMP, 1994).

ii. *Solid Content*

Fish processing wastewater contains high levels of suspended solids which are mainly proteins and lipids (Palenzuela-Rollon et al., 2002). The fish condensate has high volatile solids (VS) consisting of trimethyl amine (TMA) and volatile fatty acids (VFA). The wastewater characteristics from fish processing units depend on the composition of raw fish, the unit processes, source of processing water and additives used such as brine, oil for the caning process (Palenzuela – Rollon, 1999).

iii. *Fat, Oil and Grease (FOG)*

Fat, oil and grease (FOG) are also important parameters of fish processing wastewater. Around 60% of the oil and grease originates from the butchering process (Nova Tec, 1994).

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b) Environmental problems

The main environmental problems linked to seafood cannery industries are wastewater, emissions to the atmosphere, solid wastes (with similar characteristics to urban wastes), inert and hazardous wastes (IHOBE 1999). The main atmospheric emissions are due to the use of fuel to generate steam. In addition, there are other emissions in the form of odors, because of the presence of hydrogen sulphide and trimethylamine (Xunta de Galicia 2001).

Wastewaters generated in seafood processing factories have quite a high organic load (mainly from proteins and fats) and also high levels of salinity. These effluents exert a strong impact on the environment, being especially relevant when they are discharged into seawater with low self-purification capacity, as occurs in the estuaries of Galicia, where the largest number and highest density of these factories in Europe are located. Fish and shellfish canning industries in Galicia and also in Spain generate different liquid effluent that present a wide range of characteristics according to the raw material processed (tuna, mussel, sardine, mackerel, etc). Cooking effluents contain the highest organic matter load. The high salinity (Na^+, Cl^-, SO_4^{2-}) is caused both by the raw material (fish and shellfish) and the seawater used in the process.

c) Biological treatment processes

After suitable primary treatment the wastewater is treated through a biological wastewater treatment system where microorganisms are involved in degradation of organic matter. Biological treatment processes carried out in two ways:

Aerobic processes - biological treatment processes that occur in the presence of oxygen.

Anaerobic processes – biological treatment processes that occur in the absence of oxygen.

Anaerobic treatment converts the organic pollutants (COD, BOD_5) in wastewater into a small amount of sludge and a large amount of biogas (methane and carbon dioxide), while leaving some pollution unresolved. The main advantages, particularly for bigger plants, are i) low operating costs, ii) low space requirements, iii) valuable biogas production, and iv) low sludge production. Anaerobic systems are well suited to the treatment of fish processing wastewater because a high degree of BOD_5 removal can be achieved at a significantly lower cost than comparable aerobic systems and generate a smaller quantity of highly stabilized, and more easily dewatered, sludge. Furthermore, the methane – rich gas which is generated can be captured for use as a fuel (Johns, 1995).

d) Nitrification

Nitrification is the aerobic oxidation of ammonium to nitrate. It consists of two sequential steps carried out by two phylogenetically unrelated groups of aerobic chemolithoautrophic bacteria. Some heterotrophic bacteria can also oxidize ammonium to nitrate, but this is only a very small contribution to the overall ammonia oxidation. First, ammonium is oxidized to nitrite by the aerobic ammonia – oxidizing bacteria. Approximately 2moles of protons are produced for every mole of ammonium oxidized. Ammonium oxidization is therefore an acidifying reaction. In the second step nitrite is oxidized to nitrate by the nitrite oxidizing bacteria. No single known autotrophic bacterium is capable of complete oxidation of ammonium to nitrate in a single step. The key reactions of nitrification are given by,

$$NH_4^+ + \tfrac{3}{4}\, O_2 \rightarrow NO_2^- + H_2O + 2H^+ NO_2^- + \tfrac{1}{2}\, O_2 \rightarrow NO_3^-$$

The main objective of this study was to degrade the ammonia present in the fish processing waste water. The fish processing waste water was analyzed for total solids, dissolved oxygen, nitrite, nitrate, ammonia, BOD and COD. Microbial consortium of nitrifying bacteria was obtained from culture collection centre. The process parameters for the nitrification were optimized and using the optimized conditions a Rotating Biological Contactor (RBC) was operated.

II. Materials and Methods

a) Chemicals used

The chemicals used in this study are of the best quality and the analytical grades. Distilled water is used throughout the experimental work.

b) Experimental methods

i. *Microbial cultures and maintenance*

The nitrifying strains of *Nitrosomonas and Nitrobactor* were obtained from National Collection of Industrial Microorganism Laboratory, Pune, India. The growth medium for the strains was prepared by mixing 100ml of solution-I and 5ml of solution-II. The composition of solution-I consists of: $MgSO_4.7H_2O$-0.2 gL^{-1}; K_2HPO_4-1.0gL^{-1}; $FeSO_4.7H_2O$-0.05gL^{-1}; $CaCl_2.4H_2O$-0.02gL^{-1}; $MnCl_2.4H_2O$-0.002gL^{-1}; and $Na_2MoO_4.2H_2O$-0.001gL^{-1}. The pH of the solution was then adjusted to 8.5 using 1N Na OH. After adjusting the pH, 0.5% of $CaCO_3$ was added to solution-I used for the growth of *Nitrosomonas*. Solution-II was prepared by dissolving 6g of $NaNO_2$ and 3g of NH_4Cl in 1L of distilled water for *Nitrobactor* and *Nitrosomonas* respectively. 100ml of solution-I was transferred in to each Erlenmeyer flask, which was cotton plugged and then sterilized in an autoclave at 121°C for 20 min. After cooling, 5ml of filter sterilized solution-II was added aseptically to reach a final concentration of 150mg. The media was then inoculated with 1ml of the corresponding culture and the flasks were placed in the incubator shaker at 30°C for 5 days. The culture was stored at 4°C in refrigerator.

The fish processing wastewater samples investigated in the study were collected from the local fish market. The samples were transported in cool boxes to the laboratory for analysis.

ii. *Rotating Perforated Tubes Bio film Reactor*

Rotating perforated tubes bio film reactor is a novel bioreactor employed in this study for treatment of fish processing wastewater. Rotating biological contactors is a remediation technology used in the secondary treatment of fish processing wastewater. This technology involves allowing wastewater to come in contact with a biological medium in order to facilitate the removal of contaminants.

A rotating biological contactor (RBC) consists of discs mounted on a shaft which is driven so that the discs rotate at right angles to the flow of settled sewage. The discs are usually made of plastic (polythene, PVC, expanded polystyrene) and are contained in a trough so that about 40 percent of their area is immersed. The discs are arranged in groups or packs with baffles between each group to minimize surging or short circuiting. With small units the trough is covered and large units are often housed within buildings. This is reducing the effect of weather on the active bio film which becomes attached to the disc surfaces.

iii. *Advantages of Rotating Biological Contactors (RBC)*

- Short contact periods are required because of the large active surface.
- They are capable of handling a wide range of flows.
- Sloughed biomass generally has good settling characteristics and can be easily separated from waste stream.
- Operating costs are low because little skill is required in plant operation.
- Short hydraulic retention time.
- Low power requirements.
- Low sludge production
- High efficiency & easy to operate

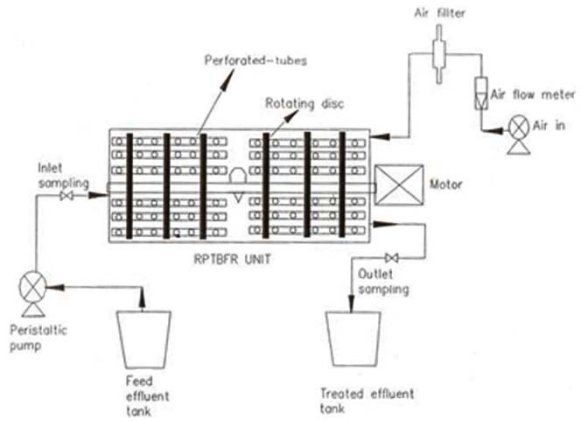


Figure 1 : Schematic diagram of rotating perforated tubes biofilm reactor

c) Assay methods

i. *Estimation of suspended solids*

100 ml of the given water sample was taken and filtered using pre weighed what man No.1 filter paper. After filtration, the filter paper along with the suspended solids was dried at 110°C in an oven and the dry weight was taken. The amount of suspended matter present in 100 ml of the given sample was obtained as the difference in weight of the filter paper and converts the value into pm.

ii. *Determination of total dissolved solids*

50 ml of the filtered water sample was taken in a pre weighed china dish. The water content in the china dish was completely evaporated by keeping in an oven at 110°C. After evaporation, the china dish was cooled and weighed. The difference in weight of the china dish was taken as the amount of total dissolved solids present in 50 ml of the filtered water sample and the value was represented in pm.

iii. *Estimation of dissolved oxygen (DO)*

The dissolved oxygen in the water sample was analyzed using Winkler titration method. A series of reagents namely manganous sulphate, concentrated sulphuric acid and alkaline iodine were added in to the sample contained in an air tight BOD bottle. Manganous sulphate when react with potassium hydroxide forms manganese hydroxide which then oxidize to manganic hydroxide by dissolved oxygen in the sample. Manganese hydroxide then converts to manganese sulphate on reaction with sulphuric acid and this in turn liberate free iodine when reacted with potassium iodide. The amount of iodine liberated is obtained by Titrating the above reaction mixture against standard sodium tio sulphate and this represents the amount of dissolved oxygen in the sample. 2.5ml of sample was diluted to 250ml using distilled water and the diluted sample was taken in a 250ml BOD incubator. 2ml of manganese sulphate was added followed by 2ml of alkaline iodine reagent immediately after collection. The tip of the pipette should be below the liquid level while adding these reagents and close with the stopper immediately. Mix well by inverting the bottle 2-3 times and allow the precipitate to settle. Then add 2ml concentrated sulphuric acid. Mix well till precipitate goes into solution. Transfer 50ml sample into a clean conical flask and titrate against standard sodium thio sulp hate until a very pale straw yellow color remains. Add 1ml of starch indicator (blue color develops). Disappearance of blue color is the end point.

iv. *Estimation of nitrite*

The nitrite content in the water sample was analyzed using a colorimetric method. In acid conditions, nitrite forms a diazo-compound with sulfanilamide. This diazo-compound reacts with N-(1-naphthyl)-ethylenediamine (NED) forming an intensely colored pink azo dye, the intensity of which is related to

the nitrite concentration and can be measured photometrically. Stock solution of nitrite was prepared by dissolving 1.5g sodium nitrate in 1000ml of distilled water. 10ml of the stock nitrite solution was diluted to 100ml with distilled water to obtain the working standard. From the working standard, 2, 4, 6, 8 and 10 ml was pipette out in to a series of volumetric flasks and made up to 100ml using distilled water. 40ml of each dilution was taken in a 50ml volumetric flask and 2ml of sulfanilamide solution was added and thoroughly mixed. After 3-6 minutes 2 ml of NED solution was added and diluted up to 50 ml and mixed well. The color developed during the reaction was read at 543 nm. The calibration chart was prepared by plotting absorbance value verses the corresponding concentrations. 40ml of filtered sample was taken in a 50ml volumetric flask and the same procedure was repeated to read the absorbance.

v. *Estimation of nitrate*

The nitrate content in the water sample is analyzed by a spectrophotometric method. The determination is based on the reduction of nitrate to nitrite in the presence of Zn/NaCl. The produced nitrite is subsequently diazotized with sulfanilamide then coupled with NED to form an azo dye which is measured at 543 nm. Stock solution of nitrate was prepared by dissolving 1.3g of sodium nitrate in 1L of distilled water. Pipetted out 10 mL of nitrate stock solution to a beaker, added 5 mL of concentrated HCl and 2mL of Zn/Na Cl granular mixture and was allowed to stand for 30 minutes, with occasional stirring to form nitrite. The solution was then filtered using what man filter paper and made up to 100ml using distilled water to obtain the working standard. 2, 4, 6, 8 and 10ml were pipette out and made up to 100ml using distilled water. The reduced nitrate was obtained by analyzing the nitrite liberated using the previous described method. Calibration chart was obtained by plotting absorbance verses corresponding nitrate concentration. 10ml of the filtered sample was taken in a beaker and the same procedure was followed to read the absorbance. The nitrate content was determined from the calibration chart.

vi. *Determination of ammonia*

Stock solution was prepared by dissolving 3.141g of ammonium chloride in 1000ml of distilled water. Working standard was prepared by diluting 10ml of the stock solution into 100ml using distilled water. 2, 4, 6, 8 and 10ml of the working standard were pipetted out and made up to 100ml. To 100ml of each dilution 1 mL of $ZnSO_4$ solution was added and mixed thoroughly. The pH of the sample was adjusted to 10.5 using 6N Na OH and allowed to stand for a few minutes and filtered. Initial 25ml was discarded and the rest was collected. 50 mL of the aliquot was taken in a Nessler tube and 0.05 mL EDTA solution or 0.1 mL Rochelle salt was added and mixed well. To this 2 mL of Nessler reagent was add and mixed well by capping Nessler tubes and allowed to stand for a few minutes. The absorbance was measured at420 nm using 1cm cuvette with a reagent blank. Absorbance was plotted against the concentration of ammonia to obtain the calibration chart. 100ml of filtered sample was taken and the same procedure was followed to read the absorbance at 420pm. The concentration of ammonia in the sample was directly derived from the calibration chart.

vii. *Determination of Biological Oxygen Demand (BOD)*

Biological oxygen demand is the amount of oxygen required by the mixed microbial population for the biological decomposition of the organic matter in water under aerobic conditions.

The method consists of filling with sample, an airtight bottle of 250ml and incubating it at 20°C for 5 days. Dissolved oxygen (DO) is measured initially and after incubation, and the BOD is computed from the difference between initial and final dissolved oxygen. Because the initial DO is determined shortly after the dilution is made, all oxygen uptake occurring after this measurement is included in the final BOD measurement. The dissolved oxygen is determined by Winkler method. 2.5ml of the effluent was diluted to 250ml using dilution water in two separate volumetric flasks. One bottle was kept a BOD incubator and incubates at 20°C for 5 day for the determination of final DO content. To the other BOD bottle, 2ml of manganese sulphate was added followed by 2ml of alkaline iodine reagent immediately after collection. The tip of the pipette should be below the liquid level while adding these reagents and close with the stopper immediately. The contents in the bottle were mixed well and the precipitate was allowed to settle. Then add 2ml concentrated sulphuric acid. Mix well until the precipitate goes into solution. Transfer 50ml sample into a clean conical flask and titrate against standard sodium thiosulphate until a very pale straw yellow color remains. 1ml of starch indicator was added which develops a blue color. Disappearance of blue color is the end point. Determine the DO in the sample on the initial day and 5th day. BOD_5 was calculated as follows:

$$BOD_5 = \frac{D1-D2}{P}$$

Where, D1-DO of diluted sample immediately after preparation (mg/L); D2-DO of diluted sample after 5 day incubation (mg/L) and P, decimal volumetric fraction of sample used.

viii. *Determination of Chemical Oxygen Demand (COD)*

Chemical oxygen demand (COD) represents the amount of oxygen required to oxidize all the organic and inorganic pollutants present in the effluent. The organic matter present in the effluent was oxidized by potassium dichromate in the presence of sulphuric acid

to carbon dioxide and water. The excess potassium dichromate remaining after the reaction is titrated with ferrous ammonium sulphate. The volume of dichromate consumed gives the oxygen required for the oxidation of organic matter. Known quantity of wastewater sample was pipetted out into a 500ml standard flask. (The sample, if highly polluted, can be suitably diluted to a known quantity and an aliquot sample is taken). 20ml of standard dichromate solution was pipetted out into the same distilling flask. With gentle mixing 20ml of concentrated sulphuric acid was added carefully. 0.2 g silver sulphate was added followed by 20ml water. Put one or two glass beads, and then attach the reflux condenser. The solution was refluxed for 2hrs over a heating mantle or Bunsen flame. After cooling, the inside of the condenser was rinsed with small amount of distilled water and collect the washings into the distilling flask.

The excess dichromate present in the solution was titrated against standard ferrous ammonium sulphate, using 0.5ml of ferro in indicator. The end point is blue to red. A blank was also performed with distilled water instead of sample of wastewater and the same procedure was followed. The COD of the sample was calculated as follows:

$$\text{COD}=\frac{(V1-V2)\times N\times 1000}{X}$$

Where, V1-the volume of titrate value with blank; V2-the volume of titrate value with sample; N-the normality of ferrous ammonium sulphate and X-the volume of water sample.

d) Optimization of parameters

i. *Response surface methodology (RSM) using Central Composite Design (CCD)*

RSM is a very effective and most popular statistical tool to optimize the variables having equal importance and influence on each other in the ammonia degradation. The effect of significant process parameters such as pH, temperature, microbial load and effluent concentration were tested for their significance on ammonia degradation.

ii. *Statistical analysis and modeling*

The data obtained from RSM on ammonia degradation are subjected to the analysis of variance (ANOVA). The experimental results of RSM are fitted through the response surface regression procedure using the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

in which *Y*, the predicted response; X_iX_j, independent variables; β_0, offset term; β_i, *i*th linear coefficient; β_{ii}, i^{th} quadratic coefficient and β_{ij}, the ij^{th} interaction coefficient. However, in this study, the independent variables are coded as X_1, X_2 and X_3.

The chosen independent variable (X_i) levels are coded as x_i according to the equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1,2,3,\ldots\ldots,k,$$

Where x_i, the dimensionless value of an independent variable; X_i, real value of an independent variable; X_0, real value of the independent variable at the center point and ΔX_i, the step change value.

Thus, the second order polynomial model equation for four factors takes the following form:

$$Y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \beta_4x_4 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{33}x_3^2 + \beta_{44}x_4^2 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{14}x_1x_4 + \beta_{23}x_2x_3 + \beta_{24}x_2x_4 + \beta_{34}x_3x_4$$

The Central-Composite design was adopted in order to optimize the important process parameters namely; pH of the medium, temperature, microbial load and effluent concentration for both mixed and isolated nitrifying culture. A five level design (-2, -1, 0, 1, 2) with seven central points was used for the study. The coded and the actual values of the parameters used in the design are presented in Table 1. The design allow to vary the factors simultaneously and to give the individual, interactive and square effects of the parameters associated with the ammonia degradation through a second order polynomial equation.

The statistical software package, MINITAB version 15.0 was used for the regression analysis of the experimental data, and also to plot the contour and response surface plots. The statistical significance of the model equation and the model terms were evaluated via the Fisher's test. The Fisher's variance ratio, F value = (Sr^2/Se^2), is the ratio of the mean square owing to regression to the mean square owing to the error. It is a measure of the variation in the data about the mean. The significance of each coefficient was determined using Student's *t*-test.

Table 1: The coded and actual values of the variables used in the design

Parameters	Coded values				
	-2	-1	0	+1	+2
pH- (A)	7	7.5	8	8.5	9
Temperature (^{0}C) – (B)	20	25	30	35	40
Microbial load (% v/v) (C)	1	2	3	4	5
Effluent concentration (%)-(D)	20	40	60	80	100

The quality of fit of the second-order polynomial model equation is expressed using the coefficient of determination (R^2) and the adjusted R^2. Coefficient of determination, R^2, is defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit. It is also the proportion of the variability in the response variables, which is accounted for by the regression analysis. When R^2 approaches unity, the better the empirical model fits the actual data. The smaller the value of R^2, the less relevance of the dependent variables in the model has in explaining the behavior variation.

The fitted polynomial equation was then expressed in the form of contour plots and also three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

e) Growth kinetics

In general, the Monod model has been used for representing the microbial fermentation kinetics. The Monod model which includes the substrate concentration limits the applicability of the model for most of the cases. Instead, the logistic model which relates the growth rate with the initial and final cell mass concentrations could be used to predict the growth behavior of many strains.

i. *Logistic model*

The logistic model states that the rate of growth of the cell is proportional to the cell mass concentration present at any time. When the cell mass reaches the stationary phase there is no growth and hence the rate becomes zero. The growth rate thus depends on how far the cell mass concentration is away from the stationary phase.

$$\frac{dX}{dt} = kX(X_S - X)$$

Where, k is a constant defined as the specific growth rate. The above Equation implies that the growth rate increases with an increase in cell mass concentration and is independent of the substrate concentration.

ii. *Substrate Utilization Kinetics*

Substrate consumption depends on the magnitude of three sink terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The assumed kinetic form is a linear combination of these terms (Weiss and Oll is, 1980) and is given by,

$$\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt}$$

This equation is used for substrate utilization kinetics. The yield coefficient of biomass ($Y_{X/S}$) is based on the substrate utilized which is defined as follows:

$$Y_{X/S} = \frac{Cell\ mass\ produced}{Substrate\ consumed} = -\frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S}$$

These equations are rearranged and integrated with the set of initial conditions $X=X_0$ and $S= S_0$ gives,

$$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right]$$

III. Results and Discussion

a) Physical-chemical characteristics of fish processing waste water

Table 2 shows the results of physical-chemical characteristics of fishery waste water. The COD (250 mg/L) and BOD (20 mg/L) values were above the permissible limits and are due to the presence of high inorganic and organic matters (Gonzalez et al, 1996). Effluent has high ammonia concentration; therefore the pH value is high. The ammonia concentration mainly depends upon the blood and slime content in the fishery waste water. The high TSS is due to high water-insoluble fats and lipids present in the wastewater (Palenzuela – Rollon, 1999). These results have been observed from the calibration chart which is discussed previously.

Table 2 : physical-chemical characteristics of fish processing waste water

Parameter	Measured value
pH	7.22
Colour	Dirty brown
Total ammonia	250 mg/L
Total nitrite	17.6 mg/L
Total nitrate	6.25 mg/L
TSS	4000 mg/L
TDS	1100 mg/L
BOD	9003 mg/L
COD	11040 mg/L
DO	41.04 l/L

b) Process parameter optimization using central composite design for ammonia degradation using mixed culture

The design matrix which consists of 31 experimental runs was constructed, in order to arrive at a second order polynomial equation to predict the ammonia degradation system. The design matrix and their corresponding experimental and the predicted values are given in Table 3 after the reference. The experimental results suggest that the maximum values of ammonia degradation were obtained for the runs with the central points. The experimental runs of 4, 12, 15,

27, 29, 30, and 31 produced the highest ammonia degradation (129 mg/l). The results were analyzed using the analysis of variance (ANOVA) and the estimated coefficients are presented in Table 4 after the reference section.

ANOVA results of the data indicate that the model terms, A, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 are significant ($P < 0.05$). The model term B only insignificant. The need for model reduction to improve the model does not arise because all the terms of the model are significant except B. Thus, it is clear that the linear, square and interactive effects of pH, microbial load, and effluent concentration are significant. Temperature is insignificant. The model F-value was 849.31. The high F-value and non-significant lack of fit indicate that the model is a good fit. The P-values for the model (<0.0001) from the analysis also suggested that the obtained experimental data was in good fit with the model. The regression equation coefficients were calculated and the data was fitted to a second order polynomial equation. The ammonia degradation using a mixed culture of *Nitrosomonas* and *Nitrobactor* can be expressed in terms of the following regression equation:

$$Y = 129-6.83A+0.83B-2.75C+12.08D-5.63AB-3.63AC-13.38AD-1.37BC-7.63\ BD+4.88CD-23.06A^2-27.31B^2-10.94C^2-22.69D^2$$

where, A: pH; B: temperature; C: Microbial Load; and D: Effluent Concentration.

The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 0.9987 (a value > 0.1 indicates the fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 99.87% of the variation in response. This is supported by the parity plot between the experimental and predicted ammonia degradation using mixed culture, as illustrated in Figure 2. The 'adjusted R^2' (0.9976) and the 'predicted R^2' (0.9927) are in reasonable agreement, which indicates that the model is good. The 'adequate precision value' of the present model was 83.687, and this also suggests that the model can be used to navigate the design space. The 'adequate precision value' is an index of the signal-to-noise ratio, and values of higher than 4 are essential prerequisites for a model to be a good fit.

Three dimensional surface plots are drawn to determine the optimum values of the four parameters and are shown in Figure 3.1 to 3.6. The three dimensional surface plot shown in Figure 3.1 explains the interactive effect of pH and temperature on the degradation of ammonia. The maximum value of ammonia degradation lies between 7.9 to 8.1 and 29°C to 31°C.

The Figure 3.2 depicts the interaction between pH and microbial load. The trend observed that maximum degradation of ammonia is obtained at the central point of microbial load and pH for it lies between 2.5 (v/v) to 3 (v/v) and 7.9 to 8.1 respectively.

The Figure 3.3 explains the interactive effect of pH and effluent concentration on the degradation of ammonia. The maximum value of ammonia degradation lies between the pH and effluent concentration range of 7.9 to 8.1 and 64 % to 72 % respectively.

Figures 3.4, 3.5 and 3.6 also showed similar trends as that of previous one. The following are the optimum values obatained by solving the second degree polynomial equation:

pH: 8; temperature: 30° C; microbial load: 3 %(v/v) and effluent concentration: 60%. These optimum values were maintained for all further studies.

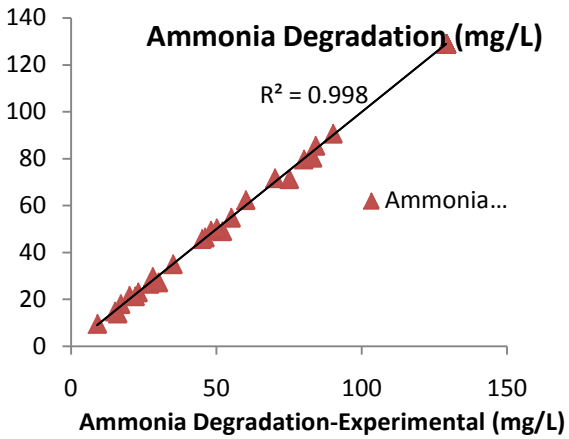
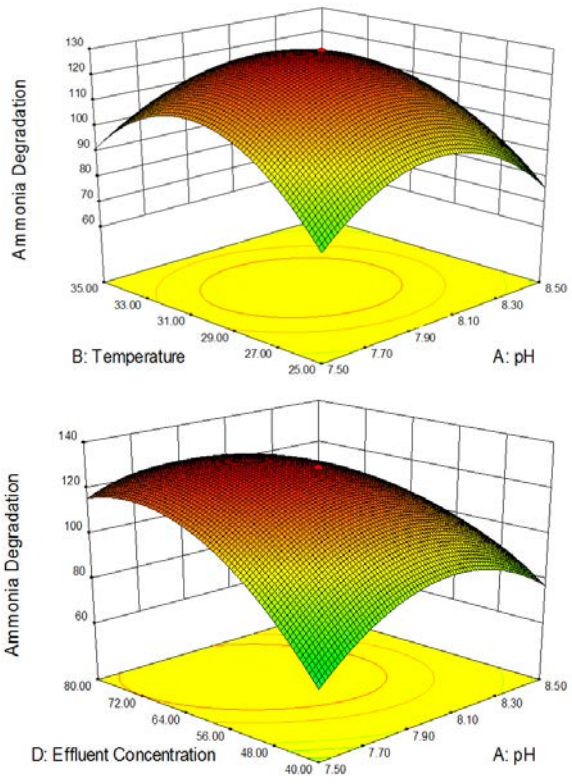


Figure 2 : Parity plot between the experimental and predicted values of Ammonia degradation using mixed culture by Central-Composite design



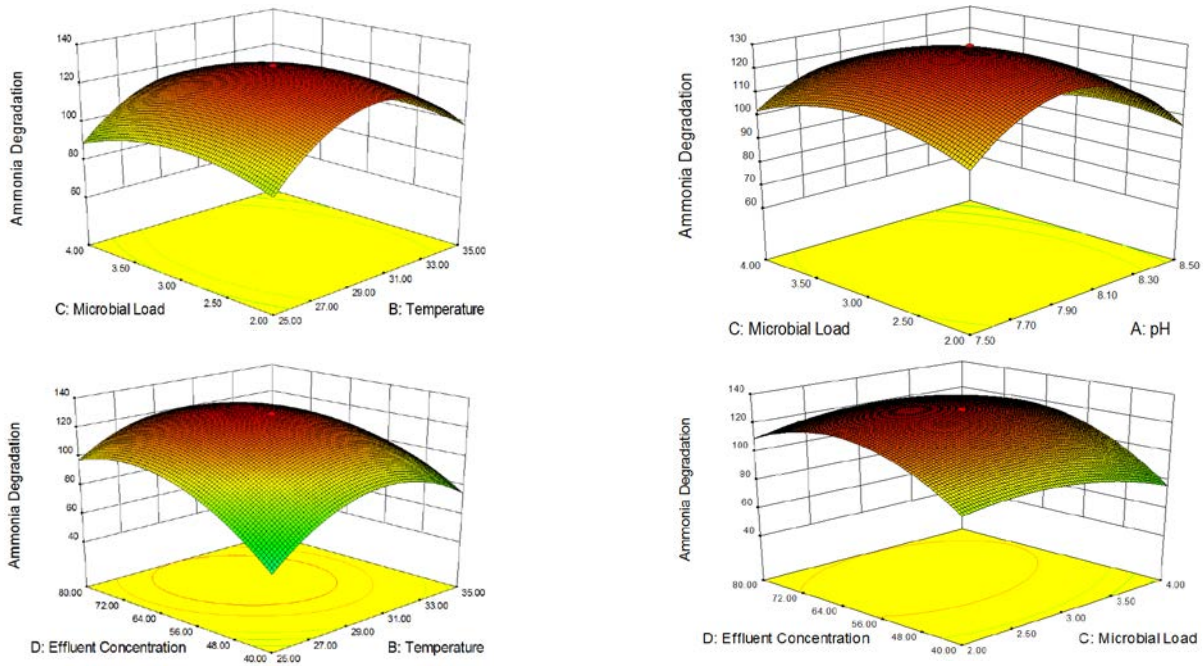


Figure 3.1 to 3.6: Plots showing the interactive effects between significant parameters on ammonia degradation

c) *Batch degradation of waste water in a Rotating Biological Contactor (RBC)*

Degradation of fish processing wastewater was carried out using mixed (*Nitrosomonas* and *Nitrobactor*) culture in a rotating biological contactor under constant operating conditions & optimized parameters. The RBC was operated with an average loading rate of 150mg/L at 15rpm. Ammonia degradation and biomass growth profile are given in Table 5. The result showed that the ammonia concentration decreases continuously with time and reaches a minimum value of 15mg/L on the twenty first day and no further degradation was observed thereafter. An exponential increase in biomass concentration was observed from the sixth day and the stationary phase was attained from the twenty first day. The maximum ammonia degradation (90%) and biomass concentrations (92g/L) were attained and the results were illustrated in Figure 4.

$$\frac{dX}{dt} = kX(X_s - X)$$

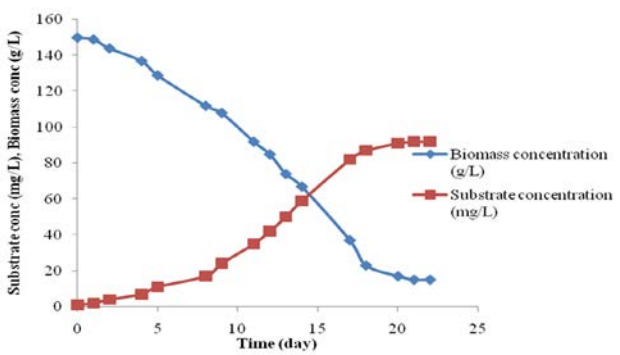


Figure 4 : Time profile of substrate and cell mass concentration in RBC

Table 5 : Time profile of ammonia degradation in RBC

Time(Day)	Ammonia degradation (mg/L)	Biomass concentration (g/L)	% degradation
0	150	1	0
1	149	2	.6
2	144	4	4
4	137	7	8.6
5	129	11	14
8	112	17	25.3
9	108	24	28
11	92	35	38.67
12	85	42	43.33
13	74	50	50.67
14	67	59	55.33
17	37	82	75.33
18	23	87	84.67
20	17	91	88.67
21	15	92	90
22	15	92	90

d) *Growth kinetic models*

i. *Logistic model*

The logistic model states that the rate of growth of the cell is proportional to the cell mass concentration present at any time. When the cell mass reaches the stationary phase there is no growth and hence the rate becomes zero. The growth rate thus depends on how far the cell mass concentration is away from the stationary phase.

Where, k is a constant defined as the specific growth rate. This equation implies that the growth rate increases with an increase in cell mass concentration and is independent of the substrate concentration. Logistic model obtained by rearranging this equation governs the growth of the cell in reality by a hyperbolic relationship and the equation is given by,

$$\frac{dX}{dt} = k\left[1 - \frac{X}{X_S}\right]X$$

Let $\beta = 1/X_S$, then this equation becomes

$$\frac{dX}{dt} = k[1 - \beta X]X$$

Integrating the above equation from $X = X_0$ at t=0 to X=X at t=t gives a sigmoidal variation of X(t) that may empirically represent both the exponential and stationary phase and is given by,

$$X(t) = \frac{X_0 e^{kt}}{1 - \beta X_0(1 - e^{kt})}$$

This Equation is used to represent cell growth for the batch kinetics, where X_0 is the initial cell mass concentration, g/L and X is the cell mass concentration at any time, g/L.

ii. *Substrate Utilization Kinetics*

Substrate consumption depends on the magnitude of three sink terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The kinetic model is given by

$$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right]$$

The time profile of biomass growth for mixed nitrifying cultures is represented in Table 6. The cell growth kinetic model given in Table 7 is used to represent the batch kinetics. The experimental data for biomass growth was fitted in to the above equations for mixed cultures. Using MATLAB the k and β values of the mixed culture were found to be 0.5284 day^{-1} and 0.0114 L/g with a correlation coefficient of 0.9974 as shown in Table 8.

Table 6 : Time profile of substrate utilization and cell mass growth

Time(Day)	Cell mass concentration (g/L)	Substrate concentration (mg/L)
0	1	150
1	2	148
2	4	146
3	7	142
4	11	138
5	13	133
6	20	125
7	28	113
8	35	99
9	48	82
10	60	73
11	73	58
12	79	49
13	82	41
14	84	36
15	86	30
16	87	25
17	88	23
18	88	21
19	88	21

Table 7 : Batch kinetic models used for cell growth and substrate utilization

Model	Equations
Logistic	$X(t) = \frac{X_0 e^{kt}}{1 - \beta X_0(1 - e^{kt})}$
Substrate utilization	$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right]$

Table 8 : Values of kinetic parameter and regression coefficient (R^2)

Model	Parameter estimation	Regression coefficient (R^2)
Logistic	k=0.5284 day^{-1} β= 0.0114 L/g	0.9974
Substrate utilization	$Y_{X/S}$=0.7162	

e) Model evaluation

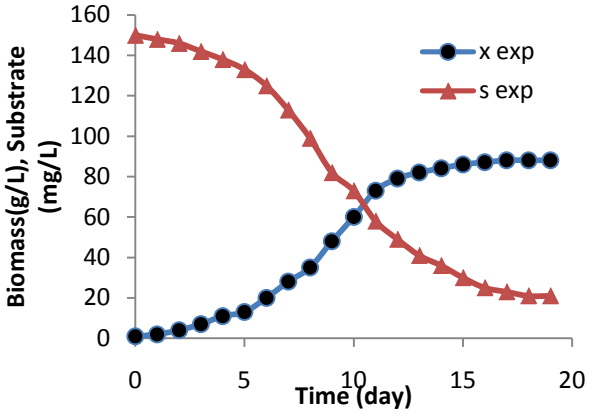
Various growth and substrate utilization kinetic models were analyzed using the experimental data for mixed nitrifying cultures. The models which showed maximum fit are represented the previous table 8 along with the estimated parameters. The other models and the estimated parameters obtained for those models during the biodegradation of ammonia by mixed nitrifying cultures were given in Table 9.

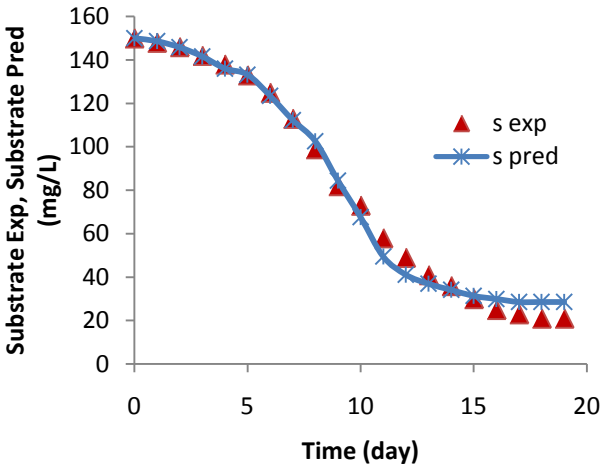
The optimized model parameter values are evaluated using MATLAB coding. With the estimated parameter values obtained using the respective models, the profiles of cell mass growth and substrate utilization are simulated for the entire time period of fermentation. Comparisons are made between the experimental data and the theoretical predictions as shown in Figure 5.1 to 5.6 for mixed nitrifying culture. The regression coefficient values (R^2) were estimated for the substrate concentration and are presented in Table 9 mixed cultures. From the regression coefficient values, it is clear that the correlation between the experimental data and the theoretical predictions are good. Thus, the Logistic model represents ammonia degradation very well for the mixed culture of *nitrobactor* and *nitrosomonas.*

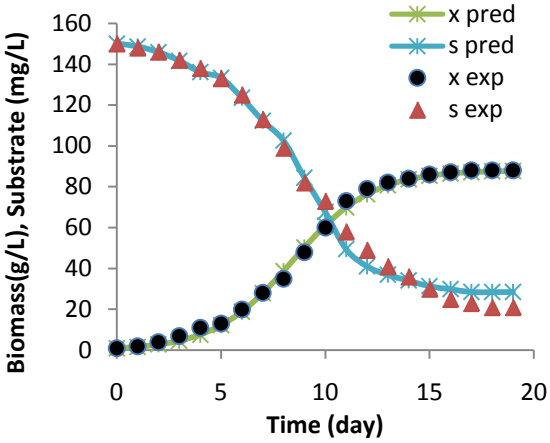
Table 9 : Growth kinetic parameter and regression for different models

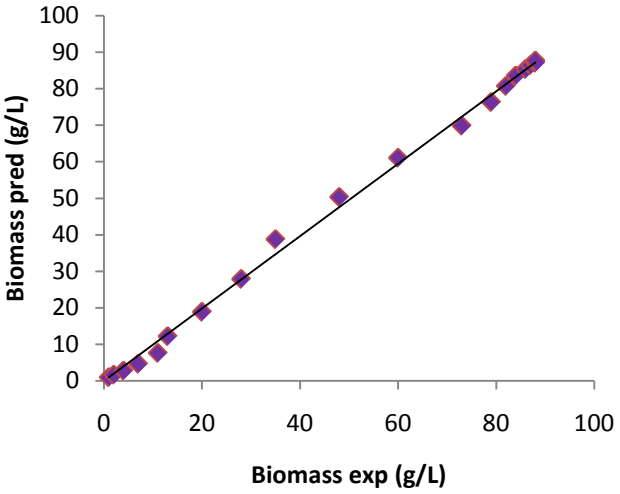
Model	Parameter estimation	Regression coefficient (R^2)
Monod model	μ_m = 0.5875 k_s = 124.1	0.4290
Herbert model	μ_m =0.5875 k_s =52.1 m = 0.2768	0.5195
Shehata & Marr model	μ_m =0.5875 μ_1 = - 80.54 k_1 =10.29 k_2 = 10.57	0.4618

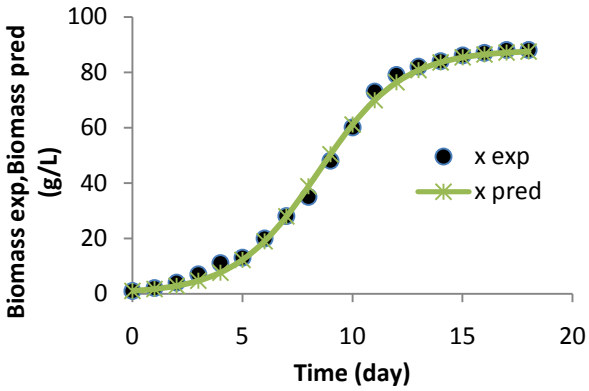
Tessier model	μ_m =0.5875 T = 0.8098	- 3.8740
Haldane model	μ_m = 0.5875 k_i = - 0.0007875 k_s = 132.8	0.4309











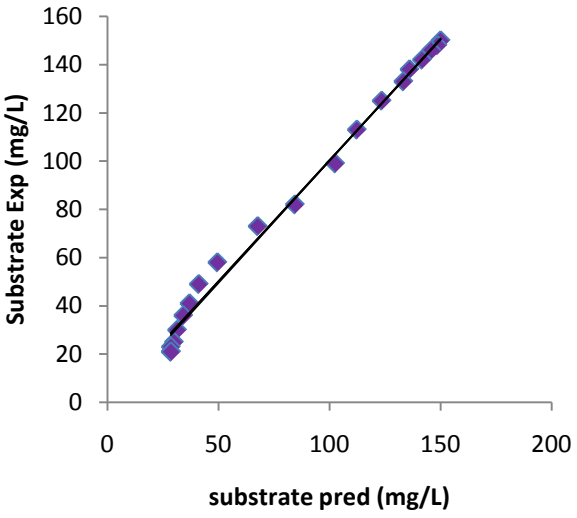


Figure 5.1 to 5.6 : Comparison between experimental data (points) and theoretical predictions (lines) of ammonia degradation

IV. Conclusion

The present work demonstrated the nitrification of fish processing waste water using a mixed culture of *Nitrosomonas* and *Nitrobactor*. The process parameters namely, the pH, temperature, microbial load and effluent concentration were optimized using Central Composite Design of RSM. The optimum conditions were found to be pH:8; temperature: 30°C; microbial load:3%(v/v) and effluent concentration:60% for mixed culture. The physical-chemical characteristics of fish processing wastewater were analyzed and it showed high values of BOD (9003 mg/L) and COD (11040 mg/L). The batch kinetic data obtained for mixed cultures were fitted into various models, namely, *Logistic, Monod, Herbert, Shehata & Marr, Tessier and Haldane* models. Among these models the Logistic model fits the data best with a correlation coefficient of 0.9974 and 0.9994 for mixed cultures of *nitrobactor* and *nitrosomonas*. Maximum ammonia degradation was observed for mixed culture, where Rotating Biological Contactor was operated on a batch basis with an average organic loading rate of 150mg/L of ammonia at 20 rpm and the experimental data were collected. The result showed that, a maximum ammonia degradation of 135mg/L was observed on twenty second day. The future research has been suggested for treating the fish processing waste water using the isolated nitrifying bacteria from the soil.

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Table 3 : Five level Central-Composite design matrix for the optimization of significant process parameters in ammonia degradation using mixed culture of *Nitrobactor* and *Nitrosomonas*

Run No.	pH	Temperature (°C)	Microbial Load % (v/v)	Effluent Concentration (%)	Ammonia degradation mg/L	
					Experimental	Predicted
1	1	-1	1	1	52	49.167
2	1	-1	1	-1	27	26.750
3	-1	1	1	-1	35	35.083
4	0	0	0	0	129	129.000
5	2	0	0	0	23	23.083
6	1	1	1	1	20	21.583
7	-1	-1	-1	-1	15	14.917
8	0	-2	0	0	17	18.083
9	-1	-1	1	-1	9	9.667
10	1	1	-1	-1	55	54.917
11	0	2	0	0	22	21.417
12	0	0	0	0	129	129.000
13	0	0	0	2	60	62.417
14	0	0	-2	0	90	90.750
15	0	0	0	0	129	129.000
16	-1	-1	1	1	84	85.583
17	1	1	1	-1	28	29.667
18	1	-1	-1	-1	46	46.500
19	-1	-1	-1	1	75	71.333
20	1	1	-1	1	30	27.333
21	0	0	2	0	80	79.750
22	-1	1	1	1	83	80.500
23	-1	1	-1	-1	45	45.833
24	1	-1	-1	1	48	49.417
25	-2	0	0	0	50	50.417
26	0	0	0	-2	16	14.083
27	0	0	0	0	129	129.000
28	-1	1	-1	1	70	71.750
29	0	0	0	0	129	129.000
30	0	0	0	0	129	129.000
31	0	0	0	0	129	129.000

Table 4 : Results of the ANOVA of the process parameter optimization data for ammonia degradation using mixed culture by Central-Composite design of experiments

Source	Coefficient	Sum of Squares	Degrees of Freedom (DF)	Mean Square	F Value	P-Value Prob > F
Model	129.00	48353.80	14	3453.84	849.31	0.0001
A-pH	-6.83	1120.67	1	1120.67	275.57	0.0001
B-Temperature	0.83	16.67	1	16.67	4.10	0.0611
C-Microbial load	-2.75	181.50	1	181.50	44.63	0.0001
D-Effluent concentration	12.08	3504.17	1	3504.17	861.68	0.0001
AB	-5.63	506.25	1	506.25	124.49	0.0001
AC	-3.63	210.25	1	210.25	51.70	0.0001
AD	-13.38	2862.25	1	2862.25	703.83	0.0001
BC	-1.38	30.25	1	30.25	7.44	0.0156
BD	-7.63	930.25	1	930.25	228.75	0.0001
CD	4.87	380.25	1	380.25	93.50	0.0001
A^2	-23.06	14588.68	1	14588.68	3587.38	0.0001
B^2	-27.31	20460.96	1	20460.96	5031.38	0.0001
C^2	-10.94	3281.25	1	3281.25	806.86	0.0001
D^2	-22.69	14118.11	1	14118.11	3471.67	0.0001
Residual		61.00	15	4.07		
Lack of fit		61.00	10	6.10		
Pure error		0.000	5	0.000		
Total		48414.80	29			