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Application of Synergetic Microorganisms to Remove Ammonia-Nitrogen and Chemical Oxygen Demand from the Effluent

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Synergetic effect is the phenomenon of living together of two or more microorganisms for the production of growth stimulatory substance. The over all objective of this study is to remove the ammonia-nitrogen and Chemical Oxygen Demand by using synergetic effect of microorganisms in the activated sludge which is the process used for the water treatment. Apart from Ammonia – Nitrogen there are wide varieties of organic compounds in surface and ground from the chemical, Pharmaceutical, synthetic polymer and fossil fuel refining industries. Effluent was processed with environment factors such as temperature and aeration. The initial and final amount of Ammonia- nitrogen and Chemical Oxygen Demand were estimated from 1-11 days. In the initial period the microbial load was determined by serial dilution method and the microorganisms were counted. After 11 days, the microbial load of the first, second and third tanks were increased as too numerous to count but in the fourth tank which kept as control contain *Bacillus* sp. From this, the microbial communities and the normal control were effectively compared. After 11 days, the samples from all the tanks were used for total protein estimation. The mixed culture of microorganisms can produce larger amount of proteins for the removal of Ammonia- Nitrogen and Chemical Oxygen Demand.

Key words: Synergetic, Ammonia- nitrogen, COD, activated sludge.

The biological treatment of waste water is the degradation of organic waste by the action of micro organisms. The degradation of organic matters results in their structural change. The degree of alterations in the organic matter determines whether biotransformation or mineralization has occurred. Biotransformation refers to the simplification of organic compounds to simpler compounds. Mineralization is the complete breakdown of organic molecules into cellular mass, carbon dioxide, water and inert residuals.

The biological treatment of any hazardous organic materials can be achieved by the establishment of proper microbial communities. There are number of factors involved in the biological treatment process. The dissolved oxygen, pH, sludge age, aeration period, and carbon/nitrogen relation are important parameters that must be controlled to obtain good results for pollutant reduction (Peavy *et al*., 2001). Several wastewaters require treatment to remove excess phosphorus and nitrogen, such as in the case of

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pharma effluents, which are highly polluted with ammonia nitrogen and high levels of chemical oxygen demand. These ammonia nitrogen and other toxic chemicals can cause disagreeable odours to the water, chloramine formation, toxicity, etc. Recently there has been increasing preoccupation about reducing these toxic chemicals in the effluents by low cost means (Ferraz *et al.,* 1994).

The most common processes used for the waste water treatment is the activated sludge process. Activated sludge process has gained its significance in the biological waste water treatment. The industrial effluent contains increased ammonia nitrogen level and high level of chemical oxygen demand can be treated effectively by means of activated sludge process (Culp *et al*., 1998). The removal of ammonia-nitrogen plays an important role because ammonia is the principal form of toxic ammonia. It has been reported that it is toxic to fresh water organisms at concentrations ranging from 0.53 to 22.8 mg/L. Toxic levels are both pH and temperature dependent. Plants are more tolerant of ammonia than animals, and invertebrates are more tolerant than fish. Toxic concentrations of ammonia in humans may cause loss of equilibrium, convulsions, coma and death.

Freshwater organisms are most at risk from exposure to ammonia. The effective nitrifications by the heterotrophic bacteria were found in activated sludge, where most of the microorganisms were heterotrophic. The result of ammonium conversion to gaseous nitrogen carried out by the same groups of bacteria comes from the fact that aerobic denitrifiers can also be heterotrophic nitrifiers (Zhao *et al*., 1997*)*. The several bacteria such as *Pseudomonas, Micrococcus, Achromobacter* and *Bacillus* promote denitrification during the agitation process (Lee *et al*., 1997). Simultaneous nitrification and denitrification of ammonia in the waste water was carried out by activated sludge process in the sequential batch reactor. The nitrifying bacteria such as *Nitrosomonas, Nitrobacter* and denitrifying bacteria such as *Pseudomonas* & *Bacillus* sp were simultaneously involved in the process (Munch *et al.,* 1996).

The conversion of toxic ammonia nitrogen into gaseous nitrogen is a two step biological oxidation process. It can be carried out in a single process by means of synergism.

$$NH_3 \xrightarrow[\text{Oxygenase}]{\text{Nitrosomonas}} NO_3 \xrightarrow[\text{Reductase}]{\text{Bacillus}} NO_2$$

MATERIAL AND METHODS

Sample Collection

The pharma effluent was collected from Matrix Laboratories, Hyderabad. The activated sludge process was carried out in plastic tanks by using it as a single stage reactor at the volume of 20 litres capacity. The four tanks were constantly aerated by the compressed air forced through a submerged diffuser. The first tank was added with 15 litre water + 3 litre cow dung sludge + Jaggery + Activated carbon+ Sodium carbonate + one litre effluent. The pH was neutralized by using Phosporous acid. The second tank was added with 15 litre water + 3 litre cow dung sludge + Activated carbon + Jaggery + one liter effluent. The pH was neutralized by using Phosporous acid The third tank was added with 15 litre water + 3 litre cow dung sludge + mixed mass culture of micro organisms + one litre effluent. The fourth tank was kept as control by adding 15 litre water + 3 liter cow dung sludge + one liter effluent. All the four tanks were aerated constantly. The pH, Ammonia-nitrogen and Chemical Oxygen Demand was measured daily by titrimetric method and open reflux method respectively.

BioChemical Tests

The isolated bacterial species were identified by using Indole test, Methyl Red, Voges – Proskauer, Citrate utilization, urease, triple Sugar iron test, Nitrate reduction test, Catalase and oxidase test.

Estimation of Total protein

An aliquot of diluted sample of total proteins was made up to one ml with distilled water. Water was used as blank and various concentration of BSA were used as standard. All the tubes were treated similarly. Alkaline copper reagent (4.5 ml) was added to all tubes, mixed well and incubated at room temperature for 10 minutes. Then 0.5 ml of Folin – Ciocalteu was added and incubated at room temperature till the blue colour developed. Then the colour was measured at 640 nm in a spectrophotometer.

Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis -SDS PAGE

Total proteins were subjected to SDS-PAGE under 6% Stacking Gel and 10% Separating Gel. The total protein yield for the scaled-up preparation was 10 mg and was referred to as whole cell extract. The separating gel was prepared by adding 10 ml of (30%) acrylamide, 7.5 ml of lower Tris Hcl (pH 8.8), 12.4 ml of distilled water, and 75µl of Ammonium persulphate and 25µl of TEMED. The solution was mixed well and poured into a glass sandwich. A fine mixture of 0.1% of SDS was layered on the gel to make an even surface. After the completion of polymerization, the SDS solution was poured off, 6% of stacking gel was prepared with 2ml of Acrylamide (30%), 3ml of Tris-HCl (pH 6.8), 4.9ml of distilled water, 50µl of APS and 15 µl of TEMED. It was mixed and poured over the separating gel. A teflon comb (1.5mm thickness) was inserted to form wells. After polymerization, the teflon comb was removed and the wells were rinsed with double distilled water. After removing the basal strip, the glass plate with the polymerized gel was fixed to the electrophoresis apparatus and the tank was filled with Running Buffer (pH 8.3). Samples of 75 µl were taken in sterile microcentrifuge tubes and mixed with 25µl of sample loading buffer. The samples were loaded into the well after keeping them in a boiling water bath for two minutes. Electrophoresis was carried out for about six hours at constant voltage of 60V in stacking and 12V in separating gel. The gel was stained by Coomasive Brilliant Blue (CBB) staining method and photographed.

Estimation of Ammonia Nitrogen by Titrimetric Method

500 ml water and 20 ml borate buffer was added to a distillation flask and adjust pH to 9.5 with 6N Sodium hydroxide solution. A few glass beads were added and the mixture was used to steam out the distillation apparatus until distillate shows no traces of ammonia.

Sample preparation

500 ml dechlorinated sample or a portion diluted to 500 ml with water. When NH_3-N concentration is less than 100 μg/L, sample volume of less than 1000 ml were used. Residual chlorine was removed by adding, dechlorinating agent. If necessary, neutralize to approximately pH 7.Then 25 ml borate buffer solutions were added and adjust to pH 9.5 with 6N sodium hydroxide solution.

Distillation

Distillation was carried out at a rate of 6 to 10 ml/min with the tip of delivery tube below the surface of acid receiving solution. Distillate was collected in a 500 ml Erlenmeyer flask containing 50 ml indicating boric acid solution.

Titration

Ammonia was titrated in distillate with standard 0.02N Sulphuric acid titrant until indicator turns pale lavender.

A blank was carried through out all steps.

Calculation:

$$\text{mg } NH_3\text{-N/L} = \frac{(A\text{-}B) \times 280}{\text{Volume of sample}}$$

A = Volume of Sulphuric acid titrated for sample (ml)
B = Volume of Sulphuric acid titrated for blank (ml).

Estimation of Chemical Oxygen Demand (Standard Methods Committee, 1985)

50 ml sample was taken in a 500 ml refluxing flask. 1 g of Mercury (I) sulfate and was added and 50 ml of sulphuric acid reagent was added slowly and mixed to dissolve Mercury (I) sulfate crystals. It was cooled during mixing to avoid possible loss of volatile materials. 25 ml of 0.0417N Potassium dichromate was added and mixed. The flask was attached to the condenser and the remaining 70 ml sulphuric acid reagent was through open end of the condenser. The open end was covered using a small beaker to prevent the entering of foreign materials into the refluxing mixture and reflux the mixture for 2 hour. The condenser was cooled and washed down with distilled water. The excess Potassium dichromate titrated with Ferrous ammonium sulphate titrant. 2 to 3 drops of ferroin indicator were added before titration. The end point of the titration was noted as sharp color change from blue-green to reddish brown.

Calculation:

$$\text{COD as mg } O_2/L = = \frac{(A - B) \times N \times 8000}{\text{Volume of sample}}$$

A = Volume of Ferrous ammonium sulphate titrant used for sample (ml)
B = Volume of Ferrous ammonium sulphate titrant used for blank (ml)
N = Normality of Ferrous ammonium sulphate titrant
8000 = Milliequivalent weight of oxygen X 1000 mL / litre

RESULTS AND DISCUSSION

In the present study, effluent were collected and treated by using activated sludge process for diminution of Ammonia-nitrogen and Chemical Oxygen Demand by using synergetic effect of microorganisms.

The activated sludge process simply involved the bringing together of wastewater and mixed culture of microorganisms under aerobic condition. The system usually includes a secondary treatment given to the settled sewage, and requires an environment in which active microorganisms were maintained in intimate contact with wastewater in the presence of sufficient oxygen. Air bubbles were formed by compressed air forced through a submerged diffuser or by mechanical aeration where turbulent mixing entrains air in the liquid. The significant activity of activated sludge microorganisms were the rate of substrate removal. The liquid suspension of microorganisms in an aeration basin was generally referred to as mixed liquor, and the biological growth were called mixed liquor suspended solids. These masses of microorganisms were observed to be very active in removing soluble organic matter from solution. Aeration control was usually performed by using feedback control based on oxygen and/or ammonia measurement in the last aerobic reactor (Lindberg and Carlsson, 1996).

The isolated Bacterial species were identified by utilizing various sugars and other organic product with enzyme produced by them. This biochemical test reactions were carried out by media containing specific substrate and their utilization were identified (Table 1).

The experiment was performed in four plastic tanks which were processed with same environmental condition such as temperature and aeration.

The micro organisms present in the first tanks were determined by serial dilution method. In the first tank microbial load were $156x10^{-6}$ / ml. The organisms present in the first tank were found to be *Escherichia coli, Pseudomonas, Klebsiella, Nitrosomonas* and *Citrobacter*.

In the second tank, the microbial load was counted as $166x10^{-6}$ /ml. The organisms present in the second tank were found to be *Escherichia coli, Pseudomonas, Nitrosomonas and Citrobacter.*

In the third tank, the microbial load was counted as $178x10^{-6}$ / ml. The organisms present in the third tank were found to be *Escherichia coli, Pseudomonas, Klebsiella, Nitrosomonas*, *Citrobacter* and *Bacillus.*

The fourth tank was kept as control and the microbial load was counted as $120x10^{-6}$ /ml. The organisms present in the fourth tank were found to be *Escherichia coli, Pseudomonas, Klebsiella, Nitrosomonas* and *Citrobacter*

The several bacteria such as *Pseudomonas, Micrococcus, Achromobacter and Bacillus* are capable of denitrifying under fully aerobic conditions (Braber, *et al*., 2001).

After 11 days the final microbial load was determined. The microbial counts were found to be increased in all the tanks when compared with the initial amount. In the first, second and third tanks microbial load were Too numerous to count (TNTC) and same thing in the other second and third tank. In the fourth tank (control) microbial load was $310x10^{-6}$ /ml (Table 2).

The predominant strains were considered and it might be responsible for the reduction ammonia nitrogen and Chemical Oxygen Demand. The first tank consists of *Nitrosomonas* and *Bacillus.* The second tank consists of *Bacillus, Pseudomonas* and *Nitrosomonas.* The third tank contains *Pseudomonas, Nitrosomonas, Bacillus* and *Citrobacter*. The fourth tank (control) contains *Bacillus.* The third tank contains mixed culture micro organisms might be responsible for the effective removal of ammonia-nitrogen and COD. The initial and final microorganisms were identified and noted (Table 3).

The Ammonia and Chemical Oxygen Demand in all the tanks were periodically measured. The Ammonia and Chemical Oxygen

Table 1. Biochemical properties of bacterial strains from the four tanks

Organisms	Gram staining	Indole	MR	VP	Citrate	TSI	Catalase	Oxidase	NRT
Pseudomonas	-	-	-	-	+	A/A	+	+	+
Bacillus	+	-	-	-	-	A/K	+	+	+
Klebsiella pneumoniae	-	-	-	+	+	A/A	+	-	+
Citrobacter	-	-	+	-	+	-	+	-	+
E.coli	-	+	+	-	-	K/K	+	-	+
Nitrosomonas	-	-	-	-	-	-	-	-	+

+ → Positive
- → Negative
A/A → Acid slant; Acid Butt
A/K → Acid slant; Alkaline Butt
K /K→ Alkaline slant/ alkaline butt

Table 2. Microbial Load in Four Tanks (Masurements taken in One day interval)

Days	Microbial Load (CFUX10^{-6}/ml)										
	1	2	3	4	5	6	7	8	9	10	11
Tank 1	178		215		240		290		310		TNTC
Tank 2	166		176		253		268		260		TNTC
Tank 3	156		175		213		273		295		TNTC
Tank 4	120		168		179		220		265		310

Table 3. Microbial Load present in all tanks (Measurements taken in One day interval)

Days	Microbes Present in the Tanks					
	1	3	5	7	9	11
Tank 1	*E.coli, Pseudomonas, Klebsiella, Nitrosomonas,* Citrobacter	*Pseudomonas, Klebsiella, Nitrosomonas,* Citrobacter	*Pseudomonas, Nitrosomonas, Bacillus,*	*Pseudomonas, Nitrosomonas, Bacillus.*	*Pseudomonas, Nitrosomonas, Bacillus.*	*Nitrosomonas, Bacillus,.*
Tank 2	*E.coli,* Pseudomonas, *Nitrosomonas, Bacillus,* Citrobacter	*Pseudomonas,,* Nitrosomonas, *Bacillus,* Citrobacter.	*Pseudomonas,* Bacillus, *Nitrosomonas,* Citrobacter.	*Pseudomonas,* Nitrosomonas, *Bacillus.*	*Pseudomonas,* Nitrosomonas, *Bacillus.*	*Pseudomonas,* Nitrosomonas, *Bacillus.*
Tank 3	*E.coli, Pseudomonas, Klebsiella, Nitrosomonas, Bacillus* Citrobacter	*E.coli, Pseudomonas, Klebsiella, Nitrosomonas, Bacillus* Citrobacter	*Bacillus, Nitrosomonas,* Citrobacter, *Pseudomonas.*	*Bacillus, Nitrosomonas,* Citrobacter, *Pseudomonas.*	*Bacillus, Nitrosomonas,* Citrobacter, *Pseudomonas.*	*Bacillus, Nitrosomonas,* Citrobacter, *Pseudomonas*
Tank 4	*E.coli, Pseudomonas, Klebsiella, Nitrosomonas, Bacillus*	*E.coli, Pseudomonas, Klebsiella, Nitrosomonas, Bacillus*	*E.coli, Pseudomonas, Nitrosomonas, Bacillus.*	*E.coli, Nitrosomonas, Bacillus*	*Bacillus.*	*Bacillus.*

Table 4. Estimation of Initial and Final amount of Ammonia - Nitrogen

Days	Ammonia - Nitrogen (mg/L)										
	1	2	3	4	5	6	7	8	9	10	11
TANK 1	81.2	78.4	70	70	70	64.4	56	56	51	47	43
TANK 2	84	75.6	81.2	92.4	89.6	75.6	74	72.6	68.3	62.4	56
TANK 3	94	72.8	84	70	70	53.2	42	25.2	5.2	4	NIL
TANK 4	84	75.6	93	72	66	64.2	54	53	55.2	51	33

Table 5. Estimation of Initial and Final Chemical Oxygen Demand (COD)

Days	(COD) (mg/L)										
	1	2	3	4	5	6	7	8	9	10	11
TANK 1	1600	760	640	520	400	360	150	64	NIL	NIL	NIL
TANK 2	1400	700	660	540	430	340	180	82	NIL	NIL	NIL
TANK 3	3800	3080	2640	1400	1280	800	800	600	360	84	NIL
TANK 4	2680	2360	2618	1240	680	540	360	180	100	76	49

Table 6. Estimation of total protein

Samples	Amount of Protein µg/mL
TANK 1	86
TANK 2	75
TANK 3	104
TANK 4	45

Demand were decreased in all the tanks but their efficiency varies. In the first tank, ammonia was reduced from 81.2 mg/L to 43 mg/L. In the second tank, ammonia was reduced from 84 mg/L to 56 mg/L. In the third tank, ammonia was reduced from 94 mg/L to 0 mg/L. In the fourth tank (control), ammonia was reduced from 84 mg/L to 33 mg/L. The Chemical Oxygen Demand in the first, second and third tanks were fully reduced, but in the fourth tank (control) Chemical Oxygen Demand was reduced from 2680 mg/L to 49 mg/L. The removal of Ammonia-nitrogen and Chemical Oxygen Demand was found to be effective in the third tank added with the mixed culture of micro organisms (Table 4 & 5, Figure 1 & 2). The use of the sequencing batch reactor must be adjusted to promote carbon consumption and ammonia, nitrite, nitrate and phosphate reduction. Some authors have obtained very good results such as 93% of COD and nitrogen reduction together with 95% of phosphorous reduction (Bortone *et al*., 1996).

After 11 days the samples from all the tanks were used for total protein estimation. The amounts of total protein were noted (Table 6).

In SDS PAGE enzyme profile were determined by the molecular weight of the protein. In lane 3 which contains sample from tank 3 shows enormous amount of protein, 36 bands were seen from 66 K Da. to 15 K Da. In other lanes shows less number of bands when compared with lane 3 indicates mixed culture of micro organisms can produce large amount of proteins for the removal of Ammonia-nitrogen and Chemical Oxygen Demand.

The presence of toxic chemical substances and hazardous waste materials in drinking water pose a large spectrum of human health risks to the general population. They range from simple ailments such as short-term skin rashes, nose and eye irritations, gastrointestinal distress and numbness in fingers, toes and to a variety of serious acute to chronic disease (Olsson and Newell, 1999). Many persistent organic pollutants (POPs) found in drinking water (that do not breakdown in the environment for weeks, months and years), such as aromatic and halogenated hydrocarbons causes developmental and nervous system disorders, reproductive

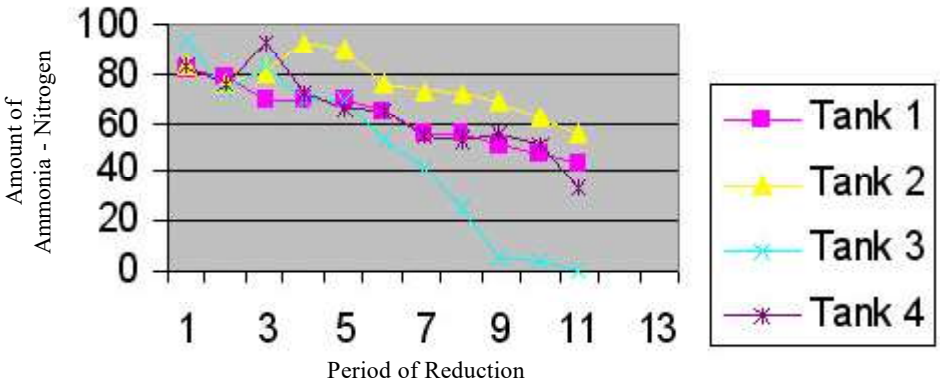

Fig. 1. Reduction of Ammonia -Nitrogen

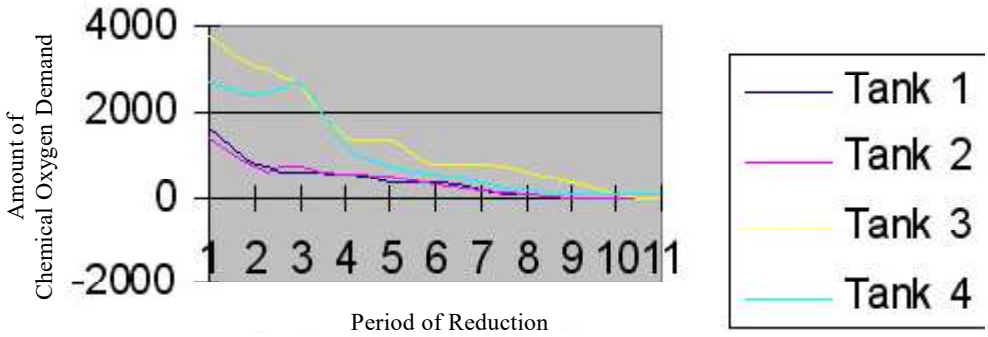

Fig. 2. Reduction of Chemical Oxygen Demand

difficulties, hepatomagaly, kidney problems, cardiovascular disorders, increased risks of childhood and adult cancers and potential genetic damages to future generations.

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