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# Bioremediation of Ammonia Using Ammonia Oxidizing Bacteria Isolated from Sewage

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**Abstract** Urbanization in the world increased the pollution levels in the environment making it for worsen life. General awareness about pollution is increasing today. To control pollution levels, biological methods are eco-safe and economical. In this study a wild strain of *Bacillus species* was isolated and optimized for bioremediation studies of ammonia in flask cultures. Strategy was tried to develop for ammonia bioremediation in a single stage aerobic nitrification and denitrification. The results reveal that the optimum pH for the isolated *Bacillus species* was 7 and optimum temperature was 30°C. At optimum pH and temperature this organism was able to remove 99.2% of ammonia in flask cultures supplemented with 5g/L of ammonium sulphate. The biomass obtained after bioremediation studies was 0.3g/L which is very low. The study on ammonia toxicity revealed that toxicity was due to pH and osmolarity.

***Keywords:*** *ammonia, sewage water, Bacillus species, growth parameters, acclimatization, bioremediation*

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## 1. Introduction

Growth of the country depends on industrialization and rapid evolution of new technologies. This brings many consequences related to environmental disturbances and imbalance in ecosystems. Industries use most of the water resources and out of all only 30% of the remaining water is let out as effluents. These effluents are highly polluted with many toxic organic and inorganic chemical compounds. Industries like chrome plating, fuel refining, coke plants, fertilizer industries etc, produce ammonia rich effluents. Ammonia when present in water exists in two forms ammonium ion ($NH_4^+$) and free ammonia ($NH_3$) depending on the pH of water (Princic. A*et al*., 1998). At higher pH ammonia is toxic to aquatic organisms and also for terrestrial organisms. These include skin irritation, mucus irritation, respiratory distress, kidney and liver failure and even death when exposed to very high concentrations. Pollution of ammonia concentrations in effluents can be minimized by strict implementation of MINAS (minimum standard limits) and CPCB discharge limits. Though many technologies are available for the removal of ammonia from industrial effluents but most of them are expensive and some are facing operational difficulties. Application of air stripping leads to accumulation of carbonate and maintenance of temperature is required. Biological methods are inexpensive and easy to maintain. Generally autotrophic removal of ammonia using traditional nitrification and denitrification requires more time and is a two stage process (Khin and Annachhatre, 2004). Microbes like chemolitho-autotrophic bacteria converts ammonia into nitrite and nitrates. These nitrates are again converts to nitrogen gas by denitrification process. More over autotrophic nitrifiers cannot tolerate higher concentrations of ammonium and organic loads (Kim et al., 2006, Joo et al., 2005) and their applications is limited in treating high strength ammonium waste waters. Generation time is more for these organisms and this is an unfavorable aspect compared to heterotrophic bacteria. Heterotrophic nitrifiying bacteria using external carbon source remediate ammonia from industrial effluents. Although heterotrophic nitrifiers have an immense potential in future bioremediation systems but further research is needed to ensure cost effective measure to meet the demands of full scale operations (Yang. X.P *et al.*, 2011). In 2006 Muller.T *et al* conducted toxicity experiments on *E.coli* and *Bacillus species* but their study was limited to concentrations of ammonia only. The main aim of the experiment was to study the growth parameters of the isolated organism in absence of organic carbon source and identify the degradation efficiency with different parameters for ammonia removal.

## 2. Materials and Methods

### 2.1. Isolation of AOB Species

Domestic sewage sample was collected from wastewater treatment plant located at Appughar, Visakhapatnam. Sample was collected and stored at 4°C.

A basal inorganic medium of Brierley and wood 2001 (pH 7.0) was used for the isolation of ammonia oxidation of bacteria.

## 2.2. Enrichment

100ml of the inorganic liquid media containing 100mg of ammonium was sterilized at 121°C for 15minutes and cooled. This media was inoculated with 2ml of the sewage and incubated at 30°C in shaking incubator at 140rpm. The $7^{th}$ day of incubation optical density at 610nm was recorded and again 2ml of the enriched culture was transferred into fresh media and same conditions were maintained. On the $4^{th}$ day the culture was tested for nitrite formation by using griess-Ilosvay method.

## 2.3. Isolation of Pure Bacterial Culture

Enriched basal inorganic media was solidified (Ford,1998) with of purified agar (Himedia Ltd) and was used for bacterial isolation. Phenol red was added to the media for the detection of nitrification products (Grunditz. C and Dalhammer. G, 2001). 1ml of the liquid enriched medium was added to prepared solid basal media and pour plate technique was followed. The petriplates were inoculated with serially diluted culture and incubated at 30°C for 98hrs. The isolated colonies were cultured in plates repeatedly by subjecting to streak plate method and transferred to agar slants and preserved with paraffin wax.

## 2.4. Identification Assays

Bacteria were subjected to Gram's staining. Further biochemical tests like Vogues Proskeur test, Mannitol test and glucose fermentation test, Catalase test, citrate test, starch hydrolysis test, gelatinase test were performed. Motility test was also performed by semisolid dip method.

## 2.5. Measurement of Bacterial Growth

1.5L of the basal inorganic media 2g/L ammonium sulphate concentration was prepared and pH was adjusted to 7. A series of 15conical flasks of 250ml each were setup and 100ml was distributed in each flask and autoclaved for 15minutes at 121°C. Media was cooled to room temperature and inoculated with isolated identified bacterial culture and incubated at 30°C in shaking incubator at 140rpm. All experiments were performed in duplicate. OD at 610nm was measured for each set at an interval of 24hrs.

## 2.6. Effect of pH

100ml of the basal inorganic medium with different pH levels ranging from 6, 6.5, 7, 7.5 8, 8.5 9, 9.5, 10 were prepared and 10ml of the liquid media was dispensed in test tubes. Media with test tubes were sterilized at 121°C for 15 minutes. Sterilized media was inoculated with fresh cultures of $OD_{610nm}$ 0.15 incubated at 30°C for 24hrs. OD was recorded at 610 nm.

## 2.7. Effect of Temperature

Fresh inorganic media was prepared and dispensed into test tubes of 10ml each and sterilized at 121°C for 15 minutes. 1ml of culture with $OD_{610nm}$ at 0.15 was used to inoculate this media and incubated at different temperatures ranging from 20, 25, 30, 35, 40, 45, 50, 55, 60°C for 24hrs. OD was recorded at 610nm.

## 2.8. Tolerance Limit for higher Concentrations of Ammonia ($NH_4^+$+$NH_3$) at pH 7

Basal inorganic media was incorporated with 0.5g/L, 1g/L, 2g/L to 10g/L concentrations of ammonium sulphate considering industrial concentrations and pH was adjusted to 7. Media was sterilized at 121°C for 15 minutes. Fresh cultures at 0.15 $OD_{610nm}$ was used to inoculate this media and incubated at 30°C for 24hrs. Growth rate was assessed by taking $OD_{610\ nm}$ on colorimeter.

## 2.9. Effect of Ammonia Toxicity at Different pH Levels

100ml of basal inorganic medium with 1g/L to 10g/L of ammonium sulphate concentrations at different pH levels ranging from 6 to 10 with 0.5 variations was prepared and 10ml quantity each was dispensed into test tubes. The media was sterilized at 121°C for 15 minutes and cooled to room temperature. Inoculation was carried out by using fresh cultures at $OD_{610nm}$ 0.15 and incubated at 30°C for 48hrs. Effect of toxicity on bacterial growth was recorded by taking OD at 610nm on colorimeter.

## 2.10. Bioremediation Ability

Fresh inorganic media was prepared with 5g/L concentration of ammonium sulphate and sterilized at 121°C for 15minutes.Inoculation was carried out by using fresh cultures with $OD_{610nm}$ 0.15 and incubated at 30°C for 48hrs.After incubation media was filtered with 0.4 µm membrane filter and this filtrate is used for ammonia, nitrite and nitrate analysis following standard APHA methods 2005.

## 2.11. Biomass Estimation

Fresh inorganic media was prepared with 5g/L of ammonia sulphate and sterilized at 121°C for 15 minutes. Inoculation of the media was carried by using fresh cultures of $OD_{610nm}$ 0.15 and incubated for 48hrs at 30°C. 0.4µm membrane filter was dried at 100°C in hot air oven and weighed on an electronic balance and weight was recorded. This filter is used for filtering the media and the filter was dried on hot air oven at 100°C and weight was measured on electronic balance and dry weight of cell was calculated.

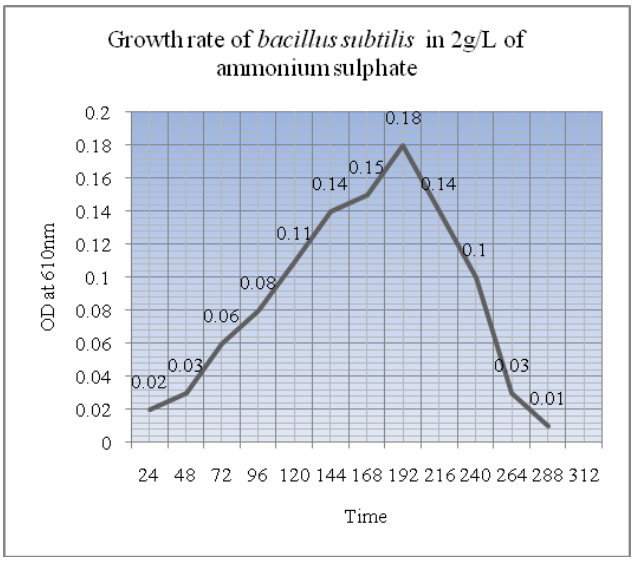
## 2.12. Analytical Methods

All the analytic methods were followed according to standard APHA methods 2005. For ammonia estimation Nesselers method was followed, for nitrite photometric method was followed, for nitrate phenol disulphonic acid (PDA) method was followed. Cell biomass was calculated by using dry cell weight estimation. Cell growth was estimated by taking optical density at 610nm calorimetrically.

# 3. Results and Discussion

## 3.1. Isolation

For isolation of *Bacillus species* from the enriched media pour plate method was followed. After incubation different colonies appeared on the media. Carefully with the sterilized inoculation loop medium sized cream colored colonies were selected and re-inoculated in a freshly prepared media and incubated at 30°C for 48 to 72hrs. After incubation single colony was taken from previous plate for re-inoculation.




**Graph 1.**

Media was incorporated with phenol red indicator to know the nitrification ability of the isolate. After the growth of the culture media color was change from orange to yellow color indicating possible acidification of the media due to the conversion of ammonia to nitrite in the media. After repeated streaking of the isolate on solid inorganic basal media along with indicator the isolate was preserved on basal inorganic media slant layered with paraffin wax for further use.

## 3.2. Identification of Isolate

**Table 1.**

| S.no | Test | Results |
|---|---|---|
| 1. | Gram's staining | Positive rods |
| 2. | Spore location | Sub terminal |
| 3. | Colony colour | Cream whitish |
| 4. | Colony morphology and size | Wrinkled outer margins, medium |
| 5. | Motility | positive |
| 6. | Methyl red | negative |
| 7. | Vogues proskeur | positive |
| 8. | Citrate | positive |
| 9. | Urease | negative |
| 10. | Starch hydrolysis | positive |
| 11. | Gelatin liquefaction | positive |

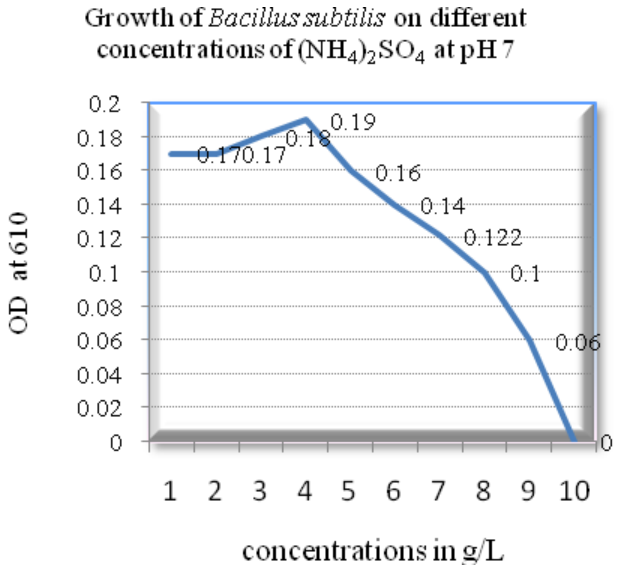
The preliminary identification of the isolate was done by using gram staining procedure. Morphology of the colony was wrinkled from outside and colony color was creamy. The results showed a sub terminal spore with gram positive rods. Starch hydrolysis was positive for the isolate (Table 1). Methyl test was negative and VP test gave positive result for the isolated bacteria. Citrate and gelatinase gave positive tests for the isolate which confirmed that isolated bacterium was *Bacillus species.*

## 3.3. Parameter Standardization

Physical parameters of a bacterial growth generally depend on pH and temperature. So for this reason these parameters were selected for optimization of bacterial growth in specified growth conditions.

## 3.4. Study of Growth Rate

The growth rate of the isolate was studied in basal inorganic medium with 2g/L of ammonium sulphate. The pH was maintained at 7. At this pH growth rate was optimum. As it can be observed from the graph maximum growth rate was obtained on the eighth day of incubation (192hrs) with an optical density $_{610nm}$ of 0.18. It was clearly observed from graph.1 that growth rate of this organism was slow and lag phase was long. This can be justified by two reasons, due to the absence of carbon source in the medium there may be slow growth and medium is purely inorganic in nature which may account for the slow growth of the organism.

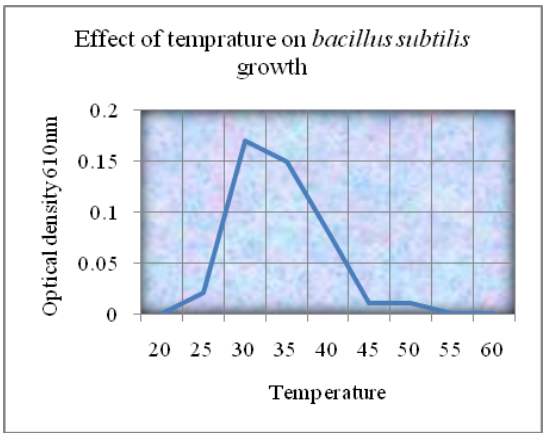



**Graph 2.**

## 3.5. Temperature

For standardization of temperature of the organism same conditions were provided keeping all the parameters constant except temperature. At temperature 20°C growth of the organism was restricted as optical density was lowered to zero. At 25°C moderate growth was observed where as at 30°C growth observed for this organism. Temperature above 30°C to 45°C growth was scanty and above that there was no growth for this organism. This shows that the isolated organism's optimum temperature was 30°C (Graph 3).

Growth was absent. These results indicate that the optimum temperature for this organism is 30°C.




**Graph 3.**

## 3.6. Effect of pH

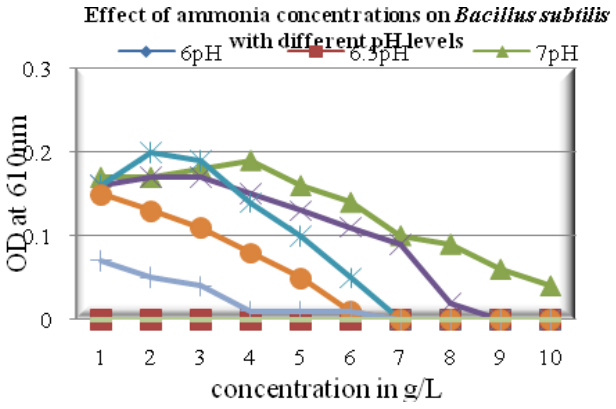
For optimization of pH levels basal inorganic media was freshly prepared and pH ranges from 6 to 10 were selected with 0.5 variation. After incubation growth of the isolate was observed. The bacterium *Bacillus species* was able to grow up to pH 9 and there was no growth of the organism above pH 9. This showed that the organism can tolerate higher concentrations of unionized ammonia which is toxic to other organisms. Most of the ammonia toxicity in waters was due to unionized $NH_3$ (princic.A *et al.*,1998). The growth of the isolated *Bacillus species* was restricted at pH 6 and 6.5 indicating it cannot tolerate acidic conditions. At neutral pH 7 the growth was normal for this organism indicating it is its optimum pH for growth in this media. At higher pH levels above 8.5 the growth observed was slow and scanty. Even after more incubation period also growth was not recovered at pH 9 to 10.

## 3.7. Tolerance Limit for Higher Concentration of Ammonia ($NH_4^+$+$NH_3$) at pH 7

Tolerance limit of ammonia for the isolated *Bacillus species* was studied at pH 7. The results indicated that at neutral pH the organism can tolerate higher concentration of ammonia. At this pH *Bacillus species* was able to grow even in higher concentrations of ammonia. In literature it was found that at pH 7 most of the ammonia exists in the form of ammonium ions which is not toxic to living cells. This may be the reason for the growth of *Bacillus species* even at concentration 10g/L. Miller *et al.*, 2006 stated that growth retardations were not due to the toxicity of ammonia but may be due to the osmolarity of the medium. In this study it was proved that growth inhibition was due to osmolarity of the medium but not the ammonium ion.

## 3.8. Toxicity of Ammonia

Ammonia toxicity was tested on the growth rate of *Bacillus species* grown in minimal media. In these experiments different concentrations of ammonium sulphate was added to the minimal medium ranging from 1 to 10g/L. To each concentration a set of pH variations were taken from 6 to 10 with 0.5 variations. Log cultures were added to the medium to minimize the incubation period. From the results it was observed that growth was restricted from pH 9 onwards showing that ammonia is the main toxicant for the growth of this organism and not the concentration of the ammonium sulphate added to the media. In the previous studies (Leejeerajumnean *et al.*,2000) it was proved that ammonia was the main cause for the inhibition of growth of *Bacillus species* not the ammonium ion. Growth of the organism was moderate at pH 7 and 7.5 even in higher concentration (10g/L) indicating that concentration was not the main criteria for growth inhibition as *Bacillus species* is growing at higher concentration. The main important cause for the growth inhibition was pH. Miller *et al.*, 2006 studied ammonia toxicity on bacterial growth but their results were not clear about the toxic effect on the growth of *Bacillus species* and in our studies it was clear that pH plays a key role on the toxicity of ammonia on the bacterial growth.




**Graph 4.**

## 3.9. Bioremediation Ability of Isolated Bacterium

The ability of isolated *Bacillus species* to remove ammonium form the autotrophic medium was analyzed by following standard APHA methods of 2005. Heterotrophic organisms require additional carbon source to removal ammonia from the waste waters but in this study without any additional carbon source the organism was able to remove 99.2% of the supplemented ammonia in the media. To know whether the organism was able to removal ammonia in higher concentration or not 5g/L of ammonium sulphate was added to the media. The available concentration of ammonia in the medium was 1365ppm. After 96 hrs of incubation at 30°C in shaking condition, the left out ammonia calculated was 10.7ppm. Trace amounts of nitrite and nitrates were estimated in the medium after incubation this showed that the isolated bacterium was able to remove ammonia even in higher concentrations. In previous works it was given that with the increasing concentrations of ammonia the efficiency of *Bacillus species* was decreased (Yang.X.P *et al.*, 2011) but in our studies it was found that this organism can tolerate higher ammonia concentrations (6000ppm) even in toxic form (at pH 7, 10g/L).

**Table 2.**

| Initial conc of ammonia | Final conc of ammonia | Nitrite conc | Nitrate conc | Biomass growth | % ammonia removal |
|---|---|---|---|---|---|
| 1365mg/L | 10.7mg/L | ------ | 2.6mg/L | 0.3g/L | 99.2% |

% ammonia removal=
[Initialconcentration-Finalconcentration/Initialconcentration]×100.

### 3.10. Biomass Estimation

Biomass estimation was carried out for the bioremediation studies. It was observed that from the results biomass production was low indicating that most of the ammonia removed from the media was not assimilated into the cell but it was removed aerobically from the media. For the biomass estimation 5g/L of ammonium sulphate was used. From the results it was deduced that biomass production was too low (0.3g/L). Generally the main disadvantage with heterotrophic nitrification is production of more amount of biomass which is difficult for full scale operations. Literature survey reveals that heterotrophic nitrification results in a large biomass production compared to autotrophic nitrifiers but in this study it was shown that heterotrophic bacteria like *Bacillus species* produce low biomass and can be opted for full scale operational treatment systems.

$$\text{Biomass dry weight estimation} = \begin{bmatrix} \text{Initial weight before filtration} \\ -\text{Final weight after filtration} \end{bmatrix}$$

## 4. Conclusion

In this study wild type *Bacillus species* was isolated from the sewage waters and characteristics of the isolated bacterium were studied. It was revealed that in absence of organic carbon source *Bacillus species* was able to grow in minimal medium irrespective of higher concentrations of ammonia in the medium provided under optimal conditions and toxicity of ammonia in presence of different pH levels on the growth rate of the *Bacillus species* was also studied. It was concluded that at optimum temperature 30°C and at pH 7 this organism can grow even at higher concentrations above 10g/L and can remove 99.2% of ammonia aerobically in flask studies. Further studies are required on impact of organic carbon source on growth rate of *Bacillus species* and sequencing at genomic level for further identification of species is required. Further research is needed with immobilization studies.

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