Simultaneous Nitrification and Denitrification of Ammonical Wastewaters Using Bacillus Species SB1 Isolated from Domestic Sewage

Sheela. B[1], Khasim Beebi. S[2], and Yellaji Rao.O[3]
[1] Department of Biotechnology, Gitam Institute of Science, Gitam University, Visakhapatnam
[2] Department of Biotechnology, Gitam institute of Technology, Gitam University, Visakhapatnam
[3] Safety and Environment, Synergies Castings Limited, Visakhapatnam
Email: sheela_bethapudi@yahoo.com

Abstract — **Industrialization in the world increased the pollution levels in the environment making life more complicated. 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 of ammonia in flask cultures. The study was attempted to develop a process for ammonia bioremediation in a single stage aerobic nitrification and denitrification of ammonia. The results reveal that the optimum pH for the isolated Bacillus species strain was 8 and optimum temperature was 300C. At optimum pH and temperature this organism was able to remove 72.7% of ammonia in flask cultures supplemented with 860 mg l-1 of ammonia. The study on ammonia toxicity revealed that toxicity was due to increase in hydrogen ion concentration and osmosis.**

Index Terms—**ammonia bioremediation, Bacillus species, aerobic nitrification, denitrification, optimization, tolerance limit.**

I. 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. Industrial effluents are highly polluted with many toxic organic and inorganic chemical compounds. Industries like tannery effluents, textile, landfill leachate, fertilizer industries etc, produce ammonia rich effluents (Jung J.Y *et al.*, 2004). Ammonia when present in water exists in two forms ammonium ion ($NH4^+$) and free ammonia (NH3) 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. 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). Chemolitho-autotrophic bacteria convert ammonia into nitrite and nitrates. These nitrates are again converted 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 high for these organisms and this is an unfavorable aspect compared to heterotrophic bacteria.

Heterotrophic nitrifying 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. Nitrification and denitrification efficiency can be easily estimated by balancing nitrogen calculations (Yang. X. P *et al.*, 2011). The main aim of the present study was to isolate an organism which can tolerate high ammonia concentrations, determine the growth parameters of the isolated organism in presence of organic carbon source characterize and identify the organism and study the degradation efficiency for different initial ammonia concentrations.

II. Materials and Methods

A. Isolation of Bacteria Using Enrichment Method

Domestic sewage was collected from wastewater treatment plant located at Apphughar, Visakhapatnam. Basal inorganic medium as given in Table I was prepared and autoclaved at 121 ^oC for 15minutes and 3ml of the collected sample was inoculated into ammonia enriched medium. This was incubated at 30 ^oC and 120 rpm for 4 days. After incubation nitrite and nitrate concentrations were analyzed by following photometric methods. The culture was repeatedly inoculated in to the enrichment medium with increasing concentrations till $3gml^{-1}$ for six times in order to eliminate the growth of other organisms.

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TABLE I MEDIUM COMPOSITION (BRIERLEY AND WOOD, 2001)

Constituent in g l^{-1}		Trace element solution l^{-1}	
$(NH_4)_2SO_4$	- 1	H_3BO_3	-2.86g
KH_2PO_4	- 0.7	$ZnSO_4.7H_2O$	-0.22g
$CaCl_2$	- 0.5	$CuSO_5.7H_2O$	-0.08g
$MgSO_4.7H_2O$	- 0.5	$MnSO_4.4H_2O$	-2.03g
Trace element solution	- 1ml	$Na_2MoO_4.2H_2O$	-1.26g

B. Isolation and Identification

Enrichment culture was serially diluted and spread on purified agar containing basal inorganic medium with phenol red indicator. Plates were incubated for 72hrs at 30 ^oC. Colonies indicating nitrification were selected and streaked on solid basal inorganic medium. Isolated cultures were stored on nutrient agar slants. Further isolated cultures were repeatedly inoculated on slid medium with increasing concentration of ammonia till10gm^{-1}. The isolated culture was stained by Gram's stain and subjected to biochemical tests following Bergey's manual of Bacteriology. KB03 strip test was performed for preliminary identification of the isolate. 24hr old cultures grown on basal liquid and solid nutrient medium were used for all biochemical tests. All tests were conducted in triplicates (n=3). 24hr old cultures were sent to Bioaxis DNA research centre, Hyderabad for 16s rRNA sequencing. The sequencing was carried out by using forward and reverse bacterial universal primers named 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3') (Zhang Q. L. *et al.*, 2012).

C. Phylogenetic Analysis

Similar 16s rRNA sequences were retrieved from gene bank database of NCBI.hlm.gov using query sequence and multiple sequence alignment with CLUSTAL W was carried out followed by maximum likelihood method with MEGA 6.06 for phylogenic tree construction. Statistical analysis was performed using bootstrap method with 1000 replicates for tree construction. The sequence query was submitted in gene bank data base and accession number KJ804254 was obtained from NCBI gene bank (Fig. 2).

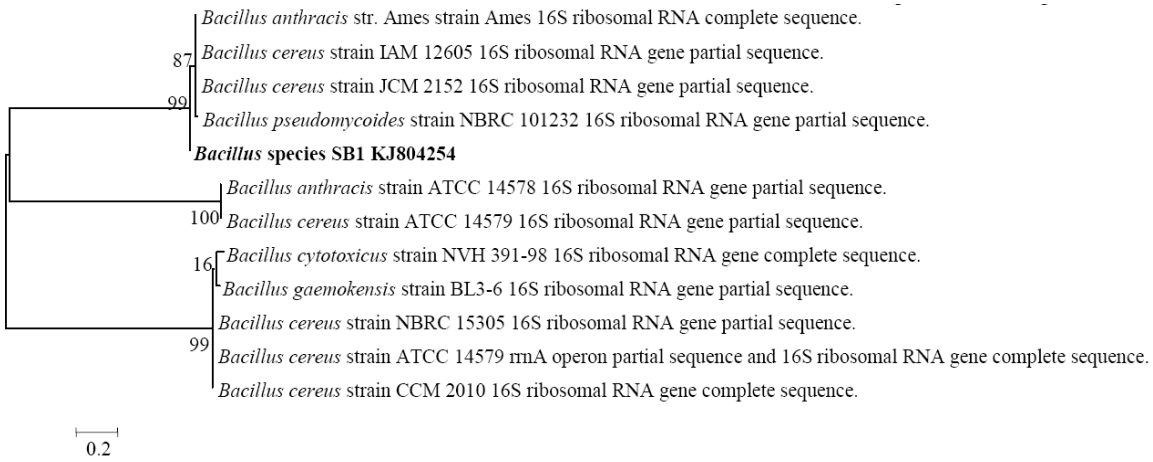


Figure. 2 Constructed Phylogenetic tree with isolate whose gene bank accession number is in bold.

D. Optimization of Parameters for Bacterial Growth

Bacterial growth parameters C:N ratio, pH, temperature, were optimized for the growth and bioremediation of ammonia. pH ranging from 6-9 with an interval of 0.5, temperatures ranging from 20 ^oC to 60 ^oC with an interval of 5 ^oC and two substrates, Acetate and glucose were used as carbon sources to grow the isolate for ammonia remediation. All the experiments were carried out in triplicates (n=3).

E. Effect of Ammonia Concentrations ($NH_4^+ + NH_3$) on the Growth of the Isolate

Effect of ammonia concentrations on the growth of the isolate was studied and along with ammonia removal efficiency. Ammonia concentrations ranging from 1-10gm l^{-1} were used with optimized parameters. Samples were periodically checked for nitrification products of the isolate at different concentrations of ammonia. All tests were performed in triplicates (n=3).

F. Analytical Procedures

Concentration of ammonia, nitrite and nitrate were estimated by Nesselerisation, photometric method and phenol disulphonic acid method respectively. The optical densities for ammonia, nitrite, and nitrate were measured using visible spectrophotometer 105, Systronics, India. Total nitrogen was determined using Total nitrogen analyzer (Kelplus-Classic DX, Pelican. India. The analytical methods were followed as per standard methods for the examination of water and waste water, APHA (2005). Cell growth was estimated by measuring optical density at 610nm on Photoelectronic colorimeter 113, Systronics, India. pH was measured by pH meter 510, EUTECH, Singapore. The liquid cultures were grown on shaking incubator ORBITEK, Scigenics Biotech, India. The experimental results were analyzed using STASTICA software version 6.0. Results were considered to be significant at $p < 0.05$.

III. Results and Discussion

A. Isolation of Bacteria Using Enrichment Method

The enrichment method was followed for selection of ammonia oxidizing bacterial isolate. The preliminary observations revealed that nitrification products, nitrite and nitrate were formed which confirmed that medium contained ammonia oxidizing organisms. At each stage of enrichment, inorganic ammonia concentration was increased in the medium to select the organism which can tolerate high concentrations of ammonia. This led to the selection of desired organism form the raw sample.

B. Isolation and Identification

For isolation of the desired bacterial isolate the enrichment medium was spread on basal inorganic medium incorporated with phenol red indicator. The colony which has bigger nitrification zone was picked for further purification process. Six isolates named A1, A2, A3, A4, A5 and A6 were selected from the culture plate. The isolate A1 was able to grow at an initial ammonia concentration of $10gml^{-1}$ and has survived for a period of 96hr. The observations of morphological biochemical characterization were given in Table II. The isolate was found to be Gram positive rod and the enteric and extracellular enzyme production tests suggests that the isolate might be Bacillus species. Further, the sugar fermentation tests suggest that the species might be cereus.

Table II: Observations and Results of Gram Staining and Biochemical Tests of the Isolate

Staining and Morphological Tests		**Enteric Tests**		**Tests for Extracellular Enzyme Production**	
Gram's staining	Positive rods	Methyl red	negative	Starch hydrolysis	Positive
Spore location	Sub terminal	Vogues proskeur	positive	Gelatin liquefaction	Positive
Colony colour	Cream whitish	Citrate	positive	**Sugar Fermentation Tests**	
Colony morphology and size	Wrinkled outer margins, medium	Urease	negative	Glucose fermentation	Positive
Motility	positive	Indole	negative	Mannitol fermentation	Negative

C. Phylogenetic Analysis

The results of phylogenetic analysis for the isolate query completely matches with the sequence of *Bacillus cereus* and sequence similarity was found to be only 99% and not 100% and the isolated organism might be a new *Bacillus species*. For future reference, the sequence of the isolate was submitted to NCBI gene bank data base with name *Bacillus* sp SB1.

D. Growth of Bacillus sps

The isolated culture has grown in an autotrophic medium with $840mgl^{-1}$ of initial ammonia concentration and the cell density was measured periodically. The optical density v/s incubation time was plotted and shown in Fig. 1. This result showed that ammonia was converted to biomass and the organism was capable of autotrophic nitrogen removal. Similar observation was made by Yang. X. P *et.al*. for another *Bacillus subtilis* A1 sps (2011). The study reveals that the isolated organism is capable of assimilating ammonia.

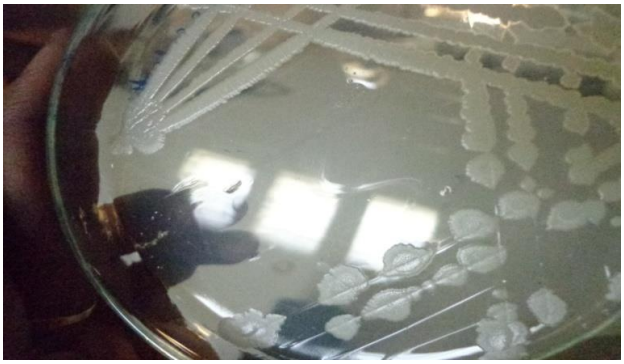


Figure 1

E. Optimization of Parameters for Bioremediation of Ammonia

1) Effect of carbon source

To study the effect of carbon source on bioremediation efficiency of the isolate, acetate and glucose were used as carbon sources. In the experiments conducted with acetate, the ammonia was not metabolized that means the organism is not utilizing the acetate for the cell growth. This might be due to the absence of acetate utilizing enzymes or inactive in state. In previous studies, acetate was not utilized by active cells but this was actively up taken during spore state (Gollakota and Halvorson, 1963). The results obtained for glucose source was given in Fig. 2. It was found from the results that glucose was best suitable for ammonia bioremediation. At C/N ratio 10, the ammonia removal rate in shake flask cultures at 48hr incubation was found to be 66.9%. The highest rate of ammonia removal was found to be 90.95% with *Methylotrophicus* strain L7 at concentrations of $1121.24mgl^{-1}$. The authors have selected $1000mgl^{-1}$ as highest ammonium concentration (Zhang *et al.*, 2012). Most of the isolates were able to remove in ammonia at low concentrations whereas the present isolate was able to remove at high concentrations i.e. at $840.6 \pm 16.17mgl^{-1}$. In all the experiments, the nitrite and nitrate concentrations were in negligible quantities. This might be due to their reduction to denitrification products. It is clearly evident from the Fig. 2 that increasing optical density resulting in increased organic nitrogen content and simultaneous decrease in inorganic ammonia concentration.

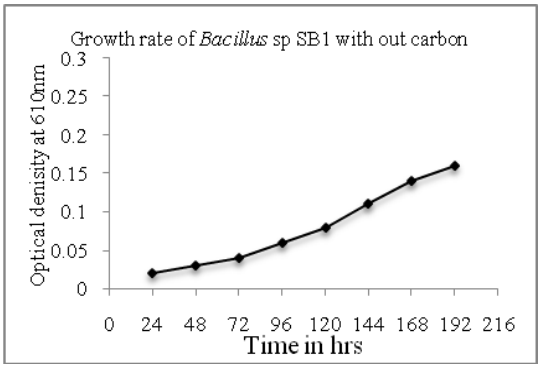
2) Effect of pH on Bacillus sps growth and ammonia bioremediation

The glucose was used as the co substrate along with ammonia for biomass multiplication. The optimum pH for bioremediation was determined by conducting experiments at different pH. The results obtained are depicted in Fig. 3. It was found that pH 8 showed

more efficiency when compared to other studied pH values. The growth of the organism was measured by optical density at 610nm. At pH 6 and 6.5 there was no significant nitrification whereas at pH 7 and 7.5 showed little growth. The earlier investigations revealed that nitrification will be inhibited below pH 6 and above pH 9. At 48hrs of incubation, the ammonia concentration was found to be 220mgl-1. It was found that pH 8 was optimum for better ammonia removal in this medium. A thermophilic *Bacillus* strain MS 30 showed maximum nitrification ability between pH 7.5-8 and maximum growth rate was obtained at pH 6-6.5(Mavel and Prieur, 2002). The results obtained in our study correlates with the results of *Bacillus Methylotrophicus* strain L7 results which states that efficient nitrification was achieved between pH 7-8 (Zhang Q. L. *et al.*, 2012).

3) Effect of temperature on Bacillus sps growth and ammonia bioremediation

For optimization of temperature for remediation, different temperatures were selected ranging from 20 to 60 ^{o}C by keeping all other parameters constant. After 48hr of incubation it was found that at 30 ^{o}C temperature, maximum ammonia removal was achieved from the liquid medium (Fig. 4). At 20 to 25 ^{o}C there was no proper growth and above 30 ^{o}C there was no change in Biomass concentration. The ammonia removal efficiency with Bacillus methylotrophicus strain L7 has increased with increase in temperature from 20 ^{o}C to 37 ^{o}C (Zhang Q. L. *et al.*, 2012). In the present study, at 37 ^{o}C ammonia removal was less as compared to 30 ^{o}C. This temperature is more favorable for their application in waste water treatments. The initial concentration of ammonia (840.6 ±16.17mgl^{-1}) was depleted to 223.3 ±29.46mgl^{-1} within 48hrs of incubation. The ammonia removal percentage was found to be 58.9%. From the total nitrogen content it was found that 32.4% was organic nitrogen and 26.5% was inorganic nitrogen.



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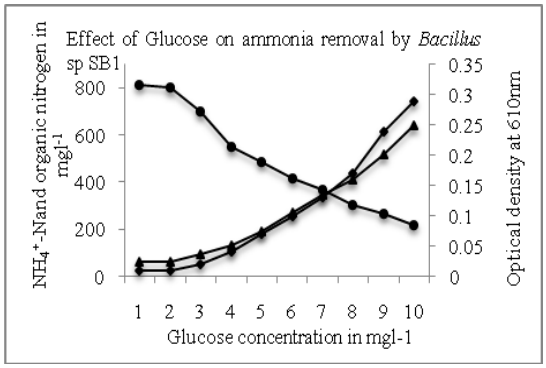

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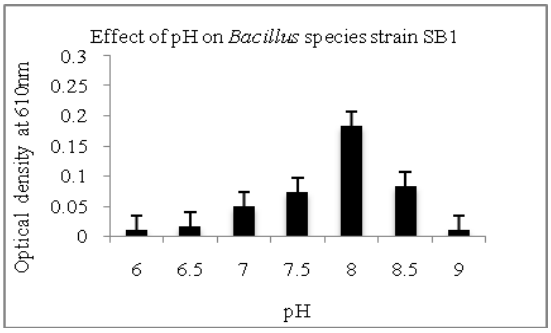

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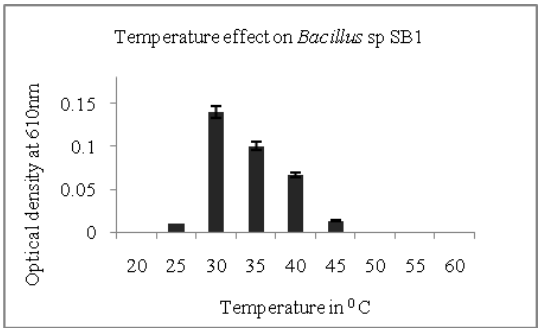

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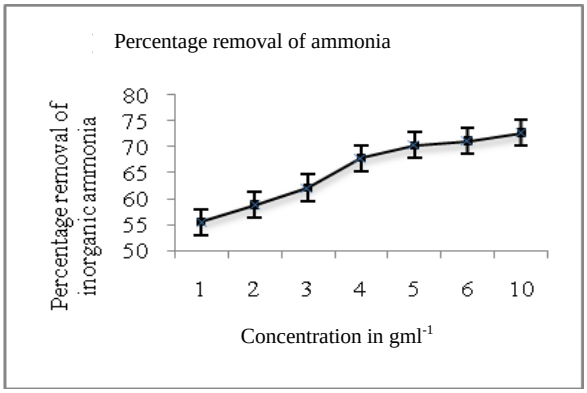

(5)

(1) Graph for growth rate of the isolate. (2) Graph with results for glucose as carbon source. (3) Graph for pH optimization. (4) Graph for temperature optimization. (5) Graph showing the percentage removal of inorganic ammonia from the medium. $NH4^+$-N (●) $NO3^-$ (◊) $NO2^-$(□) TN (■) Organic nitrogen (▲), optical density (♦).

4) Effect of ammonia concentrations ($NH4^+$ +NH3) on bioremediation

To study the effect of inorganic ammonia concentration on the growth, ammonia concentration ranging from 1-10 gl^{-1} was added into the liquid medium keeping all other parameters constant and incubated for 48hr. Total nitrogen, inorganic ammonical nitrogen, nitrite and nitrate, optical density, pH, and cell dry weight was assessed. From the results, it was found that with increasing concentration of inorganic ammonia the depletion of ammonia from the medium was followed with increasing trend (Graph.5). There was biomass growth even at high ammonia concentration. With increase in growth, the organic nitrogen content has also increased revealing ammonia assimilation in to the cell. After 48hrs of incubation, at $10gl^{-1}$ initial concentration, the inorganic nitrogen content in the medium was 48.53% and organic nitrogen content was 37.43%. The balance amount of nitrogen was assumed to be escaped from the medium as gaseous nitrogen. In all the flasks 99% of ammonia depleted only after 120-144hrs. The denitrification started after 72hrs of incubation period. Existence of two mechanisms, nitrification and denitrification in same organism can be a beneficiary aspect. The highest rate of ammonia removal was found to be 90.95% with *Methylotrophicus* strain L7 at concentrations of $1121.24mgl^{-1}$. *Bacillus subtilis* A1 was able to tolerate higher ammonium concentrations but this strain is vulnerable to higher concentrations of ammonia in the inorganic medium (Yang X. P *et al.*, 2011). Many of the isolated heterotrophic nitrifiers were able to survive only at low concentrations of ammonia but Bacillus sp SB1 isolate was able to withstand high concentrations of ammonia, and hence suitable for treating high strength ammonical waste waters.

IV. Conclusion

The bacteria isolated form sewage using ammonia enriched medium was identified as Bacillus species and named as strain SB1. Genetic sequencing of 16s rRNA was performed and sequence query was submitted to NCBI gene bank data base and an accession number was obtained from the NCBI. The isolate was utilized for ammonia bioremediation. The culture was growth in batch mode and the incubation time needed to obtain maximum concentration was found to be 96 hr. The isolate was acclimatized to higher concentration of ammonia and optimized pH, temperature and co-substrate concentration. With increasing concentration of inorganic ammonia in the medium the organism was able to convert ammonia into gaseous nitrogen products. At an initial ammonia concentration of 4483.2mg of ammonia, the isolate achieved 72.7% ammonia removal. The optimum temperature and pH for the isolated *Bacillus* sp. was 30 ^oC and 8 respectively. This organism is capable of resisting higher concentrations of ammonia and has removed 72.7% of ammonia aerobically in flask studies.

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