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ENRICHING THE CULTURE OF AMMONIA OXIDIZING BACTERIA FROM SOIL AND FISHPOND WITH BIO-FILTERS

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Abstract

A special group of microorganisms, which are called ammonia oxidizing bacteria (AOB), is capable to reduce ammonia-nitrogen from the ecosystem. However, this bacterium is slow growing and has a low yield capacity. Hence, the objective of this research was to grow the AOB using a support medium in the form of bio-filters to reduce the ammonia-nitrogen. The enrichment cultures of this AOB were performed from the inoculum of common soil and effluent from fishpond. The experiment was conducted using two sets of flasks labelled Medium A and Medium B, respectively. Each medium contained three flasks of which Flasks 1 and 2 were inoculated with the common soil and effluent from a fishpond. Flask 3 was the control, which contained neither soil nor effluent from any fishpond or bio-filter. Then the ammonia-nitrogen and the formation of nitrite-nitrogen were measured. The results showed that the enrichment culture of the effluent of the fishpond had achieved a high formation of nitrite-nitrogen (450 µg/L) in comparison with the enrichment culture of the soil (360 µg/L). A high reduction of ammonia-nitrogen (50%) was obtained from the fishpond culture rather than the soil (40%). Secondly, a higher reduction of ammonia-nitrogen and production of nitrite-nitrogen were found in the bio-filter flasks against the controls (without bio-filter). In summary, it can be concluded that the bio-filter can be a reliable support to grow AOB.

Keywords: Ammonia-oxidizing bacteria (AOB), Batch culture, Bio-filter, Effluent from fishpond, Soil.

1. Introduction

Nitrogen is essential for the synthesis of nucleic acids and proteins. This main element, together with carbon, contributes to the microbial development. Nitrogen

mainly exists in organic forms within molecules like proteins, DNA and other cellular compounds. Proteinaceous materials from nitrogen are broken down to release ammonia-nitrogen in a composting process [1], which can occur in soil. In water having living organism such as fish, the ammonia-nitrogen exists from the presence of the fish faeces.

High ammonia-nitrogen in the water bodies leads to the toxicity of fish and many other aquatic organisms. As ammonia-nitrogen is an oxygen-consuming compound, ammonia-nitrogen can cause depletion of dissolved oxygen in the water, which may harm the aquatic ecosystem [2]. Nitrogen, in the forms of ammonia-nitrogen and phosphorus that enters the water, stimulates the productivity of phytoplankton, which in turn leads to the intensive process of photosynthesis, which increases the pH during the day. This phenomenon creates a high proportion of ammonia-nitrogen and causes eutrophication [3]. Hence, it is very important to reduce the content of ammonia-nitrogen in the water, in particular, the wastewater effluent. In this case, the key nitrogen cycling process of ammonia-nitrogen is nitrification.

Nitrification involves two actions: ammonia-nitrogen being oxidized to nitrite-nitrogen, then further oxidized to nitrate-nitrogen. These processes are conducted by two autotrophic microorganisms. The first limiting step is commonly performed by ammonia oxidizing bacteria (AOB) while the second is by nitrite oxidizing bacteria (NOB). However, these tasks need to be executed under aerobic condition [4].

Noting that the AOB belonging to one of the physiological subsets of the nitrifying bacteria family [5], they play a major role in relieving ammonia-nitrogen from the ecosystem. They are the key elements in the biogeochemical nitrogen cycle in ammonia oxidation [6]. However, these bacteria have a long generation time with a slow growth rate [5]. This slow nature of growth makes the isolation of AOB in pure culture extremely difficult that requires diligence and patience [7].

Here, nitrifying bacteria in soils can be the 'starter' in ammonia oxidation. Hence, research is actively conducted to establish the AOB growth to study its oxidation activity in a culture of soil or water effluents to reduce the ammonia-nitrogen.

To do so, the basic strategy is to enrich the culture that would grow on ammonia-nitrogen. The enrichment cultivation is designed to increase the relative number of a particular AOB to encourage their growth, survival, or spatial separation from other members of the population. One of them is using bio-filters as a support media in the batch enrichment studies. Attempts to have been conducted on some species of nitrifying bacteria (AOB and NOB) which are able to complete ammonia oxidation and nitrite oxidation in the water systems and soil [8-9]. This indicates that the enrichment culture from soil and effluent from fishpond could be possible. Hence, research should be conducted combining experiments using bio-filters and elements from soil and fishpond in serial batch culture.

Nevertheless, some constraints need to be overcome by introducing the attached growth system using bio-filters. As the AOB are highly sensitive and can easily be eliminated [5]. A biofilm is an accumulation of microorganisms attached to a solid surface [10]. In the enrichment culture stages, biofilms in the support media of the bio-filters is established.

2. Materials and Methods

The study hypothesized that the attachment of the slow-growing bacteria (AOB and NOB) on the bio-filter in the culture media of soil and effluent from fishpond would reduce ammonia-nitrogen and produce nitrite-nitrogen. Hence, it attempted to establish the AOB growth and study its oxidation activity in a culture of soil and fishpond in order to reduce the ammonia-nitrogen.

This experiment employed an attached growth system (bio-filter) in a serial of flasks. Two sets of flasks were used and each set contained three flasks. The first set of three flasks was called Medium A while the second, Medium B. The three flasks were label Flask 1, Flask 2 and Flask 3 respectively. Flask 1 contained a bio-filter, while Flasks 2 and 3 did not. Flasks 1 and 2 were inoculated with soil and while Flask 3 was not (a blank or control). The soil used in this experiment was a common soil while the bio-filter was named Flocor.

The same procedure was conducted to enrich the culture of AOB using effluent from a fishpond. The first set of three flasks was named Medium A while the second, Medium B. The three flasks were also labelled Flask 1, Flask 2 and Flask 3, respectively. Flask 1 contained a bio-filter, while Flasks 2 and 3 did not. Flasks 1 and 2 were inoculated with the effluent from a fishpond, but Flask 3 was not (a blank or control). The Flocor bio-filter was also used in this fishpond culture.

2.1. Nitrification of Medium A

The formulation of Medium A nitrification was as follows: Na_2HPO_4, 13.5 g: KH_2PO_4, 0.7 g; $NaHCO_3$, 0.5 g; $MgSO_4.7H_2O$, 0.1g; $FeCl_3.6H_2O$, 0.014 g; $CaCl_2.2H_2O$, 0.18 g and 1000 mL of distilled water [7]. Aliquots of 100 mL of medium were put into 250 mL Erlenmeyer flasks, and sterile at 121^{o}C for 15 min. For ammonium oxidizers, a sterile stock solution of $(NH_4)_2SO_4$ was prepared separately from the basal medium. The stock solution of $(NH_4)_2SO_4$ was added aseptically to bring to the final concentration of 0.5 g/L., and the pH, 8.0.

2.2. Nitrification of Medium B

While the Medium B stock solution was: Na_2HPO_4, 3.5 g; KH_2PO_4, 0.7 g; $NaHCO_3$ 0.5 g; $MgSO_4.7H_2O$, 0.1 g; $FeCl_3.6H_2O$, 0.014 g; $CaCl_2.2H_2O$, 0.18 g and 1000 mL of distilled water. A sterile stock solution of $(NH_4)2SO_4$ was prepared separately from the basal medium and added aseptically to give the final concentration of 0.5 g/L as the ammonium oxidizers. The final pH was 7.8 [11].

The difference between Medium A and Medium B was their chemical measurement and final pH.

2.3. Preparation of cultural enrichment in serial batches

The bacteria were isolated from the soils by placing 0.1 to 0.3 g of it into the serial batches of the culture media in the flasks with and without bio-filters. The flasks were then incubated for two weeks in a continuous mechanical shaker at a temperature of 30^{o}C. To determine the free suspended solids, 100 mL of the liquid suspension was filtered through a Whatman filter paper (0.2 µm) and washed before being dried to a constant weight in an oven at 90^{o}C to 105^{o}C. The sample then was placed in a desiccator. The weight of the sample was taken. The flasks of serial

batch cultures with and without bio-filters were then filled again with fresh media. Ten mL of the liquid suspension, retained from previous batch cultures, was added to the media.

The enrichment procedure of the fishpond effluent was treated similarly as did of the enrichment culture of the soils. The inoculum was provided by the effluent waste of the Centre for Sustainable Aquatic Research, College of Science, Swansea University, United Kingdom.

2.4. Preparation of the aerobic liquid medium culture

The prepared media, including the bio-filters, were sterilised by autoclaving at 121ºC for 15 minutes. The two sets of flasks contained 100 mL in Medium A and Medium B. An amount of 10 mL inoculum was injected into Flask 1 and Flask 2. No inoculum was injected into Flask 3, as it was a blank control. The stoppers of the flasks were made of non–absorbent cotton and aluminium foil.

The flasks were then placed in a mechanical shaker incubator set at 180 rpm and 28ºC. The samples were taken daily to determine the concentrations of the ammonium nitrogen and nitrite-nitrogen. To encourage the surface growth on the bio-filters, the liquid medium was drained daily and replaced with the new sterile medium. The 10 mL solution was retained and the new sterile medium was placed in Flasks 2 and 3, which did not contain any bio-filters.

After the enrichment, the cultures were grown routinely by taking an inoculum, 10 mL, to grow in each flask for one week. It was taken from the active culture flasks by a syringe. The medium, after sterilization and cooling, was inoculated with nitrifying bacteria from the medium culture. Then it was injected into new sets of fresh flasks. After that, the flasks and the bio-filters were autoclaved to kill any microbes that might be present in the flasks before the nitrifying bacteria were placed in the media. This was conducted in particular to limit any possible source of contamination.

2.5. Colorimetric analysis

The ammonium-nitrogen (NH_4-N) and nitrite-nitrogen (NO_2-N) in the concentration between 5 and 1000 µg NO_2-N/L [12] was determined by a colourimetric analysis. While to determine their concentrations higher than the stated level, an ion chromatography procedure was used. The absorbance data of all samples were read by a spectrophotometer (Biorad SmartSpecPlus UV/VIS Spectrophotometer 200-800 nm).

3. Results and Discussions

Figure 1 shows the uptake of ammonia-nitrogen and Fig. 2 the production of nitrite-nitrogen. The figures show that the ammonia-nitrogen uptake and the nitrite-nitrogen production of Medium B were similar to those of Medium A. As the substrate of ammonia-nitrogen decreases, the production of nitrite-nitrogen increases, showing that the bacterial suspensions in the experiment had oxidized the ammonia-nitrogen.

Figure 1 shows the decrease of ammonia-nitrogen by nearly 40%. This shows that the ammonia-nitrogen was converted to nitrite-nitrogen in the nitrification. The

biggest decrease is in the flask containing the bio-filter in Medium B. The amount of ammonia-nitrogen introduced in every serial batch culture was 100 mg/L, which was quite high in the flasks containing 100 mL in Medium A and Medium B with 10 mL of the bacterial suspension, respectively. It is noted that the bacterial suspension was not included in the control. It was observed that a longer incubation period was needed to oxidize all ammonia-nitrogen. A high amount of nitrifying bacteria was also needed to complete the nitrification.

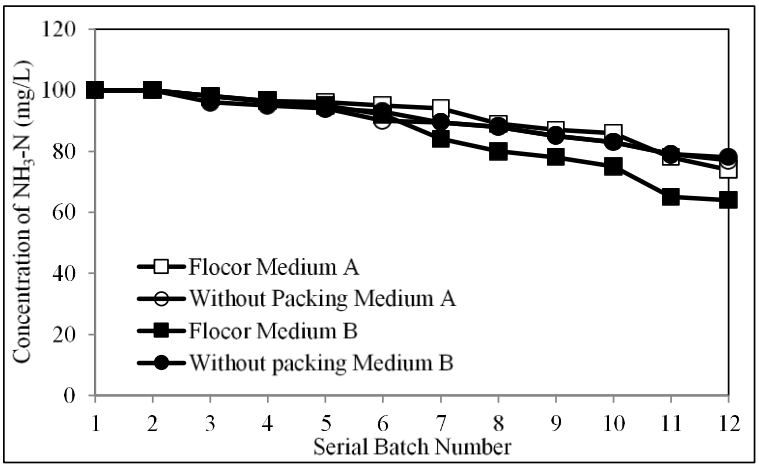

Fig. 1. Ammonia-nitrogen uptake in Medium A and Medium B in soil cultures.

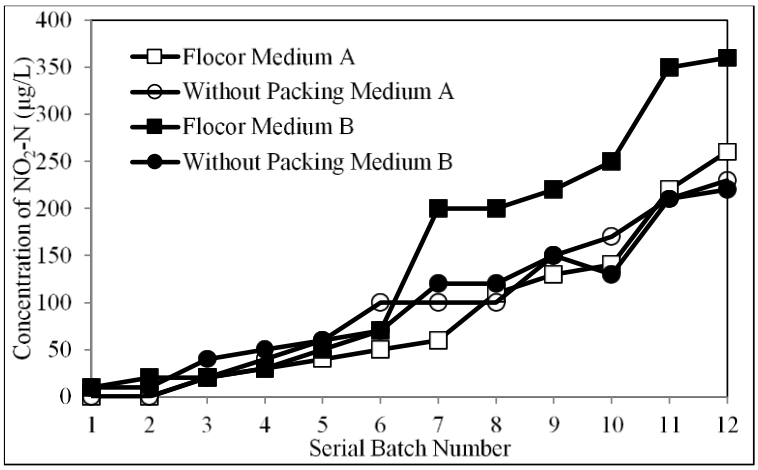

Fig. 2. The Production of nitrite-nitrogen in medium A and medium B in the soil cultures.

In comparison about the requirement of a longer incubation time, Ma et al. [13] conducted their experiment on the AOB and NOB being exposed to free nitrous acid (FNA) to evaluate their adaptation in activated sludge. Their results showed that the AOB and NOB inactivation increased as the FNA concentration increased. This was more visible as the exposure time to the FNA was

lengthened. Furthermore, the AOB did not adapt to the FNA, as the threshold of the FNA concentration to inactivate the NOB highly increased after a long-term FNA treatment.

Likewise, a study done by Dong et al. [14] had shown that the AOB were immobilized by the calcium alginate. Their results showed that a high hydrogen ion was needed to immobilize the AOB. This is to achieve a stable molecular structure of calcium alginate. Moreover, an alkaline condition must be preserved as an important factor to immobilize the AOB in calcium alginate.

In the same vein, Gregory et al. [15], studied the changes the AOB when exposed to increasing ammonia-nitrogen concentrations. Their results showed that at higher concentrations of ammonia-nitrogen, a lower percentage of ammonia-nitrogen was removed. Secondly, they also showed, on occasion, that the nitrite-nitrogen accumulation dropped in the number of operational taxonomic units (OTUs) as detected in the bacterial community; concurrently decrease in the percentage ammonia-nitrogen removal. The present study concurred with data [13-15].

To explain, the AOB, since they are slow growing, need time to adapt to the different concentrations of ammonia-nitrogen [16]. However, the AOB can predominantly grow in a large colony if the favourable environment and optimum condition are provided. Hence, in this case, Wang et al. [17] observed that the ammonia oxidizers did play an important role to treat nitrogenous wastewater.

All these results showed that ammonia-nitrogen could be reduced by bio-filters in a soil-enriched culture. As this soil contained considerable amounts of AOB, it was able to drive the nitrification. Different habitats condition did contribute to the quantity and diversity of the AOB in the nitrification process in the soil [18]. The characteristics of the soil and physicochemical properties are vital in determining the activities of the AOB and NOB [19].

Hence, three potentially distinguishing characteristics have been suggested regarding the cultivation of AOB from soil: ammonia-nitrogen affinity, mixotrophy, and the optimum pH for growth. A further analysis of soil heterogeneity and microenvironments is necessary to understand the mechanisms that control the AOB community composition and activity to increase their growth [20].

Secondly, in this experiment, a similar experiment was conducted using the fishpond effluent to enrich the culture in serial batches. The fishpond culture test also contained two sets of flasks, Medium A and Medium B. Figure 3 shows the results of ammonia-nitrogen uptake in Medium A and Medium B, while Fig. 4 shows the nitrite-nitrogen production.

Figure 3 shows that the ammonia-nitrogen concentration is reduced by nearly 50% from the initial value of 100 mg/L ammonia-nitrogen. The greatest reduction in ammonia-nitrogen is shown in three serial flasks containing bio-filters in Medium A and Medium B. In Fig. 3, the lowest decrease in ammonia-nitrogen is in the serial flask without bio-filter in Medium B.

Figure 4 shows the production of nitrite-nitrogen in every serial flask both with and without bio-filters in both Medium A and Medium B. The accumulation of nitrite-nitrogen is shown high in Medium A and Medium B in the fishpond effluent with bio-filters. However, a slow build-up of nitrite-nitrogen is shown obtained in serial flasks without bio-filter in Medium B.

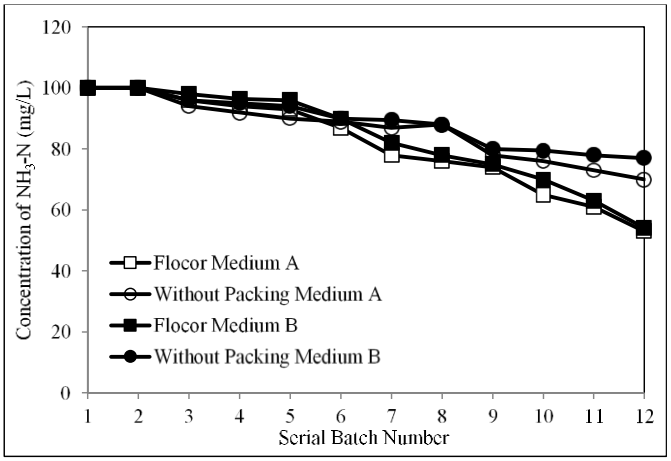

Fig. 3. Ammonia-nitrogen uptake in Medium A and Medium B in fishpond effluent cultures.

Repeatedly, the serial flasks without the bio-filter show poorer performance compared to the serial flasks with bio-filters in the effluent from fishpond culture and as well as the soil. The suspended system in serial flasks without bio-filter is seemed influenced by the vigorous mixing and/or aeration that have reduced the thickness of the liquid film surrounding the flocs. The size of the flocs reduces the effects of the mass transfer.

In the experiment, the inter and intra-phase mass transfer was insignificant, and the process was treated as a homogenous system. However, in the attached growth system in the serial flasks with bio-filters, the inter and intra-phase mass transfer were significant because the biofilm structure tended to retard the rate of transport of the substrate through the carriers. The liquid-biofilm interface is another source of resistance to the transport of substances. Therefore, biofilm systems are considered as heterogeneous systems [10].

The biofilms in the bio-filter system, called substratum, comprise 90% or more of all naturally occurring microorganisms [21]. There are three important factors that describe the efficiency of biofilm in the attached growth system. One of the factors is that the biofilm is an excellent means to retain microorganisms. Besides, in treatment technology is vital to have long retention time in particular for the slow-growing bacteria.

Secondly, there are stable gradients of substrate concentrations with microbial types. In this case, the ammonia-nitrogen is the substrate achieved by the biofilms in the bio-filter system. Here, the ammonia-nitrogen can be considered as an electron donor to the AOB for oxidation to gain energy [10]. The gradients of microbial types are stabilized by the 'cementing' action of extracellular polymeric substances (EPS). Proteins and carbohydrates from the EPS link the cells and the substratum in the form of 'glue-like' bond. This bond holds the biofilm to the substratum and keeps the different types of microorganism in their preferable locations [21].

The last factor is that the sensitive microorganisms can be protected in the biofilms due to the inhibition. Here, the slow-growing of AOB in the biofilm will be safeguarded against losses from surface detachment and predation. This is also can shield the AOB from inhibition caused by high substrate concentration. This is due to the AOB that would be located deep inside the biofilm layers [21].

Enrichment culture is used as a method to revive or start a bio-filter. It is also called a 'starter' for a bacterial population derived. Soil can be a good starter to enrich the nitrifying bacteria in aquaculture bio-filters. According to [22, 23], the 'starter' is used in the bio-filters system with ammonia-nitrogen as the substrate to create optimal conditions for the development of a nitrifying bacterial population.

The development of pre-coating nitrifying bacteria on a solid support has also been tried for use in aquaculture bio-filters to enhance nitrification, using recirculating aquaculture system (RAS) [24, 25]. Here, efficient total ammonia-nitrogen (TAN) and nitrite-nitrogen removal have been established using RAS. The hydraulic film diffusion is known to have been able to affect the nitrification rate in fixed film bio-filters quite strongly [24]. Previous studies have shown that AOB has been detected in most soils [26]. The AOB too dominate the nitrification process in agricultural soils with high ammonia-nitrogen content [27].

The most important aspects for a successful starter in bacterial amendments for aquaculture must be a lack of pathogenicity, a short lag period and tolerance to a variety of environmental conditions in ponds such as diurnal changes in temperature, radiation and salinity [28]. Hence, the nitrifying bacteria developed under extreme environmental conditions have the potential to tolerate the said environments. They, therefore, can be good starters for bio-filters in ponds under harsh environmental conditions.

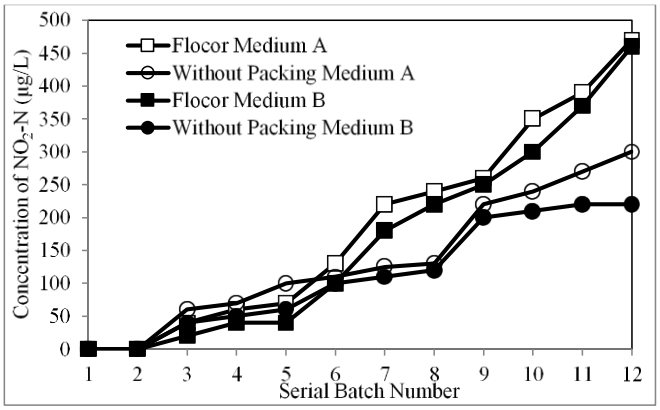

Fig. 4. Production of nitrite-nitrogen in Medium A and Medium B in fishpond effluent cultures.

The experiment displayed that a higher ammonia-nitrogen uptake and nitrite-nitrogen production were achieved in the fishpond effluent culture. This was in contrast to the soil culture with and without bio-filters. Thus it shows that the bacteria are capable to oxidize the ammonia in the fishpond effluent culture much more efficient compared to those in the soil culture.

4. Conclusions

It is concluded that the concentration of 100 mg/L ammonia-nitrogen in 100 mL of bacteria suspension was too high for a complete ammonia oxidation. For this reason, a longer incubation period is needed for the AOB to completely oxidize the ammonia-nitrogen. To optimize the growth of ammonia oxidizers in the serial batch cultures with and without bio-filters, lower concentrations of ammonia-nitrogen is needed to be investigated (<100 mg/L NH_3-N). The nitrifying bacterium also has a low maximum growth rate and biomass yield compared to other bacteria, so retaining sufficient nitrifying bacteria in a bioreactor is crucial, according to Hosseini et al. [29]. Hence, the use of bio-filters is advantageous in comparison with the system without bio-filters to retain slow-growing bacteria.

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